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Antibacterial Chemotherapy

SEBASTIAN G. B. AMYES

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Antibacterial Chemotherapy

Theory, Problems and Practice

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Antibacterial Chemotherapy

Theory, Problems and Practice

By

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Dedication

For Jackson and Thomas

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Preface

Antibiotics are one of the most important discoveries of the 20th century. Almost immediately the majority of infectious diseases caused by bacteria could be cured and it is estimated that this has increased global life expectancy by 10 years. The fear of these infections was instantly removed. Soon after the introduction of antibiotics, resistant bacteria began to emerge. These resistant bacteria were largely checked by the discovery of new antibiotics and infections caused by them continued to be controlled; however, the era of new drugs is now long past and the proportion of bacteria resistant to the current antibiotics continues to increase. This is most keenly felt in hospitals where there are now incidences of bacteria causing severe infections that are resistant to virtually every antibiotic available to treat them. The judicious use of antibiotics and the control of the spread of resistance are now the responsibility of all healthcare workers who deal with infectious diseases and no longer the duty of just the microbiologist.

Failure by all stakeholders in healthcare to recognise the problems of antibiotic resistance is likely to lead to a bleak outlook for future treatment of bacterial infections. This book not only describes the antibiotics themselves but also draws attention to the problems of resistance and how it needs to be considered when prescribing these drugs.

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Abbreviations

AUIC	Area under the inhibitory curve
CNS	Central nervous system
CSF	Cerebrospinal fluid
CXR	Chest x-ray
CLSI	Clinical and Laboratory Standards Institute
cMRSA	Community-acquired MRSA
*bla*CTX-M	CTX-M β-lactamase gene
EMB	Ethambutol
EDTA	Ethylenediaminetetraacetic acid
ECDC	European Centre for Disease Prevention and Control
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ESCMID	European Society for Clinical Microbiology and Infectious Diseases
ESBLs	Extended-spectrum β-lactamases
GISA	Glycopeptide-intermediate Staphylococcus aureus
HIV	Human immunodeficiency virus
IS	Insertion sequence
ICU	Intensive care unit
INH	Isoniazid
Cmax	Maximum concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MDR	Multi-drug resistant
MDRTB	Multi-drug resistant tuberculosis
MLST	Multi-locus sequence typing
MPC	Mutant prevention concentration
NCCLS	National Committee for Clinical Laboratory Standards
PAS	p-aminosalicylic acid
PV	Panton-Valentine
PBPs	Penicillin binding proteins

PMEN	Pneumococcal Molecular Epidemiology Network
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PZA	Pyrazinamide
RAPD-PCR	Random amplified polymorphic DNA-PCR
REP-PCR	Repetitive extragenic palindromic-PCR
RNA	Ribonucleic acid
RIF	Rifampicin
RPT	Rifapentine
ScotMARAP	Scottish Management of Antimicrobial Resistance Action Plan
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *Staphylococcus aureus*
Vd	Volume of distribution

Chapter 1

Antibiotic action—general principles

Key points

- The importance of the selective nature of antibiotics and the therapeutic index.
- The problems of oral administration rather than those administered by injection.
- The positioning of broad- and narrow-spectrum antibiotics.
- Are combinations of antibiotics more advantageous than using individual drugs?
- Given the choice, which member of an individual drug class should we use first?

Strictly speaking, an antibiotic is an antimicrobial drug that is derived from natural products. Thus penicillin is a true antibiotic, whereas synthetic compounds such as sulphonamides and trimethoprim are not. However, there is general usage of the term to cover all systemic antibacterial drugs and thus the term antibiotic will be used in the modern sense.

Selective toxicity

The earliest use of chemicals to control bacteria was the disinfectants. These were non-selective, being as toxic for human cells as they were for bacteria. Modification of disinfectants, particularly by reducing the concentration of the active components, lead to the development of the antiseptics. These are far less toxic and can be applied to the body surfaces, such as the skin, or to areas where they are not likely to be absorbed. Despite their reduced toxicity, they are still too harmful for systemic use.

Therefore, the essential property of an antimicrobial drug that equips it for systemic use in treating infection is selective toxicity, that is, the drug must inhibit the microorganisms at lower concentrations than those that produce toxic effects in humans. This may be quantified by the therapeutic index, which is the ratio of the toxic dose to the effective dose.

$$\text{Therapeutic index} = \frac{\text{Toxic dose}}{\text{Effective dose}}$$

In general, the larger the ratio the safer the drug. Some antibacterials can be given in very high doses without toxic effects, for example, penicillins, but others may produce serious toxicity at levels that are not much higher those required for treatment of infection; however, no antibiotic is completely safe.

Parenteral versus oral

Oral antibiotics have to be able to survive the acid conditions in the stomach. They may either be inherently resistant to destruction by acid or have functional groups added to form an ester, such as cefuroxime axetil. The ester is then cleaved, often by enzymes in the host, to release the pure antibiotic. The advantages of oral administration are its ease and its reduced cost. The disadvantages are that the drug has to undergo a circuitous route to reach the site of infection. Inevitably some antibiotic passes to the lower bowel where some of the highest concentrations of bacteria, anywhere in the body, are to be found. This may cause destruction of the commensal faecal flora and lead to side effects, such as diarrhoea, or, in some cases, the selection of serious pathogens such as *Clostridium difficile*. It also provides a fertile breeding ground for resistance.

Short and long half-lives

The half-life is often a constant and is a measure of the time taken for the concentration of antibiotic, usually in the plasma, to drop by 50%. The half-lives of early antibiotics were quite short, perhaps only 1 hr, so the antibiotic had to be administered many times per day. With oral versions, this causes problems with patient compliance and with parenteral versions; this becomes expensive in resources as the medical staff have to be on hand for regular medication. Increasingly, the newer antibiotics have much longer half-lives, some over 24 hr. This means that the patient needs to be dosed just once a day to maintain sufficient drug concentrations. However, there may be disadvantages as well as advantages. The longer the half-life, the longer any side effects associated with the antibiotic will persist. Also, the antibiotic will persist in the body for many days following the end of therapy, for much of that time the concentration will be below the effective dose. As will be mentioned later, the exposure of bacteria at sub-inhibitory concentrations is a fertile breeding environment for the development of resistance; and the longer the half-life the longer will be the exposure of bacteria in the body to sub-inhibitory concentrations.

Broad-spectrum and narrow-spectrum antimicrobial drugs

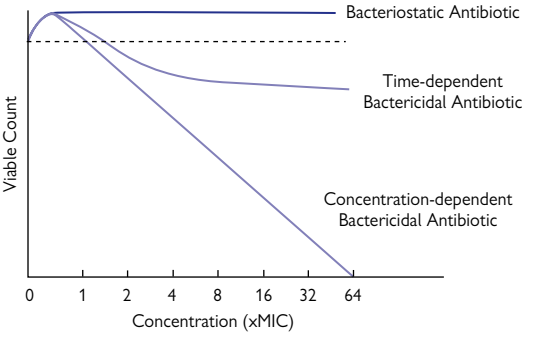
Antibiotics are often described as broad- or narrow-spectrum, according to the range of bacterial species that will be inhibited at standard therapeutic concentrations of the drug. However, no drug is specific for a particular pathogen and there will always be some effect on other bacteria. A narrow-spectrum antibiotic is usually to be preferred in treatment of an infection when the infecting species has been identified, but broad-spectrum cover may be desirable for empiric therapy if the infecting organism has not yet been identified and treatment has to be started urgently. In the past, the development of the more active antibiotics for use in hospitals focussed on antibiotics that were more active against Gram-negative bacteria. This resulted in the emergence of multi-resistant Gram-positive bacteria. In consequence, later antibiotic development focussed on the drugs active against Gram-positive bacteria and this, in turn, has resulted in the emergence of new multi-resistant Gram-negative bacteria.

Bactericidal and bacteriostatic antimicrobial action

The early principle of antibiotic usage focussed on the fact that the infection was acute and that the antibiotic merely provided a control on bacterial multiplication, the cure was provided by the patient's own defence systems as antibiotics could not provide long-term eradication or prevent infection in the absence of adequate numbers of functional white cells in the blood. Antibiotics may be bactericidal, that is, kill the bacteria, or predominantly bacteriostatic, that is, inhibit replication of the bacteria which remain viable and may start to grow when the concentration of drug falls (Fig. 1.1). Opinion differs as to whether bactericidal drugs are preferable to bacteriostatic drugs, but the decisive factor in evaluating an antimicrobial drug is the experience of its efficacy in clinical practice. Antibiotics have increasingly been used in patients who have been immunosuppressed, and some consider that bacteriostatic drugs may be less effective in controlling these infections than antibiotics that are able to kill the bacterium. Furthermore, reduction of bacterial numbers at the site of infection may reduce the capability for the bacteria to become resistant.

There are four major groups of bactericidal antibiotics; β-lactams, fluoroquinolones, diaminopyrimidimes, and aminoglycosides. Each of

Figure 1.1 Dose response to bacteriostatic and bactericidal antibiotics related to the minimum inhibitory concentration for a fixed time period



xMIC, minimum inhibitory concentration.

these groups of antibiotics does not kill the bacterium directly for, in the absence of protein synthesis, they are ineffective. Rather their inhibition of key metabolic stages in bacterial growth induces the synthesis of key enzymes that initiate a chain of events that promotes self-destruction. Of the four major groups, all except the β-lactams provide a bactericidal response that is dependent on the concentration of the antibiotic. The β-lactams produce their maximum bactericidal response at approximately 4–10 fold the minimum inhibitory concentration (MIC), so their bactericidal activity is dependent on the time the bacteria are treated with the antibiotic (Fig. 1.1).

Combinations of antibiotics

Combinations of drugs have been used for a variety of reasons but the main purpose is to overcome the presence or prevent the emergence of drug-resistant strains. This principle has been successfully used where there is an enclosed population of organisms and resistance is known to emerge during prolonged treatment of an individual patient, but there is little or no mobility of resistance genes from one strain to another; for example, in treatment of tuberculosis. The same arguments do not apply where resistance arises in a population of organisms that are freely exchanged between different patients and healthy carriers. Combinations are also used to broaden the

spectrum, especially for empiric therapy, to ensure that all likely pathogens in an infection site could be controlled.

There are many disadvantages in giving combinations of antimicrobial drugs when one drug would suffice. Some combinations of drugs can show antagonistic effects. Combinations of drugs in fixed dosage preparations may apparently be convenient for administration, but do not permit the dosage of each drug to be adjusted independently. In this case the drugs may not reach the infection site in the correct order or concentrations, which could counteract the advantages of prescribing the combination. Combinations have been successfully used to prolong and enhance the life of an individual drug. The use of a β-lactamase inhibitor with some penicillins has allowed their use long after resistance emerges to the principal drug. However, resistance to the combination inevitably occurs.

Although a broad-spectrum of cover may be required initially, it is often possible to de-escalate to a single narrow-spectrum agent when the nature of the infection is ascertained.

Short or long antibiotic courses

The accepted view of antibiotic treatment courses is that they should be prolonged and complete; in particular, the course of antibiotics should be completed even if the symptoms disappear. This principle is based on two assumptions. The first is based on the action of penicillin. The β-lactams, such as penicillin, are almost unique amongst bactericidal antibiotics; the rate at which they kill is NOT dependent on the concentration of the drug. Once a concentration of approximately four-fold greater than the MIC is reached, the ability of the antibiotic does not significantly increase. The efficacy of the drug is dependent on the time that the bacteria are exposed to concentrations above the MIC, hence the need for multi-dosing and completion of the course. These are known as time-dependent antibiotics (Fig. 1.1). Most bactericidal antibiotics are concentration dependent and the higher the concentration used, within the limits of the therapeutic index, the more effective the control. Time is not a significant factor. Some would argue that these antibiotics should be given in the highest safe doses for shorter periods of time. The second assumption is that the longer the course, the less opportunity for resistance to develop. This principle was devised when it was believed that all resistance was a result of mutation. As this is often the exception rather than the rule, then this principle needs to be re-evaluated.

Which member of antibiotic class to use first?

The general view of antibiotic usage is that, if a choice of antibiotics within a drug class is available, then the less active members of the class should be used first. The accepted wisdom behind this is that when resistance to this drug emerges, then the more active drugs can be introduced to overcome it. The flaw in this strategy is that the development of resistance to the less effective member of a drug class often results in decreased susceptibility or resistance to the more active drugs within that class. A notable example of this has been the development of resistance to the fluoroquinolones and related drugs. The use of nalidixic acid for the treatment of gastrointestinal infections in the developing world resulted in the emergence not just of nalidixic acid resistance but also decreased susceptibility to ciprofloxacin resulting with an increased rate to full ciprofloxacin resistance.

Chapter 2

Antibiotics—mechanisms of action

Key points

- The action of the penicillins and cephalosporins along with vancomycin—inhibitors of bacterial cell wall synthesis.
- The selective action of trimethoprim and inhibitors of bacterial folic acid synthesis.
- Bacterial protein synthesis, an early target for antibiotics but often now less popular particularly as they generally do not kill bacteria.
- The action of ciprofloxacin and metronidazole, still two important inhibitors of bacterial DNA synthesis.
- The recent revival of colistin has reawakened interest in antibiotics that affect cell permeability.

The action of antimicrobial agents can be considered as inhibitors in five areas of bacterial metabolism and also as moderators of all permeability.

1. Cell wall synthesis
2. Tetrahydrofolate synthesis
3. Protein synthesis
4. Ribonucleic acid (RNA) synthesis
5. DNA synthesis
6. Permeability moderators

Inhibitors of cell wall synthesis

The composition of the bacterial cell wall is unique in nature and agents, which inhibit its production, are therefore selective as they do not inhibit similar targets in mammalian cells. Cell wall synthesis goes through as series of stages; the formation of the basic sugar-pentapeptide subunit followed by its transportation to the cell surface for polymerization and final cross-linking to form the rigid cell wall. Generally, because the cell wall is disrupted, the action of the cell wall synthesis inhibitors is bactericidal.

Vancomycin

Vancomycin is a glycopeptide antibiotic active against Gram-positive bacteria. Vancomycin acts during the penultimate stage of bacterial cell wall synthesis. By binding to the pentapeptide containing D-alanyl-D-alanine, vancomycin prevents them from interacting with the active site of the enzyme peptidoglycan synthetase, ultimately inhibiting the polymerization of UDP-N-acetyl-muramyl pentapeptide and N-acetylglucosamine into peptidoglycan. A newer glycopeptide, teicoplanin, has a similar action (a more detailed analysis of cell wall synthesis and vancomycin activity and resistance can be seen in Fig 6.5).

Bacitracin

Bacitracin acts at stage two in cell wall synthesis, inhibiting conversion of phospholipid pyrophosphate to phospholipid, which is an essential reaction for the regeneration of the lipid carrier involved in cell wall synthesis. The toxicity of this compound has confined it to topical use.

β-lactams

All these antibiotics contain a β-lactam ring and act in the final step of cell wall synthesis in which strands of peptidoglycan are cross-linked via peptide side chains. β-lactam antibiotics resemble the terminal D-alanine-D-alanine of the pentapeptide and bind covalently to the active site of the transpeptidase enzyme, thereby inhibiting the transpeptidase step required for cross-linking the polysaccharide chains in cell wall peptidoglycan. The β-lactam drugs also interact with a number of other proteins at the cell membrane that are termed penicillin binding proteins (PBPs). The number and types of PBP in a cell varies among species. Some of the PBPs correspond to known enzymes involved in cell wall synthesis; however, others have not been identified.

Penicillins

Natural penicillins

Penicillin G

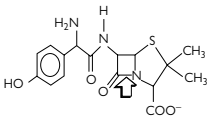
The original penicillin is acid labile and therefore must be administered by injection. It still remains the agent of choice in some infections caused by Gram-positive organisms. The drug has little activity against Gram-negative rods.

Penicillin V

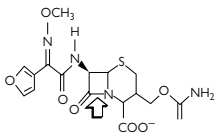
Phenoxymethyl penicillin is an acid-stable derivative of penicillin, it has much the same antibacterial spectrum as penicillin G but can be administered orally.

Figure 2.1 Structures of the important β-lactam antibiotic groups. The β-lactam ring is shown by the arrow, which also shows the carbon-nitrogen bond hydrolysed by a β-lactamase

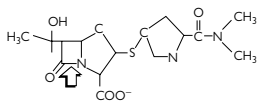
The β-lactam ring is identified by the arrow



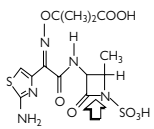
Penicillin - Example shown is amoxicillin



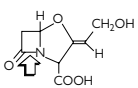
Cephalosporin - Example shown is cefuroxime



Carbapenem - Example shown is meropenem



Monobactam - Example shown is aztreonam



β-lactamase Inhibitor - Example shown is clavulanic acid

Penicillinase-resistant penicillins

Methicillin is a semi-synthetic derivative of penicillin and was the first penicillin that was stable to staphylococcal β-lactamase. Methicillin is acid labile so can only be administered by injection; flucloxacillin is similar to methicillin but is acid stable and can be given orally.

Aminopenicillins

Ampicillin is a semi-synthetic derivative of penicillin, which has an altered spectrum of activity compared to penicillin. Ampicillin is active against many Gram-negative organisms which are unaffected by penicillin G. Although less active than penicillin G against Gram-positives it retains sufficient activity to be clinically useful. Ampicillin is a broad-spectrum antibiotic which can be administered orally. However, it is poorly absorbed from the gut. This antibiotic is used far less commonly than it has in the past because of the introduction of amoxicillin.

Amoxicillin is a later derivative with the same spectrum of activity as ampicillin but with an improved pharmacokinetic profile exhibiting better absorption from the gut resulting in higher blood levels and lower residual levels in the gut.

Carboxypenicillins

Carbenicillin and ticarcillin are carboxy derivatives of penicillin G. They show increased activity against *Pseudomonas aeruginosa* which is intrinsically resistant to most β-lactam drugs and show activity against other ampicillin-resistant Gram-negative bacilli, for example, *Proteus vulgaris* and *Enterobacter* spp.

Ureidopenicillins

Mezlocillin, azlocillin, and piperacillin are again predominantly employed for their activity against *P. aeruginosa*; although mezlocillin and piperacillin can be used to treat serious Gram-negative infections in general.

Cephalosporins

The cephalosporins contain a β-lactam ring that is attached to a six-membered cephem nucleus rather than the five-membered ring in penicillins. This permits modification of the cephalosporin nucleus in two positions, rather than one as is the case with penicillins, thereby significantly increasing the scope for semi-synthetic derivatives that can be modified to alter properties. This flexibility has made the cephalosporins the largest groups of available antibiotics. Cephalosporins are normally classified in generations (Table 2.1), unfortunately there is no standardization in this and agents classified as one generation by one author may be classed as a different generation by others. One method of classification is based on the susceptibility of this group of antibiotics to β-lactamases.

The so-called 1st generation cephalosporins have a limited spectrum which include *Escherichia coli*, *Klebsiella pneumoniae,* and *Proteus mirabilis;* however, the emergence of new β-lactamases has considerably reduced the proportion of susceptible strains. These cephalosporins have no activity against *Bacteroides fragilis*, *Enterococcus* spp, methicillin-resistant *Staphylococcus aureus* (MRSA), *P. aeruginosa*, *Acinetobacter* spp, *Enterobacter* spp.

Table 2.1 Cephalosporins

Generation	Representatives	Route	Spectrum
1st	Cefalexin Cefaloridine Cefalothin	Oral Early cephalsporin Parenteral	Limited spectrum and often β-lactamase susceptible
	Cefradine Cefazolin	Oral Parenteral	
2nd	Cefuroxime	Originally parenteral but now also oral	*Escherichia coli, Klebsiella* spp, *Proteus spp, Enterobacter* spp, *Haemophilus influenzae, Moraxellus, catarrhalis, Streptococcus, Pneumoniae, Staphylococcus aureus*, (not MRSA), beta-haemolytic, Streptococci
	Cefaclor Cefodroxil	Oral Parenteral and oral	
Cephamycins	Cefoxitin	Parenteral	Gram-negative and Gram-positive bacteria, anaerobic bacteria. Not active against *Pseudomonas aeruginosa*.
	Cefotetan	Parenteral	Gram-negative and Gram-positive bacteria, anaerobic bacteria. Not active against *Pseudomonas aeruginosa*.
3rd	Cefpodoxime	Oral	Gram-positive and Gram-bacteria, but not *Pseudomonas aeruginosa. Enterococcus* spp and *Bacteroides fragilis*.
	Ceftibuten	Oral	Some Gram-positive and Gram-negative bacteria
	Cefixime	Oral	Some Gram-positive and Gram-negative bacteria
	Cefdinir	Oral	Some Gram-positive and Gram-negative bacteria
	Cefotaxime	Parenteral	Some Gram-positive and Gram-negative bacteria, but not *Pseudomonas aeruginosa, Enterococcus spp* and *Bacteroides fragilis*

Table 2.1 (*Contd.*)

Generation	Representatives	Route	Spectrum
	Ceftriaxone	Parenteral	Some gram-positive and gram-negative bacteria and chlamydia, but not *Pseudomonas aeruginosa*. *Enterococcus spp* and *Bacteroides fragilis*. Long half-life
	Ceftazidime	Parenteral	*Pseudomonas aeruginosa*
	Cefoperazone	Parenteral	*Pseudomonas aeruginosa*
4th	Cefepime	Parenteral	*Pseudomonas aeruginosa, Staphylococcus aureus* and multiple drug resistant. *Streptococcus pneumoniae, Enterobacteriaceae*
	Cefpirome	Parenteral	*Pseudomonas aeruginosa, Staphylococcus aureus* and multiple drug resistant. *Streptococcus pneumoniae, Enterobacteriaceae*
5th	Ceftobiprole	Parenteral	*Pseudomonas aeruginosa*, MRSA, penicillin-resistant *Streptococcus pneumoniae*.

Under this classification, the 2nd generation cephalosporins have a greater resistance to the earlier β-lactamases. This has increased their Gram-negative spectrum but at the cost of losing some anti–Gram-positive activity. The cephalosporins are not usually active against non-fermenting Gram-negative bacteria such as *P. aeruginosa*, *Acinetobacter baumannii*. Some members of this group have activity against anaerobic bacteria. A group related to the cephalosporins, the cephamycins, is often classified within this group. They have a similar spectrum of activity but often have greater resistance to β-lactamases including the extended-spectrum lactamases (ESBLs).

There is often more agreement of the classification of 3rd generation cephalosporins. They have a broad spectrum of activity particularly against Gram-negative bacteria and drugs within this group can be active against non-fermenting Gram-negative bacteria but this often is accompanied by a reduction, often considerable, in the ability to deal with Gram-positive infections. Early members of this group were strictly parenteral but now there are many oral formulations. The cephalosporins are able to penetrate the central nervous system making them suitable for the treatment of most bacteria responsible for meningitis. However, the increased spectrum of these cephalosporins ensured that they have become a major treatment option for nosocomial infections.

In the 4th generation cephalosporins, such as cefepime and cefpirome, the anti–Gram-positive activity found in the 1st generation drugs has largely been restored. Some of these drugs are also suitable for the treatment of meningitis and most for the treatment of *P. aeruginosa*. They also have increased resistance to β-lactamases including the ESBLs.

A 5th generation has not been established but cephalosporins, such as ceftobiprole, have a broader spectrum and may be able to control MRSA, multi-resistant *Streptococcus pneumoniae* and particularly non-fermenting Gram-negative bacteria.

Carbapenems

The nucleus of the carbapenems is similar to that of penicillins, with a five-membered side ring but differs in the replacement of sulphur by carbon. Carbapenems have the broadest spectrum of activity of any of the β-lactam family and are active against both Gram-positive and Gram-negative bacteria, aerobes, and anaerobes. The carbapenems have not emerged in generations as drugs that have been introduced more latterly are no more, and often less, active than those currently being used. The two most active drugs in common usage are meropenem and imipenem-cilastatin. More recent introductions include biapenem, doripenem, and panipenem-betamipron. The latter, like imipenem, is accompanied with a renal inhibitor to prevent the renal uptake of the carbapenem. These carbapenems are designed for hospital use. On the hand, ertapenem has a much more limited activity than the others within this group, as it is inactive against *P. aeruginosa* and *A. baumannii*. Faropenem is not a carbapenem but a penem as it retains the sulphur atom in the five-membered ring; however, the ring is unsaturated like the carbapenems and it retains some of the properties. It has been designed for oral therapy of respiratory infections but, as yet, has not been licensed in the United States.

Monobactams

Aztreonam is the only monobactam currently available clinically. It is only active against Gram-negative species and shows no activity against Gram-positive bacteria.

β-lactamase inhibitors

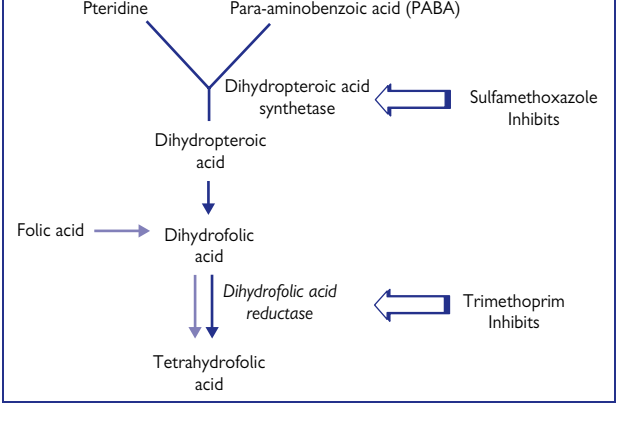
Increasing resistance as a result of β-lactamase production has led to the development of β-lactamase inhibitors. These are compounds which when co-administered with the β-lactam prevents inactivation by β-lactamases. Three β-lactamase inhibitors are available for clinical use, clavulanic acid combined with amoxicillin or ticarcillin, sulbactam which has been combined with ampicillin or cefoperazone, and tazobactam combined with piperacillin. All these inhibitors contain a β-lactam ring and function in the same way, acting largely as competitive inhibitors of β-lactamases. Clavulanic acid has an oxygen atom instead of sulphur in the five-membered ring of penicillins and the other two retain the sulphur atom but it is linked to two oxygen atoms.

Inhibitors of folate synthesis

Two groups of antibiotics act in this area: the sulphonamides and the diaminopyrimidimes, the best known is trimethoprim. Both are competitive inhibitors of enzymes in the metabolic pathway synthesizing tetrahydrofolate. Sulphonamides are structural analogues of para-amino benzoic acid and inhibit dihydropteroate synthetase, trimethoprim inhibits dihydrofolate reductase. The sulphonamides are selective in their action because the reaction catalysed by dihydropteroate synthetase does not occur in mammalian cells which utilize preformed folates. The reduction of dihydrofolate to tetrahydrofolate does occur in mammalian cells; however, trimethoprim is a selective inhibitor of bacterial dihydrofolate reductase and does not significantly inhibit the mammalian enzyme.

Because these two agents act on the same pathway several claims were made about the theoretical benefits of combining them. In particular it was claimed that the two drugs acted together synergistically and that combined usage would delay the emergence of resistance. Thus when trimethoprim was first developed it was only available in combination with sulfamethoxazole (co-trimoxazole). In fact, synergy was largely an *in vitro* observation and effectively reduced the minimum inhibitory concentrations (MICs) of each drug for the bacterium being treated. It soon became clear, however, that because the concentration of trimethoprim administered was a considerable multiple of the original MIC, in most settings, trimethoprim alone is as efficacious as the

Figure 2.2 Action of co-trimoxazole—metabolic pathway in bacteria is shown in black arrows and in man in grey arrows



combination and that the combination did not prevent resistance emerging to trimethoprim. There were some incidences where *in vivo* synergy was considered to contribute to efficacy, most notably with treatment of *Pneumocystis jiroveci* (previously *carinii*) pneumonia.

Inhibitors of protein synthesis

There are a number of important groups of antibiotics that act on protein synthesis. The basis for selective activity in many, but not all, cases results from differences in structure between bacterial and mammalian ribosomes (Table 2.2). Most of the antibiotics in this group, except the aminoglycosides, are bacteriostatic.

Table 2.2 Inhibitors of protein synthesis

Antibiotic class	Representatives	Selective action	Metabolic effect
Aminoglycosides	Streptomycin Gentamicin Tobramycin Netilmicin Amikacin	Interaction with bacterial ribosomes but not mammalian ribosomes	Bactericidal
Macrolides and related compounds	Erythromycin Clarithromycin Azithromycin	Interaction with bacterial ribosomes but not mammalian ribosomes	Bacteriostatic
Tetracyclines and glycines	Tetracycline Oxytetracycline Doxycycline Minocycline Tigecycline	Inability of drug to be transported into mammalian cells	Bacteriostatic
Streptogramins	Quinupristin/ dalfopristin	Early and late phase bacterial protein synthesis	Bactericidal/ bacteriostatic
Chloramphenicol	Chloramphenicol	Interaction with bacterial ribosomes but not mammalian ribosomes	Bacteriostatic
Oxazolidinone	Linezolid	Prevents bacterial ribosome formation	Bacteriostatic
Lincomycins	Lincomycin Clindamycin	Interaction with bacterial ribosomes but not mammalian ribosomes	Bacteriostatic

Aminoglycosides

Aminoglycosides are part of a group of antibiotics known as the aminocylitols, which also include antibiotics such as spectinomycin. The aminoglycosides interact with bacterial 70S but not mammalian 80S ribosomes. Interaction of aminoglycosides with bacterial ribosomes has a number of effects, including disruption of peptide chain formation and misreading of the genetic code. The resulting inadequate production of vital proteins has disruptive effects on many essential bacterial functions leading to cell death. Streptomycin was the first of the aminoglycosides introduced into clinical use. Today the main four aminoglycosides are gentamicin, tobramycin, netilmicin, and amikacin. Gentamicin, tobramycin, and netilmicin are very similar but amikacin remains active against isolates which display cross-resistance to the other agents.

Macrolides

Erythromycin was the first member of this group; it is a relatively narrow-spectrum drug with activity primarily against Gram-positive bacteria. Erythromycin is an inhibitor of protein synthesis, binding to a single site on the 70S ribosome. It is thought that this binding inhibits translocation by interfering with the association of peptidyl-tRNA after peptide bond formation. Erythromycin is primarily bacteriostatic in activity, although this is dose-dependent and bactericidal activity can be observed at higher concentrations. Macrolides do not bind to mammalian 80S ribosomes. Other related compounds, including azithromycin and clarithromycin, have similar actions.

Tetracyclines

Tetracyclines inhibit protein synthesis as a result of binding to prokaryotic ribosomes. This interaction prevents the binding of aminoacyl-tRNA to the acceptor site on the mRNA ribosome blocking the addition of new amino acids to the peptide chain. Tetracyclines also bind to mammalian ribosomes and the basis for their selective activity does not result from differential binding. The ability of tetracyclines to inhibit bacterial and not mammalian cells seems to result from an inability of the drug to enter mammalian cells whereas, in contrast, tetracycline appears to enter bacterial cells by both passive diffusion and active uptake. Tetracyclines exhibit a bacteriostatic effect on bacteria and have a broad spectrum of activity encompassing both Gram-negatives and Gram-positives, aerobes and anaerobes.

Doxycycline is a semi-synthetic tetracycline with a similar mode of action to tetracyclines. It has, however, a broader spectrum of activity that may include MRSA and *Acinetobacter* spp.

Tigecycline is a glycylcycline antibiotic with a similar action to tetracycline though it has a much broader spectrum. It can inhibit MRSA and *A. baumannii* but has no effect on *P. aeruginosa*.

Lincomycins

Two lincomycin antibiotics are available, lincomycin and clindamycin. Clindamycin is a synthetic derivative of lincomycin which is more active and has improved absorption from the gut. The lincomycins bind to the bacterial 70S ribosome. They appear to bind at the same site as chloramphenicol and the macrolides but the effect of the lincomycins is to prevent initiation of peptide chain formation. They are predominantly bacteriostatic drugs although under certain conditions can be bactericidal. They are active primarily against Gram-positive bacteria and anaerobes.

Chloramphenicol

Chloramphenicol interacts with 50S subunit of intact bacterial 70S ribosomes preventing protein synthesis by inhibiting peptide bond formation. The interaction of chloramphenicol with the ribosome affects the attachment of aminoacyl-tRNA preventing these compounds reacting with peptidyl transferase and stopping peptide bond formation. Chloramphenicol is a bacteriostatic agent and has a broad spectrum of activity.

Linezolid

Linezolid is an oxazolidinone and has an unusual mode of action. Like chloramphenicol, it binds to the 50S ribosomal subunit but instead of preventing peptide bond formation, it prevents the binding of the 50S to the 30S subunit to form the 70S ribosome. Thus it works at the initiation of protein synthesis. This unusual mechanism of action was thought to ensure that the antibiotic would be active against multi-resistant bacteria that had become resistant to most other drugs. Linezolid has proved active against MRSA but resistance has emerged.

Streptogramins

The streptogramins, quinupristin, and dalfopristin, are used together in the ratio of 3–7 and the two components act synergistically so their activity, *in vitro* at least, is greater than the sum of their individual activities. The components are metabolized when they enter the body and their metabolites also contribute to the antimicrobial activity of the streptogramin combination. The site of action of quinupristin and dalfopristin is the bacterial ribosome. Dalfopristin has been shown to inhibit the early phase of protein synthesis whereas quinupristin inhibits the late phase of protein synthesis. The particular attribute of this streptogramin combination is its broader spectrum. It is active against *Enterococcus faecium*, though only bacteriostatic, but has no activity against *Enterococcus faecalis*. It also has bactericidal activity against all types of *Staphylococcus aureus*, including MRSA.

Inhibitors of DNA synthesis

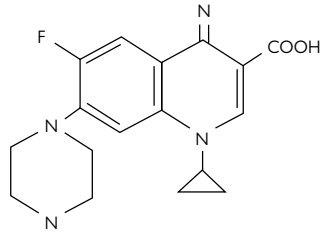
Quinolones

The original quinolone antibacterial, nalidixic acid, has enjoyed widespread clinical use since 1962; but it was only with the development of the modern quinolones the full potential of these agents has been realized. The discovery that the insertion of fluorine at the six position of the base nucleus broadened the spectrum and increased the activity of these compounds. This led to the development of the modern fluoroquinolones (Fig 2.3) which have antibacterial activities up to 1,000-fold that of nalidixic acid and were introduced into clinical medicine in 1980s. There were a number of early fluoroquinolones but most have not survived. Ciprofloxacin was the most active of these and remains a major component of modern clinical therapy. The quinolone antibacterials are bactericidal agents and kill bacteria by inhibiting more than one target. Central to their killing action is the interaction of the quinolone with bacterial DNA gyrase (topoisomerase II). DNA gyrase consists of two A and two B subunits and is the enzyme responsible for supercoiling strands of DNA into the bacterial cell. Nalidixic acid interacts with the A subunit while the newer quinolones appear to interact with both the A and B subunits. This interaction with DNA gyrase is responsible for the lethal effects of these drugs. The drugs also interact with topoisomerase IV, an enzyme very similar in structure to DNA gyrase, which is responsible for decatenation (separation) of the DNA strands following DNA replication. Although the early fluoroquinolones had a broad spectrum of activity against Gram-negative bacteria, they were not sufficiently active against most Gram-positive organisms. The development of the L-isomer of ofloxacin, levofloxacin, increased the anti–Gram-positive activity so that *S. pneumoniae* infections could be readily treated. This led to the development of even more powerful, anti–Gram positive fluoroquinolones such as moxifloxacin, gemifloxacin, and gatifloxacin, though the latter has almost completely dropped out of clinical use.

Metronidazole

Although not a classic inhibitor of DNA synthesis as such, metronidazole is included in this section as its bactericidal activity is mediated by its effects on DNA. Once metronidazole has entered the bacterial cell it undergoes reductive activation when the nitro group of the drug is reduced by low redox potential electron transport proteins. The resulting active compounds damage the cell through interaction with DNA. The activity of metronidazole is restricted to anaerobic bacteria and it is the agent of choice for many anaerobic infections.

Figure 2.3 Chemical structure of the fluoroquinolone, ciprofloxacin



Inhibitors of RNA synthesis

Only one medically important antibiotic group acts by directly inhibiting RNA synthesis. The rifamycins act by inhibiting bacterial DNA-dependent RNA polymerase. By far the most important of these is rifampicin; the importance of which lies in the fact that it is one of the cornerstones in the treatment of tuberculosis. It is selective because it does not act on the equivalent mammalian enzyme. The inhibition of RNA synthesis and its consequential inhibition of protein synthesis would indicate that this drug should be bacteriostatic; however, it has been reported to be bactericidal under certain conditions with some bacteria.

Permeability moderators

The special feature of the permeability moderators is that they are usually bactericidal, but unlike the bactericidal inhibitors of macromolecular synthesis listed above, they do not require protein synthesis to function and thus can act upon non-growing cells.

Polymyxins

Polymyxins have both hydrophilic and lipophilic moieties, which can interact with the bacterial cytoplasmic membrane, changing its permeability. This disruption of the control of influx and efflux by the cell is bactericidal. The main drug in use is colistin; it is a mixture of cyclic polypeptides colistin A and B. The membrane disruption properties were associated with adverse effects; however, the decreasing therapy options for the treatment of some non-fermenting Gram-negative such as *A. baumannii* and *P. aeruginosa* has ensured a revival of the drug as it is seen as a last resort for multi-resistant versions of these bacteria.

Daptomycin

Daptomycin is a lipopeptide antibiotic which is bactericidal against Gram-positive bacteria has been obtained from the soil saphrophyte

Streptomyces roseosporus. A number of different models have been put forward for its mechanism of action but it is believed to disrupt the function of the plasma membrane without penetrating into the cytoplasm of the bacterial cell. The acyl tail portion of the compound binds and inserts itself into the cytoplasmic membrane. This forms a channel that causes depolarization of the membrane and is associated with the bactericidal action of the drug. The channel permits the efflux of ions, particularly potassium, from the cell and prevents the cell's synthesis of essential macromolecules.

A summary of the antibacterial agents is shown in Table 2.3.

Table 2.3 Summary of the antibacterial agents

Inhibitors of cell wall synthesis		
β-lactams		
Benzylpenicillins	Penicillin G	
Phenoxypenicillins	Penicillin V	
β-lactamase resistant penicillins (antistaphylococcal)	Oxacillin Cloxacillin Dicloxacillin	Flucloxacillin Methicillin Nafcillin
Aminopenicillins	Ampicillin	Amoxicillin
Carboxypenicillins	Carbenicillin	Ticarcillin
Ureidopenicillins	Azlocillin Mezlocillin	Piperacillin
Cephalosporins (1st generation)	Cefalothin Cefazolin Cefapirin Cefradine	Cefalexin Cefadroxil Cefaclor
(2^{nd} generation)	Cefamandole Cefuroxime Cefonicid Ceforanide	Cefoxitin Cefmetazole Cefotetan
(3^{rd} generation)	Cefotaxime Ceftriaxone Ceftizoxime Ceftazidime	Cefoperazone Moxalactam Cefixime
(4^{th} generation)	Cefepime	Cefpirome
(5^{th} generation)	Ceftobiprole	
Monobactams	Aztreonam	
Carbapenems	Meropenem Imipenem	Doripenem Ertapenem
β-lactamase inhibitors	Clavulanic acid Sulbactam	Tazobactam
Other cell wall synthesis inhibitors	Vancomycin Teicoplanin	Bacitracin

Table 2.3 *(Contd.)*		
Inhibitors of protein synthesis		
Aminoglycosides	Streptomycin Gentamicin Tobramycin	Netilmicin Amikacin
Macrolides	Erythromycin Clarithromycin	Azithromycin
Tetracyclines	Tetracycline Oxytetracycline Tigecycline	Doxycycline Minocycline
Lincomycin	Clindamycin	Lincomycin
Oxazolidinone	Linezolid	
Stretpogrammins	Quinupristin plus Dalfopristin	
Other agents	Chloramphenicol	
Inhibitors of tetrahydrofolate synthesis		
Sulfonamides	Sulfamethoxazole Sulfadiazine	Sulfanilic acid
Diaminopyrimidimes	Trimethoprim	
Combinations	Co-trimoxazole	
Inhibitors of DNA synthesis		
Quinolones	Nalidixic acid	
Fluoroquinolones	Ciprofloxacin Norfloxacin Sparfloxacin Lomefloxacin	Ofloxacin Levofloxacin Gemifloxacin Moxifloxacin
Metronidazole	Metronidazole	
Inhibitors of RNA synthesis		
Rifamycins	Rifampicin	
Others		
Polymyxcins	Colistin	

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Chapter 3

Pharmacokinetics applied to antimicrobials

Key points

- The measurement of the antibiotic's distribution in the host's tissues.
- The volume of distribution (Vd) and clearance of the antibiotic.
- The importance of specific measurable parameters, such as maximum concentration.
- Linking antibiotic dosing with clearance.
- The influence of protein binding.

The use and application of pharmacokinetic principles to antimicrobial agents is a rapidly growing science. The term pharmacokinetics is used to define the time course of drug absorption, distribution, metabolism, and excretion. One of the main applications of clinical pharmacokinetics is to increase the effectiveness or to decrease the toxicity of a specific drug therapy. The term pharmacodynamics refers to the relationship between drug concentration at the site of action and pharmacologic response. However, when we apply these principles to antimicrobial therapy there are a number of factors which can alter the predicted outcome (Table 3.1).

Table 3.1 Factors which can influence therapeutic outcome

Bacterial	Pharmacokinetics
Inhibitory activity	
Sub-inhibitory activity	Absorption
Concentration-dependent activity	Distribution
Time dependent	Metabolism
Bactericidal/bacteriostatic activity	Excretion
Post-antibiotic effect	Protein binding
Resistance—phenotypic —transferability	

How the body copes with a drug is a complex mixture, in which several processes work together to affect how much of a drug gets where in the body, and at what concentrations. To understand these processes, a model of the body can be used. Such models are classified by the number of compartments needed to describe how a drug behaves. There are one, two, and multi-compartment models, which refer to groups of similar tissues or fluids. These models can be used to predict the time course of drug concentrations in the body. The highly perfused organs (e.g. heart, liver, and kidneys) are considered to be one compartment (central) whilst fat, muscle, cerebrospinal fluid (CSF), and so on are in the peripheral compartment.

There are several other key terms which are useful in understanding drug distribution. An important indicator of the extent of distribution is the Vd or Volume of Distribution. This relates the amount of drug in the body to the measured concentration in the plasma. A large Vd indicates that the drug extensively distributes into body tissues and fluids but does not specify which tissues or fluids.

$$\text{Vd} = \frac{\text{Amount of drug given (dose)}}{\text{Initial drug concentration}}$$

Other key aspects of drug handling include:

- Clearance—the removal of drug from plasma and relates the rate at which a drug is given and eliminated to the resultant plasma levels. It is expressed as Volume/Time.
- Cmax—the maximum concentration reached at the site of infection, usually taken as the peak serum level.
- Tmax—the time taken, after dosing, for the antibiotic concentration to reach the Cmax.
- Half-life (t½)—the time taken for the concentration of the drug in the plasma to decrease by half. This is often used as an indicator as to how often the drug should be administered (Fig. 3.1).

Area under the curve (AUC)—The parameter which links clearance to dosing (Fig. 3.2). It is easily calculated:

$$\text{AUC} = \frac{\text{Initial concentration}}{\text{Elimination rate constant}}$$

Figure 3.1 Short and long half-lives

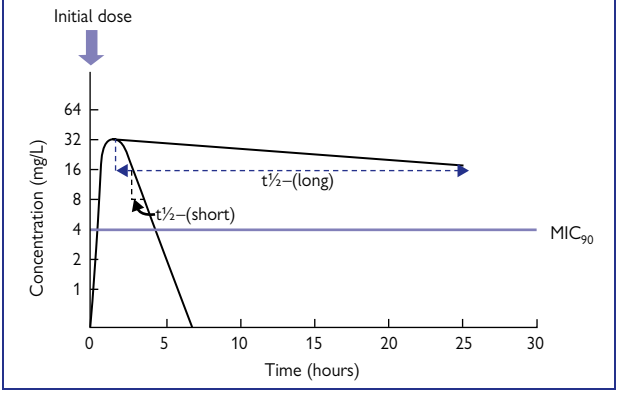
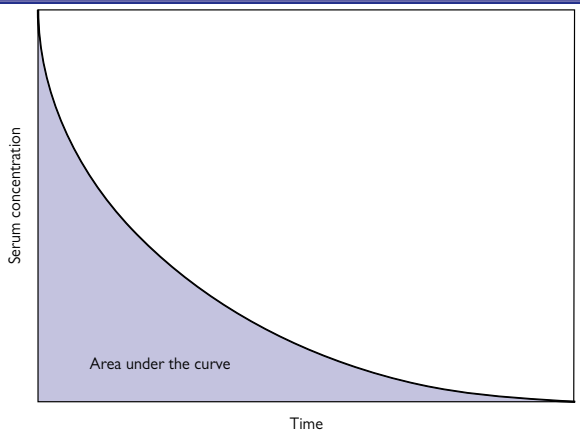


Figure 3.2 Graph showing antibiotic distribution and area under the curve



Area under the inhibitory curve (AUIC)

The extent of bacterial death with some antibiotics (e.g. fluoroquinolones) is crucially dependent on the drug concentration. However, with other antibacterial drugs (e.g. β-lactams) concentrations four-fold or above the minimum inhibitory concentration (MIC) have no increased effect. With the latter the length of time the concentration

of the antibiotic remains above the MIC is usually the most important consideration. With the former group, it is important to know the AUIC. This is an antimicrobial adaptation of AUC and refers to the concentration of the drug that is able to exert antibacterial activity over a given organism for a specific time. The AUIC is the drug concentration divided by the MIC, usually the MIC_{90} (see chapter 4), of a specific bacterial species. All AUIC values are reported for 24 hr of dosing. An AUIC of 125 is considered the lower limit of activity for a cure, the preferred value is >250.

Bearing these processes in mind antibacterials can be divided into those which have a high Vd (e.g. fluoroquinolones) and those which need more regular dosing due to short half-life (e.g. penicillins); by modifying the molecular structure of some drugs we have been able to improve absorption and thus achieve better plasma concentrations (e.g. ampicillin to amoxicillin). Table 3.2 shows a selection of serum pharmacokinetics and other factors of commonly used antimicrobials

The recent drive with antimicrobial research has been to develop agents

- which have a broad spectrum of antibacterial activity
- are given once or twice a day (at the most) so with a long half-life
- have a large Vd into specific tissues
- are well tolerated.

The principles of pharmacokinetics are being applied to achieve these aims.

Table 3.2 Serum kinetics and other factors of common antimicrobials

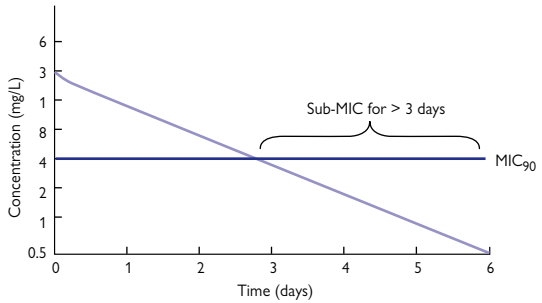
Antibiotic	Dose/route	T/2(h)	Protein-binding(%)	Bio-availability(%)
Amoxicillin	0.5g PO	0.8–2	20	80
Ampicillin	0.5g PO 0.5g IM	0.8–1.5	17–20	50
Penicillin	5m Unit IV	0.5	40–60	20–30
Cefaclor	1g PO	0.5–1.5	25	70
Cefixime	0.2g PO	3–9	65	50
Ceftriaxone	1g IM	8.0	83–95	–
Cefuroxime	1g PO	1–2	33–50	36–50
Ciprofloxacin	0.5g PO	3–6	40	60–80
Norfloxacin	0.4g PO	2–4	10–15	30–40
Erythromycin	0.5g PO	1.2–2.6	75-90	?
Metronidazole	0.5g PO	6–12	<20	>80
Clindamycin	0.15g PO	2–4	60–95	90

Antimicrobials developed within the last 20 yr have shown some remarkable pharmacokinetic profiles. Agents belonging to the fluoroquinolone and macro/azalide classes have both high volumes of distribution and half-lives in excess of 5 hr (thus allowing once or twice daily dosing) (Fig. 3.3). Whilst among the cephalosporins, ceftriaxone has half-life upto 8 hr. A better understanding of aminoglycosides has allowed the administration of these drugs once a day as a bolus dose, rather than three times daily as initially licensed. This shift has enabled these drugs to be used more safely without compromising their efficacy.

There are potential problems in that a long half-life will mean that any side effects associated with the antibiotic will persist for longer, but also that there will be a considerable period at the end of therapy where significant, but sub-inhibitory, concentrations of the antibiotic will persist. This is a potent environment for the selection of resistance not only in any cells of the original pathogen that have remained but also for any other bacteria in the body.

A feature commonly and controversially considered for an antibiotic is protein binding. This is the ability of the serum proteins to bind free antibiotics. This can vary enormously from antibiotic to antibiotic, ranging from less than 10% to greater than 90% (Table 3.2). The questions arises how does protein binding affect the availability of the antibiotics to deal with bacterial infections and whether the *in vitro* experiments performed to measure protein binding are an accurate assessment of the binding within the body. Some consider it to be crucial, for example, 90% protein binding would remove 90% of the available antibiotic whereas the opposite view is that even though the antibiotic may

Figure 3.3 The effect of a long half-life in maintaining sub-inhibitory concentrations of antibiotics



MIC, minimum inhibitory concentration.

become bound in the body it is still available as an antibacterial. Although there are many reports measuring protein binding, there is little evidence to suggest that it has a major effect on efficacy. It is, however, considered to have an influence on the half-life of the antibiotic.

Finally, the concept of an agent continuing to exert its activity long after detectable concentrations have ceased at the site of infection is known as post-antibiotic effect. Attempts to quantify this have been made by measuring the time taken by the recovering bacterium to multiply ten-fold. The greater the time, the longer the apparent post-antibiotic effect. This varies between antimicrobials and different organisms. Whether it is a useful indicator as to how long we can expect a drug to work beyond certain time points or not is still a point of conjecture. Perhaps the safest view is not to rely upon it or even consider it and assume that a drug is perceived to be effective while its concentration remains above the MIC.

Chapter 4

Sensitivity and identification tests

Key points

- Sensitivity tests on live bacteria versus tests performed using just the bacterial DNA.
- Determination of the 'gold-standard' for bacterial sensitivity, the minimum inhibitory concentration (MIC) and recent introduction of the ε test.
- Bacterial breakpoints and how should they be set.
- The introduction of automated tests.
- The importance of National and International Guidelines.
- Why it is crucial to distinguish between surveillance and epidemiology and why surveillance usually over-estimates the proportion of resistant bacteria in the population.

In vitro tests are an invaluable guide in choosing therapy, although they cannot always predict *in vivo* responses accurately; however, if an organism is found to be resistant to an antibiotic *in vitro* it is most unlikely that normal therapeutic doses of that antibiotic will be of value in eliminating the infection. The tests can be based on either the phenotypic (based on the expression of specific characteristics of the cell) or the genotypic (based usually on the DNA complement) properties. Although the latter is currently gaining more attention, there remains a need for both.

Phenotypic tests

Traditionally, there have been two major methods for non-automated sensitivity in the laboratory—dilution tests and disk tests.

Dilution tests

These are performed with doubling dilutions of antibiotic solution in the bacterial culture medium. Tube dilution tests use a liquid culture medium; a known concentration of the drug is diluted in a series of doubling dilutions so that the range of concentrations of antibiotic

obtained will cover the likely bactericidal and bacteriostatic levels of that antibiotic for the organism under test. Each tube is then seeded with a standard number of organisms and the tubes are incubated, usually overnight at 37°C. Control series of tubes should also be set up with standard organisms of known sensitivity as a check on the potency of the antibiotic preparation used and the accuracy of the dilution technique.

The bacteriostatic level of the antibiotic is read as the last tube showing no evident turbidity, that is, the highest dilution (lowest concentration) of the drug that has inhibited growth of the organism. The bacteriostatic level indicates the minimal inhibitory concentration (MIC) of the antibiotic for that particular organism.

The bactericidal level is obtained by sub-culturing a small quantity from all tubes showing no turbid growth onto an agar medium, and this is incubated again. The last of the tubes yielding no growth on that agar contains the minimum bactericidal concentration (MBC) of the antibiotic (Fig. 4.1).

More usually, dilution sensitivity tests are set up with the antibiotic incorporated into solid media in agar plates. The test is again seeking the minimum concentration of antibiotic that inhibits visible growth. The advantage of the test on solid media is that many bacterial cultures can be tested on the same agar plate. Indeed, the plate could be read with a video camera and, with suitable software, the result can be incorporated straight into a database. There are also fewer problems with contamination (Fig. 4.2).

Figure 4.1 Tube dilution test for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

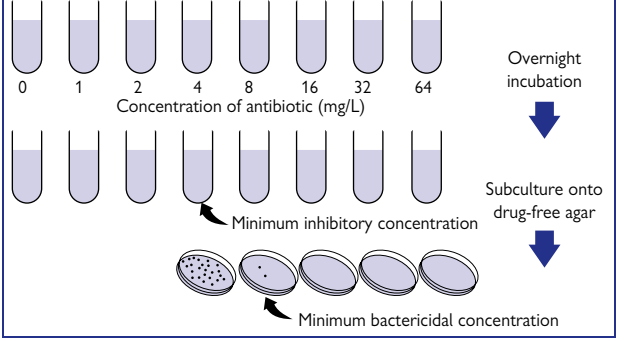
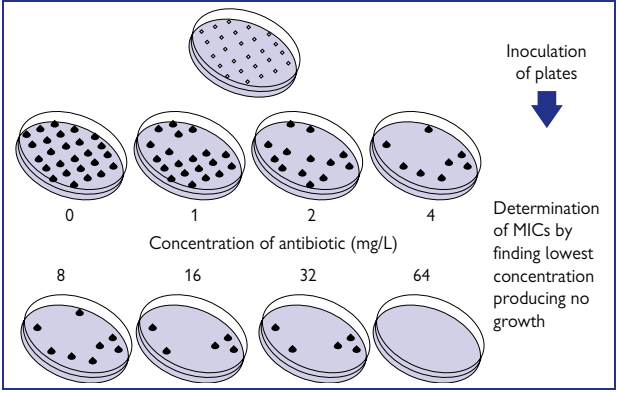


Figure 4.2 Agar dilution test for minimum inhibitory concentration (MIC) determination using multipoint inoculation



MIC range, MIC_{50}, and MIC_{90}

Often it is important to know whether the susceptibility of a whole bacterial population is sensitive or resistant. It is, therefore, convenient to describe a series of MIC results by the defined criteria of the range, the MIC_{50} and MIC_{90} values. These may be obtained by sorting the MIC results from the lowest to the highest. Some laboratories present their results as cumulative MICs, in which the MIC values tested are plotted against the percentage of bacteria inhibited by this concentration. However, most data are now usually entered into a database and this sorting can be conveniently performed by almost any database or spreadsheet computer programme (Fig.4.3).

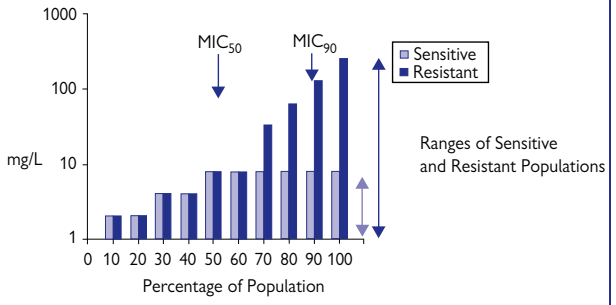
Range

The range of MICs is simply the lowest and the highest MIC value in this series and is often expressed as, for example, 1.0–512 mg/L (as in the 'resistant' population in Fig 4.3). The range of MICs establishes the spread of the results and allows easy comparison of two populations, showing whether they are similar or not. However, the range only shows the spread, it does not identify the distribution within that spread.

MIC_{50}

It is often convenient to know the median of the series. that is, the MIC value of the strain that appears 50% up the series. This MIC_{50}

Figure 4.3 Parameters associated with the expression of MICs of two populations of bacteria



MIC, minimum inhibitory concentration.

figure will allow a broad comparison of the population with others. However, most MIC data are requested to establish whether resistance is a problem or emerging, and the MIC_{50} is too crude to establish this. When resistance emerges in a bacterial population, it is often manifested by a few strains showing significant increases in MIC whereas the rest of the population remains unaffected, thus a more significant measurement would be to determine the MIC_{90} value.

MIC_{90}

The MIC_{90} is obtained in a similar manner to the MIC_{50} except that it is the MIC value of the strain that appears 90% up the series. An antibiotic is likely to be considered successful if more than 90% of the population are inhibited by it. The MIC_{90} value will show this readily. It will also show if resistance is beginning to emerge in a population. Although this will be reflected by an increase in the higher value of the range, the range will not show how many bacteria have decreased susceptibility. The MIC_{90} value will show when 10% are affected.

Breakpoints

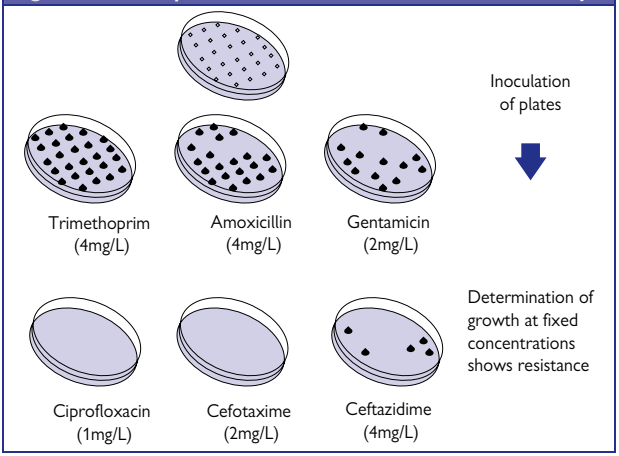
Although the MIC determination may give the maximum information about bacterial sensitivity to antibiotics, for the routine laboratory it is both expensive and time consuming. To recommend a suitable antibiotic, the essential piece of information is to identify if the causative organism is sensitive to the concentration of antibiotic at the site of infection. Thus a compromise of the agar dilution MIC determination has been reached whereby the bacteria are tested against usually a single concentration of antibiotic. Sometimes, however, this may be extended and two distinct concentrations are used, a high value and a low value. The test is set up in exactly the same manner as the MIC

determination adhering to all the conditions about the antibiotic concentrations, media, bacterial inoculum, and so on. The fixed concentrations of antibiotic may either be provided by preparing suitable concentrations in the agar plate, either from stock solutions, or by placing a fixed content tablet to the plate and adding a fixed volume of media. The agar is inoculated as before by a multiple inoculator delivering 1–2μL onto the surface of the agar plate. The plates are incubated and then examined. Essentially, the examination determines whether the bacterium has been inhibited, in which case it is considered sensitive and suitable for treatment with this antibiotic, or the bacterium has grown, in which case it is considered resistant and unsuitable for treatment (Fig. 4.4).

Choice of break points

Recommendations for breakpoints are usually based on the maximum concentration (Cmax), the Cmax of antibiotic at the site of infection. This means that breakpoints should be determined for every pathogen at every site of infection but this is rarely the case. Sometimes two breakpoints are recommended, a low and a high. Often a low breakpoint is ¼ the Cmax and the high breakpoint equals the Cmax; this value is taken after the administration of a standard dose of antibiotic. The low breakpoint applies to normal doses for general infections and the high breakpoints apply to the designation 'intermediate sensitivity' (aminoglycosides, trimethoprim, quinolones), to increased dosage or normal dosage when the antibiotic is locally concentrated (urinary tract).

Figure 4.4 Breakpoint test to determine bacterial sensitivity



The choice of breakpoints becomes particularly difficult when drug combinations are used. The use of break points infers that the Cmax and half lives of the component parts of the drug combination will be very similar; this is often a near impossibility to guarantee. The two drug combinations most often tested in the United Kingdom (though not in the United States) are the penicillin-β-lactamase inhibitor concentrations, such as amoxicillin plus clavulanic acid, and piperacillin plus tazobactam.

In many diagnostic bacteriology laboratories, disc sensitivity tests are used and these give a rapid indication of the sensitivity or resistance of infecting organisms when the greater precision of tube dilution testing is not required.

Disc sensitivity tests

Disc tests may be less accurate than dilution tests but they are rapid and convenient, and many diagnostic laboratories still use them. This technique uses filter paper discs containing a known amount of antibiotic. The discs are usually coloured or printed with code letters to allow easy identification. The discs are carefully placed on a plate that has previously been seeded with the organism to be tested. This sets up a concentration gradient. After incubation overnight, the plate is examined for zones of inhibition of growth around the discs, where the antibiotic has diffused into the medium (Fig. 4.5).

The size of the zone of inhibition depends on the sensitivity of the organism and the rate of diffusion of the antibiotic from the disc. The amount of a particular antibiotic put into a disc used for diagnostic bacteriology is such that a considerable zone of inhibition is given with a sensitive organism and no zone, or only a small zone, with a resistant organism. In other words, the disc test seeks to give a rapid prediction of the likely response of the test organism to a particular antibiotic *in vivo* (Fig. 4.5).

In diagnostic bacteriology it is important to be able to report to the clinician as rapidly as possible. It is usually possible to seed a plate with the infected pus, urine, and so on, and add discs directly to it, to have sensitivity test results available at the same time as the infecting organism is isolated. This procedure is inevitably less accurate than disc or tube tests using standard inocula of pure cultures, but if the results are interpreted with due caution they are of great value in giving early guidance to the clinician.

Etests®

In the disk sensitivity test, at the limit of the zone, the concentration of the antibiotic is equivalent to the MIC. This fact has been exploited in the Etest®. This is a plastic strip containing a predefined gradient of antibiotic concentrations. The top of the strip is graduated with concentrations of the antibiotic. Essentially the strip is placed on a lawn of bacteria in the same manner as the disk sensitivity test. After

incubation a zone of sensitivity is usually found around the end of the strip with the highest concentrations. Where the zone touches the strip, the MIC can be read directly (Fig. 4.6). This method of MIC determination does show good correlation, for most bacteria-antibiotic combinations, with the traditional disk dilution method for determining MICs (Fig. 4.6).

Figure 4.5 Diffusion of antibiotic from a paper disc

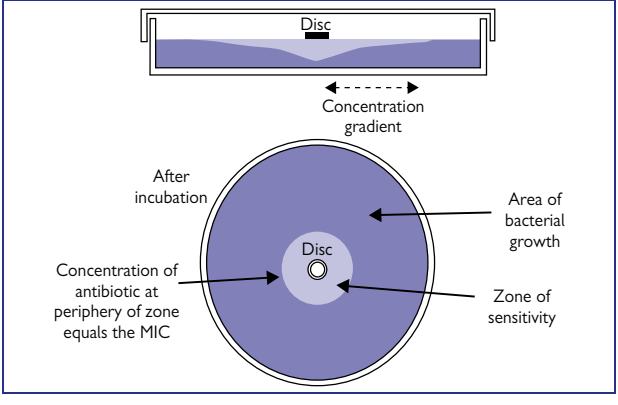
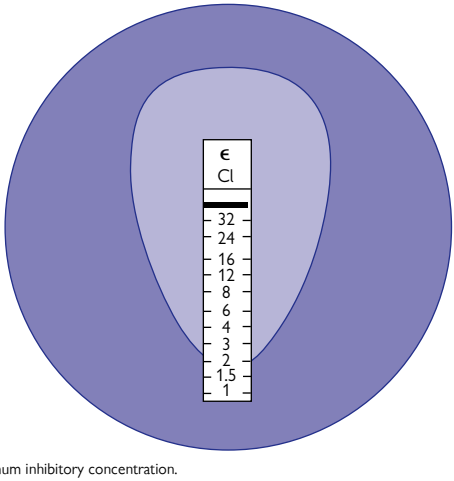


Figure 4.6 Ɛtest® for MIC determination, in this case showing the bacterium has an MIC of 2mg/L



MIC, minimum inhibitory concentration.

Automated phenotypic methods

Many laboratories have fully automated their methods for sensitivity testing; in particular, in the United Kingdom, VITEK 2 is a fully automated system that performs antibiotic susceptibility testing as well as bacterial identification. There are obvious advantages to this as it reduces the time to obtain antibiotic susceptibility results and provides more accurate bacterial identification. The method is based on a modular card system. For susceptibility tests, there is extensive data analysis using algorithms to look at a variety of parameters and test conditions to ensure accurate test results. The software can interpret and provide not just the bacterium's susceptibility profile but can also predict some resistance mechanisms; such as extended-spectrum lactamases (ESBLs).

The isolated bacterium is placed in a saline suspension to a fixed density. This is then placed in a cassette and a sample identification number is electronically entered at the bench which is transmitted to the machine by a memory chip on the cassette to give accurate tracking of the sample through to the final report. Bacterial identification and antibiotic susceptibility can be achieved concurrently. This level of automation provides rapid results which are comparable with slower, manual techniques.

β-lactamase detection

Antimicrobial susceptibility tests are not just capable of identifying existing resistances but can also measure specific resistances and the emergence of resistance problems. β-lactamase detection has been available for some time, with the use of the chromogenic cephalosporin nitrocefin. In its simplest form, nitrocefin can be incorporated in a plate and round the individual colonies; a red zone indicates β-lactamase activity.

Some of the ESBLs have been modifications of existing β-lactamases and as they have undergone several mutations to extended-spectrum activity, the intermediate steps often did not provide clinical resistance. However, these intermediate mutations can be detected so the early emergence of this resistance can be determined. Most ESBLs are class A β-lactamases and so are susceptible to inhibition with clavulanic acid; in fact they are more susceptible to inhibition than the parent enzyme. This has been incorporated into double-disk tests where disks of various cephalosporins are set up about 25mm from a disk of clavulanic acid (or co-amoxiclav). Synergy of any of the cephalosporins with the clavulanic acid, as seen by a distortion zone of sensitivity, may indicate ESBL activity. This has been refined in the Ɛtest®, where the strip becomes two strips back-to-back, a cepha-

losporin (usually ceftazidime or cefotaxime) on one side and the same cephalosporin plus clavulanic acid (4mg/L) on the other. If the MIC of the cephalosporin is reduced by eight-fold or more in the presence of clavulanic, an ESBL is to be suspected. Similarly, the class B metallo-β-lactamases capable of conferring carbapenem resistance can be predicted with a strip set up with imipenem on one side and imipenem plus ethylenediaminetetraacetic acid (EDTA) on the other. These principles can be incorporated into the automated susceptibility testing procedures, so predictions can be made alongside susceptibility results. Further modifications of principles of combinations are available for investigational use only for the detection of the transferable ampC (see chapter 6) β-lactamases. These class C β-lactamases can be identified by Ctest® with cefotetan on one strip and cefotetan plus cloxacillin on the other.

Standards and guidelines

The assignment of sensitivity and resistance by any of the phenotypic tests listed earlier is established by guideline lists of standards. These standards were originally nationally determined but more recently attempts have been made to give them international acceptance. This is an important consideration because determination of sensitivity by one set of guidelines can give a completely different set of results from the same group of bacteria tested by another. Dr Christopher Thomson and I tested *E. coli* strains for sensitivity to amoxicillin plus clavulanic acid by both the Guidelines of the British Society for Antimicrobial Chemotherapy and the French Guidelines. The former tested the amoxicillin-clavulanic acid combination in the ratio of 2:1 whereas the French Guidelines measured amoxicillin sensitivity in the presence of a fixed concentration of 2mg/L clavulanic acid. Under the BSAC guidelines, more than 98% of the strains were sensitive whereas, under the French Guideline, only 70% of the same strains were sensitive. This is probably why French laboratories were able to detect the infiltration of the emergence of the modified TEM β-lactamases capable of conferring resistance to clavulanic acid but British laboratories were far less successful.

Arguments exist, even within the same country, as to which Guidelines should be adopted. In the United States, there has been virtually universal acceptance of those of Clinical and Laboratory Standards Institute (CLSI) which was called National Committee for Clinical Laboratory Standards (NCCLS) before 2005. Many countries around the world have also adopted these Guidelines. Indeed, many VITEK 2 automated systems default to the CLSI standards. In Europe, there are many different national guidelines but pan-continental standardization is being introduced by the European Committee on Anti-microbial

Susceptibility Testing (EUCAST), which is organized by the European Society for Clinical Microbiology and Infectious Diseases (ESCMID), the European Centre for Disease Prevention and Control (ECDC) and the European national breakpoint committees. Standards have been set for breakpoints but these are not as extensive as those provided by the longer established CLSI.

Which standards are or will be best? This is a virtually impossible question to give an unbiased answer to, but the most important feature is that there should be one set of standards within a defined area, certainly within a country if national statistics are to be published. In Scotland, there were two major Guidelines used by different laboratories, CLSI and BSAC, making comparison difficult. The recommendation of the Scottish Management of Antimicrobial Resistance Action Plan (ScotMARAP) 2008 was that all laboratories within the country should test to the same set of guidelines. This allows a more significant comparison between different regions within the health area.

Identification of bacteria and molecular tests

Phenotypic identification of bacteria is often based on tests that were devised over 100 years ago. They are dependent on the expression of genes and may not be accurate but also may fail to distinguish between a pathogen and closely-related non-pathogenic strains. Phenotypic identification by automated methods, such as VITEK 2, is more accurate; however, recently a number of molecular tests have been introduced for the identification of bacteria. These are usually based on the polymerase chain reaction (PCR), which makes them very sensitive, and they are especially useful in areas where bacterial numbers are small or the organisms are unculturable. However, they are valuable for almost all identifications, particularly for surveillance of a species. Examples include the *lytA autolysin* gene of *Streptococcus pneumoniae*, the $bla_{OXA\text{-}51\text{-like}}$ β-lactamase gene of *Acinetobacter baumannii*, which distinguish these species from other species in their respective genus. More tests are continuously introduced and, no doubt, soon this information will be incorporated into automated molecular tests.

Phenotyping and genotyping

The ability to track bacteria within a hospital, a health region, or even a continent is an important adjunct to their management. Identification of closely-related bacteria at different sites is a possible indication of cross-resistance and this would trigger different measures of

control from repeated spontaneous emergence of resistant bacteria. The test has to be sensitive enough to ensure that true differences can be detected but not so sensitive that every bacterium appears completely different from all others. There is some natural evolution of a bacterium as it passes from patient to patient and this 'noise' must be taken into account when considering if one bacterium is derived from another. The use of phenotypic markers, such as serotyping, phage typing, and so on is often insufficiently sensitive as there can be considerable variation within an individual phenotype. There is much greater acceptance of molecular methods for typing bacteria, known as genotyping. Early methods were based on random PCR amplifications of different parts of the genome repetitive extragenic palindromic and random amplified polymorphic DNA (REP- and RAPD-PCR). The results were very sensitive to variations in technique. Less influenced by user practice are pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST).

PFGE relies on extraction of the bacterial DNA and its digestion with restriction enzymes that cut rarely. The resultant fragments of DNA, often similar in size, are separated through a gel by alternating the current from one side to the other to tease apart these fragments. Originally, the Tenover criteria classified bacteria as the same if they had less than three band differences. However, it is now quantified and the image of the pattern of bands produced can be captured and interpreted by a computer algorithm such as Bionumerics. From the banding patterns of different bacteria, the algorithm can produce a dendrogram which indicates the degree of similarity. Some bacteria, with genomes that readily swap information (Campylobacter, Helicobacter) are not suitable for typing by PFGE.

MLST identifies usually seven or so 'housekeeping' genes. These are genes that are considered to be essential for the strain and will not evolve quickly. PCR amplification of each of the genes and subsequent sequencing of the products means that the sequence of the seven genes can be compared. Differences in gene sequence are scored and associated with individual sequence types. The advantage of this information is that it is digital and can be transferred from centre to centre.

Patient management, surveillance, and epidemiology

The main stakeholder in the outcome of the sensitivity test is the individual patient; the primary purpose for the test is for individual patient management. Bearing this in mind, it is often more important to obtain a quick and accurate antibiotic sensitivity profile than precise identification of the organism. Thus, in the past, many laborato-

ries have only needed to classify bacteria by vague criteria, such as 'coliforms' to describe bacteria that may belong to different genera of the Enterobacteriaceae but are probably *E. coli*.

However, for surveillance, this definition is insufficient because not only do different genera of bacteria have different resistance profiles but also different species with the same genus can have completely different resistance profiles. Some believe that even within a species, there should be sub-categories depending on the resistance status, for example, the methicillin-sensitive *Staphylococcus aureus* strains are different from MRSA. To compare the sensitivities within a species or species sub-category, accurate speciation of the bacterium is essential to provide valid results. Phenotypic tests for species identification are not as accurate as molecular tests and the most meticulous surveillance is performed on bacteria that have been rigorously identified by the presence of signature molecular markers as described above.

There is an inherent flaw in surveillance, the population of bacteria in specimens sent to diagnostic laboratories, from which the surveillance data are taken, is biased. Much therapy, particularly for community infections, is empiric and often no specimen is sent to the diagnostic laboratory unless therapy fails. If it is assumed that the majority of bacteria that respond to empiric treatment are sensitive, then these are missing in the surveillance results. The proportion of these missing 'sensitive' strains will vary depending on the need for a diagnostic result; it will be small in many hospital-acquired infections but could be considerable in some community-acquired infections. Thus resistance levels are probably almost always over-estimated. This is not to suggest that surveillance is worthless for it has certainly identified the emergence and spread of new resistant bacteria, merely to identify that it is not necessarily an accurate representation of the problem.

Epidemiology would require the whole population to be tested. Thus all specimens from a particular infection site should be taken, before therapy is commenced, and then sent to the diagnostic laboratory for testing. This cannot be done for all specimens but epidemiology usually extrapolates from relatively small numbers and it would provide a more accurate view of the denominator. However, it is expensive and would require the rigorous testing of specimens that are not needed for patient management.

Chapter 5

Genetics of antibiotic resistance

Key points

- Mutations leading to resistance and the concept of the mutant prevention concentration (MPC).
- Bacterial plasmids as the transporters of resistance genes.
- The role of transposons and integrons in bringing the correct combination of resistance genes to a bacterium undergoing clinical treatment.
- The plasmid–bacterium relationship as a marriage of convenience.

Bacteria may be naturally resistant to particular antibiotics, for example, the enterobacteria are not effectively inhibited by standard concentrations of benzyl-penicillin. However, this inherent resistance has not traditionally been of great concern because the choice of antibiotic has taken it into account. Of more immediate importance clinically is the acquisition of resistance by sensitive pathogens. The mechanisms by which drug resistance may be acquired are

1. Chromosomal mutation and selection
2. Transfer of plasmid-borne resistance, usually by conjugation
3. Transposition
4. Integrons

Mutation to drug resistance

Drug resistance may arise within a culture by the selection of spontaneously occurring mutants that are resistant to increased concentrations of a particular drug. This may occur in a single large step, with the cell acquiring resistance to high levels of the drug in a single mutation (Fig. 5.1), or it may require multiple small steps with sequential selection, giving a gradual build-up in the level of resistance until it becomes high enough to be of clinical significance, such as resistance to the fluoroquinolones. Resistance to streptomycin or erythromycin may arise in a single step; resistance to tetracycline is by multiple small steps.

The emergence of mutations was the basis for the adage that a course of antibiotics should be completed. However, this was largely based on experience with the time-dependent bactericidal antibiotics such as the penicillins. It has been shown that mutations can be prevented, especially with the fluoroquinolones, as long as a certain concentration of antibiotic is reached at the site of infection. This is known as the Mutant Prevention Concentration (MPC). In Figure 5.2, the concentrations of two antibiotics (A and B) are shown over a period of time. The maximum concentration (Cmax) of antibiotic A exceeds the MPC so mutants would not be expected whereas the Cmax of antibiotic B does not and so mutations may be expected.

Figure 5.1 Chromosome mutation—selection of a resistant variant

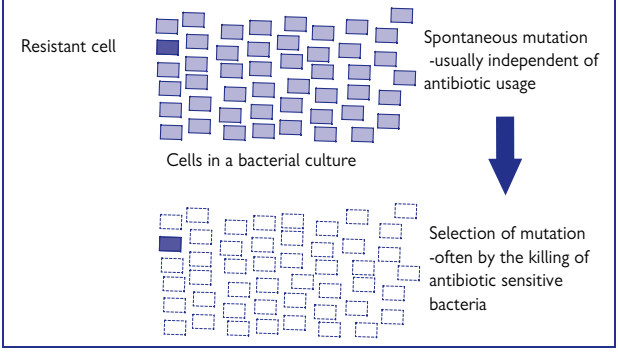
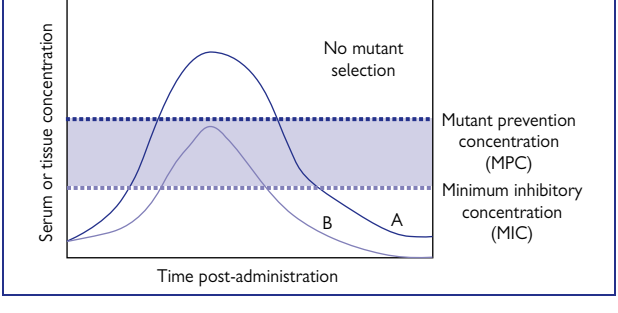


Figure 5.2 Mutant prevention concentration



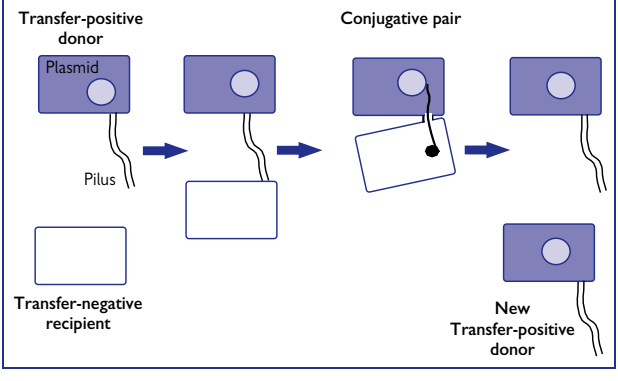
Transfer of plasmid-borne antibiotic resistance

Plasmid-mediated resistance generally causes much greater concern. In the Gram-negative rods, conjugation allows spread of R plasmids (plasmids bearing drug-resistance genes) between cells of a wide range of different strains and species of Gram-negative bacilli. *In vitro*, transfer occurs fairly readily among the Enterobacteriaceae—*Escherichia coli*, *Klebsiella* spp, *Proteus* spp, *Salmonella* spp, *Shigella* spp. These Enterobacteriaceae plasmids may also be transferred to less closely related genera *in vitro*, for example, *Pseudomonas* spp, *Vibrio* spp, *Yersinia* spp. R plasmid resistance is common in *Pseudomonas aeruginosa* and there is some exchange of R plasmids between *Pseudomonas* spp and the enterobacteria. Most clinically important drug resistance in these bacteria is found to be plasmid borne and transferred by conjugation (Fig. 5.3).

Detection of plasmid transfer

Plasmid transfer is usually an infrequent event and thus detection of the transconjugant requires positive selection. This is often achieved by the use of recipient bacteria that are resistant to antibiotics for which there has never been plasmid transfer detected, that is, rifampicin, nalidixic acid, etc. The donor and the recipient bacteria are mixed together for a fixed time period, often 1 hr. At the end of the conjugation, the mixture is placed onto selective agar plates containing two

Figure 5.3 Conjugation in Gram-negative bacteria



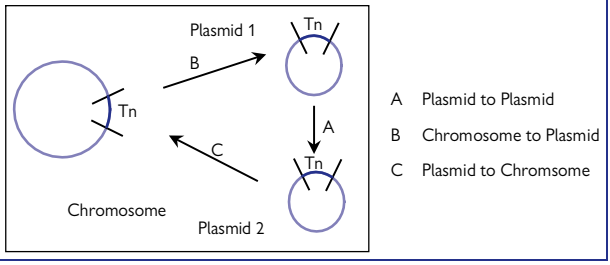
antibiotics; the first is the antibiotic for the transferable resistance gene and the second is the antibiotic to which the recipient is resistant. Controls are set up with the donor and recipient on the same selective media. The selective plates are incubated and transconjugants identified by growth on the selective plates inoculated with the conjugation mixtures provided that there is no growth on the control plates.

Transposition

Plasmids, almost identical to those now known to confer resistance, were present in bacterial pathogens long before the start of the antibiotic era. These plasmids must have subsequently acquired the genes that we now associate with resistance. The obvious chromosomal origin of several plasmid-mediated resistance genes and the widespread occurrence of hundreds of different resistance genes within a variety of bacterial genera suggested that mechanisms must exist which enable the mobilization of resistance genes from their original location to new genetic locations within clinical bacteria. The discovery of insertion sequences (IS) and subsequently transposons provides one explanation whereby genes could be 'picked up' and moved independently to new sites. These new sites can be within the same or an alternative replicon in the original cell as shown in Figure 5.4.

Long before the antibiotic era, bacterial genomes possessed short DNA sequences that were able to insert a copy of themselves from one part of the genome, into another section or on to other replicons such as plasmids. If two of these IS are inserted in close proximity, then the internal section of DNA between them could also be transferred. If the internal section carried a resistance gene, then the whole section including the IS elements becomes a class I

Figure 5.4 Transposition between plasmids and the bacterial chromosome—migration of a cluster of resistance genes



transposon. This encodes a transposon which can facilitate the ability to migrate from one DNA molecule (the donor) to another (the recipient) but are themselves unable to replicate independently. A large number of transposons and insertion sequence elements have been described in both Gram-negative and Gram-positive bacteria and the genetic basis of transposition has been elucidated for several of these.

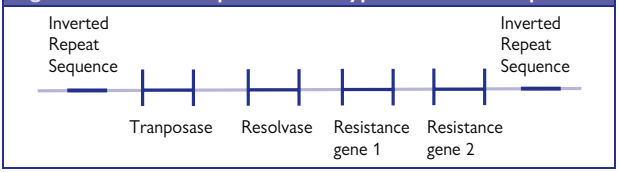
Not all transposons require IS. The common class II transposons have inverted repeat sequences at the ends of the transposon. The product of the transposon gene acts on the repeat sequences to form a co-integrate physically linking the original replicon with the target replicon. A second enzyme, a resolvase, cleaves this co-integrate so both the original replicon and the target replicon have a copy of the transposon and the resistance genes(s) (Fig. 5.5).

IS can also carry promoter sequences so that when they insert upstream of a resistance gene, that previously has not been expressed, it is able to switch on the gene.

Integrons

A further type of genetic element has been described more recently in the plasmids and transposons of Gram-negative bacteria. This element, called an integron, is characterized by conserved 5' and 3' ends which flank a variable central DNA segment. The 5' conserved end contains a functional gene coding an enzyme, integrase, which mediates site-specific integration of external DNA, often containing resistance genes, into the integron. The integron can reside within a transposon as shown in Figure 5.6. Essentially, the integron extracts segments of DNA from other replicons, so a transposon, which possesses an integron can vary the antibiotic resistance genes that it carries depending on which replicons it comes into contact. In the example (Fig. 5.6), the transposon on plasmid 2 possesses an integron. This integron produces an enzyme (integrase) which interacts with plasmid 1 and randomly extracts DNA from it. If it extracts a complete resistance gene, the transposon will now confer resistance to that antibiotic. If the host bacterium is challenged with that antibiotic, then the integron will provide a selective advantage on the cell.

Figure 5.5 Gene composition of a typical class II transposon



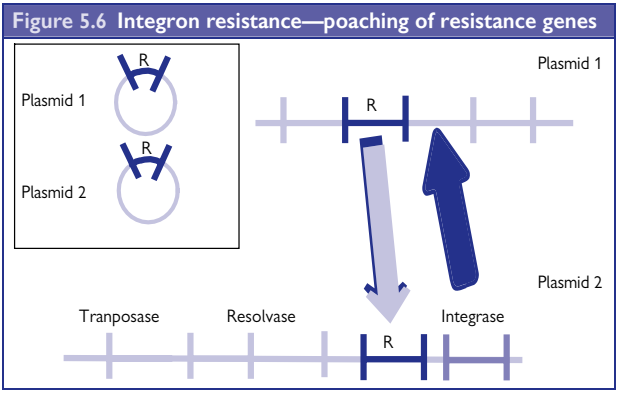


Figure 5.6 Integron resistance—poaching of resistance genes

Migration of resistance genes

The resistance genes that we find in clinical bacteria have not usually emerged in real-time. Rather they have been present in some bacterial species, often not of clinical importance, where they have evolved to overcome a local antibiotic threat. The massive antibiotic selective pressure that we have applied to clinical bacteria ensures that any migration of these resistance genes into clinical bacteria is an immediate advantage and retained. The ability to sequence the DNA of resistance genes and the flanking sequences of DNA surrounding them and their comparison with the massive DNA sequence databases has enabled the identification of the source of the resistance genes. The escape of the resistance gene from the original strain may have occurred on many occasions; for instance, the genes responsible for the CTX-M β-lactamases (blaCTX-M) have escaped from bacteria of the genus *Kluyvera* on many occasions and the methicillin resistance gene cassette (*SCCmec*) has independently entered bacteria that we now call methicillin-resistant *Staphylococcus aureus* (MRSA) on a number of occasions.

Plasmid–bacterium relationship

Plasmids were originally thought to be very promiscuous, rapidly transferring between bacteria. This is not often the case. In many cases, the plasmid migrates through the bacterial population until it enters a bacterial cell with which it has apparent empathy. This was seen many years ago with the typhoid epidemic in southern Asia, where the causative *Salmonella enterica* Serovar Typhi strain had the same *inc*HI1 resistance plasmid wherever it was isolated. More

recently, there has been a huge expansion in the isolations of *E. coli* strains harbouring the CTX-M-15 ESBLs. This expansion is explained by the spread of a cluster of *E. coli* strains harbouring *inc*FII plasmids which have a gene (*aac[6']-Ib-cr*) that can confer aminoglycoside and fluoroquinolone resistance. The close association of the plasmid with the individual bacterium is probably maintained by the presence of 'so-called' addiction genes.

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Chapter 6

Mechanisms of antibiotic resistance

Key points

- How bacteria prevent the antibiotic reaching its target.
- The rise in importance of the efflux pumps particularly in the early stages of resistance development.
- Antibiotic destruction and modification, the most successful mechanisms of resistance.
- The dominance of the β-lactamases.
- The ability of the bacterium to change the target of the antibiotic so it no longer binds.

The main mechanisms of resistance:

1. Impermeability	Where the antibiotic fails to get into the bacterial cell.
2. Efflux pumps	Where the antibiotic is removed from the bacterial cell faster then it can enter.
3. Destruction	Where the antibiotic is destroyed so that an active drug is no longer intact and thus cannot attack its normal target.
4. Modification	Where the antibiotic is modified so that an active drug is no longer intact and thus cannot attack its normal target.
5. Alteration of target	When the target of the antibiotic is modified so that the drug can no longer bind to it.
6. Additional target	The production of a second target, which is usually less sensitive to binding by the antibiotic and thus provides a by-pass to the original inhibition.
7. Hyper-production of target	When the target is produced in larger quantities than normal, thus the excess target molecules mop-up the available antibiotic. This mechanism is often used in conjunction with one of the others.

If the resistance has arisen from chromosomal mutation, then all of the resistance mechanisms could, in theory, be employed. On the

other hand, if the resistance is determined by plasmid, transposon or integron genes, the options are more limited because the resistance mechanism usually has to be a single gene product and it has to be dominant within the cell (Table 6.1).

Impermeability

This is quite a common mechanism of inherent resistance. The antibiotic just cannot penetrate the cell sufficiently to attack the target. *Pseudomonas aeruginosa* has very few porins on its outer membrane and thus antibiotics generally have difficulty in penetrating the cell. On the other hand, it is quite a rare mechanism of chromosomal mutational resistance because the changes required to provide impermeability are often very energy dependent and thus put the cell at a significant disadvantage.

Chromosomal mutational resistance to tetracycline is provided by impermeability. This large antibiotic is often actively transported in to the bacterial cell. The mutation may simply be an inactivation of the active transport mechanism.

Efflux pumps

Plasmid-mediated resistance to tetracycline is mediated by an impermeability-like mechanism. However, the plasmid does not interfere with the active transport of the antibiotic into the cell. Instead, it encodes a protein that expels the antibiotic out of the cell faster than it can get in. The dynamic equilibrium set up ensures that there

Table 6.1 Common resistance mechanisms

Resistance mechanism	Chromosome		Plasmids, transposons & integrons	
	Found	Examples	Found	Examples
Impermeability	✓	Tetracycline	✓	Tetracycline
Destruction	✓	β-lactams	✓	β-lactams
Modification	✓	Aminoglycosides	✓	Aminoglycosides Chloramphenicol
Alteration of target	✓	Aminoglycosides	✓	Macrolides
Additional target	✓	Trimethoprim	✓	Trimethoprim
Hyperproduction of target	✓	Trimethoprim	✓	Trimethoprim

is insufficient tetracycline within the cell to interfere with bacterial protein synthesis. Like the mutational chromosomal mechanism, the plasmid efflux pump mechanism only increases the resistance of the cell by 10–100-fold and is often not efficient.

Efflux pumps have increasingly become implicated in antibiotic resistance, encoded not just by plasmids but also by the chromosome. Their contribution is difficult to measure because they are often present but may not be functioning. They are part of a de-toxification system in bacteria and, although they can confer resistance to a wide range of antibiotics, they usually cannot tolerate high concentrations and may be overwhelmed. Their contribution is probably as a first defence, keeping some of the bacterial cells alive until a more potent resistance mechanism is in place.

Destruction

The only example of this mechanism is that to β-lactam drugs (penicillins, cephalosporins, and carbapenems); however, it is the most successful resistance mechanism of all. It is manifested by the production of β-lactamases that can hydrolyse and inactivate various members of this family of antibiotics. The bond that all β-lactamases hydrolyse is shown by the arrows in Figure 6.1. The same position can be identified in most of the β-lactam antibiotic groups by the arrows shown in Figure 2.1.

The β-lactamase hydrolyses the carbon-nitrogen bond of the β-lactam ring. The integrity of this ring is crucial to the activity of the antibiotic. In Gram-positive bacteria, the β-lactamase is produced within the cytoplasm of the cell and is exported through the cell membrane into the surrounding medium. It provides a drug-free blanket around the cell. It also provides protection for other microorganisms in close proximity. In Gram-negative bacteria, the antibiotic has more difficulty in penetrating. The β-lactamase is still produced in the cytoplasm; however,

Figure 6.1 Inactivation of amoxicillin with a β-lactamase

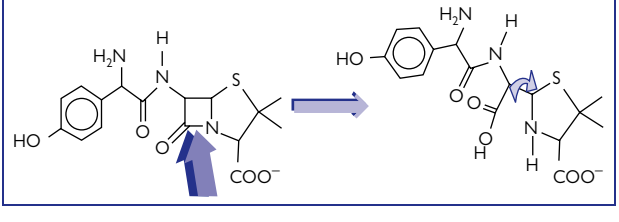
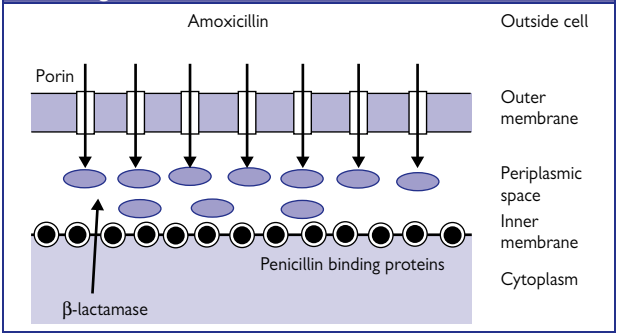


Figure 6.2 Interaction of amoxicillin with β-lactamases in Gram-negative bacteria



most of it is exported only as far as the periplasmic space, between the two membranes. It is here that the β-lactamase intercepts the incoming β-lactam drug and destroys it. It is a more directed and more efficient mechanism than that found in Gram-positive bacteria.

It has recently been suggested that there are over 750 β-lactamases found in clinical bacteria. They have conveniently been classified by their molecular structure into four groups. There is no homology between each group but significant homology exists within a group. In all β-lactamases, there is one main active site component; this can either be a serine residue that provides the catalytic basis for the hydrolysis of penicillins and cephalosporins (Classes A, C, and D) or a metal ion that provides the catalytic basis for hydrolysis, particularly for the carbapenems (Class B).

The class A enzymes have been studied in most detail. They comprise the chromosomal β-lactamases of Gram-positive bacteria and the most common plasmid-encoded β-lactamases. In any study of plasmid-encoded, β-lactamase-conferred resistance performed anywhere in the world, at least 75% of all the enzymes will be the class A β-lactamase *TEM-1*. This enzyme is highly efficient at binding and hydrolizing amoxicillin conferring high-level resistance (MIC >1,000mg/L). β-lactamase inhibitors were developed specifically to overcome the effects of this enzyme. However, the β-lactamase has been able to mutate to prevent the binding of the inhibitor. The cephalosporins were also exploited to overcome the effect of the *TEM-1* β-lactamase. Unfortunately, the TEM molecule has been able to mutate to become an extended-spectrum β-lactamase (ESBL), so that it can bind and hydrolyse the most sophisticated cephalosporins. There are now about 150 of these ESBL enzymes.

The *SHV ESBLs*, derived from an enzyme closely related to *TEM-1*, *SHV*-1, soon replaced the *TEM ESBLs*. This is probably because the former were more effective against slow-penetrating cephalosporins, such as ceftazidime, and the SHV enzymes are more effective against the faster penetrating cephalosporins such as ceftriaxone and cefotaxime. There are about 80 *SHV ESBLs*. The *SHV-2* and, to some extent, *SHV-5 ESBLs* were very prevalent but now are beginning to be replaced by another group of class A ESBLs, the *CTX-M* group. Unlike the previous two, these ESBLs did not derive from mutation of an established plasmid β-lactamase gene but were imported from various species of the *Kluyvera* genus. There are nearly 100 of these and in the United Kingdom, the most prevalent is CTX-M-15.

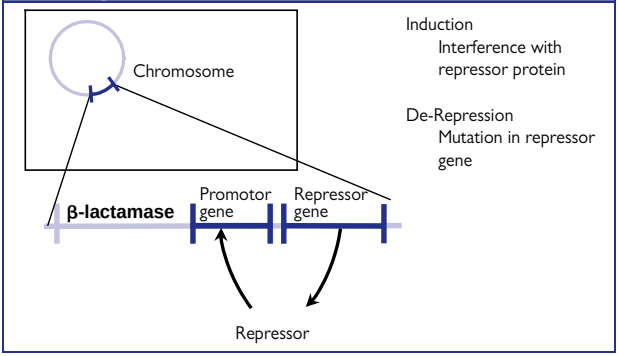
The class B enzymes are metallo-β-lactamases. *In vitro*, they are particularly active against the carbapenems, such as imipenem and meropenem. Although they are usually encoded by the bacterial chromosome, they have to be induced to produce sufficient enzyme levels to confer resistance. Even elevated levels may be insufficient to confer resistance and this type of β-lactamase has to operate in concert with another resistance mechanism, such as reduced permeability. An increasing number of the class B β-lactamases have been found to be plasmid-mediated, particularly the *IMP* and *VIM* groups, and these are not inducible but are constitutively produced.

The class C β-lactamases are predominantly the chromosomally-encoded β-lactamases of Gram-negative rods. The production of these β-lactamases also has to be induced to produce sufficient enzyme (Fig 6.3). Induction is not a very efficient long-term mechanism and the host bacteria are more successful if the repression system is disabled completely. Thus de-repression occurs with a mutation in the repressor gene so that no repressor protein is produced. This is a stable change and can only be reversed with a back mutation.

There are a few class C β-lactamase genes that have migrated onto plasmids, that is, *BIL-1* and other members of the *CMY-2* group. When this occurs, only the β-lactamase gene is present, there is no repression system so the gene is expressed constitutively. These are known as ampC β-lactamases. They are difficult to detect because they are usually present in strains that have their own, similar but less effective, class C β-lactamase.

The class D β-lactamases were originally found exclusively plasmid-encoded and predominantly acted against penicillins. However, they have been found to be the part of the chromosomal β-lactamase complement of *Acinetobacter* spp. The *OXA-51*-like β-lactamases are the chromosomal enzymes of *Acinetobacter baumannii*. In *Acinetobacter* spp the class D enzymes can confer resistance to carbapenems (see chapter 7).

Figure 6.3 Chromosomal class C β-lactamase production in Gram-negative bacteria



Modification

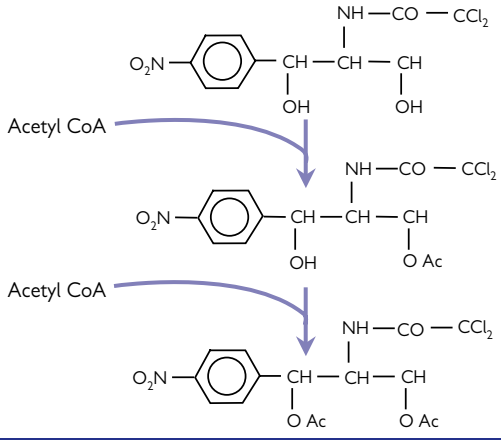
The consequence of modification on an antibiotic has the same effect as destruction, an inactive drug is created and it can no longer inhibit its target. Essentially the plasmids encode a gene that adds a functional group to the antibiotic. The enzyme can have one of three actions:

1. Acetyl transferase — The enzyme adds an acetyl group.
2. Adenyl-tranferase — The enzyme adds an adenyl group.
3. Phospho-transferase — The enzyme adds an phosphate group.

The main antibiotics that are modified in this manner are chloramphenicol and the aminoglycosides. Chloramphenicol can only have acetyl groups added and this is obtained from acetyl-CoA (Fig 6.4). Modifying enzymes are produced in the cytoplasm but often act as the antibiotic enters the cell. In the case of the aminoglycosides, the antibiotics are actively transported into the cell. At their point of entry, the molecules are modified. Only a small proportion of the incoming antibiotic is modified, often approximately 1%, suggesting that the mechanism of resistance is manifested by the modified antibiotic blocking the transport system into the cell rather than its inability to bind to the 30S ribosomal subunit.

They produce moderately high levels of resistance on their host bacterium (MIC ~ 100–500 mg/L).

Figure 6.4 Action of chloramphenicol acetyl-transferase



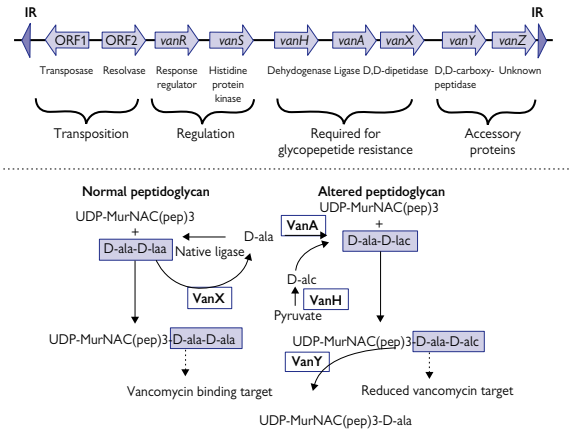
Alteration of target

This mechanism is the most common form from chromosomal mutation, such as aminoglycoside resistance. The target protein on the 30S ribosomal subunit alters so that it cannot bind the aminoglycoside. This produces very high levels of resistance (MIC > 2,000mg/L).

One major example of the alteration of a target are the fluoroquinolone-resistant mutants. In Gram-negative bacteria, the primary target of the fluoroquinolones, DNA gyrase, is composed of two pairs of subunits, A and B. Changes in the A subunit can be particularly effective in preventing fluoroquinolones binding and, in combination with secondary mutations in the decatenation enzyme topoisomerase IV, can confer high levels of resistance on the host bacterium (MIC > 64mg/L).

It used to be thought that alteration of the target was a mechanism used only for chromosomally-encoded resistance but it is the mechanism employed for the plasmid-encoded resistance to the macrolides, such as erythromycin, where the 23S ribosomal RNA is methylated preventing the binding of the antibiotic. However, vancomycin resistance in enterococci is the most sophisticated of the target alterations. The target of vancomycin is two D-alanine residues at the end of the pentapeptide in the peptidoglycan precursor

Figure 6.5 The *vanA* operon in *Enterococcus* species conferring resistance to glycopeptides including vancomycin and teicoplanin by producing an alternative biosynthetic pathway for cell wall peptidoglycan

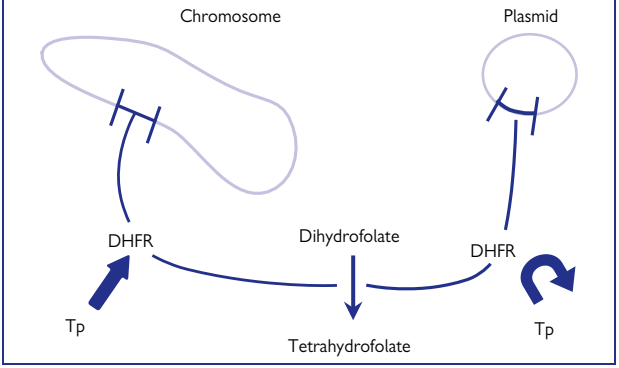


used in the synthesis of the bacterial cell wall. The resistance operon is probably derived from chromosomes of species such as *Streptomyces toyacaensis* and *Amycolatopsis orientalis*; essentially there are three genes. In the *vanA* operon, the product of the first (*vanX*) reverses the ligation of two D-alanine residues to form D-alanine-D-alanine. The second (*vanH*) gene product dehydrogenates pyruvate to D-lactate and the product of the third (*vanA*) ligates the D-lactate to the free D-alanine to give D-alanine-D-lactate, which is incorporated into the peptidoglycan precursor. The substitution of D-alanine-D-lactate for D-alanyl-D-alanine, provides a much weaker binding target for vancomycin (and teicoplanin), thus conferring resistance (Fig. 6.5).

Additional target

This is usually a plasmid-mediated mechanism of resistance. The antibiotic binds to its normal target but the plasmid produces an additional target, which is less susceptible to the antibiotic. This mechanism of resistance can only work if the quantity required of the product of the inhibited step is low. This is the case for the co-factor tetrahydrofolate as trimethoprim selectively inhibits bacterial dihydrofolate reductase (Fig. 6.6).

Figure 6.6 By-pass mechanism of plasmid-encoded trimethoprim resistance—production of an additional dihydrofolate reductase (DHFR)



The plasmid produces an additional dihydrofolate reductase that cannot readily bind the drug but can still reduce dihydrofolate to tetrahydrofolate. Often the plasmid-encoded enzyme binds the drug around 10,000-fold less effectively than the chromosomal enzyme and there is a corresponding increase in resistance on the host bacterium (MIC > 1,000 mg/L). Similar mechanisms are seen with sulphonamide resistance and the plasmid production of altered dihydropteroate synthetase.

Hyper-production of target

Chromosomal dihydrofolate reductase can be hyperproduced 100-fold so that it is able to bind many trimethoprim molecules. Even if 99% of the produced dihydrofolate reductase molecules are inhibited by trimethoprim, there are enough enzyme molecules to reduce dihydrofolate to provide sufficient tetrahydrofolate for the cell's needs. It is a highly expensive mechanism of resistance in energy so host cell has a selective disadvantage once the antibiotic has been removed.

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Chapter 7

Multi-drug resistant (MDR) bacteria and healthcare-acquired infections

Key points

- The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) and the recent appearance of vancomycin resistance.
- The extended-spectrum β-lactamases (ESBLs) in the Enterobacteriaceae and the fall of the cephalosporins.
- Carbapenem resistance in Gram-negative bacteria, a disaster currently occurring.
- *Clostridium difficile*, a bacterium that has not developed resistance but which antibiotics have selected.
- Why multi-drug resistant (MDR) bacteria spread clonally.

Currently, the major problem with resistance is the emergence and spread of MDR bacteria such as MRSA, vancomycin-resistant enterococci (VRE), carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, penicillin-resistant *Streptococcus pneumoniae* and, probably soon, carbapenem-resistant *Klebsiella pneumoniae*. In this group, we should also include the anaerobe *C. difficile*, not because it has developed resistance during antibiotic exposure but rather because it has been selected by antibiotics that are active against aerobic bacteria. Most of these bacteria are causes of healthcare-acquired infections, normally in hospital, and mostly they are represented by bacteria from a relatively small number of genetic clusters, often referred to as clones.

Staphylococcus aureus and MRSA

The organism that we refer to as MRSA is not the same as the MRSA which emerged in the late 1970s, following the introduction of methicillin to deal with β-lactamase producing *S. aureus*. Then the bacteria were treated with aminoglycosides and rapidly decreased. The bacteria that emerged resistant to methicillin and aminoglycosides

were the harbingers of MRSA we see today. They are multi-resistant bacteria, rather than just methicillin-resistant.

The sequencing of 14 complete *S. aureus* genomes has shown that the diversity across the species is approximately 28%; however, this diversity is considerably reduced in the main epidemic MRSA clones (EMRSA-15 and EMRSA-16 in the United Kingdom), which show little genomic variation within the clone and considerably less that 1% diversity between then. With the increase of MRSA to 40% of all hospital *S. aureus* isolations within 5 yr up until 2002 in the United Kingdom, it appears that the majority of the species has been suppressed by antibiotic therapy and that a few, fitter bacteria have been selected by continuous antibiotic selection. These bacteria have been able to spread easily, colonize healthcare workers and survive in the environment when other bacteria would have been eradicated.

Treatment has relied upon the glycopeptides, particularly vancomycin and teicoplanin, but as new anti–Gram-positive antibiotics, such as linezolid and daptomycin, came on stream, these have been used as well. MRSA has become adept at developing resistance to these drugs. Furthermore, MRSA can become insusceptible to the glycopeptides as well. Firstly, in the mid-1990s, intermediate resistant strains (VISA or GISA for vancomycin-intermediate or glycopeptide-intermediate *Staphylococcus aureus* respectively) emerged, initially in Japan. The cell wall production was increased and the intermediate strains were able to raise the MIC to about 8mg/L. These have since been found in Europe and North America. In the United States, an even more disturbing development occurred when, in 2002, the vancomycin resistance operon from VRE (Fig. 6.5) migrated to MRSA on a 60-kb plasmid to give vancomycin-resistant *Staphylococcus aureus* (VRSA). The number of VRSA strains is still, fortunately, very small. It appears that the 60-kb plasmid carrying the vancomycin resistance operon from enterococci was very unstable in MRSA and only if the operon can transpose onto an indigenous MRSA plasmid, before the host plasmid is destroyed, does the operon have any chance of survival. So currently, we appear to be protected from the emergence of widespread VRSA emergence by an inability of the carrier plasmid to survive in *S. aureus*. This suggests that it may just be a matter of time until VRSA strains become established, which may render these MRSA virtually impossible to treat.

MRSA is perceived mainly as a nosocomial infection but it is found in all healthcare centres, including convalescent homes and sheltered housing for the elderly. Within a health district, the epidemic strains can also spread round these facilities. However, a great concern is the emergence of community-acquired MRSA (cMRSA). These are not the same as their hospital counterparts and their genomes are not closely related. The cMRSA can carry the *lukF-PV* and *lukS-PV* genes that encode the Panton-Valentine (PV) leukocidin components. This

leukocidin has a powerful toxic effect on human white blood cells and is strongly associated with severe forms of pneumonia (necrotic pneumonia) caused by community-acquired *S. aureus* strains.

The clonal nature of nosocomial MRSA suggests that prevention of its spread, with infection control measures, are at least as important as appropriate antibiotic therapy. In the Netherlands, they managed to prevent MRSA becoming established by screening all patients coming into hospital. This proved extremely effective and kept the infection rate below 1%. However, at the time of writing, no country has yet managed to reduce the burden of MRSA to that level after it has already become established within the hospitals.

VRE

Enterococcus faecalis and *Enterococcus faecium* are commensal bacteria in the human gut and were not considered to be particularly pathogenic. However, as patients were immunosuppressed for advanced medical procedures, enterococci were increasingly found causing infections in the intensive care unit (ICU). Soon these bacteria became resistant to most antibiotics used to treat them, including the glycopeptides, which were considered to be the final defence against these bacteria. Interestingly the most prevalent species of sensitive enterococci is *E. faecalis* but among vancomycin-resistant strains, *E. faecium* predominates. Vancomycin resistance is conferred by a collection of closely related operons probably derived from *Streptomyces toyacaensis* and *Amycolatopsis orientalis*. The most widely distributed are conferred by the *van*A (see chapter 6) and vanB operons but operons *van*C–*van*G have been also found a variety of species of *Enterococcus* and the soil organism *Paenibacillus*. Most of the operons function after induction. The *van*A operon can be induced by both vancomycin and teicoplanin whereas the vanB operon can only be induced by vancomycin but not teicoplanin (though if already induced it can confer resistance to teicoplanin).

VRE tend to be clonal, spreading around through a hospital population, thus infection control is important in their control. Ten years ago, these bacteria were almost impossible to treat as they had become resistant to most antibiotics; however, *E. faecium* is sensitive to quinupristin plus dalfopristin, whereas *E. faecalis* is resistant. Similarly tigecycline has been used to treat these bacteria. They were initially sensitive to linezolid as well but resistance has since emerged.

Carbapenem-resistant *P. aeruginosa*

P. aeruginosa is inherently resistant to many antibiotics but was susceptible to the cephalosporin ceftazidime and fluoroquinolones such

as ciprofloxacin. Resistance to these has risen over the years. Approximately 3%–5% of *P. aeruginosa* strains appear inherently resistant to carbapenems. This is probably due to hyperproduction of the chromosomal class C β-lactamase though some have acquired the IMP and VIM class B transferable β-lactamases. Resistance to colistin is found but generally the bacteria are sensitive.

P. aeruginosa is a particular problem in patients with cystic fibrosis as they often succumb to lung infections caused by it. These bacteria are often MDR, probably because the condition is chronic and frequent repetitive dosing is required. Furthermore, the environment is very specialized and individual clones of *P. aeruginosa* have been shown not only to spread from patient to patient but also to migrate over large areas. On the other hand, *P. aeruginosa* can be an opportunistic pathogen in the hospital infecting a myriad of sites if the patient is compromised in some manner. In this case, although the bacteria may become resistant, often the strains are not related to one another and have probably derived from diverse sources. The reason for this is not clear as many nosocomial MDR pathogens are very clonal, but it may be because *P. aeruginosa* was already inherently resistant to most drugs and the continuous use of antibiotics did not change the distribution of bacteria whereas, in other species, the more sensitive strains had been eradicated.

Carbapenem-resistant *A. baumannii*

A. baumannii is a product of the antibiotic era. Although species of the *Acinetobacter* spp have been identified for many decades, the rise of the species *A. baumannii* occurred only after extensive use of antibiotics. Originally perceived as a pulmonary pathogen, it is also a potent cause of bacteraemia. Already resistant to 2nd generation cephalosporins, the introduction of the 3rd generation cephalosporins was quickly followed by resistance because of the inherent chromosomal class C β-lactamase. The challenge by the fluoroquinolones rendered a species that was originally ciprofloxacin sensitive, completely resistant.

So the drugs of choice became the carbapenems. However, carbapenem resistance has emerged and, in some cases, spread significantly. The mechanism of carbapenem resistance was different from all other bacteria including *P. aeruginosa*; the bacteria encoded a class D β-lactamase capable of hydrolyzing carbapenems. The first of these, OXA-23, was found in a isolate in Edinburgh Royal Infirmary over 20 yr ago and they have now been found all over the world. Currently in the south of England, there is an epidemic of two clones of *A. baumannii* carrying the OXA-23 β-lactamase. So, the progression to MDR in this organism, is accompanied by the rise of individual

clones. There are about five other OXA β-lactamases, related to a lesser or greater extent to OXA-23, that have been shown to confer carbapenem resistance; the most important of these is OXA-58, largely found outside Europe.

There is a further point of concern. Twelve years ago a strain from Argentina was found to have a different OXA β-lactamase (called OXA-51). It was subsequently found that all *A. baumannii* strains carry a closely related β-lactamase (collectively called OXA-51-like). There are over 50 of these OXA-51-like β-lactamases, some of which may express carbapenem hydrolysing activity. However, their genes are not normally expressed and thus the bacterium is sensitive. It has subsequently been shown by Poirel and Nordmann in Paris that if the insertion sequence IS*Aba*1 is present upstream of the *blaOXA-51*-like gene, the carbapenemase will be expressed because the IS*Aba*1 element carries a gene promoter. Indeed, the same element is believed to promote the expression of the chromosomal class C β-lactamase to give cephalosporin resistance. Insertion sequences such as IS*Aba*1 may be pivotal in providing *A. baumannii* with its MDR phenotype.

ESBLs and carbapenem resistance in *K. pneumoniae* and *E. coli*

Extended-spectrum β-lactamases (ESBLs) emerged in nosocomial *Klebsiella* spp in the early 1980s. These were exclusively modifications of the TEM and SHV β-lactamases. The TEM ESBLs, usually with a pivotal mutation at amino acid 164, seemed to emerge after exposure to slow-penetrating cephalosporins such as ceftazidime. The SHV ESBLs, which soon became more prevalent, were more successful against the faster penetrating cephalosporins, such as cefotaxime, presumably because of the pivotal mutation at amino acid 238. Both ESBLs from the TEM and SHV groups migrated into *Escherichia coli*, but it was in nosocomial *Klebsiella* spp they comprised the biggest problem. The SHV-2 ESBL often predominated and, in some hospitals, the plasmid carrying this ESBL migrated into a few key clones, which then spread. Huge number of patients became infected and this severely compromised the future use of cephalosporins.

Therapy had to be switched to 4th generation cephalosporins, fluoroquinolones, piperacillin plus tazobactam, and the carbapenems. All were effective against *Klebsiella* spp which did not possess ESBLs, but doubts were raised about their long-term capability to deal with ESBL producers. Strains became fluoroquinolone resistant and there continuing doubts about the efficacy of 4th generation cephalosporins and piperacillin plus tazobactam, ensured highly-active carbapenems, became the drug of choice. The success of this group of β-lactams encouraged the use of a newer carbapenem, ertapenem,

for the treatment of infections caused by *Klebsiella* spp containing ESBLs. This drug is not effective against Gram-negative non-fermenting bacteria, so its use was primarily designed for community-acquired infections where the risk of super-infection by these bacteria is often negligible. However, in hospitals, the risk is high and less-active carbapenems may promote the emergence and spread of Gram-negative non-fermenting bacteria.

This has become acute as carbapenem resistance has begun to emerge in *Klebsiella* spp. In some strains, no detectable β-lactamase could be found to be responsible for carbapenem resistance and this was associated with reductions in permeability caused by porin loss. However, in many strains, a new, plasmid-encoded class A β-lactamase capable of mediating resistance to extended-spectrum cephalosporins and carbapenems was identified. This β-lactamase, KPC-1, first emerged in the United States in 2001 but has now been found on other continents as have different variations of it (KPC-2–7). Fluoroquinolone and carbapenem-resistant ESBL-producing *Klebsiella* spp represent a real threat to continued successful treatment and care should be taken not to promote them.

ESBLs in *E. coli* did not comprise a major problem when they were derived from TEM and SHV. However, the emergence of the CTX-M ESBLs, particularly CTX-M-15, has caused significant problems in community and nosocomial *E. coli*. Furthermore the *blaCTX-M-15* gene is often linked, on the same plasmid, with the *aac(6')-Ib-cr* gene, responsible for decreased susceptibility to aminoglycosides (kanamycin, amikacin, and tobramycin) as well as to ciprofloxacin. This results in *E. coli* that have to be treated with carbapenems, but carbapenem resistance has already been detected so considerable caution needs to be exercised to ensure that an appropriate carbapenem is used.

C. difficile

C. difficile is a bacterium inherently resistant to most antibiotics. It is an anaerobic bacterium, which allows it to grow in the oxygen-depleted environment of the gut. Normally it can only proliferate when the normal intestinal bacteria have been removed by antibiotics. Under these conditions it produces two toxins (A and B) that damage the cells lining the intestine. Hospitalized patients, particularly the compromised and the elderly, who have been treated with broad-spectrum antibiotics, are of *C. difficile* disease. Most of those affected are elderly patients with serious underlying illnesses. Most infections occur in hospitals (including community hospitals), nursing homes and so on; but it can also occur in primary care settings. It spreads by cross-infection, from another patient, either through direct patient to patient contact, via healthcare staff, or via a con-

taminated environment. It is further complicated by the fact that it produces spores so that it can survive for a long time in more hostile environments than most other bacteria. Patients who have *C. difficile* diarrhoea excrete large numbers of the spores, which can contaminate the general environment around the patient's bed.

C. difficile is genotyped by ribotyping, with over 100 types identified. However, the rapid emergence of Type 027 has caused particular problems as it produces much more of the toxins than most other types because a mutation has knocked out the gene that normally restricts toxin production. It causes a greater proportion of severe disease and appears to have a higher mortality.

Penicillin-resistant *S. pneumoniae* (PRSP)

Most of the causative bacteria above are predominantly problems, but not exclusively, in hospitalized patients. *S. pneumoniae* is the cause of the most severe form of common community-acquired pneumonia besides causing otitis media, meningitis as well as invasive infections. The rise to MDR status in *S. pneumoniae* has occurred almost exclusively in the community. *S. pneumoniae* remained exquisitely sensitive long after the introduction of penicillin but suddenly resistance emerged and then increased. The cause of this could be related to the use of aminopenicillins such as amoxicillin. However, penicillin resistance is a misnomer as these strains are resistant to cephalosporins as well, and it has been suggested that the introduction of oral cephalosporins, especially 3rd generation, has driven the proliferation of these MDR bacteria. The mechanism of β-lactam resistance is an alteration of the penicillin-binding proteins, which can give varying degrees of insusceptibility. These are classified as intermediate if the MIC of penicillin ranges from 0.06–2mg/L and resistant if the MIC is greater than 2mg/L. Furthermore, PRSP are not resistant just to β-lactam antibiotics, they are usually resistant to macrolides and, possibly also, to fluoroquinolones, so these are truly MDR bacteria.

Like the MDR hospital pathogens, as *S. pneumoniae* become MDR, individual clones have emerged and spread around the world. These are classified by the Pneumococcal Molecular Epidemiology Network (PMEN), which has currently identified 43 clones. These were originally classified by serotyping but more latterly they have been categorized by genotyping, although they have retained their serotyping roots; i.e. country first identified serotype—sequential numbering in network—subsequent described serotype (Spain23F-1-19F. Spain = country in which the clone was first identified [based on publication], 23F = serotype of the clone first identified, 1 = clone number 1, 19F = serotype 19F variant of Spain23F-1 clone).

MDR bacteria

Many of the MDR bacteria currently causing problems are disseminating clonally through the clinical population. This may be caused by a variety of factors. It may indicate that some strains within a bacterial species are more capable of becoming resistant; they may have greater mutation capabilities, increasing the mutation rate to resistance or even the acceptance of mobile DNA. These mutators can either be a transient state or more permanent. In either case, they give the strains an advantage. In the non-fermenting Gram-negative bacteria, such as *P. aeruginosa* and *A. baumannii*, alterations in the *mutS* gene have been associated with increased mutation rate and MDR status.

In the case of the CTX-M-15 β-lactamase, there is a close association of the plasmid with a particular strain of *E. coli*; the strain is dependent on the carriage of the plasmid not just for resistance but its actual survival. Similarly, in some strains, the close association of resistance genes with sections of DNA that encode the pathogenicty of the organism may ensure that the resistance and pathogenicity may become interdependent.

The insertion sequence IS*Aba*1 is seen to be crucial for the expression of important resistance genes in *A. baumannii* because of the promoter that it carries. Strains that are able to move these insertion sequences to promote genes that are already present in the cell, but may be switched off, are likely to become particularly successful.

Therefore, in conclusion, most MDR bacteria do not appear to be sensitive strains that have become resistant but rather a 'fitter' subspecies that possess a variety of characteristics that have allowed them to rise as the sensitive strains are eradicated by our use of antibiotics.

Chapter 8

Anti-mycobacterium therapy

Key points

- The cause of the 'white death' still infects a third of the world's population.
- The antibiotics currently available to treat tuberculosis.
- The emergence of resistance is rapid and leads to treatment failure.
- The development of resistance has changed therapeutic strategy.
- The regimens now recommended are complicated but guidelines are available.

The genus *Mycobacterium* contains over 40 species; however, few actually cause disease in humans. The most prevalent is *M. tuberculosis* which is estimated to infect over 1.7 billion people—nearly a third of the world's population; of these around 8 million develop clinical disease each year. The co-called 'atypical' species that cause human disease include *M. kansasii*, *M. marinum*, *M. avium-intracellulare*, *M. fortuitum,* and several others.

Tuberculosis has been a recognized clinical entity since ancient Greek times, killing young and old, the obscure and the famous including the writers Anton Chekov, all three Brontë sisters, Somerset Maugham, Robert Louis Stevenson, D. H. Lawrence, George Orwell, the composer Frederick Chopin and much more recently, the actress Vivien Leigh. It is over 100 years since Robert Koch published his treatise which linked the tubercule bacillus to the devastating disease. Within the last 40 years considerable progress in the knowledge of the pathogenesis, epidemiology, prevention, and treatment of tuberculosis has been made.

Anti-tubercle therapy has improved markedly since 1944 when the discovery of streptomycin was shown to be effective in the treatment of human disease. Some 5 yr later the combination of p-aminosalicylic acid (PAS) and streptomycin was demonstrated to be effective against streptomycin resistant strains of *M. tuberculosis*. This combination therapy approach is now the mainstay of tuberculosis treatment programmes.

By the early 1950s isoniazid was shown to be active against *M. tuberculosis* and it replaced PAS/streptomycin as the mainstay of therapy. Its main benefit has been to shorten the course of therapy, which previously had been 12 months or longer in some patients.

By the 1960s further new antituberculosis agents were introduced, these included pyrazinamide, ethambutol, and rifampicin. These drugs permitted shorter regimens to be instituted and for intermittent therapy to be utilized. The latter approach has proven to be of particular value in developing nations where supervised daily drug administration is not standard.

Anti-mycobacterial agents

Streptomycin

Streptomycin is an aminoglycoside which can only be given parenterally. It acts on the protein synthetic pathway, in the growing phase of the bacterial life cycle. Although it penetrates into the appropriate tissues the toxic side effects of streptomycin therapy, particularly vestibular damage, mean that it is now used as a last-resort agent.

PAS

PAS is an inhibitor of folic acid synthesis and is a foul tasting drug with marked gastrointestinal side effects, as such is not in regular use today.

Isoniazid

Unusually this drug is active only against tubercle bacilli; it is highly potent and bactericidal. It has become the mainstay of therapy due to its activity within the macrophage, it is well tolerated, it penetrates well into tissues and it is inexpensive. Isoniazid is given orally in a single dose; it can achieve high-peak concentrations. Adverse reactions are uncommon; they include disturbances in liver enzymes, blurred vision, slurred speech, and occasionally hepatitis (0.1%). Resistance is unusual but can develop if used as a single agent.

Pyrazinamide

Pyrazinamide has a remarkable sterilizing effect on tubercle bacilli contained within the macrophage. It can be given orally yielding high serum and cerebrospinal fluid (CSF) levels. Owing to these factors it has found a place in short-course regimen. Adverse reactions are unusual, these include hepatic problems, dermal hypersensitivity and photosensitivity may also occur.

Ethambutol

Ethambutol has effectively replaced PAS as a combination agent for the treatment of both tubercle and other mycobacteria. It is a bacteriostatic agent which acts on growing organisms. After oral administration it achieves high serum and tissue levels. The major side effect

of ethambutol therapy is that of optic damage by a neuritis which affects visual acuity and colour vision. These changes are dose-dependent but are normally reversible.

Rifampicin

Rifampicin is a broad-spectrum antibacterial with potent activity against mycobacteria. It has been shown to be a great value in primary therapy as well as for the treatment of relapses. Side effects associated with rifampicin include elevated liver enzymes, central nervous system (CNS) disturbances, allergic reactions, and worrying to the patient, but not significantly, orange colouration of saliva, sweat, tears, urine, and stool. To date primary resistance is rare in the United Kingdom (<1%); however care must be employed as single step mutation can lead rapidly to resistance development.

Other agents

Other agents which may be resorted to when primary or first line therapy fails include ethionamide, prothionamide, cycloserine, kanamycin, capreomycin, and thiacetazone. Each has significant disadvantages.

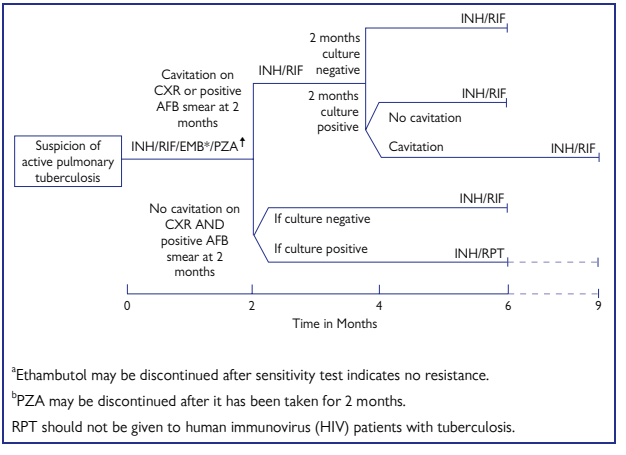
Treatment regimens

The treatment regimens for particularly pulmonary tuberculosis vary not only between countries but also from health district to health district. Therapeutic agents for tuberculosis should ONLY be instituted by an appropriately qualified physician and usually follow full clinical investigations to confirm the presence of acid-alcohol fast bacilli and, in the case of pulmonary tuberculosis, a chest X-ray. Two priorities are to ensure that therapy is administered quickly after tuberculosis is suspected but also to consider the risks of the emergence of resistance. The latter has dominated the strategies used to treat tuberculosis for decades. The bacterium is an enclosed system, with no migration of resistance genes from one strain to another. Therefore, it is a good candidate for combination therapy, as plasmids or other mobile elements will not import resistance genes from other bacteria. However, resistance has emerged and the extremely slow growth of the strains and subsequent delay in culture-based sensitivity tests mean that therapy has be started empirically. To ensure that some of the antibiotics are active, quadruple therapy is unusually initiated and when the results of the sensitivity tests are obtained (e.g. at 2 months), some of the drugs are removed from the cocktail. An example of this strategy is shown in Fig. 8.1, which is an algorithm for tuberculosis treatment devised by the Center for Disease Control in the United States. The ability of mycobacteria to develop single or multiple resistance mechanisms is now the cause of

major epidemics of multi-drug resistant tuberculosis (MDRTB) in the United States and South East Asia. These outbreaks create huge infection control problems as presently there are no known effective treatment regimens, even six or seven drug cocktails are proving ineffective.

For the treatment of 'atypical' mycobacteria it is preferred to tailor the regimen to match the sensitivity pattern of the isolated strain. However, as frequently the atypical species infect the immunocompromised patient, such as those with HIV infection, the instituted therapy may be in vain. For the immunocompetent patient infections such as those caused by *M. marinum* (fish-tank disease) are fairly easily, but lengthily, treated with conventional agents.

In the United Kingdom tuberculosis therapy is still active against the vast majority of the 6,000 or so cases seen annually.



[a]Ethambutol may be discontinued after sensitivity test indicates no resistance.

[b]PZA may be discontinued after it has been taken for 2 months.

RPT should not be given to human immunovirus (HIV) patients with tuberculosis.

Figure 8.1 WHO treatment regimen for pulmonary tuberculosis to overcome resistance. CXR—chest X-ray, EMB—ethambutol, INH—isoniazid, PZA—pyrazinamide, RIF—rifampicin, RPT—rifapentine. Cited by Center for Disease Control as Fig. 1 at http://www.cdc.gov/mmwr/preview/mmwrhtml/rrs2llal.htm (accessed 11th September 2009).

Chapter 9

Clinical use of antibiotics to prevent or control resistance

Key points

- Choice of appropriate antibiotics is currently based primarily on individual patient need.
- Preservation of bacterial sensitivity needs understanding of how antibiotics select resistance.
- 'Ten commandments' which might be considered carefully when a choice has to be made for antibiotic prescribing.
- The crucial importance of the fact that all healthcare personnel should take ownership of the need to prescribe appropriately and to practice effective infection control.
- A realization that antibiotics may not be capable for the tasks required of them and eventually, with widespread resistance, may be incapable of the task they do today.

There is much debate about the use and misuse of antibiotics. It is often difficult to actually define what misuse is. Clearly, if an antibiotic is given for an infection that is clearly of viral origin, then this could be construed as inappropriate. However, a significant proportion of therapy is empiric and this is often based on clear guidelines, such as those in the British National Formulary, which identifies recommended treatment options with doses.

It should be remembered that there are infections that antibiotics may not resolve, either because of resistance or because the infection has simply developed too far for an antibiotic to be fully effective. Thus, in some cases, we do not have the antibiotics that are needed to deal with certain infections, particularly in patients that are immunocompromised and/or those in the intensive care units.

Prescribers should attempt to tailor therapy to the needs of the individual patient. There is a complex interaction between patient, organism, and antibiotic. However, 20 years ago the prescriber was not faced with either the level of resistance that we now have or the underlying resistance genes threatening to emerge or spread. The

suggestions below outline general principles for initial treatment only and they are just a personal view of the potential opportunity for resistance development. It is always essential to seek specialist/microbiologist advice.

The following points should be considered and may be helpful in decision making.

1. Which antibiotic to use. Doses and individual recommendations have deliberately not been included in these tables. Refer to National Formularies (such as the British National Formulary), National Guidelines (such as the NICE guidelines) and their various appendices for information on drug interactions, use in pregnancy, and so on. However, some general principles might apply. The greatest threat at the moment to the continued use of antibiotics is the development of resistance. Therefore, where possible, this should be considered while prescribing.
   a) Best in Class. Resistance develops quickly when the patient is dosed with an insufficient concentration of antibiotic, particularly if it falls below the inhibitory concentration; therefore the most active member of a drug class is probably less likely to select out resistant bacteria than a less active one. Previously, it was considered that less active drugs should be used first so that the more active members could be introduced when resistance to the 'first generation' drugs developed. However, more often than not, resistance to the 'first generation' drugs also provides some resistance to the more active drugs. There are many examples of this; the early quinolones were powerful selectors of resistance to the later drugs. Similarly with the cephalosporins, some members are powerful selectors of the extended-spectrum β-lactamases (ESBLs) and now more latterly, some of the newer, less-effective carbapenems are selecting resistance to more powerful members of this class such as imipenem and meropenem.
   b) Long or short half-life. It has long been considered that a long half-life antibiotic should be preferred. This is convenient for the administrator of the antibiotic. An antibiotic with a long half-life should maintain a concentration of antibiotic above the inhibitory concentration for the whole period between dosing; however, once dosing ceases there is a considerable period of time, often lasting many days, where the antibiotic concentration slowly decreases leaving the remaining bacteria exposed to sub-inhibitory concentrations, a most powerful selection environment for the development and spread of resistance.

c) I/V or Oral dosing. I/V dosing is recommended for severely unwell patients. Often, a switch is made to oral therapy as the patient improves. I/V therapy often controls the infection more quickly simply because of the speed at which the antibiotic can reach the target from the blood. Whatever route is chosen, it must be ensured that the dose and dose interval are appropriate and that the antibiotic had been given at the correct intervals.

2. Surveillance versus epidemiology. Be aware of local antibiotic sensitivity patterns as this is likely to give an indication of whether therapy, particularly empirical, is likely to succeed. These figures are usually carefully prepared. The best figures ensure that repeats from the same patients are removed which would give a clearer indication of the susceptibility rates. However, most of these figures are obtained from surveillance not from epidemiological studies; therefore they are reactive to the specimens sent to the laboratory. Epidemiological studies would ensure that all specimens are sent to the laboratory. If empirical therapy has been successful and no specimen was sent, then the strain was presumably clinically sensitive; thus local antibiotic sensitivity patterns can over-estimate the proportion of resistant isolates. It depends on what proportion of patients, with a specific infection, had a specimen taken before therapy was begun.
3. Local antibiotic sensitivity patterns. The definition of local is important; at the very most for clinical purposes it should probably include only a single hospital. It is well known that there can be considerable variation between hospitals in the same area, even if they are in close proximity. It will depend on the clinical units that the hospital has. Thus the best figures would be for the individual units in which the patient is located. The figures for infections in the community may be more accurate, as long as they do not include patients from residential homes. Most empirical therapy is given in the community, so the local sensitivity pattern for a given area may be a good guide.
4. Clinical microbiologists, infectious disease specialists, and so on. The best advice regarding antibiotic prescribing is available from microbiologists, infectious disease specialists, clinical pharmacologists and pharmacists. These can certainly help with local information particularly if there is considerable variation between one section of a hospital and another. It is part of their remit to control the spread of multi-resistant pathogens and the most up-to-date advice should be obtained from them.

5. Empirical therapy. This should be chosen with regard to the likely pathogen, 'best guess' antibiotic, pharmacology of the agent and the possibility of drug toxicity or interaction. Often it is important to ensure that the antibiotic will be effective and not lead to resistance problems, so the Best in Class (Section 1a) may often be the best option. In some cases, identification of the pathogen is not so important but in other infections it is crucial. So knowledge of the local sensitivity patterns for individual pathogens that may cause critical infections is likely to be very significant; for example, the different organisms that can cause meningitis can vary considerably in their susceptibility patterns.
6. Local prescribing policies and protocols. Most hospitals have local guidelines even if they do not have defined policies. These have been developed to satisfy local needs and problems often to try and contain the spread of resistant bacteria. There may also be a financial element attached to this as most hospital pharmacies cannot afford to carry every antibiotic nor would it necessarily be desirable to do so. Conforming to these policies should satisfy the needs of most patients and will allow those in charge of infectious diseases and infection control to manage antibiotic resistance by the use of appropriate drugs.
7. Microbiological specimens. Where possible, microbiological specimens should be taken before starting treatment. There is a good reason for this because any specimen taken after the start of therapy is likely to be biased if the antibiotics have eradicated some or all of the pathogens. It could be argued that no specimen taken during or shortly after antibiotic therapy should be included in the surveillance results. For this reason, it is important to identify recent exposure to antibiotics and to specify this on the request. Furthermore, if empirical therapy is started at the time of the specimen being taken, then this would be valuable information for the clinical microbiologist to have. Many specimens are analysed by automated phenotypic identification systems; in many cases these are accurate and the susceptibility patterns that they provide are the best that can be achieved within the routine laboratory. However, the accuracy of these identification systems is not uniform for all bacteria and the successful identification of some pathogens may be relatively low compared with the success rate for more common organisms. This may be important in the decision-making process and, certainly with unusual or rare pathogens, the therapeutic options should be discussed with the microbiologist.

8. Should an antibiotic be used at all? There are many cases where an antibiotic may not be the best method to deal with an infection. Apart from the obvious inappropriate treatment of viral infections with antibiotics, systemic antibiotics are usually inappropriate for topical treatment. The antibiotic would simply not reach sufficient concentrations to deal with the infection. Similarly, drainage of a collection of pus is usually more effective than antibiotic therapy though it could be argued that there is a case for giving antibiotics as well.
9. Antiseptics and disinfectants. These are chemicals and, as such, bacteria can become resistant to them. Indeed many of the multi-resistant 'superbugs' carry resistance genes to some of the antiseptics at least. This is a problem for two reasons; the first is that the antiseptics could become less effective in the future but secondly, and more importantly, resistance genes tend to congregate together and thus the continuous selection of resistant strains with antiseptics may inadvertently continue to select resistance genes to powerful antibiotics. The use of antiseptics, like antibiotics, comes with a cost and they should be used cautiously. Unlike antibiotics, no tests are routinely performed for antiseptic sensitivity so we have no idea the proportion of resistant strains. The argument is often used that antiseptics are used in such high concentrations that they kill the organism; two features mitigate against this view. The first is that they are often used inappropriately and thus low concentrations may come into contact with the bacteria and second they are very selective and are hardly effective against certain types of bacteria, thus providing a powerful selective environment.
10. Infection control. When we observe an increase in resistance, mostly we are observing the increased frequency of bacteria that have already become resistant and that are spreading from patient to patient. The emergence of new resistance genes emerging during therapy is a relatively rare event. Bearing this in mind, prevention of infection by rigid infection control procedures is almost certainly the most effective method to control the spread of resistant bacteria. Rigid guidelines have been implemented in many areas and it is vital that they are adhered to. Simple failures in hygiene can have a devastating ability to spread resistant bacteria. Many patients acquire infections from the healthcare workers and thus they carry a huge responsibility to ensure that infectious material is not carried by them from one patient to another.

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