

# MULTIPLE DRUG RESISTANCE

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## Introduction

Bacteria are ubiquitous in the environment, including on the skin and mucous membranes, and within the gastrointestinal tracts of animals and people. Their ubiquity is due, in part, to their ability to survive hostile conditions and their amazing capacity to adapt to changes in the environment. Clearly, development of antimicrobial resistance is not a recent phenomenon, but an inevitable and irreversible consequence of bacterial cell adaptation. Indeed, the ability of bacteria to transfer resistance to other bacterial species was first described by Lederberg and Lederberg back in 1952, not long after the introduction of sulfonamides and penicillin.

Current concerns relating to antimicrobial resistance stem principally from the rapid rate of development of resistance relative to the slow rate at which new mechanistic groups of antibiotics are introduced and the conviction that development of resistance is significantly accelerated by overuse of antibiotics. Furthermore, the ability of bacteria to acquire and transfer multidrug resistance is alarming. Aside from the medical challenges posed by antimicrobial resistance, it is estimated that resistant bacterial infections increase healthcare costs in the US alone by \$4 billion annually. This problem is not only relevant to human health, but also to the maintenance of animal health and production. Indeed, the two are inextricably linked because of the risk of transfer of multiple resistance from animals to human consumers of food animals, such as beef from feedlot cattle. It is estimated that more than half of all antibiotics manufactured in the US are administered to animals and several high-profile conferences, studies, scientific reports, and federal regulatory initiatives have alleged that such use increases the occurrence of resistant bacterial infections in human consumers. Considering the importance of this issue to veterinary medicine, it is incumbent on the disciplines of internal medicine and pharmacology to play an active role in assessing the risk of antibiotic use in food animals, and to develop strategies to avoid possible adverse consequences of such use. Furthermore, if we are to retard the development and spread of resistance, it is necessary that we understand the mechanisms bacteria employ to resist multiple classes of antibiotics and the processes whereby this capacity is acquired and transferred. That is the goal of this presentation.

## Types of Multiple Drug Resistance

Broadly defined, multiple drug resistance may result from: (1) acquisition of multiple gene operons encoding for multiple mechanisms of resistance; and/or (2) the presence of a single gene operon encoding for a single mechanism that confers resistance to multiple antibiotics. An example of the former is resistance against both penicillin and tetracyclines conferred by the expression of genes encoding for production of beta-lactamases and alteration in the ribosomal target binding site for tetracyclines. The latter usually is due to the presence or absence of outer membrane porins or efflux pumps that are capable of transporting different types of antimicrobial agents. Both porins and efflux pump systems largely determine the permeability of the cell wall to antibiotics and the ability of the drug to achieve inhibitory concentrations at a particular site of action.

## Acquisition and Survival of Multiple Resistance Genes

The problem of multiple drug resistance lies not only in the capacity of individual isolates to resist the actions of many different classes of antibiotics, thus limiting the number of agents available for therapy, but it is due also to the ease with which bacteria acquire such resistance and then transfer it to other previously susceptible strains. Generally, antibiotic exposure will not cause a susceptible strain to mutate to a resistant one, although antibiotics may affect the expression of existing resistance genes. Nevertheless, the likelihood of a resistant mutant arising is still quite high, considering the replication rate of bacteria: mutation rates for resistant phenotypes may be as high as 1 in  $10^6$  to 1 in  $10^9$  divisions. Such chromosomal mutations generally result in small step-wise increases in resistance.

Once resistance has been acquired by mutation, it can be transferred by several processes:

Transformation. Bacterial incorporation of naked, resistance-carrying DNA that is free in the environment.

Transduction. Resistance-carrying DNA is incorporated within bacteriophages and passed from resistant to sensitive organisms.

Conjugation. Transfer of a resistance-carrying plasmid via an intercellular bridge:

Conjugation is particularly relevant to this topic because of the frequency with which it transfers multiple resistance. The entire process is encoded by a plasmid and involves transfer of the plasmid, or circular extrachromosomal DNA, via an intercellular bridge from a donor to a recipient bacterium. Initial production of a pilus is followed by contact with the recipient and formation of a mating pair, cutting of a single strand and transfer of this strand to the recipient bacterium, replication of both DNA strands and, finally, separation of bacteria. In this manner, multiple resistance genes can be transferred between bacteria of different strains, species, and possibly even genera.

Although these newly acquired resistance genes can be expressed from the plasmid, the stability of such expression may be considerably enhanced by inclusion of these genes in the bacterial chromosome. This often involves a recombination event referred to as transposition.

Transposons are DNA elements that can transpose or hop from one place in DNA to another, from and to plasmids and chromosomes. Referred to as "jumping genes", they allow nonhomologous recombination, thus avoiding the requirement that the DNA strands involved in breaking and rejoining have complementary base pairing. Transposases, encoded by the transposon, cut out donor DNA and insert this in the recipient. Such DNA may include genes for antimicrobial resistance, thus providing a high degree of mobility that enhances the spread and expression of antimicrobial resistance, insuring that the organism is able to adapt rapidly to changes in the environment.

To understand the roles of these mechanisms in transfer of multiple drug resistance, it is necessary that we go one step further and consider the role of integrons. An integron is a genetic unit with a site-specific recombination system that provides for assembly of multiple resistance genes, called cassettes, within single transposons and/or plasmids. Components of an integron include the CS regions, integrases, a promoter, and a cassette receptor site. The integron may accumulate and express several cassettes, which are mobile genetic elements containing an antibiotic resistance gene and an integrase-specific recombination site. Once acquired by random mutation, the collaborative function of conjugation, transposons, and integrons greatly facilitate transfer and expression of multiple drug resistance, thus demonstrating that bacteria have a phenomenal adaptive ability to survive the insult of antibacterial therapy.

Although antimicrobial agents apparently cannot induce mutations to resistant phenotypes, it appears that exposure to these drugs nevertheless promotes development of resistance. Classically, resistance in a bacterial population can be identified by the existence of at least two distinct sub-populations separated on the basis of MIC values. Survival of the relatively resistant sub-population is promoted by exposure to concentrations of antibiotics that inhibit only the susceptible sub-population. Antibiotic exposure not only promotes the survival of drug-resistant pathogenic bacteria, but also increases the population of drug resistant nonpathogenic bystanders, thus increasing the reservoir of resistance in the bacterial population as a whole, raising the odds that this resistance may be spread to pathogenic bacteria by processes such as conjugation.

The following observations have been presented in support of there being a causal relationship between antibiotic exposure and development of resistance (Shales *et al.* (1997) *Clin. Infect. Dis.* 25;584):

- Changes in antimicrobial usage are paralleled by changes in prevalence of resistance.
- Antimicrobial resistance is more prevalent in nosocomial bacterial strains than in those from community-acquired infections.
- During outbreaks of nosocomial infections, patients infected with resistant strains are more likely than control patients to have received prior antimicrobials.
- Areas within hospitals that have the highest rates of antimicrobial resistance also have the highest rates of antimicrobial use.
- Increasing duration of patient exposure to antimicrobials increases the likelihood of colonization with resistant organisms.

Although this causal relationship is generally accepted to exist, the specific dynamics of these interactions have yet to be confirmed experimentally, thus necessitating further research investigation of the issue because it is crucial to the arguments in support of limiting antibiotic use in food animals. Furthermore, the effects of antibiotic withdrawal on prevalence of resistance also need to be studied.

### Multidrug Efflux Pumps

Multidrug efflux pumps have been the focus of considerable research and may have important implications relating to the prophylactic and metaphylactic use of antibiotics in food animals. Two types of systems exist in bacteria: (1) ATP-binding cassette (ABC) pumps, which are similar to P-glycoprotein systems in mammalian cells, have narrower substrate specificities, and appear to be less important with regard to antibiotic resistance; and (2) secondary pumps that are widespread, rely on proton motive force, occur in both gram-positive and gram-negative bacteria, and tend to have rather wide substrate activities. Secondary systems are classified into four major families: the MFS (Major Facilitator Superfamily), SMR (Small Multidrug Resistance family), RND (Resistance-Nodulation-Cell Division family), and MATE (Multidrug And Toxic compound Extrusion family) families. These transporters are well represented in a variety of notable bacterial pathogens that are frequently multidrug resistant, including *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa*. Individual bacterial species may have multiple transporter types, but RND systems appear to occur only in gram-negatives. Generally, multidrug efflux pumps transport drugs only across the inner cytoplasmic membrane, but RND systems span both outer and inner membranes. The RND transporter interacts closely with several other proteins (membrane fusion proteins [MFP] and an outer membrane channel [OM]), all of which are encoded by genes contained in an operon under the control of a regulator gene. In most instances, the

regulator gene acts as a repressor, e.g., AcrR repressor of *E. coli*. RND systems have wide substrate activity, including many different chemical classes and other exogenous agents, such as dyes, and endogenous agents, such as bile salts.

Considerable research effort has been invested in studying multidrug resistance (MDR) systems in bacteria causing infections in humans. Of course, many of these, such as those in *E. coli* and *Pseudomonas*, are relevant also to animal diseases. It appears that these mechanisms may be found also in bacteria that are of exclusive interest to animal health practitioners, such as *Mannheimia haemolytica*. Recent research conducted at Oklahoma State University identified a 45 kDa membrane protein (PmrC) that is recognized by bovine immune serum against live *M. haemolytica*. This discovery led to the identification of an operon consisting of several genes, *pmrR*, *pmrC*, and *pmrD*, the deduced Pmr protein sequences of which are similar to RND family efflux pumps in *Haemophilus influenzae* and *E. coli*. Presence of a MDR system in *M. haemolytica* has not yet been confirmed, but homology with RND systems of other bacteria provides very strong circumstantial evidence of its existence. Ongoing studies involve construction of *pmr*-deficient and over-expressing mutants, which will be used *in vitro* and *in vivo* to study the role of this putative system in antibiotic resistance.

However, further research suggested that this putative RND system in *M. haemolytica* may be less important than initially suspected. Using a number of isolates collected from a single herd of cattle involved in an outbreak of BRD, the presence of the *pmr* operon and its expressed proteins were correlated with antimicrobial resistance patterns. Chromosomal DNA was extracted and the *pmrC* and *pmrR* genes were amplified using appropriate primers. The products were then run on an agarose gel and the bands stained with ethidium bromide. Western blots were also conducted: isolates were cultured and whole cell lysates were run on SDS PAGE, the proteins transferred to a nitrocellulose membrane, and probed with antibodies produced against proteins expressed by *pmrC* and *pmrR* clones (strains of *E. coli* containing the genes on plasmids). Despite a wide variation in MIC values for these isolates, they all were positive for the presence of the genes, and appeared to express the Pmr proteins to a similar degree, suggesting that in this instance differences in antibiotic sensitivity were not related to the presence of the *pmr* operon. These *M. haemolytica* isolates then were studied further by culturing them *in vitro* in the presence of antibiotics: selection of multidrug resistant mutants is generally easily accomplished by culturing bacteria on solid media containing a gradient of antibiotic concentration. Indeed, resistant mutants were isolated that had increased MIC values for a range of different chemical classes of antibiotics. When the protein expressions of the original wild-type and resistant mutants were compared, the absence of an expressed protein in the resistant mutant profiles suggested the loss of an outer membrane porin, the other permeability-associated system relevant to this discussion of multiple drug resistance. Ongoing research will involve comparison of this protein with proteins expressed by other bacteria, to explore whether the protein constitutes part of a recognized outer membrane porin.

### Impact of Multiple Drug Resistance

Multidrug resistance causes increased morbidity, mortality, and virulence of animal infectious diseases as well as increased cost of therapeutic intervention. The possible impact of antimicrobial resistance in food animals on the health of human consumers is the focus of much current attention, particularly development of resistance that may be promoted by prophylactic use of antimicrobials. The assumption has been that as long as agents used prophylactically in animals are not used therapeutically in people, such prophylactic use will not promote an increase in antimicrobial resistant infections in human patients. However, circumstantial evidence, based on the following correlations between resistance of human infections and antimicrobial use in animals, suggests that selection of a resistant genotype by use of a particular agent may confer resistance to a different agent used therapeutically in people:

- Fluoroquinolone resistance in *Campylobacter* and use of enrofloxacin in poultry
- Ceftriaxone resistance in *Salmonella* and use of ceftiofur in cattle
- Synercid (quinupristin/dalfopristin) resistance in enterococci and agricultural use of virginiamycin
- Vancomycin resistance in enterococci and use of avoparcin

Considering the wide substrate activities of multidrug efflux pumps and outer membrane porins, it is conceivable that prophylactic use of one antimicrobial substrate may result in selection of a bacterial population also resistant to other therapeutically important antimicrobials. Clearly, bacteria have the molecular/cellular mechanisms of multidrug resistance necessary for such selection.

### Prevention and Control of Multiple Drug Resistance

Strategies employed to prevent and control multiple drug resistance are similar to those relating to single drug resistance:

- Use combinations of antimicrobial agents when the mutant selection window (difference between the minimum inhibitory concentration [MIC] and mutant prevention concentration [MPC]) is wide.
- When the mutant selection window is narrow, doses that result in tissue concentrations that exceed MPC should be employed.

- Lower antimicrobial consumption by restricting antimicrobial use to well defined indications for control of susceptible target microorganisms.
- Rotate use of different antimicrobial agents.
- Development of pharmacological agents designed to inhibit multidrug resistance systems.
- Institute surveillance measures.

Although the benefit of these strategies has been promoted for some time, only recently has there been a coordinated effort by national governments, producer groups, scientific associations, and biomedical health professions to address the issue. With specific reference to multiple drug resistance and the use of antibiotics in food animals, it is imperative that the veterinary profession and particularly the specialty disciplines of internal medicine and clinical pharmacology play a proactive role in generating and employing scientific data in the development of sound policy.

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