

How do minute microorganisms actually resist antimicrobial actions? What enables them to do this? How do previously susceptible bacteria gain resistance? How is antimicrobial resistance detected in bacterial populations? This module addresses the intricate science behind the antibiotic resistance phenomenon.

The Microbiology Module will explain what takes place within the bacterial cell to enable antimicrobial resistance, and how antimicrobial resistance can be detected and measured. These basic principles should be a useful resource for client education and for reinforcing the veterinarian's role in protecting the public's health.

### Module Objectives:

This module aims to introduce the microbiological aspects of antimicrobial resistance through a discussion of:

1. bacterial strategies involved in resisting antimicrobial actions;
2. the molecular basis for bacterial resistance to antimicrobial actions;
3. the common laboratory methods for detecting and measuring antimicrobial resistance.

### Pharmacology Module Outline

- I. Antimicrobial resistance
- II. Bacterial resistance strategies
  - a. Reducing entry of antimicrobial agents
  - b. Expulsion of antimicrobial agents
  - c. Inactivation of antimicrobial agents
  - d. Modification of antimicrobial targets
- III. Molecular basis of resistance
  - a. Intrinsic resistance
  - b. Acquired resistance
    1. mutation
    2. horizontal gene transfer
- IV. Detecting antimicrobial resistance
  - a. Introduction
  - b. Lab approaches and strategies
  - c. Test methods in detecting resistance
    1. Dilution methods (broth, agar)
    2. Disk-diffusion method
    3. E-test
    4. Automated methods
    5. Mechanism-specific tests such as beta-lactamase detection test and chromogenic cephalosporin test
    6. Genotypic methods such as PCR and DNA hybridization methods
- V. Module summary
- VI. References

## Antimicrobial Resistance

The introduction of antimicrobials transformed human and animal health systems by revolutionizing our weaponry in the war against infectious diseases, resulting in improved survivability for both humans and their domestic animals. However, this health triumph was immediately ebbd by the subsequent realization that bacterial populations could quickly modify themselves to resist antimicrobials, propagate these resistance traits, and even share resistance genes with other contemporary bacteria within their environment. Such abilities have seriously compromised the usefulness of antibiotics in the war against microbes and warn of a future when antimicrobials may have very limited usefulness to control bacterial infection

**Antimicrobial resistance** is the ability of a microorganism to survive and multiply in the presence of an antimicrobial agent that would normally inhibit or kill this particular kind of organism. Antimicrobial resistance is just one of the many adaptive traits that resilient bacterial subpopulations may possess or acquire, enabling them to out-compete and out-survive their microbial neighbors and overcome host strategies aimed against them. This phenomenon is nearly as old as the discovery of antimicrobials themselves, having been described by pioneers like Ehrlich for trypanosomes<sup>8</sup> and Fleming for staphylococci<sup>10</sup>. What is most alarming today is the rate at which antibiotic resistance often develops and how quickly it spreads across the globe and among different species of bacteria.

Furthermore, as a result of sequential, cumulative acquisition of resistance traits against different antibiotics, more bacterial pathogens with **multiple-drug resistance** are being reported worldwide. As a consequence, many bacterial organisms, including major human and animal pathogens such as *Mycobacterium* and *Salmonella* species, have become resistant to antibiotics which were previously quite efficacious

## the antibiotic timeline

<b>2001 to 2008</b>	Introduction of broader spectrum fluoroquinolones (2001), Telithromycin (2002), Tigecycline (2006)	Emergence of vancomycin-resistant staphylococcal infections; Spread of extended-spectrum beta-lactamase among Gram negatives; Emergence of more multi-drug resistant organisms
<b>1991 to 2000</b>	Introduction of oral extended spectrum cephalosporins (1998), Quinupristin-dalfopristin (1999), linezolid	Emergence of vancomycin-resistant enterococci; emergence of multi-drug resistant <i>Mycobacterium tuberculosis</i> ; global emergence of multi-drug resistant <i>Salmonella enteric</i> serovar Typhimurium DT 104
<b>1981 to 1990</b>	Introduction of cefotaxime (1981), clavulanic acid-amoxicillin (1983), imipenem-cilastatin (1985), norfloxacin (1986), aztreonam (1986)	Spread of methicillin-resistant staphylococcus infections; emergence of AIDS-related bacterial infections
<b>1971 to 1980</b>	Introduction of carbenicillin (1973), cefoxitin (1978), cefaclor (1979)	Increasing trend of nosocomial infections due to opportunistic pathogens; Ampicillin-resistant infections become frequent
<b>1961 to 1970</b>	Introduction of gentamicin (1963), ampicillin (1966), cephalothin (1966), amikacin (1970)	Emergence of gentamicin-resistant <i>Pseudomonas</i> (1968); emergence of methicillin-resistant staphylococcal infections (1968)
<b>1951 to 1960</b>	Introduction of erythromycin, vancomycin, tylosin and methicillin	Penicillin-resistant infections become clinically significant
<b>1941 to 1950</b>	Introduction of streptomycin (1944), chloramphenicol (1946) and chlortetracycline (1948)	Penicillin made available to the public; widespread use in animals by 1950.
<b>1930 to 1940</b>	Introduction of sulfonamide	Efficacy of penicillin in humans shown; sulfonamides introduced in food animal use
<b>Before 1930</b>	Discovery of penicillin (1929)	

Resistance to single antibiotics became prominent in organisms that encountered the first commercially produced antibiotics. The most notable example is resistance to penicillin among staphylococci, specified by an enzyme (penicillinase) that degraded the antibiotic. Over the years, continued selective pressure by different drugs has resulted in organisms bearing additional kinds of resistance mechanisms that led to multidrug resistance (MDR), novel penicillin-binding proteins (PBPs), enzymatic mechanisms of drug modification, mutated drug targets, enhanced efflux pump expression, and altered membrane permeability. Some of the most problematic MDR organisms that are encountered currently include *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella pneumoniae* bearing extended-spectrum  $\beta$ -lactamases (ESBL), vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant MRSA, and extensively drug-resistant (XDR) *Mycobacterium tuberculosis*.

Alekshun MN and Levy SB. 2007. Molecular Mechanisms of Antibacterial Multidrug Resistance. Cell 128:1037-1050.

## Bacterial Resistance Strategies

To survive in the presence of an antibiotic, bacterial organisms must be able to disrupt one or more of the essential steps required for the effective action of the antimicrobial agent (see Pharmacology Module, Mechanisms of Action).

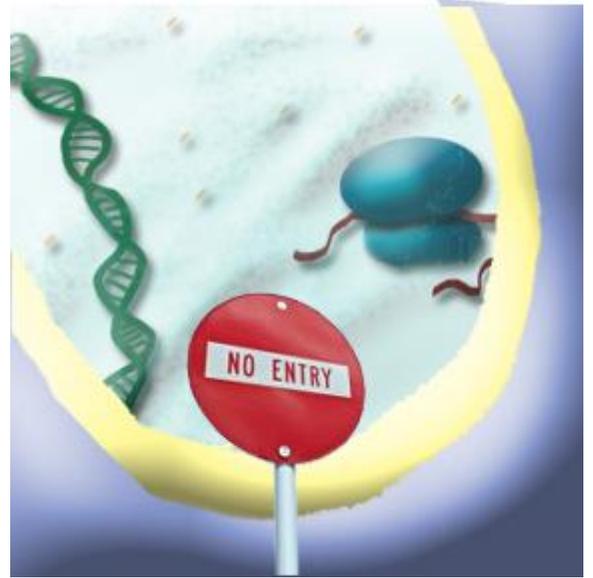
The intended modes of action of antibiotics may be counter-acted by bacterial organisms via several different means. This may involve preventing antibiotic access into the bacterial cell or perhaps removal or even degradation of the active component of the antimicrobial agent. No single mechanism of resistance is considered responsible for the observed resistance in a bacterial organism. In fact, several different mechanisms may work together to confer resistance to a single antimicrobial agent.

### Four major bacterial resistance strategies:

- (1) By prevention of the antimicrobial from reaching its target by reducing its ability to penetrate into the cell
- (2) By expulsion of the antimicrobial agents from the cell via general or specific efflux pumps
- (3) By inactivation of antimicrobial agents via modification or degradation
- (4) By modification of the antimicrobial target within the bacteria

**(1) By prevention of the antimicrobial from reaching its target by reducing its ability to penetrate into the cell**

Antimicrobial compounds almost always require access into the bacterial cell to reach their target site where they can interfere with the normal function of the bacterial organism. Porin channels are the passageways by which these antibiotics would normally cross the bacterial outer membrane. Some bacteria protect themselves by prohibiting these antimicrobial compounds from entering past their cell walls. For example, a variety of Gram-negative bacteria reduce the uptake of certain antibiotics, such as aminoglycosides and beta lactams, by modifying the cell membrane porin channel frequency, size, and selectivity. Prohibiting entry in this manner will prevent these antimicrobials from reaching their intended targets that, for aminoglycosides and beta lactams, are the ribosomes and the penicillin-binding proteins (PBPs), respectively.



This strategy have been observed in:

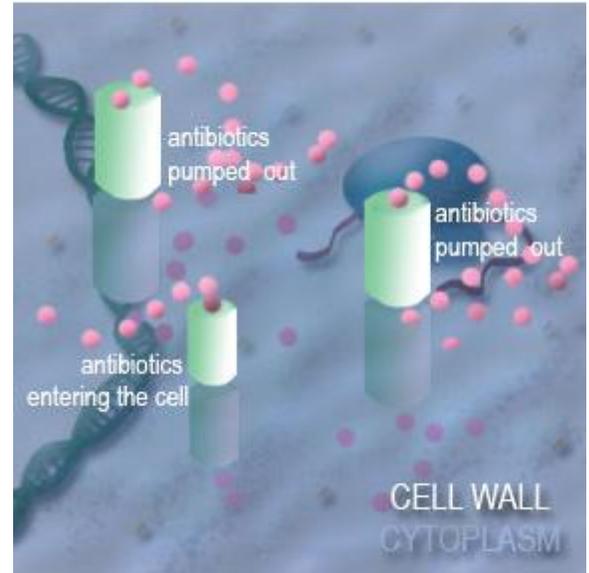
- *Pseudomonas aeruginosa* against imipenem (a beta-lactam antibiotic)
- *Enterobacter aerogenes* and *Klebsiella* spp. against imipenem
- Vancomycin intermediate-resistant *S. aureus* or VISA strains with thickened cell wall trapping vancomycin
- Many Gram-negative bacteria against aminoglycosides
- Many Gram-negative bacteria against quinolones

(2) By expulsion of the antimicrobial agents from the cell via general or specific efflux pumps

To be effective, antimicrobial agents must also be present at a sufficiently high concentration within the bacterial cell. Some bacteria possess membrane proteins that act as an export or efflux pump for certain antimicrobials, extruding the antibiotic out of the cell as fast as it can enter. This results in low intracellular concentrations that are insufficient to elicit an effect. Some efflux pumps selectively extrude specific antibiotics such as macrolides, lincosamides, streptogramins and tetracyclines, whereas others (referred to as multiple drug resistance pumps) expel a variety of structurally diverse anti-infectives with different modes of action.

This strategy has been observed in:

- E.coli and other Enterobacteriaceae against tetracyclines
- Enterobacteriaceae against chloramphenicol
- Staphylococci against macrolides and streptogramins
- Staphylococcus aureus and Streptococcus pneumoniae against fluoroquinolones



#### Trivia: Efflux pumps

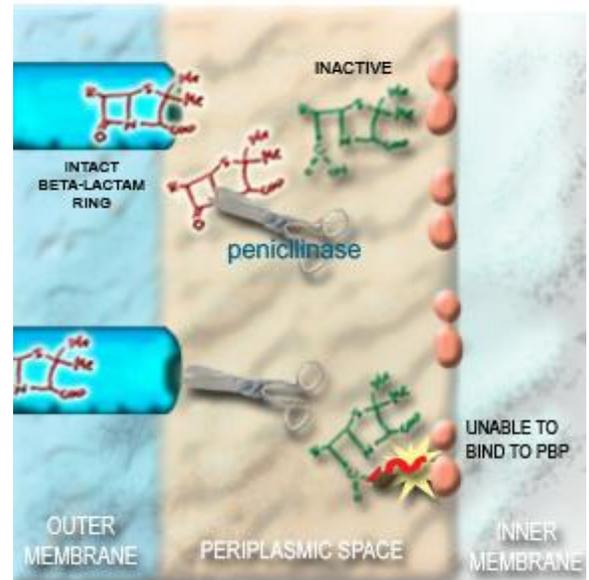
These efflux pumps are variants of membrane pumps possessed by all bacteria, both pathogenic and non-pathogenic, to move lipophilic or amphipathic molecules in and out of the cells. Some are used by antibiotic producers to pump antibiotics out of the cells as fast as they are made, and so constitute an immunity protective mechanism for the bacteria to prevent being killed by their own chemical weapons (Walsh, 2000).

**(3) By inactivation of antimicrobial agents via modification or degradation**

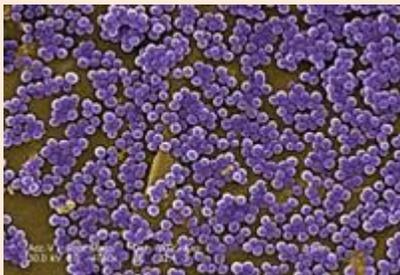
Another means by which bacteria preserve themselves is by destroying the active component of the antimicrobial agent. A classic example is the hydrolytic deactivation of the beta-lactam ring in penicillins and cephalosporins by the bacterial enzyme called beta lactamase. The inactivated penicilloic acid will then be ineffective in binding to PBPs (penicillin binding proteins), thereby protecting the process of cell wall synthesis.

This strategy has also been observed in:

- Enterobacteriaceae against chloramphenicol (acetylation)
- Gram negative and Gram positive bacteria against aminoglycosides (phosphorylation, adenylation, and acetylation)

**Trivia: first evidence of antimicrobial resistance**

The first antibiotic resistance mechanism described was that of penicillinase. Its presence and activity was first reported by Abraham and Chain in 1940 shortly after its discovery (Abraham, E. P. and E. Chain. 1940. An enzyme from bacteria able to destroy penicillin. Nature 146: 837)

**Resistance Mechanisms Keeping Up**

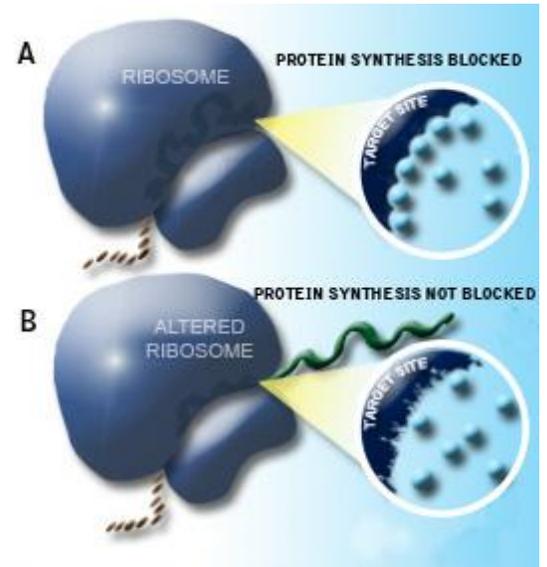
Less than 10 years after the clinical introduction of penicillins, penicillin-resistant *Staphylococcus aureus* was observed in a majority of Gram-positive infections in people. The initial response by the pharmaceutical industry was to develop beta-lactam antibiotics that were unaffected by the specific beta-lactamases secreted by *S. aureus*. However, as a result, bacterial strains producing beta-lactamases with different properties began to emerge, as well as those with other resistance mechanisms. This cycle of resistance counteracting resistance continues even today (Bush, 1988. Beta-Lactamase Inhibitors from Laboratory to Clinic. Clinical Microbiology Reviews. 1(1):109-123.

**(4) By modification of the antimicrobial target within the bacteria**

Some resistant bacteria evade antimicrobials by reprogramming or camouflaging critical target sites to avoid recognition. Therefore, in spite of the presence of an intact and active antimicrobial compound, no subsequent binding or inhibition will take place.

This strategy has been observed in:

- Staphylococci against methicillin and other beta-lactams (Changes or acquisition of different PBPs that do not sufficiently bind beta-lactams to inhibit cell wall synthesis.)
- Enterococci against vancomycin (alteration in cell wall precursor components to decrease binding of vancomycin)
- Mycobacterium spp. against streptomycin (modification of ribosomal proteins or of 16s rRNA)
- Mutations in RNA polymerase resulting in resistance to the rifamycins;
- Mutations in DNA gyrase resulting in resistance to quinolones

**Some Examples Of Bacterial Resistance Due To Target Site Modification <sup>4, 18</sup>:**

- **Alteration in penicillin-binding protein (PBPs) leading to reduced affinity of beta-lactam antibiotics** (Methicillin-Resistant *Staphylococcus aureus*, *S. pneumoniae*, *Neisseria gonorrhoeae*, Group A streptococci, *Listeria monocytogenes*)
- **Changes in peptidoglycan layer and cell wall thickness resulting to reduced activity of vancomycin:** Vancomycin-resistant *S. aureus*
- **Changes in vancomycin precursors reducing activity of vancomycin:** *Enterococcus faecium* and *E. faecalis*
- **Alterations in subunits of DNA gyrase reducing activity of fluoroquinolones:**
- Many Gram-negative bacteria
- **Alteration in subunits of topoisomerase IV leading to reduced activity of fluoroquinolones:** Many Gram positive bacteria, particularly *S.aureus* and *Streptococcus pneumoniae*
- **Changes in RNA polymerase leading to reduced activity of rifampicin:** *Mycobacterium tuberculosis*

## Mechanisms of Resistance Against Different Antimicrobial Classes

(Forbes et al., 1998; Berger-Bachi, 2002)

ANTIMICROBIAL CLASS	MECHANISM OF RESISTANCE	SPECIFIC MEANS TO ACHIEVE RESISTANCE	EXAMPLES
<b>Beta-lactams</b> Examples: penicillin, ampicillin, mezlocillin, peperacillin, cefazolin, cefotaxime, ceftazidime, aztreonam, imipenem	Enzymatic destruction	<b>Destruction of beta-lactam rings by beta-lactamase enzymes.</b> With the beta-lactam ring destroyed, the antibiotic will no longer have the ability to bind to PBP (Penicillin-binding protein), and interfere with cell wall synthesis.	Resistance of staphylococci to penicillin; Resistance of <i>Enterobacteriaceae</i> to penicillins, cephalosporins, and aztreonam
	Altered target	<b>Changes in penicillin binding proteins.</b> Mutational changes in original PBPs or acquisition of different PBPs will lead to inability of the antibiotic to bind to the PBP and inhibit cell wall synthesis	Resistance of staphylococci to methicillin and oxacillin
	Decreased uptake	<b>Porin channel formation is decreased.</b> Since this is where beta-lactams cross the outer membrane to reach the PBP of Gram-negative bacteria, a change in the number or character of these channels can reduce betalactam uptake..	Resistance of <i>Enterobacter aerogenes</i> , <i>Klebsiella pneumoniae</i> and <i>Pseudomonas aeruginosa</i> to imipenem
<b>Glycopeptides</b> Example: vancomycin	Altered target	<b>Alteration in the molecular structure of cell wall precursor components</b> decreases binding of vancomycin so that cell wall synthesis is able to continue.	Resistance of enterococci to vancomycin
<b>Aminoglycosides</b> Examples: gentamicin, tobramycin, amikacin, netilmicin, streptomycin, kanamycin	Enzymatic modification	<b>Modifying enzymes</b> alter various sites on the aminoglycoside molecule so that the ability of this drug to bind the ribosome and halt protein synthesis is greatly diminished or lost entirely.	Resistance of many Gram-positive and Gram negative bacteria to aminoglycosides
	Decreased uptake	<b>Change in number or character of porin channels</b> (through which aminoglycosides cross the outer membrane to reach the ribosomes of gram-negative bacteria) so that aminoglycoside uptake is diminished.	Resistance of a variety of Gram-negative bacteria to aminoglycosides
	Altered target	<b>Modification of ribosomal proteins or of 16s rRNA.</b> This reduces the ability of aminoglycoside to successfully bind and inhibit protein synthesis	Resistance of <i>Mycobacterium</i> spp to streptomycin
<b>Quinolones</b> Examples: ciprofloxacin, levofloxacin, norfloxacin, lomefloxacin	Decreased uptake	<b>Alterations in the outer membrane</b> diminishes uptake of drug and/or activation of an "efflux" pump that removes quinolones before intracellular concentration is sufficient for inhibiting DNA metabolism.	Resistance of Gram negative and staphylococci (efflux mechanism only) to various quinolones
	Altered target	<b>Changes in DNA gyrase subunits</b> decrease the ability of quinolones to bind this enzyme and interfere with DNA processes	Gram negative and Gram positive resistance to various

## ANTIBIOTIC MODES OF ACTION AND BACTERIAL MECHANISMS OF RESISTANCE

	 <p>ANTIBIOTIC MODE OF ACTION</p>	 <p>BACTERIAL MECHANISM OF RESISTANCE</p>
Beta-Lactams	Target and bind to penicillin-binding proteins (PBPs), inhibiting bacterial cell wall synthesis.	<ul style="list-style-type: none"> <li>- Enzymatic destruction of beta-lactam rings</li> <li>- Target (PBP) modification</li> <li>- Reduced intracellular accumulation</li> </ul>
Glycopeptides	Inhibit the last stages of cell wall assembly by preventing cross-linking reactions.	<ul style="list-style-type: none"> <li>- Target modification</li> <li>- Production of false targets</li> </ul>
Quinolones	Targets DNA gyrase and topoisomerase IV of the bacteria and inhibit the necessary step of supercoiling.	<ul style="list-style-type: none"> <li>- Target modification</li> <li>- Reduced intracellular accumulation</li> </ul>
Aminoglycosides	Target and bind to the 30s ribosomal subunit to cause misreading of the genetic code which results in inhibition of protein synthesis	<ul style="list-style-type: none"> <li>- Antibiotic (structural) modification</li> <li>- Target modification</li> <li>- Reduced uptake</li> </ul>
Macrolides	Target and bind to 50S ribosomal subunit to inhibit translocation and transpeptidation process, resulting in inhibition of protein synthesis	<ul style="list-style-type: none"> <li>- Reduced intracellular uptake</li> <li>- Target modification</li> </ul>
Tetracyclines	Target and bind to 30S ribosomal subunit to prevent aminoacyl-tRNA to attach to RNA-ribosome complex, inhibiting protein synthesis.	<ul style="list-style-type: none"> <li>- Reduced intracellular accumulation</li> <li>- Target modification</li> </ul>
Rifampicins	Interacts with the beta-subunit of the bacterial RNA polymerase to block RNA synthesis.	<ul style="list-style-type: none"> <li>- Target modification</li> </ul>
Sulfonamides	Targets dihydropteroate synthase (DHPS) and prevents addition of para-aminobenzoic acid (PABA), inhibiting folic acid synthesis	<ul style="list-style-type: none"> <li>- Target modification</li> </ul>

### Molecular mechanisms of resistance

The abilities of bacterial organisms to utilize the various strategies to resist antimicrobial compounds are all genetically encoded. [Intrinsic resistance](#) is that type of resistance which is naturally coded and expressed by all (or almost all) strains of that particular bacterial species. An example of intrinsic resistance is the natural resistance of anaerobes to aminoglycosides and Gram-negative bacteria against vancomycin.

Changes in bacterial genome through mutation or horizontal gene acquisition, on the other hand, may consequently lead to a change in the nature of proteins expressed by the organism. Such change may lead to an alteration in the structural and functional features of the bacteria involved, which may result in changes leading to resistance against a particular antibiotic. This is referred to as [acquired resistance](#), which is limited to selected isolates of that particular species or group of microorganisms.

For example, we know that methicillin resistance of *Staphylococcus aureus* is primarily due to changes that occur in the penicillin binding protein (PBP), which is the protein which beta-lactam antibiotics bind and inactivate to consequently inhibit cell wall synthesis. This change is actually rendered by the expression of a certain *mecA* gene in some strains of these bacteria, which is hypothesized to have been induced by the excessive use of penicillin. Expression of this *mecA* gene results in an alternative PBP (PBP2a) that has a low affinity for most  $\beta$ -lactam antibiotics, thereby allowing these strains to replicate in the presence of methicillin and related antibiotics.

Some antimicrobial resistance is brought about by multiple changes in the bacterial genome. For example, Isoniazid resistance of *Mycobacterium tuberculosis* results from changes in the following genes: *katG* gene which encodes a catalase; *inhA* gene which is the target for isoniazid; the *oxyR* gene and neighboring *aphC* gene and their intergenic region.

#### Biological Versus Clinical Resistance

Biological resistance refers to changes that result in the organism being less susceptible to a particular antimicrobial agent than has been previously observed. When antimicrobial susceptibility has been lost to such an extent that the drug is no longer effective for clinical use, the organism is then said to have achieved clinical resistance. It is important to note that often, biologic resistance and clinical resistance do not necessarily coincide. From a clinical laboratory and public health perspective it is important to realize that biologic development of antimicrobial resistance is an ongoing process, while clinical resistance is dependent on current laboratory methods and established cut-offs. Our inability to reliably detect all these processes with current laboratory procedures and criteria should not be perceived as evidence that they are not occurring. (Forbes et al., 1998)

## Intrinsic Resistance

Intrinsic resistance is the innate ability of a bacterial species to resist activity of a particular antimicrobial agent through its inherent structural or functional characteristics, which allow tolerance of a particular drug or antimicrobial class. This can also be called “insensitivity” since it occurs in organisms that have never been susceptible to that particular drug. Such natural insensitivity can be due to:

- lack of affinity of the drug for the bacterial target
- inaccessibility of the drug into the bacterial cell
- extrusion of the drug by chromosomally encoded active exporters
- innate production of enzymes that inactivate the drug

**Table 2.2** Examples of intrinsic resistance and their respective mechanisms (From Forbes et al., 1998, Giguere et al., 2006)

ORGANISMS	NATURAL RESISTANCE AGAINST:	MECHANISM
Anaerobic bacteria	Aminoglycosides	Lack of oxidative metabolism to drive uptake of aminoglycosides
Aerobic bacteria	Metronidazole	Inability to anaerobically reduce drug to its active form
Gram-positive bacteria	Aztreonam (a beta-lactam)	Lack of penicillin binding proteins (PBPs) that bind and are inhibited by this beta lactam antibiotic
Gram-negative bacteria	Vancomycin	Lack of uptake resulting from inability of vancomycin to penetrate outer membrane
<i>Klebsiella</i> spp.	Ampicillin (a beta-lactam)	Production of enzymes (beta-lactamases) that destroy ampicillin before the drug can reach the PBP targets
<i>Stenotrophomonas maltophilia</i>	Imipenem (a beta-lactam)	Production of enzymes (beta lactamases) that destroy imipenem before the drug can reach the PBP targets.
Lactobacilli and <i>Leuconostoc</i>	Vancomycin	Lack of appropriate cell wall precursor target to allow vancomycin to bind and inhibit cell wall synthesis
<i>Pseudomonas aeruginosa</i>	Sulfonamides, trimethoprim, tetracycline, or chloramphenicol	Lack of uptake resulting from inability of antibiotics to achieve effective intracellular concentrations
Enterococci	Aminoglycosides	Lack of sufficient oxidative metabolism to drive uptake of aminoglycosides
	All cephalosporins	Lack of PBPs that effectively bind and are inhibited by these beta lactam antibiotics

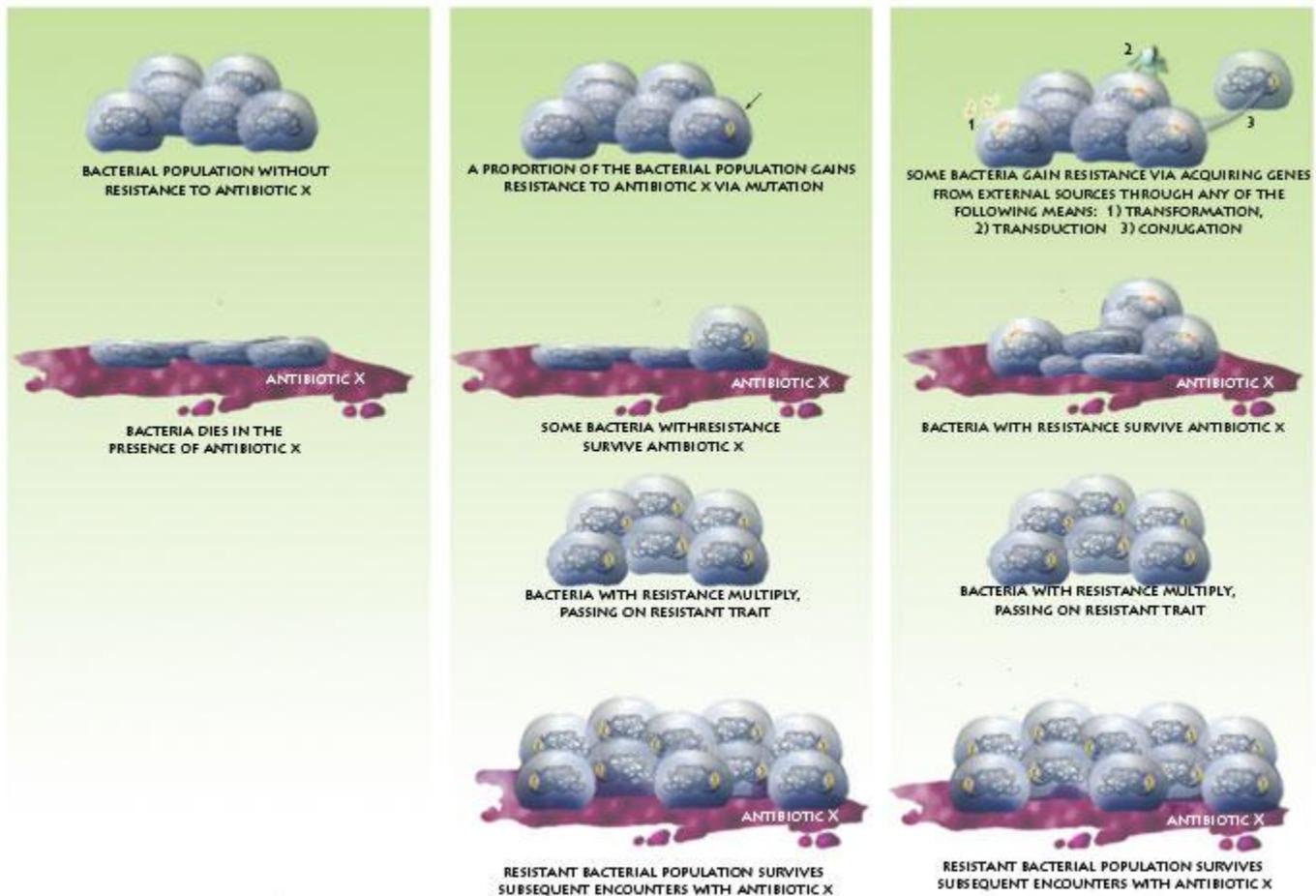
### Clinical implications: Intrinsic Resistance

Knowledge of the intrinsic resistance of a pathogen of concern is important in practice to avoid inappropriate and ineffective therapies. For bacterial pathogens which are naturally insensitive to a large number of classes of antimicrobials, such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*, this consideration can pose a limitation in the range of options for treatment and thus consequently further increase the risk for emergence of acquired resistance.

## Acquired Resistance

Acquired resistance is said to occur when a particular microorganism obtains the ability to resist the activity of a particular antimicrobial agent to which it was previously susceptible. This can result from the mutation of genes involved in normal physiological processes and cellular structures, from the acquisition of foreign resistance genes or from a combination of these two mechanisms.

Unlike intrinsic resistance, traits associated with acquired resistance are found only in some strains or subpopulations of each particular bacterial species. Laboratory methods are therefore needed to detect acquired resistance in bacterial species that are not intrinsically resistant. These same methods are used for monitoring rates of acquired resistance as a means of combating the emergence and spread of acquired resistance traits in pathogenic and non-pathogenic bacterial species. Acquired resistance results from successful gene change and/or exchange that may involve: mutation or horizontal gene transfer via transformation, transduction or conjugation.



## Acquired Resistance

Table 2.3 Examples of acquired resistance through mutation and horizontal gene transfer

ACQUIRED RESISTANCE THROUGH:	RESISTANCE OBSERVED	MECHANISM INVOLVED
Mutations	<i>Mycobacterium tuberculosis</i> resistance to rifamycins	Point mutations in the rifampin-binding region of <i>rpoB</i>
	Resistance of many clinical isolates to fluoroquinolones	Predominantly mutation of the quinolone-resistance-determining-region (QRDR) of GyrA and ParC/GrlA
	<i>E.coli</i> , <i>Haemophilus influenzae</i> resistance to trimethoprim	Mutations in the chromosomal gene specifying dihydrofolate reductase
Horizontal gene transfer	<i>Staphylococcus aureus</i> resistance to methicillin (MRSA)	Via acquisition of <i>mecA</i> genes which is on a mobile genetic element called “staphylococcal cassette chromosome” (SCCmec) which codes for penicillin binding proteins (PBPs) that are not sensitive to $\beta$ -lactam inhibition
	Resistance of many pathogenic bacteria against sulfonamides	Mediated by the horizontal transfer of foreign <i>fofP</i> genes or parts of it
	<i>Enterococcus faecium</i> and <i>E. faecalis</i> resistance to vancomycin	Via acquisition of one of two related gene clusters VanA and Van B, which code for enzymes that modify peptidoglycan precursor, reducing affinity to vancomycin.

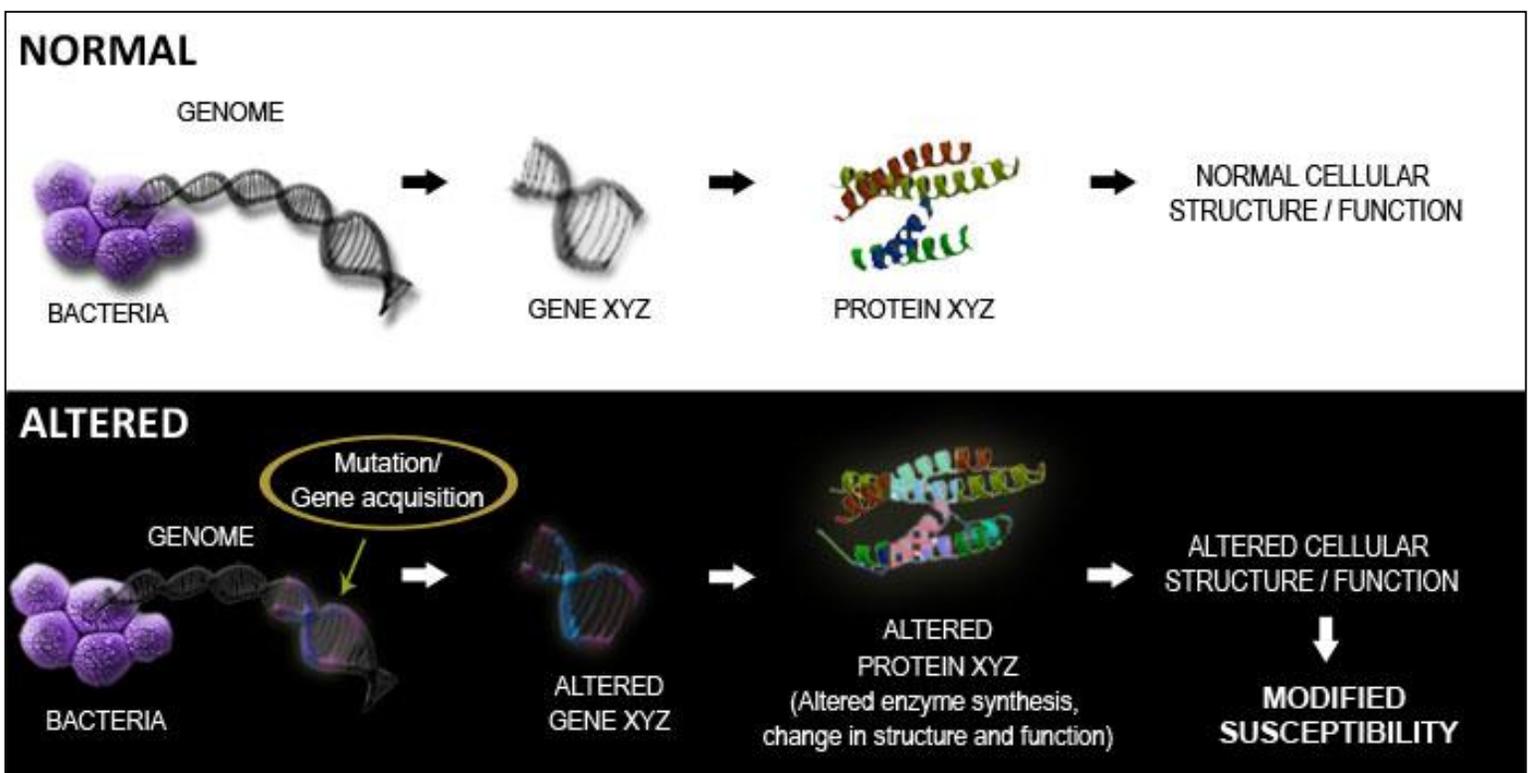
**Antibiotics exert selective pressure on bacterial populations**, killing susceptible bacteria while allowing strains with resistance to that particular antibiotic to survive and multiply. Traits for such resistance are then vertically passed on to daughter cells, subsequently creating a resistant population which can then spread and be further sources of resistance genes for other strains.

Because resistance traits are not naturally eliminated or reversed, resistance to a variety of antibiotics may be accumulated over time. This can lead to strains with multiple drug resistance, which are more difficult to kill due to reduced treatment options.

### Mutation

A **mutation** is a spontaneous change in the DNA sequence within the gene that may lead to a change in the trait which it codes for. Any change in a single base pair may lead to a corresponding change in one or more of the amino acids for which it codes, which can then change the enzyme or cell structure that consequently changes the affinity or effective activity of the targeted antimicrobials.

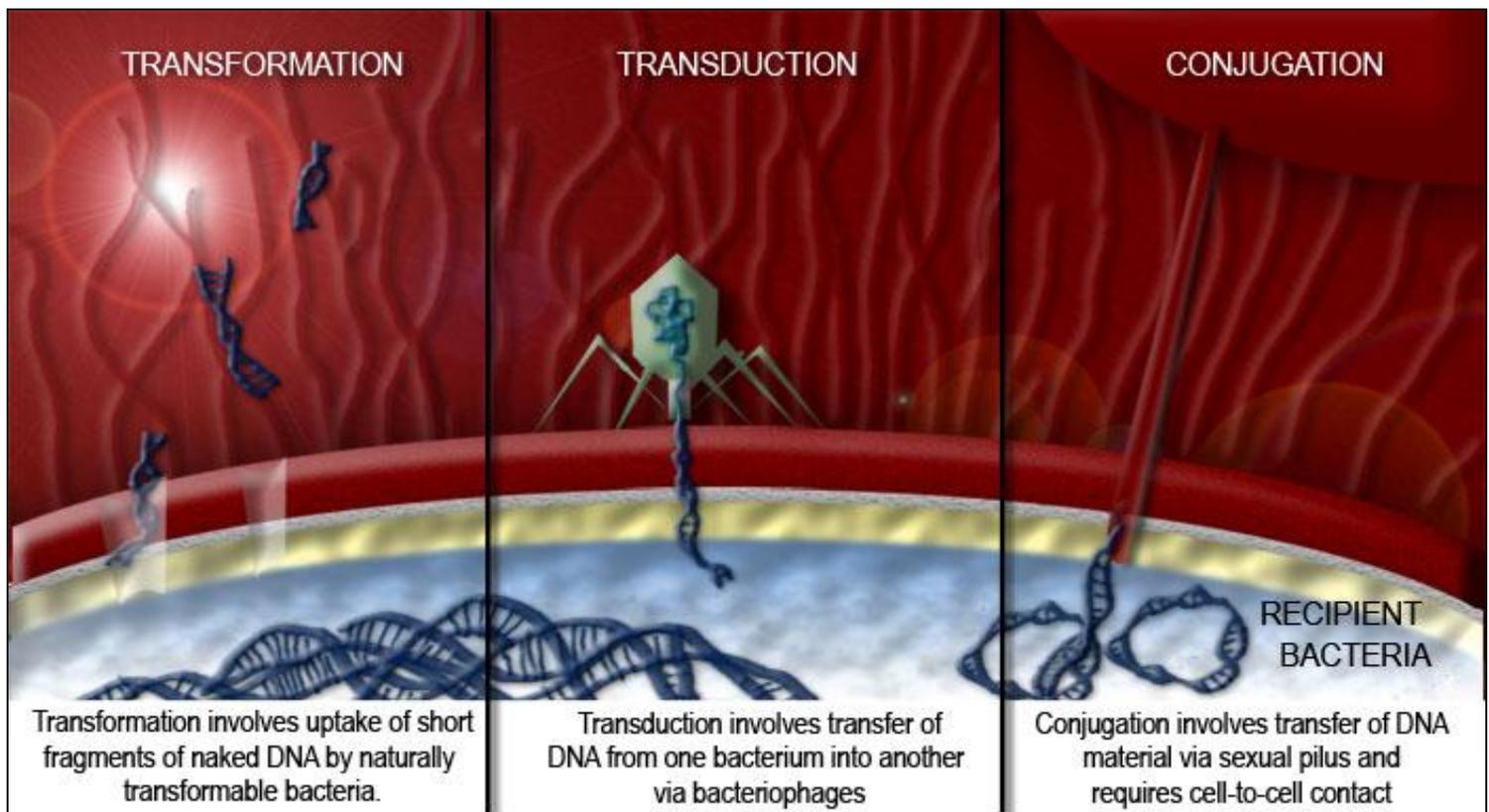
In prokaryotic genomes, mutations frequently occur due to base changes caused by exogenous agents, DNA polymerase errors, deletions, insertions and duplications. For prokaryotes, there is a constant rate of spontaneous mutation of about 0.0033 mutations per DNA replication that is relatively uniform for a diverse spectrum of organisms. The mutation rate for individual genes varies significantly among and within genes (Gillespie, 2002).



### Horizontal Gene Transfer

**Horizontal gene transfer**, or the process of swapping genetic material between neighboring “contemporary” bacteria, is another means by which resistance can be acquired. Many of the antibiotic resistance genes are carried on plasmids, transposons or integrons that can act as vectors that transfer these genes to other members of the same bacterial species, as well as to bacteria in another genus or species. Horizontal gene transfer may occur via three main mechanisms: transformation, transduction or conjugation.

**Transformation** involves uptake of short fragments of naked DNA by naturally transformable bacteria. **Transduction** involves transfer of DNA from one bacterium into another via bacteriophages. **Conjugation** involves transfer of DNA via sexual pilus and requires cell-to-cell contact. DNA fragments that contain resistance genes from resistant donors can then make previously susceptible bacteria express resistance as coded by these newly acquired resistance genes.



#### Trivia: Conjugation and Plasmids

Conjugation was first described in 1946 by Lederberg and Tatum, based on studies showing that the intestinal bacteria *E. coli* uses a process resembling sex to exchange circular, extrachromosomal elements, now known as plasmids. (Torrence and Isaacson, 2003)

## Detecting antimicrobial resistance

Historically, veterinary practitioners prescribed antibiotics based on expected mode of action, spectrum of activity and clinical experience. With the emergence and spread of antimicrobial resistance, treatment of bacterial infections has become increasingly difficult and is no longer as straightforward as it was many years prior. Practitioners now need to consider that the particular pathogen they wish to treat may be resistant to some or all of the available antibiotics, thus making antimicrobial susceptibility testing a standard procedure.

Antimicrobial susceptibility testing methods are in vitro procedures used to detect antimicrobial resistance in individual bacterial isolates. Because these laboratory detection methods can determine resistance or susceptibility of an isolate against an array of possible therapeutic candidates, antimicrobial susceptibility testing results can be a useful clinical guideline in selecting the best antibiotic treatment option for each particular patient. These same methods can also be used for monitoring the emergence and spread of resistant microorganisms in the population.

Clinical Breakpoints are threshold values established for each pathogen-antibiotic (i.e., bug-drug) combination indicating at what level of antibiotic the isolate should be considered to be sensitive, intermediate or resistant. The interpretative criteria for these are based on extensive studies that correlate laboratory resistance data with serum achievable levels for each antimicrobial agent and a history of successful and unsuccessful therapeutic outcomes. Although veterinary laboratories originally based interpretations on standards established using human pathogens, it became apparent by the early 1980s that such an approach did not reliably predict clinical outcomes when applied to veterinary practice. Subsequently, groups within organizations that set standards were created for the purpose of developing veterinary-specific standards<sup>28</sup>.

Standard conditions for these assays have been established based on extensive batteries of laboratory testing. Guidelines and recommendations for these are continuously updated by certain organizations worldwide, such as CLSI, EUCAST, OIE, BSAC, SFM, SRGA and CDS (see box, right). Of these, those which specify antimicrobial testing methods and interpretative criteria for veterinary pathogens are: the CLSI in the USA, OIE in EU and CDS-AST in Australia<sup>26, 28</sup>.

### Organizations Publishing AST STANDARDS<sup>28</sup>

**United States:** Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS)  
[www.clsi.org](http://www.clsi.org)

**European Union:** European Committee of Antimicrobial Susceptibility Testing (EUCAST)  
[www.eucast.org](http://www.eucast.org)

**European Union:** Office International des Épizooties (OIE)  
[www.oie.int](http://www.oie.int)

**United Kingdom:** British Society for Antimicrobial Chemotherapy  
[www.bsac.org.uk](http://www.bsac.org.uk)

**Germany:** Deutsches Institut für Normung  
[www.beuth.de](http://www.beuth.de)

**France:** Société Française de Microbiologie  
[www.sfm.assoc.fr](http://www.sfm.assoc.fr)

**Sweden:** Swedish Reference Group for antibiotics  
[www.srga.org](http://www.srga.org)

**Australia:** CDS disk diffusion method  
[www.med.unsw.edu.au/pathology-cds](http://www.med.unsw.edu.au/pathology-cds)

### Lab approaches and strategies

Some points to consider when deciding whether or not to conduct antimicrobial susceptibility testing should include:

- clinical relevance of the isolate
- purity of the isolate
- logical panel of antimicrobial agents to be tested (i.e., do not include antibiotics to which the isolate is known to have intrinsic resistance)
- availability of test methodology, resources, and trained personnel
- standardization of testing
- valid interpretation of results
- cost efficiency
- effective means to communicate results and interpretation to end-users

Most often, interpretation is reduced to whether the isolate is classified as susceptible, intermediately susceptible, or resistant to a particular antibiotic.. It should, however, be remembered that these *in vitro* procedures are only approximations of *in vivo* conditions which can be very different depending on the nature of the drug, the nature of the host and the conditions surrounding the interaction between the antibiotic and the target pathogen. One critical aspect is following standardized procedures that can generate reproducible results, i.e. quality control. Aspects of quality control include:

- standardized bacterial inoculum size
- culture conditions (growth medium, pH, cation concentration)
- blood and serum supplements and thymidine content)
- incubation conditions (atmosphere, temperature, duration)
- concentration of antimicrobials for testing.

Because of the required culture time, antimicrobial susceptibility testing may take several days, which is not ideal particularly in critical clinical cases demanding urgency. Often, practitioners may utilize locally established antibiograms as guideline for therapy. An antibiogram is a compiled susceptibility report or table of commonly isolated organisms in a particular hospital, farm, or geographic area, which can serve as a useful guideline in therapy before actual culture and susceptibility data becomes available for reference.

### Test Methods in Detecting Antimicrobial Resistance

There are several antimicrobial susceptibility testing methods available today, and each one has their respective advantages and disadvantages. They all have one and the same goal, which is to provide a reliable prediction of whether an infection caused by a bacterial isolate will respond therapeutically to a particular antibiotic treatment. This data may be utilized as guidelines for chemotherapy, or at the population level as indicators of emergence and spread of resistance based on passive or active surveillance. Some examples of antibiotic sensitivity testing methods are:

- Dilution method (broth and agar dilution method)
- Disk-diffusion method
- E-test
- Automated methods
- Mechanism-specific tests such as beta-lactamase detection test and chromogenic cephalosporin test
- Genotypic methods such as PCR and DNA hybridization methods

Selection of the appropriate method will depend on the intended degree of accuracy, convenience, urgency, availability of resources, availability of technical expertise and cost.. Interpretation should be based on veterinary standards whenever possible, rather than on human medical standards, which may not always be applicable. Among these available tests, the two most commonly used methods in veterinary laboratories are the agar disk-diffusion method and the broth microdilution method.

## Examples of Antibiotic Sensitivity Testing Methods

### 1. DILUTION METHODS

The Broth dilution method involves subjecting the isolate to a series of concentrations of antimicrobial agents in a broth environment. Microdilution testing uses about 0.05 to 0.1 ml total broth volume and can be conveniently performed in a microtiter format. Macrodilution testing uses broth volumes at about 1.0 ml in standard test tubes. For both of these broth dilution methods, the lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) is recorded as the minimal inhibitory concentration or MIC. The MIC is thus the minimum concentration of the antibiotic that will inhibit this particular isolate. The test is only valid if the positive control shows growth and the negative control shows no growth.

A procedure similar to broth dilution is agar dilution. Agar dilution method follows the principle of establishing the lowest concentration of the serially diluted antibiotic concentration at which bacterial growth is still inhibited.

### 2. DISK DIFFUSION METHOD

Because of convenience, efficiency and cost, the disk diffusion method is probably the most widely used method for determining antimicrobial resistance in private veterinary clinics.

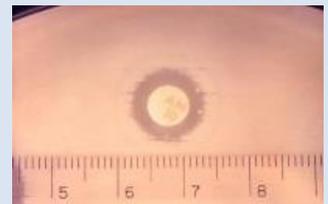
A growth medium, usually Mueller-Hinton agar, is first evenly seeded throughout the plate with the isolate of interest that has been diluted at a standard concentration (approximately  $1$  to  $2 \times 10^8$  colony forming units per ml). Commercially prepared disks, each of which are pre-impregnated with a standard concentration of a particular antibiotic, are then evenly dispensed and lightly pressed onto the agar surface. The test antibiotic immediately begins to diffuse outward from the disks, creating a gradient of antibiotic concentration in the agar such that the highest concentration is found close to the disk with decreasing concentrations further away from the disk. After an overnight incubation, the bacterial growth around each disc is observed. If the test isolate is susceptible to a particular antibiotic, a clear area of “no growth” will be observed around that particular disk.

The zone around an antibiotic disk that has no growth is referred to as the zone of inhibition since this approximates the minimum antibiotic concentration sufficient to prevent growth of the test isolate. This zone is then measured in mm and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediately susceptible or resistant. MIC measurement cannot be determined from this qualitative test, which simply classifies the isolate as susceptible, intermediate or resistant.



On this agar plate, a bacterial isolate is tested for resistance to each of twelve different antibiotics. The clear zones around each disc are the zones of inhibition that indicate the extent of the test organism's inability to survive in the presence of the test antibiotic. (A) The disk shows a large zone of inhibition; whereas (B) shows no zone of inhibition, indicating resistance of the isolate to the test antibiotic

Presence of zone of inhibition is not automatically interpreted as susceptibility to the antibiotic; the zone width has to be measured and compared against a reference standard which contains measurement ranges and their equivalent qualitative categories of susceptible, intermediately susceptible or resistant.



For example, this *E.coli* isolate on the right has a zone of inhibition of 10.1mm around ampicillin (AM); since the zone diameter interpretation chart is as follows:

- Resistant: 13mm or less
- Intermediate: 14-16 mm
- Susceptible: 17 mm or more

This particular *E.coli* isolate is read as resistant to ampicillin.

### **3. E-TEST**

E-test (AB Biodisk, Solna, Sweden) is a commercially available test that utilizes a plastic test strip impregnated with a gradually decreasing concentration of a particular antibiotic. The strip also displays a numerical scale that corresponds to the antibiotic concentration contained therein. This method provides for a convenient quantitative test of antibiotic resistance of a clinical isolate. However, a separate strip is needed for each antibiotic, and therefore the cost of this method can be high.

### **4. AUTOMATED ANTIMICROBIAL SUSCEPTIBILITY TESTING SYSTEMS**

Several commercial systems have been developed that provide conveniently prepared and formatted microdilution panels as well as instrumentation and automated reading of plates. These methods are intended to reduce technical errors and lengthy preparation times.

Most automated antimicrobial susceptibility testing systems provide automated inoculation, reading and interpretation. These systems have the advantage of being rapid (some results can be generated within hours) and convenient, but one major limitation for most laboratories is the cost entailed in initial purchase, operation and maintenance of the machinery. Some examples of these include: Vitek System (bioMerieux, France), Walk-Away System (Dade International, Sacramento, Calif.), Sensititre ARIS (Trek Diagnostic Systems, East Grinstead, UK), Avantage Test System (Abbott Laboratories, Irving, Texas), Micronaut (Merlin, Bornheim-Hesel, Germany), Phoenix (BD Biosciences, Maryland) and many more.

### **5. MECHANISM-SPECIFIC TESTS**

Resistance may also be established through tests that directly detect the presence of a particular resistance mechanism. For example, beta lactamase detection can be accomplished using an assay such as the chromogenic cephalosporinase test (Cefinase disk by BD Microbiology Systems, Cockeysville, MD and BBL DrySlide Nitrocefin, Becton Dickinson, Sparks, MD) and detection for chloramphenicol modifying enzyme chloramphenicol acetyltransferase (CAT) may utilize commercial colorimetric assays such as a CAT reagent kit (Remel, Lenexa, Kansas).

### **6. GENOTYPIC METHODS**

Since resistance traits are genetically encoded, we can sometimes test for the specific genes that confer antibiotic resistance. However, although nucleic acid-based detection systems are generally rapid and sensitive, it is important to remember that the presence of a resistance gene does not necessarily equate to treatment failure, because resistance is also dependent on the mode and level of expression of these genes<sup>11</sup>.

Some of the most common molecular techniques utilized for antimicrobial resistance detection are as follows:

- Polymerase chain reaction (PCR) is one of the most commonly used molecular techniques for detecting certain DNA sequences of interest. This involves several cycles of denaturation of sample DNA, annealing of specific primers to the target sequence (if present), and the extension of this sequence as facilitated by a thermostable polymerase leading to replication of a duplicate DNA sequence, in an exponential manner, to a point which will be visibly detectable by gel electrophoresis with the aid of a DNA-intercalating chemical which fluoresces under UV light.
- DNA hybridization. This is based on the fact that the DNA pyrimidines (cytosine and thymidine) specifically pair up with purines (guanine and adenine; or uracil for RNA). Therefore, a labeled probe with a known specific sequence can pair

up with opened or denatured DNA from the test sample, as long as their sequences complement each other. If this “hybridization” occurs, the probe labels this with a detectable radioactive isotope, antigenic substrate, enzyme or chemiluminescent compound. Whereas if no target sequence is present or the isolate does not have the specific gene of interest, no attachment of probes will occur, and therefore no signals will be detected.

- Modifications of PCR and DNA hybridization. With these basic principles, several modifications have been introduced which further improve the sensitivity and specificity of these standard procedures. Examples of such development were the use of 5'-fluorescence-labeled oligonucleotides, the development of molecular beacons, development of DNA arrays and DNA chips, among many others.

## Module Key Points

- Antimicrobial resistance is the ability of a microorganism to survive and multiply in the presence of an antimicrobial agent that would normally inhibit or kill this species of microorganism.
- It is not a new phenomenon, but in the recent years the global increase in incidence and prevalence of antimicrobial resistance has raised concerns. More bacterial pathogens have also developed multiple drug resistance, severely limiting therapeutic options for infections in both animals and people.
- Bacteria are able to resist the effects of antimicrobials through preventing intracellular access, immediately removing antimicrobial substances through efflux pumps; modifying the antimicrobial agent through enzymatic breakdown or modifying the antimicrobial targets within the bacterial cell to render the substance ineffective. Successful development of resistance often results from a combination of two or more of these strategies
- Antimicrobial resistance traits are genetically coded, and can either be intrinsic or acquired.
- Intrinsic resistance is due to innately coded genes which create natural “insensitivity” to a particular antibiotic. Innate resistance is normally expressed by virtually all strains of that particular bacterial species.
- Acquired resistance is gained by previously susceptible bacteria either through mutation or horizontally obtained from other bacteria possessing such resistance via transformation, transduction or conjugation. Acquired resistance is limited to subpopulations of a particular bacterial species and may result from selective pressure exerted by antibiotic usage.
- Antimicrobial susceptibility testing (AST) methods are in vitro procedures used to detect antimicrobial resistance in individual bacterial isolates. Because these laboratory detection methods can determine resistance or susceptibility of an isolate against an array of possible therapeutic candidates, AST results can be a useful guideline in selecting the best antibiotic treatment option for each particular patient.
- Examples of AST methods are: broth (and agar) dilution methods, disk-diffusion test, E-test, automated detection using various commercially available detection kits, mechanism-specific methods such as those which detect specific enzymes that bring about resistance, and by applying genotypic methods which detect antibiotic resistance genes.

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