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REVIEW ARTICLE

Use and Misuse of Antimicrobial Drugs in Poultry and Livestock: Mechanisms of Antimicrobial Resistance

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ABSTRACT

Food safety begins on the farm with management practices that contribute to an abundant, safe, and affordable food supply. To attain this goal antimicrobials have been used in all stages of food animal production in the United States and elsewhere around the world at one time or another. Among food–production animals antimicrobials are used for growth promotion, disease prophylaxis or disease treatment, and are generally administered to the entire flock or herd. Over many decades bacteria have become resistant to multiple antimicrobial classes in a cumulative manner. Bacteria exhibit a number of well characterized mechanisms of resistance to antimicrobials that include: 1) modification of the antimicrobial; 2) alteration of the drug target; 3) decreased access of drug to target; and 4) implementation of an alternative metabolic pathway not affected by the drug. The mechanisms of resistance are complex and depend on the type of bacterium involved (e.g. Gram–positive or Gram–negative) and the class of drug. Some bacterial species have accumulated resistance to nearly all antimicrobial classes due to a combination of intrinsic and acquired processes. This has and will continue to lead to clinical failures of antimicrobial treatment in both human and animal medicine.

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INTRODUCTION

Bacteria reside in complex ecological habitats and many commercially available antibiotics were first isolated from soil dwelling, Actinobacteria. Shortly after antibacterial agents were discovered, it was determined that some microorganisms were resistant to their effects (Furuya and Lowy, 2006). Often this was to provide immunity to a strain from its own antibiotic. Alternatively, susceptible bacteria in the same ecological niche acquired various mechanisms to protect themselves from exogenously produced antibiotics. Many self–protection genes were originally present as operons on the bacterial chromosome (D'Costa *et al.*, 2006). The vast array of bacteria in the environment and within animals and humans provides an infinite reservoir of antimicrobial resistance genes (Sommer *et al.*, 2009; Sommer *et al.*, 2010; Forsberg, *et al.*, 2012). There are different origins to the many types of resistance genes in the environment that are now referred to as the antibiotic resistome (D'Costa *et al.*, 2006; Fajardo *et al.*, 2008; Davies and Davies, 2010). Analysis of the antibiotic resistome has predicted the possibility of over 400 putative antibiotic resistance genes (Bailey *et al.*, 2010; Davies and Davies, 2010; Sommer *et al.*, 2010).

Although understanding the biochemical mechanisms of antimicrobial resistance are important, of equal importance is elucidating the ability of these genetic traits to disseminate globally. If a bacterium acquires resistance genes, but resides in a dead end niche where it does not come in contact with a transmissible vector, then it is of little consequence to human or animal populations. However, the extensive use of antimicrobials in food animal production has produced a reservoir for multiple drug resistant (MDR) commensals and pathogens in the gastrointestinal tracts of food animals (Alexander *et al.*, 2011; Sparo *et al.*, 2012). Many of the antimicrobial resistant bacteria do not cause disease in the food animals, but are pathogenic to humans and may be transmitted to humans via a fecal to oral route. Many epidemiological studies have been done to demonstrate the progression of pathogens through the food chain to humans (Bywater 2005; Hauser *et al.*, 2010; Depoorter *et al.*, 2012; Gomes-Neves *et al.*, 2012; Merchant *et al.*, 2012). Antimicrobial

resistance gene transfer has been experimentally demonstrated in the gastrointestinal tracts of poultry (Poole *et al.*, 2006), lesser mealworm beetles and larvae that infest poultry houses (Crippen and Poole 2009; Poole and Crippen, 2009). This was shown by introducing both recipient and donor strains to the target host. Therefore, in order to mitigate dissemination, all reservoirs need to be identified and controlled if not eliminated.

Mechanisms of dissemination: Mobile genetic elements have provided a rapid method of dissemination of antimicrobial resistance genes at the molecular level. The ease by which multiple drug resistance has emerged exemplifies the genetic fluidity that exists within microbial communities and represents an important mechanism that drives bacterial evolution. Many antimicrobial resistance genes that were chromosomal in origin have been translocated to mobile genetic elements such as broad–host range plasmids, transposons, integrons and phage, with plasmids playing the most significant role in horizontal dissemination (Smillie *et al.*, 2010).

Plasmids are covalently closed circular pieces of DNA that replicate independently from the bacterial chromosome and may be transferred across genus and species boundaries (Carattoli, 2009). They vary in size from a single kilobase to 250 kilobases or more. It is estimated that 25% of known plasmids are conjugative, 25% are mobilizable and 50% are non–mobilizable (Smillie *et al.*, 2010). Most of the very large plasmids are non–mobilizable, they carry many essential genes, and may be considered secondary chromosomes (Smillie *et al.*, 2010). Horizontal transfer of plasmids, and other macromolecules, to other bacterial cells is mediated by bacterial type IV secretion systems (T4S) (Christie *et al.*, 2005; Frost *et al.*, 2005). During the transfer process a nucleoprotein particle that is covalently bound to the DNA to be transferred is formed (Christie *et al.*, 2005). If this process is interrupted the plasmid may not be transferred.

Plasmids may have the capacity to replicate in different genera, but in the Enterobacteriaceae plasmids with related replication elements cannot replicate in the same cell. This characteristic was used to classify this group of plasmids based on their compatibility/ incompatibility (Inc) groups (Couturier *et al.*, 1988). Multiple replication elements can exist on the same plasmid or on different plasmids in the same cell if they are unrelated. Until 2005 determining the classification of a plasmid was done by the labor intensive method of Southern blot hybridization (Couturier *et al.*, 1988). In 2005, a PCR–based replicon typing method was developed that greatly simplified plasmid typing in Enterobacteriaceae (Carattoli *et al.*, 2005). This technique has greatly facilitated knowledge in the epidemiology of Inc plasmid dissemination. Certain Inc types are now associated with multiple drug resistance and foodborne disease outbreaks (Lindsey *et al.*, 2009). The IncA/C replicons are associated with extended–spectrum β–lactamases (ESBLs) and Ampicillin C family (AmpC) of β–cephalosporinases. The AmpC β CMY–2 (bla_{CMY-2}) is prevalent among *Salmonella enterica* serovars and *Escherichia coli* in the United States (Lindsey *et al.*, 2009; Poole *et al.*, 2009; Call *et al.*, 2010). *Salmonella enterica* Newport MDR–AmpC, was first reported as an outbreak in the United States in 2002 (CDC, 2002) and has been recognized as an epidemic strain in humans and animals and has spread across the United States in the last decade (Cobbold *et al.*, 2006).

The development of on–farm management methods that would decrease the spread of antimicrobial resistance may be developed if one of two mechanisms of dissemination was determined to be the most prevalent: 1) horizontal transfer of resistance genes to other bacterial cells at the molecular level or, 2) clonal expansion of the population of MDR bacterial strains. It seems likely that both mechanisms occur simultaneously.

Flavophospholipol has been shown to inhibit conjugation frequency in vitro (George and Fagerberg, 1984; Poole *et al.*, 2006). *In vivo* flavophospholipol may have inhibited conjugative transfer from the exogenous donor strains plasmids, but it did not prevent the recipient from acquiring naturally occurring plasmids (Poole *et al.*, 2006). This was evident by the presence of gentamicin resistance not present in the donor strain. It would be preferable to find nutritional supplements that could inhibit conjugative transfer that would be low in toxicity and are not antimicrobial in nature.

The IncA/C plasmid has a number of different plasmid backbones (Fricke *et al.*, 2009; Call *et al.*, 2010). In some IncA/C plasmid backbones that are bla_{CMY-2} positive, a bla_{CMY-2}–hyb repeat region has integrated in between *traA* and *traC* genes responsible for conjugative transfer (Fricke *et al.*, 2009; Poole *et al.*, 2009). In *Salmonella* Newport isolated from cattle that carried only the bla_{CMY-2} positive IncA/C plasmid, the plasmid was non–conjugative. In strains that carried bla_{CMY-2} negative IncA/C plasmids, the plasmids were conjugative. Strains possessing the bla_{CMY-2} positive IncA/C plasmids, with certain co–resident plasmids, transferred the deficient IncA/C plasmids with the co–resident plasmid (Poole *et al.*, 2009). This demonstrated that conjugative transfer negative strains of *Salmonella* Newport with bla_{CMY-2} positive IncA/C plasmids were able to spread across the United States in epidemic fashion regardless of its inability to directly transfer its IncA/C plasmid (Poole *et al.*, 2009).

Other mobile genetic elements that may be located on plasmids include transposons and integrons (Stokes and Hall, 1989; Liebert *et al.*, 1999). Transposons can excise themselves from a segment of DNA and integrate into another segment of DNA. This includes transfer to and from chromosomal and plasmid DNA. Transposons are characterized by repeats that flank an intervening section of DNA that contains a transposase, resolvase and antimicrobial resistance genes. The repeat segments may or may not be inverted (Liebert *et al.*, 1999).

Integrative Conjugative Elements (ICEs) and Integrative Mobilizable Elements (IMEs) are elements that integrate into the bacterial chromosome. For in–depth reviews on ICEs and IMEs see (Burrus and Waldor, 2004; Doublet *et al.*, 2005). Recently it has been shown that many of the conjugative IncA/C MDR plasmids specifically mobilize the *Salmonella* Genomic Island 1 (SGI1) (Douard *et al.*, 2010). SGI1 has previously been defined as an IME (Doublet *et al.*, 2005).

Independently mobile DNA elements called integrons are located on many transposons and may transfer to a plasmid or chromosomal location. Integrons encode a RecA–independent site specific integrase that allows

acquisition of gene cassettes that may carry antimicrobial resistance genes (Hall and Collis, 1995). An integron also includes an (*attI*) site necessary for recombinant recognition. MDR Enterobacteriaceae has been highly associated with the presence of Class 1 integrons with multiple integron–associated resistance gene cassettes in the conserved segment (CS) (Martinez-Freijo *et al*., 1998; Leverstein-van Hall *et al*., 2002). *Aeromonas hydrophia* isolated from diarrheic swine was found to have a number of antimicrobial resistance genes including a macrolide inactivation gene cluster downstream of a Class 1 integron. In contrast, *Aeromonas hydrophia* isolates from the White River in Indiana, in the United States possessed Class 1 integrons, but the CS segment did not carry integron–associated resistance gene cassettes or any other genes (Poole *et al*., 2006). This may have been due to a lack of antimicrobial selection pressure in the river.

In addition to dissemination of mobile genetic elements between bacterial species or strains, clonal expansion of antimicrobial resistant isolates is a factor in the epidemic spread of some strains. Clonal expansion of resistant bacteria often occurs when clinical use of antimicrobials disrupts the ecological balance allowing strains exhibiting antimicrobial resistance phenotypes to multiply to a much higher population than would be observed in antimicrobial free environmental niche (Levy, 1997). This may open an ecological niche, shifting the overall ecology. In some cases, the niche may return to its previous bacterial diversity once antimicrobials have been removed. Antimicrobial use may also select for MDR bacteria due to the presence of multiple resistance genes on the same mobile element. Global use of antimicrobials for the last several decades has resulted in the accumulation of multiple antimicrobial resistance traits within single bacterial strains (Glenn *et al*., 2012).

Mechanisms of Resistance: Most antimicrobial drugs act on essential metabolic or structural processes of the bacterial cell, these include: inhibition of cell wall synthesis; nucleic acid replication and synthesis; protein synthesis or disruption of cell wall structure (McDermott *et al*., 2003).

Resistance to antimicrobials fall into two broad categories: intrinsic or acquired. Intrinsic resistance refers to tolerance of an antimicrobial due to the natural physiology of that particular genus or species with regard to the chemical structure of the drug. In some cases this occurs if a bacterial genus or species does not possess the metabolic or structural target for inhibition (McDermott *et al*., 2003). For example, Gram–negative bacteria are intrinsically resistant to glycopeptides (e.g. vancomycin) and macrolides (e.g. tylosin) because these drugs are structurally too large to penetrate the outer membrane that exists among all Gram–negative bacteria. Alternatively, *Enterococci* are intrinsically resistant to cephalosporins due to insufficient binding affinity to penicillin binding proteins (PBPs) (Williamson *et al*., 1985). *Leuconostoc*, *Pediococcus*, *Enterococcus gallinarum* and Lactobacilli are Gram–positive bacteria that possess an alternative pathway for cell wall construction and are resistant to glycopeptides. Intrinsic resistant may be displayed as low level resistance which is the case of *E. gallinarum* to vancomycin (Leclercq *et al*., 1992). The genetic determinants that confer intrinsic resistance are generally located on the bacterial chromosome, since they encode essential structural or biochemical functions for the species, and are not easily transferred horizontally or expressed in other bacterial strains. However, some of genetic determinants capable of expression have become translocated to mobile genetic elements, thus, increasing the spread of resistance.

Acquired resistance is exemplified by two general mechanisms: 1) when a mutation has occurred in a gene, often located on the bacterial chromosome, or 2) when the bacterium acquires exogenous genes on mobile DNA elements as discussed above.

Cross resistance occurs when one mechanism confers resistance to multiple antibiotics or classes of antibiotics. To understand this, and other mechanisms of resistance, it is necessary to understand the biochemical pathways that antimicrobials target.

Modification of the Antimicrobial: Drug modification occurs when an enzyme catalyzes a structural change of the drug such that its mechanism of action is no longer effective for bacterial inhibition. Antibiotic classes that are inhibited by this mechanism include: β–lactams, aminoglycosides, chloramphenicol, streptogramins and macrolides (McDermott *et al*., 2003).

β–lactam antimicrobials act by binding to cell wall synthesizing proteins PBPs which effectively inhibits cell growth. The most commonly encountered mechanism of resistance for β–lactam antibiotics in Gram–negative bacteria is hydrolysis of the β–lactam ring by β–lactamase enzymes (Rice and Bonomo, 2011). Newer β–lactam antimicrobials have been developed that possess an extended spectrum of activity and bacteria have developed resistance to these in the form of ESBLs and AmpC β–cephalosporinases. An extensive family of β–lactamases exist and have been previously described (Bradford, 2001; Bush and Jacoby, 2010; Pfeifer *et al*., 2010; Smet *et al*., 2010). These enzymes have been frequently identified in the microbiota of food producing animals (Smet *et al*., 2010).

There are three mechanisms of aminoglycoside inactivation, these include drug modification by: acetylation, adenylylation and phosphorylation. Each mechanism is represented by a family of multiple enzymes (Shaw *et al*., 1993; Davies and Wright, 1997). Among these are ATP–dependent *O*–phosphorylation by phosphotransferases (APHs), ATP–dependent *O*–adenylation by adenylytransferases or nucleotidyl transferases (ANTs) and acetyl CoA–dependent *N*–acetylation by acetyltransferases (AACs). These enzymes are widespread and have been identified in most Gram–negative and Gram–positive bacteria (Shaw *et al*., 1993; Murray and Shaw, 1997).

Chloramphenicol acetyltransferases (CAT) enzymes are the most frequently observed mechanism of resistance to chloramphenicol identified among pathogens. CAT enzymes catalyze the acetyl–S–CoA–dependent acetylation of chloramphenicol.

Macrolides and lincosamides are structurally distinct; however, they share a similar mechanism of action and spectrum of activity. Lincosamides do not possess the lactone ring that constitutes the macrolide class of

antimicrobials (Leclercq, 2002). Hydrolysis of the lactone ring of macrolides by esterases inhibits the antibiotic and was first observed in 1984 (Barthelemy *et al.*, 1984). The *E. coli ereB* gene encodes erythromycin esterase II that hydrolyzes the lactone ring of erythromycin A and oleandomycin (Ounissi and Courvalin, 1985; Andremont *et al.*, 1986). Resistance to macrolide antimicrobials, but not lincosamides or streptogramins could occur as the result of enzymatic phosphorylation of the drug (Noguchi *et al.*, 1995; Noguchi *et al.*, 2000). Macrolide 2'–phosphotransferase (MPH) has been found in clinical *E. coli* isolates (Noguchi *et al.*, 2000) and *Aeromonas hydrophila* a fish pathogen that can also cause hemolytic uremic syndrome (HUS) in humans (Poole *et al.*, 2006). The macrolide inactivation observed in *E. coli* and *A. hydrophila* MPH exists as a gene cluster including *mphA–mrx–mphR* and is located downstream of a class 1 integron and is present on a TN*21*–like transposon. The presence of the esterases and phosphotransferases in Enterobacteriaceae has little significance because this family is intrinsically resistant to macrolide antimicrobials due to drug efflux transporters (Leclercq, 2002). However, this operon could, if expressed in Gram–positives, confer a more clinically significant resistance to macrolides. *Staphylococcus aureus,* that produces *mphC,* does inactivate macrolide antimicrobials. Nucleotidyltransferases encoded by *lnuA* and *lnuB* produced by *Enterococcus faecium* inactivates streptogramins.

Modification of the Drug Target: Structural modification of the antimicrobial drug target may render the drug ineffective, particularly if binding of the drug to the target is necessary. The interaction between the target molecule and antimicrobial is very specific and small structural changes, induced by point mutations that encode a different amino acid, may affect the binding affinity of the antimicrobial to its target (McDermott *et al.*, 2003; Giedraitiene *et al.*, 2011). A post–translational modification of the target molecule by an enzyme may also reduce the efficacy of the drug. Antimicrobials that are inhibited by target modification include: β–lactams, aminoglycosides, macrolides, lincosamides, streptogramins, quinolones, rifampicin, trimethoprim, tetracyclines, and mupirocin (McDermott *et al.*, 2003).

As previously discussed, hydrolysis of the β–lactam ring of β–lactam antimicrobials is a common resistance mechanism among Gram–negative bacteria; for Gram–positive bacteria, target modification is the most commonly encountered mechanism of resistance for β–lactam antibiotics. Bacteria that produce PBPs that have a reduced binding affinity to β–lactam antibiotics display a resistant phenotype (Rice and Bonomo, 2011). In contrast, it has been long known that enterococci are intrinsically resistant to penicillin due to the production of low–affinity BPBs (Williamson *et al.*, 1985).

Aminoglycosides primarily act by binding to the 16S rRNA that recognizes the aminoacyl–tRNA; this action inhibits bacterial protein synthesis (Magnet and Blanchard, 2004). Target modification by ribosomal mutations or enzymatic modifications of ribosomal components inhibit the action of aminoglycosides (Davies and Wright, 1997). A number of Actinomycetes that produce aminoglycosides also produce 16S rRNA methylases that protect them from the inhibitory effects of the antibiotic (Magnet and Blanchard, 2004). Two rRNA methyltransferases (*rmtA* and *rmtB*) had primarily been identified only in aminoglycoside producing microorganisms until 2003 and 2004 (Magnet and Blanchard, 2004), when they were identified on plasmids–borne transposons in human Gram–negative pathogens (Yokoyama *et al.*, 2003; Doi *et al.*, 2004). By 2010 seven 16S rRNA methyltransferase genes had been identified: *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD, rmtE* and *npmA*.

Cross–resistance to macrolides, lincosamides and streptogramins (MLS) occurs by methylation of the ribosomal target. There are a number of erythromycin ribosome methylase (*erm*) enzymes that confer macrolide resistance to Gram–positive bacteria, spirochetes and anaerobes (Leclercq, 2002).

Tetracyclines inhibit bacterial protein synthesis by preventing the attachment of t–RNA to the ribosome (Chopra and Roberts, 2001). Tetracycline resistance due to target modification is mediated by ribosomal protection proteins (RPP) that represent a widely distributed class of resistance genes (Thaker *et al.*, 2010). There are 11 type of RPPs found among Gram–positive and Gram–negative genera. Tet(O) and Tet(M) are the most prevalent and well–studied. The individual RPP genes include: *tet*(O), *tet*(M), *tet*(S), *tet*(W), *tet*(32), *tet*(32), *tet*(36), *tet*B(P), *otr*(A) and *tet* (Thaker *et al.*, 2010).

Fluoroquinolones are broad spectrum antibiotics that act by inhibiting bacterial DNA replication by binding to two essential enzymes, DNA topoisomerase IV consisting of two subunits of each ParC and ParE and DNA gyrase composed of two subunits of each GyrA and GyrB (Hopkins *et al.*, 2005). Accumulation mutations in *parC* and *parE* and/or *gyrA* and *gyrB* genes, primarily in the quinolone resistance–determining regions, may confer resistance to fluoroquinolones by reducing the binding affinity of the drug (Rice and Bonomo, 2011). Multiple mutations in these chromosomal genes are generally required to confer clinically significant resistance.

In Enterobacteriaceae, plasmid mediated quinolone resistance (PMQR) is encoded by the *qnr* gene and was first identified in an integron–like element (Stokes *et al.*, 1993). In *E. coli*, Qnr protects DNA gyrase from inhibition by ciprofloxacin; however, it does not protect topoisomerase IV (Hopkins *et al.*, 2005).

Decreased Access of Drug to Target: Decreased access to the intracellular drug target is primarily a consequence of active drug efflux. This involves the extrusion of noxious substances out of the cell resulting in sub toxic intracellular concentrations of the antimicrobials. This process may be very broad or narrow in substrate specificity depending on the type of efflux pump. Broad spectrum activity includes the expulsion of dyes and other inhibitory compounds (Nikaido and Pages, 2012). For this reason, expression of efflux proteins often confers resistance to multiple drugs simultaneously. Drug efflux is a highly prevalent mechanism of resistance among bacteria and there are primarily five types. These include: 1) the major facilitator superfamily (MFS); 2) the small multidrug resistance family (SMR); 3) the resistance nodulation cell division family (RND); 4) the ATP binding cassette superfamily (ABC) and 5) the multidrug

and toxic compound extrusion family (MATE). In some cases multiple types of efflux proteins are present in one bacterial strain resulting in high–level resistance when neither protein alone confers resistance (Rice and Bonomo, 2011). Efflux may also enhance other mechanisms of resistance leading to clinically relevant resistance (Nikaido and Pages, 2012). Efflux pumps are usually encoded chromosomally and gene expression is activated by environmental signals or by mutations in regulatory genes that control expression (Levy, 2002). Efflux is active against all clinically relevant antimicrobial classes (Nikaido and Pages, 2012).

Alternative Metabolic Pathway: Some bacteria possess or acquire a different metabolic pathway that by–passes the pathway the antimicrobial inhibits. In enterococci the peptidoglycan component of the cell wall is formed when two molecules of D–Ala–D–Ala are added to UDP–*N*–acetylmuramyl–tripeptide to form the UDP–*N*–acetylmuramyl–pentapeptide. This is subsequently incorporated into the nascent peptidoglycan providing the structure for formation of cross–bridges in the peptidoglycan layer (Cetinkaya, *et al.*, 2000). Glycopeptide antibiotics inhibit cell wall synthesis in Gram–positive by binding to the D–Ala–D–Ala precursor, thus, blocking their addition to the nascent peptidoglycan chain (Cetinkaya *et al.*, 2000). An example of low level intrinsic vancomycin resistance, VanC resistance, is exhibited in motile *Enterococcus casseliflavus, E. gallinarum and E. flavescens*. This is due to the ability to substitute D–Ala–D–Ala with D–Ala–D–Ser at the carboxyl terminus of the peptidoglycan precursor analogues this in turn lowers the affinity for vancomycin (Arthur *et al.*, 1996). These species remain susceptible to teicoplanin (Arthur *et al.*, 1996).

Conclusion: In Agriculture there is a need to employ management strategies that would minimize the use of antimicrobials from farm to fork. Eliminating antimicrobial use in agriculture globally is unlikely. Dissemination of MDR commensal and pathogenic bacteria worldwide is continually reducing the efficacy of available antibiotics. Very few new antibiotics are under production, and for the most part, new antibiotics in the pipeline are derivatives of currently available drugs. It is therefore likely that cross–resistance will render the new drugs less effective. New drugs that target new mechanisms of bacterial cell metabolism are necessary. The antibiotic resistome may provide novel targets for new antimicrobials. One such antimicrobial peptide has recently been discovered by examining the Panda genome (Yan *et al.*, 2012). A cathelicidin–like peptide derived from mining the Panda genome was synthesized and found to possess broad spectrum activity against Gram–negative and Gram–positive bacteria as well as fungi (Alexander *et al.*, 2011).

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