ABSTRACT

Title of Thesis: SOURCES AND OCCURRENCE OF ANTIBIOTIC

RESISTANCE IN THE ENVIRONMENT

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SOURCES AND OCCURRENCE OF ANTIBIOTIC RESISTANCE IN THE
ENVIRONMENT

by

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Introduction

Antibiotics have long been considered the "magic bullet" that would end infectious disease. Although they have improved the health of countless numbers of humans and animals, many antibiotics have also been losing their effectiveness since the beginning of the antibiotic era. Bacteria have adapted defenses against these antibiotics and continue to develop new resistances, even as we develop new antibiotics. In recent years, much attention has been given to the increase in antibiotic resistance. As more microbial species and strains become resistant, many diseases have become difficult to treat, a phenomenon frequently ascribed to both indiscriminate and inappropriate use of antibiotics in human medicine. However, the use of antibiotics and antimicrobials in raising food animals has also contributed significantly to the pool of antibiotic resistant organisms globally and antibiotic resistant bacteria are now found in large numbers in virtually every ecosystem on earth.

There is no doubt that the use of antibiotics provides selective pressure that results in antibiotic resistant bacteria and resistance genes. While some resistant bacteria are found naturally in the environment, pathogens and non-pathogens are released into the environment in several ways, contributing to a web of resistance that includes humans, animals, and the environment, essentially the biosphere. Reviewed here are the history and scope of both antibiotics and resistance, the mechanisms of resistance, and evidence for the spread of antibiotic resistant organisms and resistance genes through humans, animals, and the environment.

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History of antibiotic development

In order to comprehend the problem of antibiotic resistance as it exists today, it is useful to understand the history and development of both antibiotics and antibiotic resistance. Antimicrobial drugs have generally been classified into two categories, one includes the synthetic drugs, such as the sulfonamides and the quinolones, and the second, antibiotics, synthesized by microorganisms. In recent years, increasing numbers of semi-synthetic drugs have been developed which are chemical derivatives of antibiotics, thereby blurring the distinction between synthetic and natural antibiotics.

Interest in antimicrobial chemotherapy was kindled as soon as microorganisms were understood to be agents of infectious disease. In earlier times, plant products were sometimes used successfully in the treatment of disease, but neither doctors nor patients knew the basis for the action of these therapeutic agents. Many early medicines were used to cure protozoan diseases, rather than bacterial diseases. As early as 1619, it was known that malaria could be treated with the extract of cinchona bark (quinine) and that amoebic dysentery could be treated with ipecacuanha root (emetine) (91, 100). Only a few antibacterials, such as mercury, which was used to treat syphilis, were in use when the era of true chemotherapy began.

It was in the early 1900's when Paul Ehrlich first hypothesized that dyes could be used as antimicrobial drugs, based on their differential affinities for

various tissues. In 1904, Ehrlich and Shiga discovered that a red dye called trypanrot was effective against trypanosomes (183). It was around this time that arsenicals drew Ehrlich's interest. Ehrlich, along with Sahachiro Hata in 1909, found that arsphenamine (named Salvarsan) was active against spirochetes and, therefore, was an effective cure for syphilis (100).

The first truly effective class of antimicrobial drugs were the sulfonamides, discovered by Gerhard Domagk (73). In 1932, two scientists at the Bayer company, Mietzsch and Klarer, synthesized Prontosil red, a red dye bound to a sulfonamide group. Domagk (73) showed, in 1935, that infections in mice caused by hemolytic streptococci were cured by Prontosil red (91, 100). Unfortunately for Bayer, Prontosil red was shown to have no antibacterial activity *in vitro*. This lack of activity was explained by Tréfouël *et al*. (259) when they showed that Prontosil red is split *in vivo* into its component dye and sulfanilamide, the active antibacterial agent and a previously described molecule that was already in the public domain. From that point, sulfanilamide was manufactured by a number of companies and work was begun to modify the molecule to enhance performance, leading to decreased side effects and a broader spectrum of action.

Although penicillin was the first natural antibiotic to be discovered, the idea of using microorganisms therapeutically was not new. Fungi had been used in poultices for many years, and by 1899, a product called pyocyanase, which was an extract from *Pseudomonas aeruginosa*, was used in the treatment of wounds (91). Penicillin was first isolated from *Penicillium notatum* in 1928 by Alexander

Fleming (81), but he was unable to isolate and purify enough drug to be of any use. By 1941, Ernst Chain, Howard Florey, and Norman Heatley had shown the therapeutic value of penicillin (49), but they were also unable to produce enough penicillin for commercial use. Collaboration with Andrew Moyer and Robert Coghill (191) at the USDA's Northern Regional Research Laboratory in Illinois led to much higher production yields of penicillin by 1943. After a worldwide search for *Penicillium* strains that could produce more penicillin, Raper and Fennel (219) found a strain of *Penicillium chrysogenum* on a moldy cantaloupe at a local market that was capable of even higher yields of penicillin (70).

A series of different antibiotics were quickly discovered after penicillin came into use. In 1940, Selman Waksman began searching for antibiotic compounds produced by soil microorganisms (100). In 1943, one of Waksman's students discovered streptomycin (234), leading to a flood of researchers combing the world for new drugs. It was in this same period that Rene Dubos (117) discovered gramicidin, the first antibiotic active against gram-positive bacteria. Chlortetracycline, chloramphenicol, and others were discovered shortly thereafter (91). Many discoveries were of drugs that were too toxic for human use, or that had already been discovered. Nevertheless, this work did lead to many new drugs and within only 10 years, drugs comprising the major classes of antibiotics were found (100). In addition to soil, many of these drugs were discovered by isolating the producing microorganisms from interesting and unusual sources. For example, some antibiotic-producing bacteria were isolated from a wound

infection and others from sewage, a chicken's throat, and a wet patch of wall in Paris (91).

In 1962, one of the later discoveries was a synthetic drug, nalidixic acid, the first of the quinolones to be described, and although not therapeutically important by itself, modification of nalidixic acid led to the production of the highly effective fluoroquinolones. Members of this class, such as ciprofloxacin, norfloxacin, enrofloxacin, and ofloxacin, have become very important in the treatment of diseases in both humans and animals (183). Since the 1960's, there have been few discoveries of new antibiotic drugs. The drugs developed since have mostly been chemical modifications of existing drugs. These modifications have been very useful in treating infectious diseases, leading to enhanced killing of pathogens, increased spectrum of action, reduced toxicity, and reduced side-effects. Unfortunately, since the 1970's, only one new class of antibiotics has been introduced (155) and a recent trend in antibiotic therapy has been to employ combinations of drugs with different mechanisms of action, in order to increase their effectiveness and to overcome the problem of drug resistance.

History and scope of resistance

There is evidence that although resistant microorganisms existed in nature before the use of antibiotics, such microorganisms were mostly absent from human flora (119). However, in the intervening years, antibiotic resistant microorganisms have become frighteningly common. Almost as soon as

antibiotics were discovered, researchers began to find microorganisms resistant to the new drugs. Even by 1909, when Ehrlich first began to study dyes and arsenicals, he found drug resistant trypanosomes (100). Resistant strains of *Staphylococcus aureus* in hospitals grew from less than 1% incidence, when penicillin first came into use, to 14% in 1946, to 38% in 1947, to more than 90% today (100). Worldwide, ampicillin and penicillin resistance can be found together in more than 80% of *S. aureus* strains (199). After World War II, sulfonamides were widely used to treat *Shigella* infections in Japan, but by 1952, only 20% of isolates were susceptible. As the Japanese began to switch to tetracycline, chloramphenicol, and streptomycin, *Shigella* strains that were multiply-resistant quickly began to appear (80). Within 30 years of their discovery, sulfonamides ceased to be an effective treatment for meningococcal disease (199). In the years since, reports of resistance have grown increasingly common, and pathogens that are resistant to almost all antibiotics have been found. It has become painfully obvious that antibiotic resistance is reaching a crisis stage and some clinicians have even forecasted that we are facing a return to the devastating diseases of the pre-antibiotic era (25, 119, 155).

In general, as stated above, resistance has been found in many organisms, but some pathogens are of particular recent concern. These pathogenic organisms are becoming increasingly more common, especially with the greater frequency of travel worldwide and increase in the population of the elderly, both in the United States and in many of the developed countries worldwide. Some specific

examples of microbial species that have developed significant resistance are cited as follows.

Enterococcus spp.:

The enterococci are a group of gram-positive cocci that are part of the normal resident flora of both humans and some animals (179, 261). They are generally not considered virulent; however, their intrinsic resistance to many antibiotics (including cephalosporins, penicillin, and aminoglycosides) has made them important opportunistic pathogens and one of the most common causes of nosocomial infections. In fact, 12% of nosocomial infections in the United States are caused by enterococci, with *Enterococcus faecium* and *Enterococcus faecalis* the most important and common species (261, 269). Although the enterococci are opportunistic pathogens, they are a frequent cause of urinary tract infections, bacteremia, and endocarditis, all of which can be difficult to treat due to resistance. Mortality rates in enterococcal bacteremia can reach 70% (179).

The traditional method of treatment has been a combination of an aminoglycoside and ampicillin or a glycopeptide. By the 1970's, only ampicillin and vancomycin (a glycopeptide) were effective treatment options in most cases (83). As of 2000, high level resistance to ampicillin and aminoglycosides was common, leaving vancomycin as the treatment of last resort (128). In one study in New York City, Frieden *et al.* (83) found that 98% of vancomycin resistant enterococci (VRE) infections were acquired nosocomially and 19% of these were resistant to all antibiotics. Although vancomycin has been used in humans for more than 40 years, VRE were not generally considered a clinical problem until

recently. The first report of VRE in the lab was in 1969, but the first clinical

cases were not seen until 1986 in England, and in 1988 in New York City (28,

83). Since then, the frequency of VRE infections has vastly increased, becoming

a major health problem. Enterococcal glycopeptide resistance in intensive care

units in the United States grew from 0.4% in 1989 to 16% in 1997. Furthermore,

from 1989 to 1993, there was a 20-fold increase in nosocomial VRE infections

(48, 141). In 1998, it was reported that approximately 20% to 40% of

enterococcal nosocomial infections were vancomycin resistant (140). So far, in

the United States, clinical VRE isolates have been mostly confined to the hospital

setting, with no reports of community carriage (169). In Europe, there have been

many reports of community-acquired infections, with community carriage rates

ranging from 2% in England to 28% in Belgium (28, 169). This difference in

source has so far been unexplained, but some attribute it to the use of

glycopeptide antibiotics in food animals in Europe. A recently approved drug for

the treatment of VRE is the streptogramin combination of

quinupristin/dalfopristin. Although only approved since 1999, there have already

been reports of sporadic resistance in human isolates and resistance is quite

common on retail foods and in food animals (242).

Staphylococcus spp.:

The staphylococci are another group of gram positive cocci that are often

associated with infections, especially nosocomial infections, and antibiotic

resistance. In the clinical setting, they are divided into two groups: the coagulase-

positive and the coagulase-negative staphylococci. The most important species of

the coagulase-negative staphylococci is *Staphylococcus epidermidis*. These are the most common of the skin microbiota and are generally believed to be simply opportunistic pathogens.

In contrast, the most important pathogen of the staphylococci is *Staphylococcus aureus*. *S. aureus* is coagulase-positive and is commonly found on the skin and in the nasal passages of carriers. *S. aureus* infections are often nosocomial or opportunistic and can cause pimples, furuncles, impetigo in newborns, pneumonia, endocarditis, and septicemia (149). There are several virulence factors that determine the pathogenicity of *S. aureus*. These include several toxins that can cause such effects as toxic shock syndrome and food poisoning.

Antibiotic resistance in staphylococci is very common and was observed very early in the antibiotic era. When general use of penicillin began, nearly all staphylococcal isolates were susceptible when tested in the laboratory, but resistance to penicillin and other β-lactam antibiotics, including ampicillin, in hospitals began to appear almost immediately, so that by 1948, 59% of *S. aureus* strains isolated from patients in hospitals were resistant. By 1950, the majority of the strains from most hospitals around the world were penicillin resistant (91). Although the first penicillin resistant *S. aureus* strains were generally only found in hospitals, the pathogen moved rapidly into the community. Today, more than 90% of *S. aureus* and between 50% and 70% of coagulase-negative staphylococci are resistant (100, 199). In fact, in one recent community prevalence study of healthy young volunteers in Portugal, Sá-Leão *et al.* (231) found that 94% of

S. aureus strains were resistant either to penicillin alone, or to penicillin and erythromycin.

Resistance to other antibiotics similarly appeared shortly after their introduction. By 1953, staphylococcal resistance to streptomycin, tetracycline, chloramphenicol, and novobiocin had been reported. Through the 1950s, a pandemic was caused by a particularly virulent and penicillin resistant strain of *S. aureus* called phage type 80/81. After the discovery of penicillinase-resistant drugs such as methicillin, the pandemic was brought under control (224). Although these resistances were reported in separate strains and sometimes together in single, highly epidemic strains, it was believed that such resistances were rare and of little significance (91). Even resistance to newer drugs, such as the fluoroquinolones and quinupristin/dalfopristin, was reported (100, 250). In the early 1960s, *S. aureus* strains resistant to supposedly penicillinase-resistant β-lactam drugs, such as methicillin, began to appear in hospitals (91). Methicillin resistant *S. aureus* (MRSA) quickly spread around the world and are now a major nosocomial pathogen. In 2000, 47% of *S. aureus* and 75% of coagulase-negative staphylococcal isolates from intensive care units in the U.S., and 48% of hospital isolates in Portugal were methicillin resistant (231, 250). Until recently, MRSA was mainly a hospital problem; however, there have been reports of community-acquired infection in several countries. MRSA among children in day-care centers, sports teams, and other groups have been reported, including several deaths (231). Although community prevalence appears to be low (1%-2%), there is increasing concern over the threat of MRSA, since many strains are resistant

not only to methicillin, but to all antibiotics except the glycopeptides, making them very difficult to treat (100).

Currently, the only available drug for treatment of MRSA infections is vancomycin. In 1997, in Japan, vancomycin resistant *S. aureus* (VRSA) was first isolated (168). This discovery was quickly followed by VRSA isolates in the U.S. and other countries (250). The discovery of VRSA prompted fears that vancomycin resistance had come from VRE, especially after it was reported that the vancomycin resistance gene *vanA* could be transferred from *E. faecalis* to *S. aureus in vitro* (195). For several years, no connection to VRE could be found (250), but as of 2004, strains of VRSA containing the *vanA* gene have been isolated from patients in the U.S. (227, 274).

There has been some disagreement over the designation VRSA, as many of the strains that have been isolated do not meet the NCCLS standards for resistance. Therefore, the term, vancomycin intermediate *S. aureus* (VISA), has come into use. It is important to note, however, that even if VISA strains may not meet the criteria for resistance *in vitro*, infected patients frequently fail to respond to vancomycin, effectively indicating bacterial resistance (227, 230). Although VRSA isolates, to this point in time, have been treatable by other antibiotics, it is easy to see the danger inherent in such resistant organisms, especially if one considers the possibility of transfer of the resistance to other bacterial species.

Streptococcus spp.:

Streptococcal infections are a major cause of disease around the world. The three main pathogens of concern are *Streptococcus pneumoniae*,

Streptococcus pyogenes, and *Streptococcus agalactiae*. *S. pneumoniae*, or pneumococcus, is the leading cause of both meningitis and septicemia. In addition, *S. pneumoniae* can cause pneumonia, sinusitis, otitis media, and other respiratory infections and is responsible for 40,000 deaths each year in the U.S. alone (127, 198). In young children, the elderly, and immunosuppressed individuals, *S. pneumoniae* infections are particularly dangerous. Pneumococcal infection is the main cause of the 5 million pneumonia deaths worldwide among children under age five (127).

S. pyogenes also causes serious, sometimes fatal disease. This species is the cause of scarlet fever, impetigo, otitis media, meningitis, puerperal fever, skin infections, and is the most common cause of bacterial pharyngitis. More serious infections include streptococcal toxic shock syndrome and necrotizing fasciitis. *S. pyogenes* can also lead to post-infection complications, including rheumatic arthritis, rheumatic heart disease, and acute glomerulonephritis. Furthermore, there may be an association between infection and Tourette's syndrome (60).

S. agalactiae, also known as Group B streptococcus (GBS), was first recognized as an important cause of puerperal fever. While it sometimes causes bacteremia, pneumonia, and tissue infections in adults (especially in those with diabetes or cancer), it is the leading cause of neonatal sepsis (18). In the 1970s, the mortality rate of GBS among newborns was found to be 55% (72), prompting the medical community to seek an effective treatment. The Centers for Disease Control and Prevention (CDC) has since issued guidelines directing that

intrapartum prophylaxis be given to mothers at high risk. These measures have resulted in an attack rate of only 0.02% and a mortality rate of 5% (18, 72).

By the time penicillin came into use, both *S. pneumoniae* and *S. pyogenes* were commonly resistant to the sulfonamides. Unlike in other organisms, penicillin resistance was very slow to develop and, at least until 1971, it was believed that neither organism was capable of developing resistance (91). In 1977, however, penicillin resistant strains of *S. pneumoniae* were isolated from fatal meningitis cases in South Africa (199). Since then, resistance to penicillin has been on the rise throughout the world, reaching even to Iceland and New Guinea, with rates as high as 50% (168, 204). In Europe, penicillin resistance rates of *S. pneumoniae* vary by country with Spain (40%) being the highest and the U.K. the lowest (4%). Most countries have rates less than 10% (26). Resistance to other antibiotics has also developed in *S. pneumoniae*. Worldwide, there have been reports of streptococcal resistance to tetracycline, chloramphenicol, and erythromycin. As in other organisms, antibiotic resistance generally follows the same pattern as antibiotic usage. In Spain, chloramphenicol resistance occurred in 70% of hospital isolates in 1976, but decreased to 36% in 1983 after the use of chloramphenicol in that country was reduced when serious side effects were revealed (26).

Many strains have become multiply resistant and susceptible only to vancomycin (168). In 1999, vancomycin tolerance was first reported in a clinical isolate of *S. pneumoniae* (198). Since then, there have been reports of tolerant isolates in many countries, including Italy, Sweden, the U.S., and China (38, 110).

To date, there have been no reports of vancomycin resistance in *S. pneumoniae*, however, vancomycin tolerance is a very important threat (38, 110, 204). There are three notable concerns with vancomycin tolerance. First, it is expected that tolerance will eventually result in full-blown resistance, as has occurred with VISA and VRSA (198). Second, it is difficult to detect vancomycin tolerance in the laboratory using routine susceptibility tests, because such strains appear to be susceptible (38). Third, and most critical, vancomycin tolerance generally leads to therapeutic failure, especially in cases of meningitis, which often have high mortality rates anyway (38). One bright note is that there is a pneumococcal vaccine currently available that provides moderate protection from most strains causing infection. Included among the strains against which the vaccine is effective are the majority of antibiotic resistant strains. However, an improved vaccine is needed (127).

To date, *S. pyogenes* remains almost completely susceptible to penicillin, but resistance to other drugs, such as tetracycline and erythromycin, has been reported (40, 91). Erythromycin resistance is increasing and has been associated with streptogramin resistance as well. Erythromycin resistance rates in Italy reached as high as 31% by 1995 (40). Penicillin tolerance has been reported in recent years, but it is relatively uncommon and treatment with amoxicillin and clavulanic acid or cephalosporins remains feasible (60).

The treatment of choice for *S. agalactiae* infection remains penicillin, and resistance to this drug continues to be low. However, resistances to several antibiotics have been reported. Resistance to ampicillin, cefazolin, penicillin, and

vancomycin has been reported to be less than 2%, while clindamycin resistance is between 8% and 15%, and erythromycin resistance is between 16% and 19% (18, 35, 212).

Food and waterborne illnesses:

The effect that food and waterborne diseases have had on world populations cannot be overstated. Through history, millions of people have died, armies have faltered, and populations have been changed by diseases transmitted through food or water. Today, gastrointestinal diseases continue to cause malnutrition and death in developing countries. In the U.S. and other industrialized nations, while there are fewer deaths, many people are sickened each year by these diseases. Some of the major food and waterborne bacterial pathogens are *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., *Vibrio* spp., and *Escherichia coli*. These species cause disease around the world, and antibiotic resistance is an increasing problem in the treatment of the diseases they cause.

The campylobacters are gram-negative, microaerophilic, rod-shaped bacteria that are frequently present in the gut of many animals, especially birds. They are also found in natural water sources and in many foods. In fact, *Campylobacter* can be isolated from up to 98% of retail foods. The two most important pathogenic *Campylobacter* species are *Campylobacter jejuni* and *Campylobacter coli* (13). Since the 1980s, campylobacters have been the most common cause of bacterial gastroenteritis in both the U.S. and the U.K. (13, 84). Although it is difficult to determine the actual number of cases, since many go

unreported, between 2.1 and 2.5 million cases of campylobacteriosis are believed to occur in the U.S. each year (13, 182). Symptoms include diarrhea, cramping, and fever. Complications such as bacteremia sometimes occur, and campylobacteriosis is also associated with post-infection diseases, i.e. Guillain-Barre syndrome and Reiter syndrome (13). Most outbreaks of campylobacteriosis are sporadic rather than epidemic, and many times the source goes unrecognized (84).

Fortunately, *Campylobacter* infections are usually self limiting and antibiotic treatment is not always required. However, in serious cases and especially in cases of *Campylobacter* bacteremia, treatment is advised, usually administration of erythromycin or fluoroquinolones. Antibiotic resistance in campylobacters has increased drastically in recent years. Fluoroquinolone resistance was first reported in the late 1980s, but since 1990, the incidence of resistance has increased. For example, in the Netherlands, resistance went from 11% in 1990, to 30% in 1991 (218). In a study of U.S. troops in Thailand in 1994, most isolates were resistant to ciprofloxacin (a fluoroquinolone) and 33% were resistant to azithromycin (an erythromycin derivative) (13).

Salmonella spp. are gram-negative rods that inhabit the intestines of mammals, birds, and reptiles. They are capable of surviving for long periods in water, soil, and food (17). The two major diseases associated with *Salmonella* spp. are typhoid fever and salmonellosis. Typhoid fever, caused by *S. typhi*, has characteristic features, namely, after an incubation period of two weeks, a high fever occurs, followed by headache and diarrhea. Rather than multiplying in the

intestinal epithelium, *S. typhi* invades the phagocytes and spreads throughout the body. The disease can lead to perforation of the intestinal wall, peritonitis, and septicemia and if any of these occurs, there is a high mortality rate (78). Although typhoid fever has decreased significantly in incidence in the developed world, 16.6 million cases occur annually worldwide, in developing countries predominantly, as well as 600,000 deaths (241). In some parts of Asia, 30%-40% of hospital blood cultures are *S. typhi* (168). Cases do occur in the U.S., but most of these are acquired during international travel.

Salmonellosis caused by *Salmonella* species other than *S. typhi*, is usually a less severe illness. It is often self-limiting and symptoms include diarrhea, fever, and abdominal cramps. The most common sources of *Salmonella* infections are undercooked poultry or eggs and most cases go unreported. It is estimated that in the U.S., there are 1.4 million cases of salmonellosis each year (17). Due to the nature of the disease, antibiotics are usually not necessary for treatment; however, salmonellae can invade the bloodstream or other sites in the body and cause serious illness, requiring antibiotic treatment. Ampicillin and chloramphenicol have historically been the main treatments for *Salmonella* infections (241). Since 1988, though, ciprofloxacin has been the most prescribed drug for these infections (17).

Multiple drug resistance in non-typhoidal *Salmonella* infections was common by the end of the 1960s and has increased drastically since then. There have been several outbreaks of multiply resistant *S. typhimurium* associated with cattle and poultry (254). In 1988 in England, a multiply resistant *S. typhimurium*

strain, DT104, was first isolated. During the following decade, 90% of human

DT104 isolates were found to be multiply resistant, i.e. ampicillin,

chloramphenicol, streptomycin, sulfonamides, and tetracycline resistant. This

strain has since spread through Europe, the U.S. and many other parts of the

world. From 1997 to the present, resistance to trimethoprim and fluoroquinolones

has appeared in DT104 isolates (140, 254). Since 1986, there has been an

increasing number of reports of multiply resistant *S. typhi* throughout the world.

In Mexico, chloramphenicol resistance first appeared, followed by resistance to

ampicillin, streptomycin, sulfonamides, and tetracycline. Fluoroquinolone

resistance has also been reported in *S. typhi* in developed countries (239).

Ominously, many DT104 isolates resistant to ampicillin, chloramphenicol,

streptomycin, sulfonamides and tetracycline are now exhibiting decreased

susceptibility to fluoroquinolones (17).

      Shigella spp. are gram-negative rods that are very similar to species of the

genus *Escherichia*. Four species of *Shigella*, *S. flexneri, S. sonnei,*

S. dysenteriae, and *S. boydii*, are pathogenic. In the U.S., most shigellosis is

caused by *S. sonnei*, but around the world, *S. flexneri* is the most common.

Shigellae are capable of surviving the acidity of the stomach, and consequently,

the infectious dose is low (129).   Invasion and tissue destruction in the intestinal

epithelial layer leads to watery diarrhea, then bloody, mucoid diarrhea, as well as

cramps and fever. Usually, shigellosis is limited to gastrointestinal illness, but it

can progress to bacteremia. *S. dysenteriae* is often the culprit in serious cases and

can have a mortality rate of 20% (23). Throughout the world, 165 million cases

of shigellosis occur each year, almost all of which are in developing countries. However, 20-30 thousand cases occur in the U.S. annually. Around the world, shigellosis is the cause of 1.1 million deaths and 69% of the cases are in children under age five (129). Transmission is by the oral-fecal route and in developing countries is attributed to poor sanitation and lack of clean water.

Antibiotics are useful in treatment of shigellosis since they reduce the length of time of the symptoms, as well as bacterial shedding, but antibiotic treatment is necessary in cases of bacteremia and severe diarrhea. Early antibiotic resistance was detected in Japan, where dysentery caused by *Shigella* infection was a large problem. By 1950, 80%-90% of *Shigella* isolates were resistant to sulfonamides, and by 1965, resistance to tetracycline, chloramphenicol, and streptomycin had been reported (183). Since then, resistance continues to increase. In Israel, the incidence of resistance to ampicillin is currently 85% and to trimethoprim-sulfamethoxazole, 94%, with resistance to tetracycline increasing from 23% in 1992 to 87% by 2000 (23). Ciprofloxacin resistance has also been reported (23).

Escherichia coli is a very common gram-negative rod normally found in the intestinal tract of mammals. Not all strains are pathogenic, but those that are can cause sepsis, urinary-tract infections, meningitis, and gastroenteritis. Most gastroenteritis caused by *E. coli* is self-limiting and requires no antibiotic treatment. However, other diseases caused by *E. coli*, cited above, and *E. coli* enterohemorrhagic gastroenteritis are serious and do require antibiotic treatment.

As in the case of the microorganisms discussed above, antibiotic resistance is on the rise in *E. coli*. O'Brien *et al.* (199) reported an incidence for sulfonamide resistance of between 21% and 85%, ampicillin resistance between 17% and 72%, tetracycline resistance between 24% and 60%, and trimethoprim resistance between 1% and 4%. Resistance to fluoroquinolones was rare for many years, but since the early 1990s, this to, has been on the rise. In one study in Spain, the incidence of resistance was shown to have reached 22% and more than a third of those isolates were resistant to three or more other antibiotics (90). In Taiwan, 11.3% of isolates are reported to be fluoroquinolone resistant and 21.7% with reduced susceptibility. Not surprisingly, it is believed that reduced susceptibility will lead to increased incidence in resistance (171).

Other pathogens:

There are many other antibiotic resistant microbial pathogens that have emerged in recent years. *Neisseria gonorrhoea*, the causative agent of gonorrhea, is highly resistant to antibiotics in many countries of the world. In Southeast Asia, many strains are fluoroquinolone resistant, and in Thailand, almost all strains are penicillin and tetracycline resistant (168). Resistance and reduced susceptibility to penicillin are common in both Europe and the U.S., and this phenomenon has led to a change in the recommended treatment (100). As the number of HIV infected persons has increased, tuberculosis has re-emerged as a serious problem, and multi-drug resistance in *Mycobacterium tuberculosis* is on the rise. Even *Yersinia pestis*, the causative agent of plague, is acquiring antibiotic resistance. An isolate in Madagascar, obtained from an infected boy,

was found to contain a plasmid with resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, sulfonamide, and tetracycline (168). Although many scientists who were working in the beginning of the antibiotic era were unconcerned and almost cavalier about microbial resistance to antibiotics, no one can deny that resistance is a major public health threat worldwide.

Mechanisms of antibiotic action and resistance

There are several major classes of antibiotics that can be categorized based on their mode of antibacterial action. In general, antibiotics can be defined as those that inhibit cell wall synthesis, those that inhibit protein synthesis, and those that inhibit nucleic acid synthesis. See table 1 for a summary of the major antibiotic classes. The selective toxicity of antibiotics lies in the differences in cellular structures between eukaryotic and prokaryotic cells. However, differences in cellular structure among bacterial species can lead to resistance to certain antibiotics.

The definition of bacteria as resistant or susceptible is critical for clinicians. It is also very important to note the difference between intrinsic and acquired resistance to an antibiotic. Intrinsic resistance can best be described as resistance of an entire species to an antibiotic, based on inherent (and inherited) characteristics requiring no genetic alteration. This is usually due to the absence of a target for the action of a given antibiotic or the inability of a specific drug to reach its target. For example, mycoplasmas are always resistant to β-lactam

antibiotics since they lack peptidoglycan (which the β-lactams act upon). Similarly, the outer membrane of gram negative cells can prevent an antibiotic from reaching its target. For example, *Pseudomonas aeruginosa* exhibits high intrinsic resistance to many antibiotics due to its drug efflux pumps and restricted outer membrane permeability.

Acquired resistance can arise either through mutation or horizontal gene transfer. Presence of the antibiotic in question leads to selection for resistant organisms, thereby shifting the population towards resistance. The major mechanisms of acquired resistance are the ability of the microorganisms to destroy or modify the drug, alter the drug target, reduce uptake or increase efflux of the drug, and replace the metabolic step targeted by the drug.

Inhibitors of Cell Wall Synthesis:

There are two major groups of cell wall synthesis inhibitors, the β-lactams and the glycopeptides. As bacterial cell walls are wholly unlike the membranes of eukaryotes, they are an obvious target for selectively toxic antibiotics. The β-lactams include the penicillins, cephalosporins, and the carbapenems. These agents bind to the penicillin binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall. In gram negative cells, this leads to the formation of fragile spheroplasts that are easily ruptured. In gram positive cells, autolysis is triggered by the release of lipoteichoic acid (100).

Table 1. Major Classes and Examples of Antibiotics

Inhibitors of:

Cell Wall Synthesis	β-lactams	Glycopeptides			
	Penicillins	Vancomycin			
	Cephalosporins	Avoparcin			
	Carbapenems				
Protein Synthesis	Aminoglycosides	Chloramphenicol	Tetracyclines	Macrolides	Streptogramins
	Streptomycin			Erythromycin	Virginiamycin
	Neomycin			Azythromycin	Quinupristin-Dalfopristin
	Kanamycin			Clarithromycin	Pristinamycin
	Gentamicin				
Nucleic Acid Synthesis	Sulfonamides (diaminopyrimidines)	Quinolones			
	Sulfamethoxazole-trimethoprim	Ciprofloxacin			
		Norfloxacin			

The mechanism of β-lactam resistance is via the action of the β-lactamases. These enzymes catalyze hydrolysis of the β-lactam ring and, thereby, inactivate these antibiotics. Many bacteria contain chromosomally encoded β-lactamases necessary for cell wall production and it is only through over-production of these enzymes that resistance occurs (100). β-lactamases encoded on plasmids or other transmissible elements can lead to such over-production and, therefore, to resistance (197). There are also some bacteria that possess altered PBP's that result in reduced penicillin binding (100).

Since the discovery of penicillin and resistant bacteria, various new versions of the β-lactams have been used that have different spectrums of activity and different susceptibility to β-lactamases. Since the 1970s, several compounds, such as clavulanic acid, have been discovered that have the ability to bind irreversibly to β-lactamases and, thereby, inhibit their action. Combinations of these compounds with β-lactam drugs have been very successful in treatment of disease (42).

The glycopeptides are a group of antibiotics that include vancomycin, avoparcin, and others that bind to acyl-D-alanyl-D-alanine. Binding of this compound prevents the addition of new subunits to the growing peptidoglycan cell wall. These drugs are large molecules that are excluded from gram negative cells by the outer membrane, thus limiting their action to gram positive organisms.

Glycopeptide resistance was long thought to be rare, but has recently been shown to be quite common (42). Resistance in enterococci has developed through newly discovered enzymes that use D-alanyl-D-lactate in place of acyl-D-alanyl-D-alanine, allowing cell wall synthesis to continue. Other mechanisms of resistance involve the over-production of peptidoglycan precursors which overwhelm the drug (100).

Inhibitors of protein synthesis:

There are many types of antibiotics that inhibit bacterial protein synthesis. These drugs take advantage of structural differences between bacterial ribosomes and eukaryotic ribosomes

The aminoglycoside antibiotics are a group whose mechanism of action is not completely understood. The three major groups of aminoglycosides are the streptomycins, neomycins, and kanamycins. These drugs enter bacterial cells by an active transport that involves quinones that are absent in anaerobes and streptococci, thus excluding these organisms from the spectrum of action. Streptomycins act by binding to the 30S ribosomal subunit. Kanamycins and neomycins bind to both the 50S subunit and to a site on the 30S subunit different from that of streptomycin (100). Activity involving initiation complexes and cell membrane proteins that contribute to cell death plays a role in the action of these antibiotics, but this is poorly understood (42, 100).

There are three mechanisms of aminoglycoside resistance that have been identified to date. The first involves only streptomycin. Since streptomycin binds to one particular protein on the ribosome, alteration of this protein, even by a single amino acid in its structure, confers high-level resistance to the drug (42). The other mechanisms involve decreased uptake of the antibiotic and in one of these the cell membrane is altered, preventing active transport of the drug. In the other, one of many enzymes alters the antibiotic as it enters the cell, causing a block in further active transport (42).

Chloramphenicol is a broad-spectrum antibiotic that, although naturally occurring, is produced by chemical synthesis. Chloramphenicol inhibits peptide bond formation on 70S ribosomes (42). This drug is especially useful in that it can penetrate eukaryotic cells and cerebrospinal fluid, making it a drug of choice for treatment of meningitis and intracellular bacterial infections such as those caused by chlamydia. It is not in widespread use, however, because of potentially fatal side-effects, namely, aplastic anemia (100).

Resistance to chloramphenicol is conferred by the enzyme chloramphenicol acetyl-transferase. A number of these enzymes have been discovered, each altering the chloramphenicol molecule to prevent binding to the bacterial ribosome. Chloramphenicol resistance in gram negative cells can also arise from alteration in outer membrane permeability that prevents the drug from entering the cell (42).

The tetracyclines are another group of broad-spectrum antibiotics that inhibit bacterial protein synthesis. They are brought into the cell by active transport and, once there, bind to the 30S subunit to prevent binding of aminoacyl tRNA (223).

Resistance to the tetracyclines occurs via three mechanisms. First, production of a membrane efflux pump removes the drug as rapidly as it enters and there are several genes encoding these pumps. Second, several ribosome protection proteins act to prevent tetracycline from binding to the ribosome, thus conferring resistance. Third, a protein found only in *Bacteroides* spp., enzymatically inactivates tetracycline (223). Interestingly, efflux pump inhibitors have recently been discovered that may allow combinations of these inhibitors and tetracyclines to be used against previously resistant strains (55).

The macrolides are a group of antibiotics commonly used to treat gram positive and intracellular bacterial pathogens. Erythromycin was the first of these, and several other important macrolides have been discovered since, including clarithromycin and azithromycin. Azithromycin has a longer plasma half-life which allows treatment with a single dose for some pathogens or a once daily dose for others. Clarithromycin has enhanced absorption and causes less gastrointestinal discomfort (92). It was originally believed that erythromycin inhibited protein synthesis by competing with amino acids for ribosomal binding sites, but newer research shows several mechanisms are involved (91). The macrolides are now believed to promote dissociation of tRNA from the ribosome,

inhibit peptide bond formation, inhibit ribosome assembly, and prevent amino acid chain elongation (92).

There are two major mechanisms of macrolide resistance. First, an efflux pump has been found that removes the drug from the cell. Second, modification of the ribosome can confer resistance. Mutations at several sites of the ribosome can allosterically prevent macrolide binding and a common alteration is dimethylation of one nucleotide on the 23S rRNA. This dimethylation not only prevents macrolide binding, but also confers resistance to lincosamide and streptogramin antibiotics (92).

The streptogramins are another class of antibiotic that inhibits bacterial protein synthesis, mostly in gram positive organisms (due to decreased permeability of the gram negative outer membrane). These antibiotics are actually combinations of structurally different drugs, types A and B, that act synergistically. These compounds bind to separate sites on the 50S subunit. Type A drugs block attachment of substrates at two sites on the 50S subunit, whereas type B drugs cause release of incomplete protein chains. The synergistic effect arises from a conformational change induced by the binding of a type A drug which significantly increases affinity of type B drugs (133). Streptogramins currently in use include virginiamycin, pristinamycin, and quinupristin/dalfopristin.

Resistance to streptogramin antibiotics can be found in several forms. Efflux pumps for both type A and B streptogramins have been identified. Type A

streptogramins can be inactivated by one of the virginiamycin acetyl-transferases,

and several enzymes have been identified that can inactivate type B

streptogramins. Alteration of bacterial ribosomal proteins or RNA can also confer

resistance. A common mutation is the dimethylation of one nucleotide on the 23S

rRNA, mentioned previously, that gives rise to resistance to type B drugs, as well

as macrolides and lincosamides (133).

Inhibitors of nucleic acid synthesis:

The sulfonamides and the diaminopyrimidines should be discussed

together, in that both only indirectly inhibit nucleic acid synthesis by inhibiting

folate synthesis. Folate is a coenzyme necessary for the synthesis of purines and

pyrimidines. Although both types of drugs are useful on their own, they exhibit a

synergistic effect when combined. Sulfonamides are currently not used

commonly in medicine, but the combination drug trimethoprim-sulfamethoxazole

is sometimes used in the treatment of urinary tract infections. Sulfonamides serve

as an analog of *p*-aminobenzoic acid. Therefore, they competitively inhibit an

early step in folate synthesis. Diaminopyrimidines, of which trimethoprim is the

most common, inhibit dihydrofolate reductase, the enzyme that catalyzes the final

step in folate synthesis (100).

There are several resistance mechanisms microorganisms employ against

each of the anti-folate drugs. For example, sulfonamides are rendered ineffective

by over-production of *p*-aminobenzoic acid or production of an altered

dihydropteroate synthetase. The substrate for dihydropteroate synthetase is

p-aminobenzoic acid, and the altered form has a much lower affinity for sulfonamides than for *p*-aminobenzoic acid (253). Trimethoprim resistance can also result from several mechanisms, e.g., over-production of dihydrofolate reductase or production of an altered, drug-resistant form can lead to resistance (42). In addition, both drugs can be enzymatically inactivated, resulting in resistance (253).

The quinolones are a chemically varied class of broad-spectrum antibiotics widely used to treat many diseases, including gonorrhea and anthrax. Drugs in this class include nalidixic acid, norfloxacin, and ciprofloxacin. These drugs are commonly used and, worldwide, more ciprofloxacin is consumed than any other antibacterial agent (6). Quinolones inhibit bacterial growth by acting on DNA gyrase and topoisomerase IV, which are necessary for correct functioning of supercoiled DNA (100). Although quinolones target both enzymes, in gram negative organisms the primary target is DNA gyrase and, in gram positive organisms, the primary target is topoisomerase IV (228).

There are three main mechanisms of resistance to quinolones. Resistance to some quinolones occurs with decreased expression of membrane porins. Cross-resistance to other drugs requiring these porins for activity also results from these changes. A second mechanism of resistance is expression of efflux pumps in both gram negative and gram positive organisms (197) and the third is alteration of the target enzymes. Several mutations have been described in both quinolone target proteins that result in reduced binding affinities (228). It is

believed that high-level quinolone resistance is brought about by a series of successive mutations in the target genes, rather than a single mutation (197).

Sources of Resistance in the Environment

Concern over resistance was originally confined to acquisition of resistance by microorganisms which cause epidemic disease and was an issue only with respect to clinically isolated strains. However, in recent years, antibiotic resistant bacteria have been isolated from virtually every environment on earth. This came as a surprise to many clinicians, because resistance was found in regions never exposed to human impacts. Even as awareness of environmental resistance has increased, many investigators have continued to restrict their concern to only those pathogens that survive in the environment. It was believed that they posed a danger to humans only if the disease they caused involved resistance to antibiotics. For many years, the focus of research on resistance in the environment reflected this viewpoint. However, we now know that resistance genes can be spread far wider than once believed and a pool of resistance is developing in non-pathogenic organisms found in humans, animals, and the environment. These non-pathogenic organisms serve as a source from which pathogens can acquire genes conferring resistance, and in turn, they can become resistant by acquiring genes from pathogens discharged into the environment, e.g. via sewage or agricultural runoff. Thus, dissemination of

resistant bacteria is not only a problem of the resistant pathogens themselves, but also availability of resistance genes to pathogens via gene transfer.

Although resistant organisms can be found naturally in the environment, most resistance is associated with man-made impacts of some type, either agricultural or direct human impact. Antibiotic use in humans can lead to resistance in the environment via discharge of domestic sewage, hospital wastewater, and/or industrial pollution. In addition to use in humans, antibiotics are added to animal feed to treat infections, as prophylactics, and in sub-therapeutic doses as growth promoters. Although no definitive numbers are available, some authors have published estimates and, by 1980, almost half of the antimicrobial agents used in the United States were used in animal feed (75). In Denmark in 1994, a total of only 24 kg of vancomycin was used to treat infections in humans versus 24,000 kg for animals (275). According to Levy (151), in 1998 in the U.S., half of the 50 million pounds of antibiotics produced were used for agricultural applications. There are a variety of positive effects from using antibiotics in animal feed, namely, inhibition of harmful gut flora which leads to increased growth rates and decreased mortality. This has allowed more concentrated farming and an estimated $3.5 billion savings in production costs per year in the United States alone (75). However, the practice has resulted in selection of antibiotic resistant organisms in the guts of food animals. From there, these organisms enter the human food chain via contamination during slaughtering, or the environment via waste discharge. Resistance has been found

to follow closely the use of any given antibiotic (1). Although some investigators

dispute any danger being posed by selection of resistant flora within the guts of

animals, there is no doubt that such antibiotic use leads to higher concentrations

of resistant pathogens and non-pathogens, as well as resistance genes, throughout

the farm environment and nearby environments affected via runoff from farms.

As will be discussed later, once resistant organisms are spread into the

environment, they pose a health risk if they colonize or spread resistance genes to

bacteria that colonize humans.

Agricultural impacts

Poultry production:

The poultry industry has become one of the largest livestock industries

throughout the world, with a 44% production increase in the U.S. between 1982

and 1994 (273). In 1991, 600 billion eggs and 40 million tons of poultry meat

were produced globally (273). The same year, the U.S. alone produced 68 billion

eggs and 6.1 billion broiler chickens (243).

Antibiotics are used heavily throughout the poultry production process.

Although antibiotic use statistics are difficult to obtain for the poultry production

industry, one early study reported that 80% of poultry raised in the U.S. have been

fed antibiotics (75). Though many classes of antibiotics are used,

fluoroquinolones, avoparcin, and virginiamycin are of particular concern, since

resistance to these drugs is associated with resistance the human therapeutic drugs

ciprofloxacin, vancomycin, and quinupristin/dalfopristin, respectively.

Enrofloxacin has been frequently used in the first week of life of chickens to

alleviate disease arising from contamination during vaccination and in the third

and fourth week to prevent respiratory infections, primarily those caused by

E. coli (123) and avoparcin has been used as a growth promoter (144). Although

some of these antibiotics are no longer employed for these applications, others

remain in use, and the resulting resistant organisms have been discharged into the

environment for many years.

The use of antibiotics in poultry leads to resistant organisms within the

chickens themselves and throughout the production environment. Resistant

strains of many organisms, including *Staphylococcus*, *Streptococcus*, *Clostridium*,

Pseudomonas, and *Aeromonas*, have been isolated from these sources (137).

Many surveillance studies have been done to measure prevalence of resistance in

organisms of concern.

Resistant *E. coli* are frequently isolated from live chickens and strains with

multiple resistance to tetracycline, streptomycin, sulfonamides, gentamicin,

fluoroquinolones, and virtually all other antibiotics, have been isolated (27, 34).

Even after discontinuing an antibiotic, resistance persists. In the 1970s, an

experimental group of chickens was given oxytetracycline and compared to a

control group. In 12 weeks, 70% of the *E. coli* isolates in the experimental group

were resistant to more than two antibiotics, including streptomycin and

ampicillin (151).

Campylobacter spp. are also of particular concern since colonization rates in chickens have ranged from 50% to 100%. A longitudinal study carried out in Denmark showed prevalence on two farms to be 56% and 91% (124). In 1998, Hein *et al*. (108) reported a prevalence of 45%. Fluoroquinolones are most often used to treat campylobacteriosis and resistance to quinolones in *Campylobacter* spp. has increased greatly in the last 20 years. In Austria, 50% of *Campylobacter* isolates from poultry were resistant to ciprofloxacin (108). In The Netherlands, a 1997 study found quinolone resistance in 29% of campylobacters from live poultry (124).

Salmonella spp. are another important pathogen found in live chickens and antibiotic resistance in these species is on the rise. In 1996, in Spain, 62% of isolates were resistant to at least one antibiotic and, in 2000, 82% were resistant (260). In Denmark, in 1999, quinolone resistance was approximately 26.5% (276).

One of the most recent threats is resistance in *Enterococcus* spp. isolated from poultry. Avoparcin has been used extensively throughout the world by poultry farmers and we now know that resistance to avoparcin also confers resistance to vancomycin. Large numbers of VRE have been found in poultry, leading to the banning of avoparcin in animal production by several countries. In a study conducted from 1995 to 1997, VRE were found in 30% of broiler carcasses (144). After the ban of avoparcin, resistance decreased. For example, in Denmark, a decline in VRE in chickens occurred from 80% to 5% (24). A new drug, quinupristin/dalfopristin, has come into use against human VRE infections.

Although quinupristin/dalfopristin has never been approved for poultry use, isolates resistant to it have been obtained from broiler carcasses. Quinupristin/dalfopristin is an analog of virginiamycin which has been in agricultural use as a growth promoter for many years. In a 2000 German study of turkey manure and broiler chicken carcasses, all of the manure isolates and 46% of chicken carcasses were positive for quinupristin/dalfopristin resistance. In addition, resistance to other antibiotics in carcass isolates were 100% for erythromycin, 90% for oxytetracycline, 50% for ciprofloxacin, 20% for chloramphenicol, 20% for streptomycin, 5% for vancomycin, and 5% for ampicillin (271). In an American study of poultry, quinupristin/dalfopristin resistant *Enterococcus faecium* was isolated from 77% of litter and transport containers sampled and 51.2% of cloacal swabs (107). In a Michigan study of turkeys, virginiamycin resistance in *E. faecium* was as high as 100% (270).

With the increase in poultry production, management of poultry waste is a serious problem. In 1990, 408 million kg of manure were produced by broiler chickens in Maryland alone (187). The components of poultry waste have been well studied, showing nitrogen and phosphorus content to be higher than in most animal manures (243). Heavy metals, such as arsenic, copper, iron, selenium, and zinc, are often added to poultry feed to improve weight gain and to prevent disease (186). Since most of the metals are not absorbed, they pass through the bird and are excreted with the waste (186). In one study, Morrison (189) found arsenic levels of 15-30 ppm in the litter of chickens treated with the arsenic drug roxarsone. Concentrations of metals in litter can range greatly, between 11 mg/kg

of lead and 1000 mg/kg of iron, and are directly related to the metal content of the feed (186, 187, 243). Kunkle *et al.* (146) has shown that levels of metals in poultry feed are concentrated by at least 2.75 to 3.25 times in the litter.

In addition to heavy metals, poultry manure contains large numbers of microorganisms, including *Salmonella* spp., *Streptococcus* spp., *Staphylococcus* spp., *E. coli*, *P. aeruginosa*, *A. hydrophila*, *C. jejuni*, and others (137). Viable plate counts from fresh manure have ranged from 10^8 to 10^{11} CFU/g dry waste (243). Numbers are high even in litter stored for more than three months (196). Nodar *et al*. (196) reported that aerobic heterotrophic bacteria counts averaged 1.1×10^6 cells/g dry waste and total viable microorganisms were 6.3×10^7 cells/g dry waste over the course of a 14 week study. In another study, Kelly *et al.* (137) showed that, although storage for four months resulted in significant declines in bacterial populations, *Yersinia*, *Aeromonas*, *Campylobacter*, *Pseudomonas*, and fecal coliforms could still be isolated.

Many of these isolates from poultry manure are antibiotic and heavy metal resistant. As in live chickens, resistance to virtually all classes of antibiotics can be found, and resistance to those antibiotics used as growth promoters is common. In feces from broiler farms, studies have shown resistance to ampicillin, chloramphenicol, clindamycin, erythromycin, streptomycin, tetracycline, and virginiamycin, ranging from 2% to 94.8%. Tetracycline and erythromycin were highest at 94.8% and 89.7% respectively (280) with large percentages of these organisms resistant to multiple antibiotics.

The most common method of utilization of poultry waste is land application as manure. It has been estimated that greater than 90% of the poultry manure generated in the U.S. is applied to agricultural lands as fertilizer, mainly (187). Runoff from manure application is increasingly being recognized as a serious environmental problem. Concentrated animal feeding operations impair more miles of river than the pollutants of all other industry sources and municipal sewers combined (273).

Microorganisms, heavy metals, and antibiotic residues can be found in runoff from poultry manure. The type of soil, rainfall amount, and method of manure application have a large impact on the fate of bacteria in manure applied to land. Runoff was found to contain large numbers of bacteria after rainfall events (109). In studies of soil cores, *E. coli* 0157:H7 as well as other *E. coli* strains isolated from poultry litter were found to remain viable (culturable), moving through the soil column. The organisms continued to leach from several types of soil during rainfall events for more than two months (85, 176). Giddens and Barnett (94) reported that total coliforms were a good indicator of pollution from poultry litter. They showed that higher application rates of litter led to higher coliform counts and that rainfall led to increased runoff. They also showed that if runoff was directed over grassed waterways, coliform numbers were reduced, although levels still exceeded water quality standards. Other studies have also shown that coliform water quality standards are often exceeded, regardless of management practices (29). The organisms in runoff may be associated with increased antibiotic resistance in the aquatic environment.

Swine Production:

Pigs are grown in confinement much like poultry, and both pneumonia and diarrhea are common problems. Most pigs are given antibiotics through their feed for growth promotion or disease prevention. In such instances, tylosin, sulfonamides, and tetracyclines are the most commonly used antibiotics, but other drugs are also employed (174). Most reports on antibiotic resistance in isolates from swine involve *Enterococcus* spp. In 1998 and 1999 in Belgium, 82% of swine samples yielded *E. faecium* isolates resistant to tylosin and 97% resistant to oxytetracycline (44). Quinupristin/dalfopristin resistant enterococcal isolates were found in all pig manure samples in a German study and enterococcal resistance to other drugs was 100% for erythromycin, 100% for tetracycline, 28.6% for ciprofloxacin, 4% for vancomycin, and 61.9% for streptomycin (271). In American swine farms, multiply resistant *E. coli* are common, even on farms with low antibiotic use. For example 17% of isolates from high antibiotic use farms and 5% of sows from low antibiotic use farms had single resistance to tetracycline. At the same farms, multiple resistance was found in 81.7% and 79.3% of isolates, respectively (166).

Rising resistance in the 1990s led several European countries to ban several antibiotics as growth promoters. Beginning in 1995, Denmark banned avoparcin (24), then in 1998, virginiamycin (4). By 1999, Danish food producers voluntarily stopped use of all growth promoters (4). Similar bans have gone into effect throughout the European Union (2), leading to several studies to gauge the

impact on antibiotic resistance. The results showed that in Denmark, 80% of *E. faecium* isolates from pigs were resistant to erythromycin in 1995, peaking at 93.1% in 1996, but decreasing to 46.7% in 2000. A similar decrease was observed in virginiamycin resistance, i.e., from 60% in 1995 to 22.5% in 2000. Vancomycin resistance was 20% in 1995 and dropped to just 6% in 2000 (4). A study of Swiss pig farms involved comparison of enterococcal isolates just before the 1999 ban of growth promoters to several months after the ban. Erythromycin resistance went from 96% to 34%, tetracycline resistance from 79% to 45%, and streptomycin resistance from 18% to 3% (36). However, vancomycin resistance increased from 2% to 3% (36). The lack of decrease in vancomycin resistance is not surprising since vancomycin was banned earlier, in 1997. Yet, it is interesting that resistance was still detectible even after two years without vancomycin usage.

As with poultry manure, swine manure is disposed of by application to farmland, a method that places large numbers of organisms, which may be antibiotic resistant, into the environment. This was illustrated by Sengelov *et al.* (240) in 2003, in a study to monitor the effects of application of pig manure to farmland. The authors found the starting concentration of tetracycline resistant bacteria in the manure was 8.75×10^7 cells per milliliter and, after initial manure application, the levels of tetracycline resistant bacteria decreased until they matched the levels in control plots, five months later. The authors also measured streptomycin and erythromycin resistance. Even though neither drug had been used on any of the farms, the manure contained 1.6×10^6 erythromycin resistant

bacterial cells per milliliter and between 2.3×10^7 and 4.6×10^7 streptomycin

resistant bacterial cells per milliliter (240).

Cattle Production:

Antibiotics are used in cattle production in much the same way as in

poultry and swine production. The chief difference is in the concentration of

waste from a pasture versus a chicken house but the same issues with resistance

arise. Cattle production includes beef, veal, and dairy cows with beef cows

generally shipped to feedlots and kept in large groups. Antibiotics are

administered individually or to groups of animals via food and water to achieve

growth promotion or to prevent diseases, i.e., pneumonia and diarrhea (174). In

1999, 83% of feedlots dispensed at least one antibiotic in food or water; these

included virginiamycin, tetracycline, tylosin, and neomycin (174). In 1999, 90%

of veal calves and 60% of beef cows were estimated to have been fed antibiotics

(174). Dairy calves are also housed in groups and are often administered

antibiotics, including penicillin, cephalosporin, erythromycin, and tetracycline.

Although these antibiotics are not usually administered in feed, most cows are

routinely treated with antibiotics applied to the udder to prevent mastitis (101,

174).

As in poultry, many species of resistant bacteria can be isolated from

cattle and the pathogens of particular concern include *E. coli*, *Salmonella*,

Enterococcus, and *Campylobacter* spp. In 1998 and 1999, Butaye *et al.* (44)

reported on resistance of enterococci in ruminants, with twenty percent of

Enterococcus faecium isolates resistant to ampicillin and 80% to tetracycline. Resistance to avoparcin, virginiamycin, and streptomycin was low. In a study of cattle in Ireland, *Salmonellae* were isolated from 7.6% of carcasses and 2% in both fecal and rumen samples and included *S. dublin*, *S. typhimurium*, and *S. agona*. All of the *S. typhimurium* isolates were resistant to chloramphenicol, streptomycin, and tetracycline; all of the *S. agona* isolates were resistant to nalidixic acid and streptomycin; and 15% of *S. dublin* isolates were resistant to streptomycin (173). *S. typhimurium* DT104, a serious pathogen in humans, was one of the strains isolated from these samples. All of the isolates were resistant to multiple drugs, but susceptible to quinolones (173). *S. dublin* is increasingly a problem in the U.S. In a study of California dairy farms, 10% of *S. dublin* isolates were resistant to chloramphenicol (206). In cattle in Pennsylvania and New York, McDonough *et al.* (172) found almost all isolates of *S. dublin* to be resistant to ampicillin, chloramphenicol, tetracycline, and neomycin.

Antibiotic resistant bacteria are also capable of spreading through the cattle farm environment, even without antibiotic use. In a 1990 study, Marshall, Petrowski, and Levy (164) were able to show that cows and pigs not given antibiotics, but fed resistant *E. coli*, continued to shed resistant bacteria for four months. In addition, the test bacteria were recovered from bedding material, mice, flies, and animal caretakers for more than 4 weeks.

Dairy calves have been shown to be colonized with sensitive *E. coli* as long as they were kept with their dams, but become colonized with multiple drug-resistant *E. coli* shortly after being moved to other pens. Multiple drug-resistant

E. coli also colonize veal calves that have not been treated with antibiotics but housed with other animals that were treated (114).

Aquaculture:

Fish farming has become a large agricultural business worldwide. In the United States, the major crops are salmon, shrimp, rainbow trout, catfish, and tilapia (174). Both fresh and saltwater farms are common and, as with other agricultural endeavors, antibiotics are generously applied. However, antibiotic growth promoters are not approved in both the U.S. (174) and Denmark (236). Ormetoprim-sulfadiazine and oxytetracycline are used to treat infections in the U.S. (174) and oxolinic acid, sulfadiazine-trimethoprim, amoxicillin, oxytetracycline, and florfenicol, in Denmark (174, 236). Treatment of fish in aquaculture with antibiotics is accomplished by the administration of medicated feed. This application has a direct effect on the aquatic environment since the food is added to the water and uneaten food settles to the sediment or is dispersed. Thus, antibiotics not metabolized by the fish move directly into the environment (138). One investigator reports that 70% to 80% of antibiotics applied to fish farms end up in the environment (105).

A major fish pathogen in aquaculture is the motile aeromonad, especially *Aeromonas salmonicida* (147). In treating disease caused by these bacteria, it is difficult to determine the true effects of the addition of antibiotics to the aquaculture farm, since the aquatic environment is so complex. However, some studies have attempted to measure these effects. Yet, most studies are in

agreement that isolates from fish farms or the effluent of fish farms have higher levels of resistance, including multiple resistance, than isolates from other areas. DePaola *et al.* (71) found oxytetracycline resistance in 58% - 83% of aeromonads isolated from catfish farms. Similarly, 69% of aeromonads isolated from several Danish farms were oxytetracycline resistant (235). Another Danish study demonstrated 43% sulfadiazine-trimethoprim resistance in aeromonads and 20% resistance to oxolinic acid. Among *Flavobacterium* isolates, resistance to oxolinic acid was 100%, and resistance to amoxicillin was 36% (236). In Italy, sediments surrounding fish farms were found to have higher ampicillin, tetracycline, and streptomycin resistance (53), whereas reports from Scottish farms have indicated resistance among *A. salmonicida* to be between 50% and 55% for oxytetracycline, and between 31% and 54% for oxolinic acid (247).

A different type of fish farming has developed in southeast Asia. In integrated fish farming, chickens or pigs are housed in cages over fish ponds. These animals defecate into the water, where the nutrients support plankton growth which provides food for the fish. Generally, the fish are given no extra feed and no antibiotics. The chickens or pigs, however, do receive antibiotics in their feed, either as growth promoters or for prophylaxis. *Acinetobacter* isolates from the water and sediment of these farms have been shown to have increased levels of resistance (213). Another study of *Aeromonas* and *Enterococcus* isolates from fish showed increased resistance among *Enterococcus* spp. but not among *Aeromonas* spp., leading the authors to suggest that *Aeromonas* are poor indicators of differences in antibiotic resistance (214).

Human impacts

In addition to the effects of agricultural uses of antibiotics, humans can have significant impact on the occurrence of antibiotic resistance in the environment. Antibiotic resistant organisms from the human gastrointestinal tract, as well as unabsorbed antibiotics, can enter the environment via sewage. Domestic wastewater, however, often has less effect than hospital wastewater, since, in the latter, heavy antibiotic concentrations increase the impact.

Antibiotics consumed by humans can act to select for resistant organisms in the human gut. Both the resistant microorganisms and antibiotic residues are excreted, entering the sewage system. Although most people consider the environment to be generally safe from contamination with untreated sewage, breaches occur frequently where leakage or overflow into groundwater or natural waters occurs (106). Raw domestic sewage contains high numbers of bacteria, often including antibiotic resistant bacteria. One report showed that, in healthy people, 80.5% of fecal samples contained resistant organisms (220). Although VRE in the U.S. is associated with hospitals, in Europe, VRE is widespread in the community. See tables 2 and 3.

In Czechoslovakia, a study comparing resistance in municipal wastewater to that of clinical isolates showed higher resistance rates in wastewater for

Table 2. Low-level Vancomycin Resistant Enterococci from Domestic Wastewater (33, 121, 237)

Country	Percentage of samples
Spain	90[a]
Sweden	60[b]
U.K.	52
Germany	16[c]

[a] strains were also resistant to erythromycin (100%)

[b] strains were also resistant to erythromycin (26%)

c strains were also resistant to ampicillin (62%) and ciprofloxacin (2.5%)

Table 3. High-level Vancomycin Resistant Enterococci from Hospital Wastewater (106, 258)

Country	Percentage of samples
Spain	0.4
U.S	6.25
U.S.	5.21

the same species. Gentamicin resistance in *E. coli* was 13.9% and 30% for

Klebsiella and *Enterobacter* strains, respectively (143).

Although sewage treatment processes reduce the numbers of bacteria in

wastewater, the effluent will still generally contain large numbers of both resistant

and susceptible bacteria. Schwartz *et al.* (237) showed a decrease in VRE from

16% in untreated wastewater to 12.5% at the outlet. High numbers of resistant

coliforms have also been found in treatment plant effluents (220) and rivers that

receiving effluent from treatment plants have higher numbers of resistant organisms. Even as early as 1983, Bayne, Blankson and Thirkell (31) found a significant increase in resistance to tetracycline, erythromycin, streptomycin, ampicillin, and penicillin in enterococci, when comparing isolates downstream from a treatment plant to those upstream. Coliforms isolated from sites downstream of a treatment plant in the Tama river in Japan showed significant increases in resistance to ampicillin and tetracycline (122). In Spain, resistance in *Aeromonas* isolates increased from 50% upstream to 90% downstream of a treatment plant effluent, and resistance in enterobacteria increased from 30% to 50% (96). In Sweden in 2003, erythromycin resistant enterococci were isolated from 63% of receiving water samples and VRE were isolated from 3% (33).

Due to heavy antibiotic use, hospital wastewater contains larger numbers of resistant organisms than domestic wastewater. In Florida, vancomycin resistant *E. faecium* were isolated, without enrichment, from hospital wastewater (106). VRE were found in 35% of the hospital sewage samples in two Swedish studies (33, 121, 237). Twenty-five percent of enterococci were vancomycin resistant in a German study of biofilms from hospital wastewater, and of these, many were multiply resistant (237). Reinthaler *et al.* (220) showed significantly higher percentages of *E. coli* in the inlet water of a treatment plant receiving hospital waste than two other treatment plants. A study of *Acinetobacter* showed that an increase in the prevalence of oxytetracycline resistance was correlated with hospital wastewater (102).

Industrial pollution can also influence the incidence of antibiotic

resistance, with pharmaceutical plants yielding a particularly strong effect. Until

the 1970s, it was common for pharmaceutical plant waste to be disposed of in

regular landfills, but drug residues leaching from these landfills were detected in

nearby groundwater systems (105). Guardabassi *et al*. (102) found high levels of

multiply resistant *Acinetobacter* in pharmaceutical plant effluents. Higher levels

of antibiotic resistance have also been associated with heavy metals from

industrial pollution. McArthur and Tuckfield (167) were able to show that

antibiotic resistance was correlated with the heavy metal content of sediments

downstream of a nuclear reactor complex.

Resistant organisms in the environment

The preceding discussion leads to the conclusion that there are several

sources for antibiotics and antibiotic resistant organisms in the environment, but

where can they be found, and at what levels? Antibiotic bacteria can be found in

almost every place on earth. And although levels are higher in areas impacted by

agriculture and humans, there are, nevertheless, still resistant organisms in far

reaches where human impacts are negligible. This should not be surprising since

antibiotics are natural microbial products. Those bacteria that live in the

environment are subjected to numerous antagonistic effects from plants, fungi,

and bacteria that produce antimicrobial chemicals in an effort to compete and

survive. In addition, antibiotic resistance genes may not have always served the

same purpose as they do presently. An alteration of membrane permeability for one purpose may also confer resistance. Heavy metals may also be found in the environment due to natural processes. If a particular metal resistance is linked to drug resistance on a plasmid, there is an obvious selective pressure, even in the absence of human impacts.

Soil ecosystems:

Soils can contain high numbers of antibiotic resistant bacteria. These numbers are generally higher in regions affected by pollution or agriculture, but there are unaffected areas that contain high levels as well, perhaps from natural production of antibiotics by soil bacteria. Tropical soils have been found to contain antibiotic resistant *Rhizobium*, even in the absence of pollution (272). *Pseudomonas aeruginosa* isolated from various soils in Spain were resistant to many antibiotics and had higher levels of resistance than isolates from nearby surface waters. Four of the isolates were resistant to ampicillin, cephalothin, chloramphenicol, furadantin, kanamycin, nalidixic acid, streptomycin, tetracycline, fosfomycin, sulfadiazine, gentamicin, neomycin, lead, arsenic, cadmium, uranium, molybdenum, thallium, zinc, tungsten, and chromium (163).

Increased resistance has been found in soils after application of manure. Jensen *et al.* (131) found higher levels of resistance in *Pseudomonas* and *Bacillus* isolates after the application of pig manure. Although these organisms were not present in the manure, evidence of gene transfer was not observed, implying that antibiotics in the manure were applying selective pressure on autochthonous soil

bacteria. In Norway, fields that were without antibiotic application for 10 years, nevertheless had high levels of resistant organisms. Resistance in organic soil was 72% and resistance in sandy soil was 74%, including resistance to chloramphenicol, tetracycline, ampicillin, and streptomycin (41).

Groundwater:

Many people, especially in rural areas, rely on untreated groundwater for their water supplies. Few studies have been done to determine the antibiotic resistance of isolates from groundwater. Unfortunately, agricultural applications of manure can affect groundwater supplies. Chee-Sanford *et al.* (52) were able to show that tetracycline resistant enterococci could be isolated from groundwater underneath swine farms. Even in wells 250 meters downstream from the farms, resistant bacteria were found and the same resistance genes in bacteria from the farm were isolated from soil bacteria in the groundwater, suggesting transfer of the resistance genes. In West Virginia, coliforms in groundwater were found to have high levels of resistance. That is, 87% of the coliforms were resistant to at least one antibiotic and 60% were multiply resistant. Thirty percent of these isolates were resistant to tetracycline and nitrofurantoin, an antibiotic that is not commonly used (175). Bacteria isolated from deep aquifer sediments in Washington state also had high levels of resistance to penicillin, ampicillin, carbenicillin, streptomycin, and tetracycline. The investigators suggested that most of the resistance was intrinsic, but ca. one-third of the isolates contained plasmids, suggesting plasmid-mediated antibiotic resistance (82). Enterococcal

isolates from wells in Alabama, Florida, Maryland, and Oregon were also found

to be resistant to streptomycin (2.2%) and kanamycin (26%) (221).

Lotic and lentic ecosystems:

Most rivers are impacted by humans to a great extent. Therefore,

antibiotic resistant bacteria in those rivers may derive from wastewater effluent as

discussed above. In samples collected from river sites downstream of cities

throughout the U.S., antibiotic resistant organisms are common. For example,

resistance to ampicillin ranged from 12% in the Ohio river to 60% in the Colorado

river. Resistance to other drugs, such as cephalothin (25% to 98%) and

amoxicillin/clavulanic acid (0% to 68%) was also detected (22). Resistance is

common in polluted European rivers as well. High quinolone resistance in

Aeromonas (59% to nalidixic acid, 2% to ciprofloxacin) was reported to occur in

rivers in Spain and France (97). In polluted rivers in Morocco, coagulase-

negative staphylococci resistant to combinations of ampicillin, chloramphenicol,

tetracycline, and erythromycin were isolated, some with resistance to all of the

above drugs (139). Isolates of *Vibrio cholerae* (non-01) and *Aeromonas* from the

river Gomati in India were found to have multiple antibiotic resistance. Thirty-

nine percent of *V. cholerae* isolates were resistant to two drugs, while 47% of

aeromonads were doubly-resistant (211). In the Niger river delta, between 55%

and 72% of isolates from standard plate count agar were resistant to ampicillin.

Resistance was also found to chloramphenicol, tetracycline, and streptomycin and

multiple resistance was common (202). In Korea, 53.6% of coliform isolates

from the Sumjin river were resistant to at least one antibiotic, e.g., 21.3% to

quinolones (207). Even though most rivers are affected to some extent by

pollution, resistant organisms have been found even in remote locations.

Pseudomonas isolated from a remote upland stream by Magee and Quinn (160)

were resistant to ampicillin (18%), chloramphenicol (11%), kanamycin (50%),

erythromycin (72%), tetracycline (6%), and cotrimoxazole (23%). Multiple

resistance was common but tetracycline resistance was observed to occur only

with resistance to four other drugs.

Resistant bacteria can also be found in high numbers in lakes. In a study

of two Spanish lakes, 71% of isolates were resistant to at least one antibiotic

including erythromycin (31.1%), tetracycline (17.8%), chloramphenicol (22.2%),

and penicillin (68.9%) (14). Lobova *et al.* (156) found resistance percentages in a

large brackish lake in Russia that were two to three times higher at a heavily used

resort area near the shore than in the center of the lake. In contrast, a study in

England showed higher resistance in remote tarns than in a lake receiving sewage

effluent. The authors attribute this result to differences in species

composition (135).

Marine and estuarine ecosystems:

Numerous studies have shown antibiotic resistant bacteria in coastal and

estuarine environments, especially the Chesapeake Bay. Resistant *Aeromonas* is

easily isolated, though McNicol *et al.* (177) found only singly resistant species

that did not contain R plasmids. Morgan *et al.* (188) isolated several gram

negative species that were resistant to combinations of ampicillin, chloramphenicol, kanamycin, sulfadiazine, streptomycin, and tetracycline. Although resistance is generally greater in areas with more human impact, isolates of resistant environmental bacteria are common. *V. cholerae* (non-01) and *Pseudomonas*, *Erwinia*, and *Bacillus* spp. resistant to ampicillin, chloramphenicol, and streptomycin have been isolated (10, 89).

Resistant *Bacillus*, *Vibrio*, and *Aeromonas* isolates have been isolated from samples collected in the New York Bight, a heavily polluted ocean area off of the New York and New Jersey coasts (256). Coastal waters in Italy yielded resistant *Vibrio* and *Aeromonas* spp., and resistance to ampicillin, erythromycin, and ciprofloxacin was detected in isolates of enterococci in samples collected off the coast of Greece (20, 74). In marine sediments from Norway, resistant *Aeromonas* spp., *E. coli*, and *Vibrio* spp. have been isolated (16). In samples collected from the coasts of Spain, Egypt, Puerto Rico, Maryland, North Carolina, and in the Gulf of Mexico, sites receiving runoff from sewage treatment plants, had a higher percentage of resistant organisms than sites with less human impact (30, 68, 229, 244). In the Apalachicola Bay of Florida, 82% of *E. coli* isolates were resistant to at least one antibiotic (209). Resistance has also been found to be higher at the marine air-water interface than in bulk water (111). Ampicillin resistance has even been found in plasmid-carrying isolates isolated from sea ice and sediments in the Antarctic (142).

Thus, many studies have shown the presence of resistant organisms throughout the world. That resistant organisms can be found in places receiving little human impact should not be surprising, since antibiotics are natural products. However, the evidence suggests that human and agricultural activity have a greater impact on the levels of resistant organisms in all environments, from rivers to pastures and between.

Spread of resistance

Historically, researchers have assumed that the danger posed by resistant organisms in the environment would be minimal, since bacteria (especially pathogens) introduced to an environment were generally believed to survive only for a relatively short time. In the last 20 years, however, work by many investigators (11, 45, 210, 226) has shown that many bacterial species can survive far longer than once thought, and that organisms such as *Vibrio cholerae*, long considered to have a reservoir only in humans, not only survive, but are actually autochthonous to aquatic environments (57, 58). This drastically increases the probability that humans will come into contact with resistant pathogens from runoff or sewage. Additionally, even resistant non-pathogens can have a large impact on human health, serving as a source of resistance genes to indigenous organisms.

Gene transfer:

There is ample evidence to suggest that horizontal transfer has spread genes farther than was previously thought possible. A bacterium can gain antimicrobial resistance in at least one of two ways, either via mutation or horizontal gene transfer. Mutations in chromosomal genes leading to antibiotic resistance occur at different rates, depending on the organism and the mechanism of resistance. Resistance due to the inactivation of a gene will occur at a higher rate than resistance arising from mutation of a protein to a specific binding site.

Resistance due to chromosomal mutations can be a single-step mutation that immediately leads to a high level of resistance or multi-step mutations, which successively lead to higher minimum inhibitory drug concentrations being required to prevent growth. β-lactam resistance is a particularly common result of single-step mutations. Since many organisms already produce β-lactamase at a low-level, mutations that lead to over-production are frequent. Newer, extended spectrum β-lactam drugs are chemical derivatives to which β-lactamases are unable to bind. However, a change in a single base-pair can alter the affinity of a β-lactamase sufficiently to enable binding and inactivation of broad-spectrum drugs (66).

An example of a single-step mutation that is more common in certain organisms occurs in *Mycobacterium tuberculosis*. Streptomycin resistance results from altered 16S ribosomal RNA or ribosomal protein S12 that results in reduction of the binding affinity of streptomycin. There are multiple copies of these genes in *E. coli* and other fast growing bacteria, so that resistance arising

from a mutation in a single gene is rarely observed. In *M. tuberculosis*, there is only one copy of each of these genes, increasing the chances for resistance arising from mutation (66).

Multi-step mutations leading to resistance occur after a series of mutations to targets in the cell. For example, fluoroquinolone resistance is often the result of mutations in four genes that lead to increased expression of efflux pumps. Mutations in a single gene have only a minor effect on resistance, however, as successive mutations occur, resistance builds to a high level (197).

Once resistance has developed, the genes can spread widely through horizontal gene transfer. Horizontal gene transfer can occur through one of three ways: transduction, transformation, and conjugation. Transduction is the transfer of genetic material by incorporation of bacterial DNA by a bacteriophage during packaging, and the subsequent infection of another bacterium. Transfer of resistance by transduction was first shown with penicillin resistance in staphylococci in 1958 (91). Although phages generally have a restricted host range, they are common in many environments and may therefore play an important role in transfer of resistance genes.

Transformation refers to the uptake and incorporation of naked DNA from the microenvironment. Naked DNA is made available through secretion or after cell lysis. Although DNA can easily be destroyed in the environment, it can also be stabilized by binding to particles (67). In order to be transformed, a bacterium must be competent. There are many bacterial species that are either naturally

competent or in which competency can be induced by environmental conditions (67).

Conjugation refers to the transfer of genetic material through direct cell-to-cell contact accomplished by a pilus. Originally thought to be a highly specific interaction that could only occur between closely related bacterial species, we now know that conjugation occurs between diverse species and even between gram positive and gram negative bacteria (59).

In the 1940s and early 1950s, mutation and subsequent selection of resistant organisms was not given serious attention. New drugs were being developed, and the frequency of mutation to drug resistance was low. As each drug came into use, resistance developed and other new drugs were sought to replace the old. However, it was soon apparent that mutation alone could not explain the patterns of resistance that were developing.

In Japan, after the second world war, outbreaks of dysentery caused by *Shigella* were treated by sulfonamides but, by 1952, these drugs were ineffective as 80% of isolates were resistant. Tetracycline, streptomycin, and chloramphenicol were introduced and resistance to these drugs soon was detected. At first, the isolates were resistant to only a single antibiotic, but in 1956, a strain was isolated with resistance to sulfonamides, tetracycline, streptomycin, and chloramphenicol. Soon, multiply resistant isolates became common (80). Given the frequency of mutation, it was unlikely that these resistances could have developed in single strains during the time in which they appeared. In addition,

both multiply resistant and susceptible isolates of the same strain could be isolated from a single patient. Multiply resistant strains were found in patients that had previously excreted susceptible strains and that were treated with only one drug. These observations could only be explained by transfer of drug resistance from one strain to another. In 1959 and 1960, Ochiai (201), Akiba (8), and later, Mitsuhashi (184), separately published experiments showing that resistance could be transferred between *Shigella* and *E. coli*, both *in vitro* and *in vivo*. These "Resistance Factors" were found to be similar to the F factor, in that transfer occurred through conjugation. Watanabe *et al.* (267, 268), showed that resistance factors were episomal in nature and proposed the term "Resistance Transfer Factor". In the decade that followed, it was through the work of Watanabe, Datta and others (15, 64, 65, 180, 181, 268) that we learned the nature of R factors. Datta, Anderson, and Kontomichalou were also able to demonstrate transmissible resistance in several of the *Enterobacteriaceae* (15, 64, 65). We now know that R factors are plasmids that carry antibiotic resistance genes.

Plasmids are circular molecules of DNA that are separate from the chromosome. They often carry genes that provide antibiotic resistance, the ability to use alternate substrates, produce toxins, or other genes that can offer a survival advantage. Plasmids that carry the genes necessary for conjugation are called conjugative plasmids, while non-conjugative plasmids can only be spread during conjugation brought on by a conjugative plasmid. In a study of a large collection of enteric bacteria isolated before the discovery and use of antibiotics, Hughes and Datta (119) found that, although there was little antibiotic resistance among

these strains, 24% contained plasmids, suggesting that, although plasmids are useful in spreading resistance, their presence does not necessarily mean an organism is resistant.

Other mobile genetic elements include transposons and integrons. Transposons are sequences of DNA that are capable of transposing or moving from one region to another or between plasmids and chromosomes. Transposons can contain one or more genes, including those necessary for translocation, and often contain either resistance genes or genes necessary for degradative enzymes (238). These genes are flanked by insertion sequences that allow insertion of the DNA into the chromosome or plasmid.

Integrons are mobile elements that consist of conserved sequences of DNA bordering "cassettes" of genes. The conserved sequences contain the genes necessary for integration, promotion, and capture of cassettes. Gene cassettes are promoter-less units that contain the genes for antibiotic resistance or for virulence factors (225). Integrons are capable of carrying several gene cassettes and are associated with multiple antibiotic resistance and pathogenicity islands.

In recent years, through the study of genomics, we have come to learn that horizontal gene transfer occurs far more widely and often then we ever imagined. As complete genomes have been sequenced, evidence is mounting that not only do bacteria share genes with one another, but transfer occurs between all three domains of life, the Archaea, Eubacteria, and Eukaryotes. In 1998, Lawrence and Ochman (148) showed that 18% of the *E. coli* genome had been acquired through horizontal transfer. It has been shown that archaeal genes

comprise 24% of the *Thermatoga maritima* genome. This is particularly

surprising since *T. maritime* is thought to be among the slowest evolving, and on

the deepest branch, of the Eubacteria (192). Eukaryotes apparently trade genes

with bacteria as well. In an investigation of *Deinococcus radiodurans*, Makarova

et al. (161) found several eukaryotic genes including human, yeast, and fly genes,

some of which have only ever been found in eukaryotes. *Mycobacterium*

tuberculosis has also been reported to contain eukaryotic genes (87).

Transfer in the environment

Soil environments:

Although there is a great deal of direct evidence showing gene transfer in

the laboratory, the evidence for transfer in various environments is ample, though

indirect, involving the comparison of gene sequences from different species or the

use of microcosms or plasmid analysis. Many studies have reported the presence

of plasmids in environmental isolates. Though this is not proof of horizontal gene

transfer, it is strongly suggestive that it can occur especially in the presence of

selective pressure. Using phylogenetic analysis in Brazilian soil isolates, Weiner,

Egan, and Wellington (272) found evidence of transfer of the streptomycin

resistance gene *strA* between streptomycete species. In another study using

streptomycetes in microcosms, transfer of plasmids containing streptomycin

resistance genes was found (112). Plasmids have been found in opportunistic

pathogenic bacteria isolated from residential garden soil and dairy farm soil in

Florida (77). Plasmids were also found in 20% and 15% of isolates from two agricultural fields with no history of antibiotic application (41). In samples from soil and sewage sludge, Top *et al.* (257) found transfer of broad host range plasmids from *E. coli* to *Alcaligenes eutrophus*. In a study of soil samples taken from a pasture before and after the application of pig manure, Jensen *et al.* (131) found that resistance in soil bacteria temporarily increased after the application of manure. Götz and Smalla (99) were able to show a 10-fold increase in plasmid transfer from *E. coli* to *Pseudomonas putida* in agricultural soil after the application of manure.

Studies focusing on the dangers of releasing genetically modified bacteria into the environment have been performed to examine the transfer of genes in the rhizosphere. Antibiotic resistance markers were used and demonstrate not only horizontal gene transfer in the rhizosphere, but also horizontal transfer of resistance genes. Transfer has been found to occur between several species under various environmental conditions. In the wheat rhizosphere, *Pseudomonas* has been shown to transfer plasmids to indigenous bacteria (263). Disturbingly, fecal bacteria are able to transfer genes to natural soil bacteria, as in the case of transfer from *E. coli* to *Rhizobium* spp. (222). A criticism of some of these studies has been that they were done using microcosms and non-indigenous organisms with non-indigenous plasmids. However, in a study using *Pseudomonas fluorescens* applied to sugar beet seeds, Lilley and Bailey (153) were able to demonstrate that *Pseudomonas* was able to colonize leaf and root structures and also acquired plasmids from indigenous bacteria. Smit, Wolters, and van Elsas (245) were

similarly able to show transfer of indigenous plasmids in the wheat rhizosphere and Sullivan *et al.* (252) that seven years after *Rhizobium loti* was applied to a field that had no natural rhizobia, non-symbiotic rhizobia were isolated containing several gene sequences that could only have been acquired via horizontal transfer from the original strain.

Aquatic environments:

As with studies of other environments, the evidence for horizontal gene transfer in marine and aquatic environments is largely circumstantial, relying on microcosm studies, *in vitro* transfer between aquatic organisms, and the presence of plasmids. Antibiotic resistant organisms have been isolated from seawater samples collected from both sewage affected sites and control sites off the Spanish and U.S. coasts. Plasmids were found in many of these isolates and strains from polluted areas had both more plasmids and higher levels of resistance (30). In another investigation, 34% of environmental *Vibrio*, *Aeromonas*, *E. coli*, and *Pseudomonas* isolates from Chesapeake Bay and Bangladesh were found to contain plasmids (178). Hermansson, Jones, and Kjelleberg (111) found a very high percentage of multiply resistant bacteria (91%) in the marine air-water interface. Of the pigmented and non-pigmented bacteria isolated, 44% and 39% respectively, contained plasmids. Falbo *et al.* (79) were able to show multiply resistant *Vibrio cholerae* isolates from a cholera outbreak in Albania and Italy contained integrons that were transmissible. Other *V. cholerae* isolates were shown to have a transposon containing resistance to trimethoprim, 0/0129,

streptomycin, and spectinomycin (95). Although the isolates were clinical in origin, *V. cholerae* is known to be autochthonous to the estuarine environment. Plasmids have also been found in bacteria isolated from sediments and seawater from Antarctica. Forty-two percent of sediment isolates and 20% of water isolates carried plasmids, some of which were associated with antibiotic resistance (142).

Several studies have shown gene transfer between marine organisms or between marine organisms and enteric bacteria *in vitro*. Adams *et al.* (7) used the fish pathogen *Aeromonas salmonicida* to transfer a plasmid encoding tetracycline resistance to *E. coli*. In offshore seawater and sediment samples, Sizemore and Colwell (244) found antibiotic resistant bacteria in most samples, including those collected 100 miles offshore and from depths of 8200 meters. Isolates considered autochthonous to the marine environment were examined for plasmids and used in mating experiments. Of the 16 isolates studied, 50% contained plasmids. Several of these were able to transfer plasmids to *E. coli* (244).

Vibrio parahaemolyticus strains isolated from the Chesapeake Bay were also found to be antibiotic resistant and to contain plasmids. Mating experiments showed that resistance plasmids could be transferred from *E. coli* to *V. parahaemolyticus in vitro* (103). In presumptive *Vibrio* spp. isolated from an oil field and unpolluted controls sites in the Gulf of Mexico, Hada and Sizemore (104) found that 18% of control isolates and 27% of oil-field isolates contained plasmids.

Transformation and transduction are theorized to occur in the marine environment, but few studies have been done explicitly to demonstrate the phenomenon. However, the presence of large numbers of virus make it likely that transduction is an important means of gene transfer in the environment. In three studies of different marine environments, similar counts of viruses were found. Wommack *et al.* (277) obtained counts of 2.5 x 10^7 viruses ml^{-1}, while Bergh *et al.* (32) found 2.5 x 10^8 viruses ml^{-1}, and Børsheim *et al.* (39) found 1.8 x 10^7 viruses ml^{-1}. Using marine bacteriophages and marine bacterial isolates, Jiang and Paul (132) were able to show that transduction can occur *in vitro*.

Transfer of a tetracycline resistance plasmid from *A. salmonicida* to indigenous bacteria in marine sediment microcosms has also been demonstrated, even without selective pressure from the presence of tetracycline. When tetracycline was present, however, 86.8% of isolates had acquired the plasmid, while only 45.8% without tetracycline (232). Using green fluorescent protein (GFP), conjugative transfer was demonstrated between *P. putida* and indigenous bacteria in both artificial seawater and seawater samples (62), even under nutrient-limiting conditions (63). Some investigators have speculated that conjugal transfer can occur only under excess nutrient conditions, but this has been shown not to be the case. In microcosms and on plates, *P. fluorescens* transferred plasmids to marine isolates, even when in the viable but non-culturable (VBNC) state (50). Additionally, in an artificial seawater microcosm, both *E. coli* and marine *Vibrio* sp. S14 were capable of conjugation with another *Vibrio* S14 strain after nutrient starvation (98).

Horizontal gene transfer of antibiotic resistance also occurs in freshwater environments. In an investigation of staphylococcal isolates from polluted streams in Morocco, Kessie *et al.* (139) found that several of the isolates from different species contained a tetracycline resistance plasmid of the same size. In addition, one strain of *Staphylococcus hyicus* contained all but one of the plasmids that were found in the other isolates (139). In a Korean river, 12.6% of coliform isolates contained integrons (207) and in sediment collected from a river in Wales, 9.4% of isolates from unpolluted sampling sites and 15% of isolates from polluted sampling sites contained plasmids, 86% of which were large enough to be conjugal plasmids (43).

As stated above, transduction provides a means of gene transfer in freshwater environments. In environmental chambers suspended in a freshwater lake, transduction of streptomycin resistance occurred in *P. aeruginosa* strains (190). Using the same chamber system and different plasmids and phages, transduction was also shown both in the presence and absence of natural bacterial communities (233).

Although 14% of isolates from bulk freshwater and 16.2% of isolates from the air-water interface contain plasmids, higher percentages of multiply resistant bacteria have been found at the air-water interface in freshwater lakes. *P. aeruginosa* transferred plasmids to indigenous bacteria isolated from the air-water interface when cultured in microcosms. Jones, Baines, and Genther (134) were able to show 100 times more conjugation at the interface than in the bulk freshwater, even under conditions of nutrient starvation. Freshwater isolates can

serve as recipients of conjugation with *P. aeruginosa*, as demonstrated in

laboratory experiments (93). *P. aeruginosa* is also capable of conjugation when

tested in environmental chambers suspended in lake water, although the number

of transconjugants were less in the presence of indigenous bacterial populations

(203). Epilithic bacteria have also been shown to be capable of conjugative

transfer when examined in *in vitro* mating experiments with *P. putida* (113).

Animal environments:

Horizontal gene transfer occurs throughout animal environments,

including farms and in the farm animals themselves. Animals in the wild and

insects also contribute to the spread of antibiotic resistance genes. Integrons have

been isolated from samples collected throughout poultry farms and poultry

processing plants, using total microbial DNA rather than culturable organisms

(225). In a study of enteric bacteria isolated from horses, cattle, poultry, and

reptiles, Goldstein *et al.* (6) found Class 1 integrases in 46% of the isolates tested.

E. coli carrying transferable trimethoprim and ampicillin resistance plasmids from

chickens carried the plasmids, even in the absence of selective pressure (51).

Several studies of gene transfer and antibiotic resistance in wild animals,

both from areas heavily impacted by humans and in remote regions have been

done. In a moose and voles from Finland, low levels of resistance were found,

but, one isolate from a moose was capable of transferring streptomycin resistance

to *E. coli* by conjugation (205). Souza *et al.* (249) examined *E. coli* from wild

rodents, cats, reptiles, and marsupials from Mexico and Australia, showing

plasmids in isolates from most of the samples, although those nearest to urban areas had significantly higher numbers (249).

In enterococcal isolates from pigs, both streptogramin resistance and vancomycin resistance were found to be transferable *in vitro* (2, 271). Of evernimicin resistant *Enterococcus faecium* isolates from pigs and broilers, all were capable of transferring resistance to a susceptible strain of *E. faecium* (3). In one study, three of four *E. coli* isolates from diseased chickens were capable of transferring streptomycin and streptomycin, tetracycline, sulfa resistance to other *E. coli* strains (278). Gene transfer has also been shown to occur among bacteria isolated from pens of calves. Calves carrying *Salmonella typhimurium* type DT193 with a multiple resistance plasmid were found also to have a large number of *E. coli* of different antigenic groups in the same plasmid. When muzzled calves were fed donor and recipient strains, no transconjugants were isolated, but when the calves were allowed to lick objects in their environments, transconjugants appeared in their feces (154, 255).

There is good evidence of transfer in the poultry gastrointestinal tract. For example, rifampin resistant *E. faecium* fed to broiler chicks transferred resistance to indigenous flora at a higher rate than that observed *in vitro* (193). In another study, *E. coli* isolated from diseased poultry demonstrated high levels of multiple resistance and integrons. The integrons were found to be part of the transposon Tn21, a transposon widespread among the *E. coli* isolates, as well as in *Salmonella* strains isolated at the same time (27).

Annelids and arthropods also play a role in gene transfer and antibiotic resistance. In soil columns, earthworm activity was found to increase gene transfer from *P. fluorescens* to indigenous soil bacteria from several genera including *Agrobacterium*, *Acinetobacter*, *Pasteurella*, and *Xanthomonas* (61). The contribution of invertebrates to gene transfer in the environment is not limited to earthworms. The epiphytes *Enterobacter cloacae* and *Erwinia herbicola* have been shown to transfer plasmids in the guts of silkworms (266). *Erwinia herbicola* is also capable of transferring plasmids to resident gut flora in silkworms (265). Strains of *Bacillus thuringiensis* were able to transfer plasmids containing toxin production genes in the guts of two species of moth larvae (125). Strains of *Enterobacter cloacae* were also able to transfer an antibiotic resistance plasmid in the gut of gutworms (19).

The animal-environment-human web of resistance

Ultimately, it is the prospect of being unable to treat human disease with antibiotics that is most disturbing. Clearly, the use of antibiotics leads to increased antibiotic resistance in humans, animals, and the environment. Resistance genes can spread via horizontal gene transfer in practically every environment. This raises the question of whether antibiotic resistance in the environment leads to antibiotic resistance in humans. There is sufficient evidence to support the hypothesis that resistant organisms and resistance genes spread from humans to animals and to the environment, and vice versa. Much of the

evidence comes from the presence of similar gene sequences in bacteria isolated from humans and animals and from examination of antibiotic resistant pathogens isolated from diseased humans that were traced to animals.

In a study of human isolates of *Bacteroides* and isolates of *Prevotella* (normally found in the rumen of livestock) from different areas, sequences of the tetracycline resistance gene *tetQ* were almost identical (21, 194). In a CDC investigation of salmonellosis outbreaks from 1971 to 1983, researchers found that of 38 outbreaks, where the source could be identified, food animals were the source of 69% of the antibiotic resistant strains, and 46% of susceptible strains (116). In 1984, an outbreak of resistant salmonellosis across four Midwestern states was traced to beef cattle from South Dakota that had been fed antimicrobials. The isolates were all resistant to ampicillin, carbenicillin, and tetracycline. Interestingly, it is believed that the patients were asymptomatically infected and that disease was initiated after the consumption of antibiotics for other reasons allowed for selection of the resistant strains (115). *Salmonella* isolates have also been examined for their serotype and plasmid content. Within groups of serotypes, plasmids from both humans and animals were generally identical. One serotype and plasmid combination was found in 26 infected persons and in cattle from 20 states. The epidemiology of the cases suggests that they were derived from a food source (200). *Salmonella typhimurium* DT014 is generally resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. In Denmark in 1998, an outbreak of DT104 occurred in which the isolates were also resistant to nalidixic acid and had reduced susceptibility to

fluoroquinolones. This reduced susceptibility was caused by a single mutation in the gyrase gene. After tracing the outbreak to a pig farm, investigators found the same mutation in *Salmonella* isolated from the pigs and were unable to find the mutation in other isolates (185). A 1995 outbreak of DT104 in England and Wales occurred in which a major risk factor was determined to be contact with sick calves. Ten percent of the cases occurred on farms in a region where most residents live in urban areas (264). A 1980 outbreak of *Salmonella heidelberg* that was resistant to chloramphenicol, sulfamethoxazole, and tetracycline occurred in infants in a hospital nursery. The mother of one of the infants also developed diarrhea from the same strain. She apparently had worked on a farm where there were sick calves before coming to the hospital to deliver her baby (159). CDC data have shown that, in the U.S., the resistance patterns of *Salmonella* are more closely related to antibiotics given to farm animals that to those given to humans (17).

Spread of resistant pathogens, other than *Salmonella*, from animals to humans is common. In Taiwan, a large proportion of *E. coli* hospital isolates were either resistant to or had reduced susceptibility to fluoroquinolones (171). Human fluoroquinolone use was not high enough to explain the findings, especially in children, for whom fluoroquinolones are not approved and where the percentage of reduced susceptibility was as high as the rest of the population. However, large quantities of fluoroquinolones are used in poultry production, and a high percentage of food chickens purchased in Taiwanese markets are positive for fluoroquinolone resistant *E. coli* (171). In Spain, Garau *et al.* (90) obtained

similar results. Quinolone resistance was high in *E. coli* from both adults and children, even those never before exposed to quinolones. Yet, 90% of chicken and 45% of pig *E. coli* isolates were quinolone resistant (90).

In The Netherlands, it is likely that quinolone resistance in *Campylobacter* developed between 1985 and 1987. During this time, enrofloxacin was introduced for animal use and norfloxacin was introduced for human use. Enrofloxacin was used extensively in poultry and in pigs, whereas norfloxacin was used in humans, mostly to treat urinary tract infections (76). Quinolone resistance in campylobacters isolated from poultry went from 0% in 1982, to 14% in 1989, and in humans from 0% to 11% during that time (76). A study of *Campylobacter jejuni* in Minnesota showed that the proportion of quinolone resistant isolates increased from1.3% in 1992, to 10.2% in 1998. Although some outbreaks could be contributed to foreign travel, most were attributed to increased resistance in poultry (246).

Enterococcal resistance can also be spread from animals to humans. In France, the prevalence of VRE is much higher in cattle-rearing areas than in other regions of France and Europe. However, the authors qualified their conclusions by saying that they may have found higher numbers due to an enrichment step during isolation (86). A Dutch study of broiler farmers, laying-hen farmers and slaughterers showed that VRE in the human samples was correlated to VRE in the chickens. Also suggestive of transfer from chickens to farmers was the fact that amoxicillin resistant enterococci were isolated only from broiler chickens and broiler farmers, not the other samples. The transposon encoding vancomycin

resistance was also similar between isolates from farmers and chickens (261). In Norway, VRE were isolated from 97% of chicken houses exposed to avoparcin and only 18% of non-exposed chicken houses. Similarly, VRE was found in 60% of farmers from exposed farms and none from non-exposed farms (144). Using pulsed field gel electrophoresis to compare poultry and human VRE isolates in The Netherlands, van den Braak *et al.* (262) found that poultry and human isolates were not clonal, however, there were two major genotypes of the vancomycin resistance gene *vanA*. Forty-two percent of poultry isolates were of one type, 58% were of the other type. All of the human isolates had the second phenotype (262). Vancomycin resistance in enterococci is generally conferred by the transposon Tn1546. In Europe, VRE and Tn1546 are widespread in the community, animals, and environment. In the U.S., VRE have only been found in hospital acquired infections. Simjee *et al.* (242) isolated a gentamicin resistant VRE strain from a canine urinary tract infection that contained Tn1546 identical to Tn1546 until now found only in human isolates. Also studying Tn1546 in 271 VRE isolates, Jensen (130) was able to show that European VRE have a base pair variation in the *vanX* gene, with either a G or a T in position 8234. All poultry isolates had the G type, while all except one of the swine isolates had the T type. Human isolates had both types with one or the other dominating in a particular region (130). Vancomycin has been heavily used in U.S. hospitals, and less so in European hospitals, whereas avoparcin, which is cross-resistant with vancomycin, has been used heavily in European agriculture, and has never been approved in

the U.S. This is likely the reason for high community carriage in Europe and high

nosocomial infection rates in the U.S.

Quinupristin/dalfopristin is a streptogramin antibiotic that was approved in

the U.S. and Europe in 1999. However, three resistant streptococcal strains had

been isolated from patients in 1997 (145). Quinupristin/dalfopristin is cross-

resistant with virginiamycin which has been in use as an animal growth promoter

for 20 years. Within less than two years of approval, enterococcal isolates

resistant to quinupristin/dalfopristin have been found with high frequency in

poultry environments, food, humans, and wastewater (107, 136, 170, 242, 271).

In Germany during the 1980s, nourseothricin, a streptothricin antibiotic, was used

in pigs. Though streptothricin antibiotics were not used in humans,

nourseothricin resistant *E. coli* could be isolated from pig farmers and people

living in pig farming areas (120).

II. Chesapeake Bay Research

Antibiotic Resistance in *Vibrio cholerae* Isolated from the Chesapeake Bay

The Chesapeake Bay and its watershed provides a unique site for study of the spread of resistance genes and resistant microorganisms. The largest estuary in the U.S., the bay has a watershed of 167,000 km^2 that includes both rural and urban areas (37). Environmental parameters vary greatly from the mouth of the bay to the upper reaches and the Bay comprises a number of different ecosystems and habitats. Many studies of the microbiota of the bay have been done and several document antibiotic resistance and plasmid content of various microbial genera isolated from the bay. McNicol *et al.* (177, 178) found plasmids in 21% of *Aeromonas hydrophila* and 18% of *Vibrio cholerae* strains isolated from bay water and resistance to erythromycin, polymyxin, streptomycin, and tetracycline was detected (177, 178). Studies by Allen (9, 10), Guerry (103), and Morgan *et al.* (188), showed antibiotic and heavy metal resistance in bacteria isolated from Chesapeake Bay water, sediment and shellfish. Morgan *et al.* (188) examined antibiotic resistant coliforms isolated from water and shellfish, finding larger numbers of resistant bacteria in samples collected from near Baltimore Harbor than from other, less populated areas of Chesapeake Bay. Of the strains isolated, 73.7% were resistant to both ampicillin and streptomycin, and 25.9% were resistant to both ampicillin and tetracycline (188). Guerry (103) isolated multiply resistant gram-negative bacteria including *V. parahaemolyticus* from water and shellfish in the bay. As in other studies, numbers of resistant organisms were much higher near Baltimore harbor. Thirty-seven percent of isolates were capable

of transferring antibiotic resistance markers to *E. coli* (103). Allen (9, 10) found antibiotic resistant *Pseudomonas*, *Aeromonas*, *Vibrio*, *Flavobacterium*, *Staphylococcus*, and enteric species in samples from water and sediment. Of the resistant isolates, 96% were resistant to two or more antibiotics.

Long thought to be found only in contaminated waters, *Vibrio cholerae*, the etiological agent of cholera, is now recognized as autochthonous to marine and estuarine waters throughout the world, including regions not currently affected by cholera. To observe natural antibiotic resistance patterns, 256 strains of *V. cholerae* isolated between 1998 and 2000 were examined for antibiotic resistance. The Chesapeake Bay geographical area represents a region free from epidemic cholera for almost 90 years. Despite the lack of epidemic cholera, *V. cholerae* can be detected in bay water by a variety of methods, especially during the summer months, because its natural habitat includes estuaries. The antibiotic resistance patterns of the Chesapeake Bay strains provide a reference pattern and may also provide better understanding of the ecology of antibiotic resistance in an estuarine environment.

As part of a larger study on the occurrence of *V. cholerae* in the Chesapeake Bay, 256 strains of *V. cholerae* isolated between 1998 and 2000 were examined for antibiotic resistance (89, 157). Sampling was done from January 1998 to February 2000 at coastal sites throughout the upper Chesapeake Bay (see figure 1) and samples were collected from surface water, about 1m from the surface. A surface water fraction and two plankton fractions also were collected using sterile plastic containers at each of the shore sampling sites. Samples were

Figure 1. Location of Bay Sampling Sites
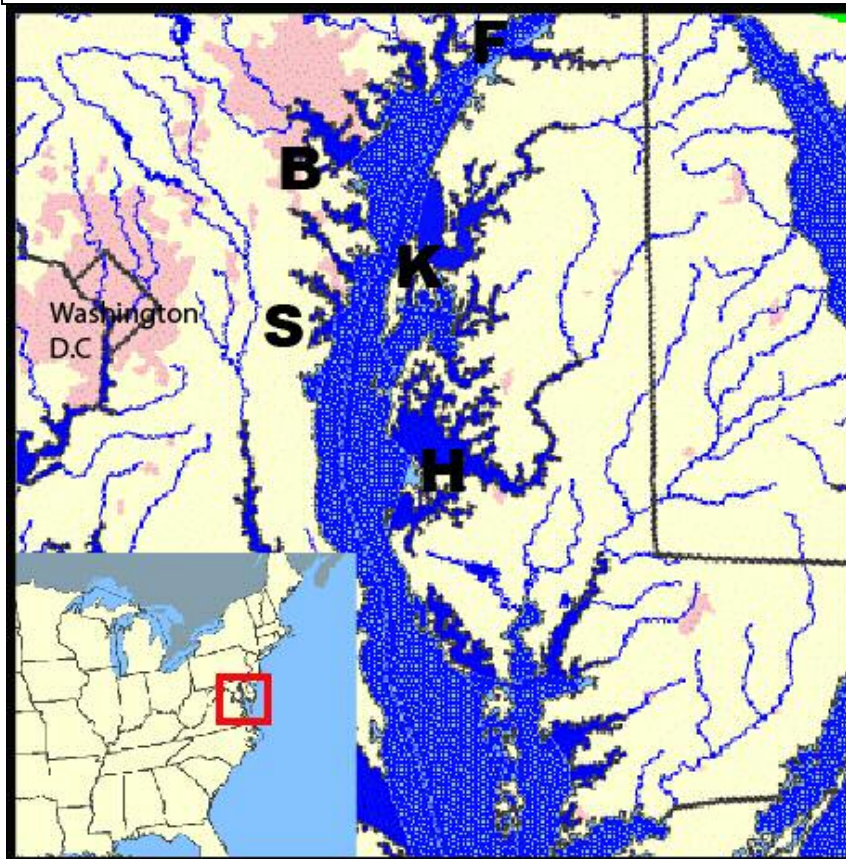
Sampling site locations are as follows:

Site B = Baltimore Harbor
Site F = Susquehanna Flats
Site K = Kent Island
Site H = Horn Point Laboratory
Site S = Smitsonian Environmental Research Center
Map courtesy of the National Atlas of the United States,
http://nationalatlas.gov

transported to the laboratory at ambient temperature and processed within 6h of

collection. Surface water temperature and salinity were measured at the sites and

V. cholerae was isolated using alkaline peptone water enrichment (54).

Presumptive *V. cholerae* isolates were confirmed by PCR, using *V. cholerae*-

specific primers (56) or dot blotted onto Luria-Bertrani agar, transferred to filter

paper, and hybridized with a ^{32}P-labelled *V. cholerae* probe. Positive colonies were identified by autoradiography. *V. cholerae* 01 and *Vibrio mimicus* grown on the same petri dish and processed along with presumptive colonies, served as positive and negative controls, respectively. The Kirby-Bauer standard antibiotic disk diffusion test was used to assess antibiotic resistance using NCCLS breakpoints for *Enterobacteriaceae* as tentative standards for resistance and susceptibility (150).

Of the 15 strains that were resistant to ampicillin, 8 (53.3%) were also intermediately resistant to streptomycin. See table 4. Twenty (57%) of the strains intermediately resistant to ampicillin were also intermediately resistant to streptomycin. Ciprofloxacin resistant and intermediately resistant strains were also resistant to ampicillin and intermediately resistant to streptomycin. The *V. cholerae* isolates that were cultured were non-01 and non-0139, but *V. cholerae* 01 strains were detected by direct fluorescent antibody assay.   There were not enough strains isolated to determine seasonality or sample site differences. In a similar study in 1979, Allen (9) investigated the prevalence of antibiotic resistant bacteria isolated from the Chesapeake Bay. Some results are presented in table 5. In addition, Allen found multiple resistance in 96% of resistant strains. Although Allen did not identify all genera to the species level, the *Vibrio* isolates she found had much higher levels of resistance. It should be noted that the two studies are not directly comparable since only *V. cholerae* were investigated here and Allen did not identify isolates to the species level.

Table 4. Antibiotic Resistance of *V. cholerae* Isolated from Chesapeake Bay

Number of Strains (percent)	Resistant	Intermediate	Susceptible
Ampicillin	15 (5.8)	35 (13.7)	206 (80.5)
Chloramphenicol	0	0	256 (100.0)
Ciprofloxacin	1 (0.39)	1 (0.39)	254 (99.2)
Streptomycin	4 (1.6)	76 (29.7)	176 (68.7)
Sulfamethoxazole/ trimethoprim	0	0	256 (100.0)
Tetracycline	0	0	256 (100.0)

Table 5. Antibiotic Resistance in Genera Isolated from Chesapeake Bay

Percent Strains Resistant	*Pseudomonas*	*Aeromonas/Vibrio*	*Enterobacteriaceae*
Ampicillin	74%	34%	22%
Chloramphenicol	72%	53%	40%
Streptomycin	77%	86%	62%
Tetracycline	17%	34%	27%

data from Allen (9)

Evaluation of Media and Culture Conditions for Enumeration of Heterotrophic Bacteria from Rivers in Maryland

The Delmarva Peninsula of the Chesapeake Bay, comprising Maryland's Eastern Shore, a portion of Virginia, and the state of Delaware is one of the nation's most concentrated chicken producing areas. Poultry production accounted for 70% of the agricultural income of Delaware in 1991, and 31% of Maryland's agricultural income in 2000 (37, 243). Maryland ranked eighth in number of broilers produced with 283 million chickens totaling $462 million in 2000 (165). Poultry manure from these farms is applied to 42% of the cropland

on the Eastern Shore, and unfortunately, most of the drainage from that cropland flows into the Chesapeake Bay (251).

The poultry industry has been an economic boon to Maryland and other regions, but a vast amount of waste is produced by this industry and contributes to runoff into the Chesapeake Bay. Eutrophication of Chesapeake Bay by both point and non-point sources of pollution has been well documented (37, 126, 162, 251). Nitrogen and phosphorus have been recognized as contributing to hypoxia and blooms of toxic dinoflagellates, leading to a decrease in submerged aquatic vegetation (37, 251) and significant attention has been given to nutrients in agricultural runoff. Nutrient management regulations have been established to mitigate damage but little attention has been paid to other possible effects of runoff (63), since the management practices deal mainly with reducing nitrogen and phosphorus input.

In an effort to understand the relationship between poultry farms and antibiotic resistance in the river system of the Eastern Shore and the watershed of the Chesapeake Bay, a preliminary study was carried out (88). Enumeration and isolation of bacteria from the aquatic environment by heterotrophic plate count has long been practiced in environmental microbiology. Total viable, aerobic, heterotrophic bacterial counts are useful in ecological studies as a baseline for study of other organisms, as well as a first step in characterizing the population dynamics and ecology of a particular niche. Such counts also allow further study of the phylogenetic and physiological diversity of various aquatic environments. There are many specialized types of media available for such counts. Most are

limited in nutrient concentration in order to simulate the oligotrophic conditions most often encountered in aquatic and marine systems. Although there are many such media available, little work has been done to determine which yields the largest number of bacteria. Plate Count Agar (PCA), Heterotrophic Plate Count Agar (HPC), and R2A Agar (R2A) were compared for successful recovery to culture.

A randomized complete block study was used, with water samples collected in November 2002, and May 2003, from four sites each in the Patuxent (Site numbers 1-4) and Pocomoke (Site numbers 5-8) rivers in Maryland. See figure 2. These rivers receive runoff from both agricultural and urban sources and drain into the Chesapeake Bay. The Patuxent river is located on the Western Shore of the Chesapeake Bay and receives runoff from urban sources, while the Pocomoke river is on Maryland's Eastern Shore and receives mostly agricultural runoff, including a significant amount of runoff from poultry farms.

Samples were collected below the surface at the shoreline, using sterile bottles, and transported to the laboratory in an insulated cooler. Surface water temperature and salinity were measured at the sites using a field thermometer and a refractometer. In the laboratory, samples were serially diluted in sterile saline and spread plates, in duplicate, were prepared on appropriate media. PCA, HPC, and R2A plates were incubated at either 30º C for 24 hours or at 15º C for 6 days (88).

Figure 2. Location of Riverine Sampling Sites.
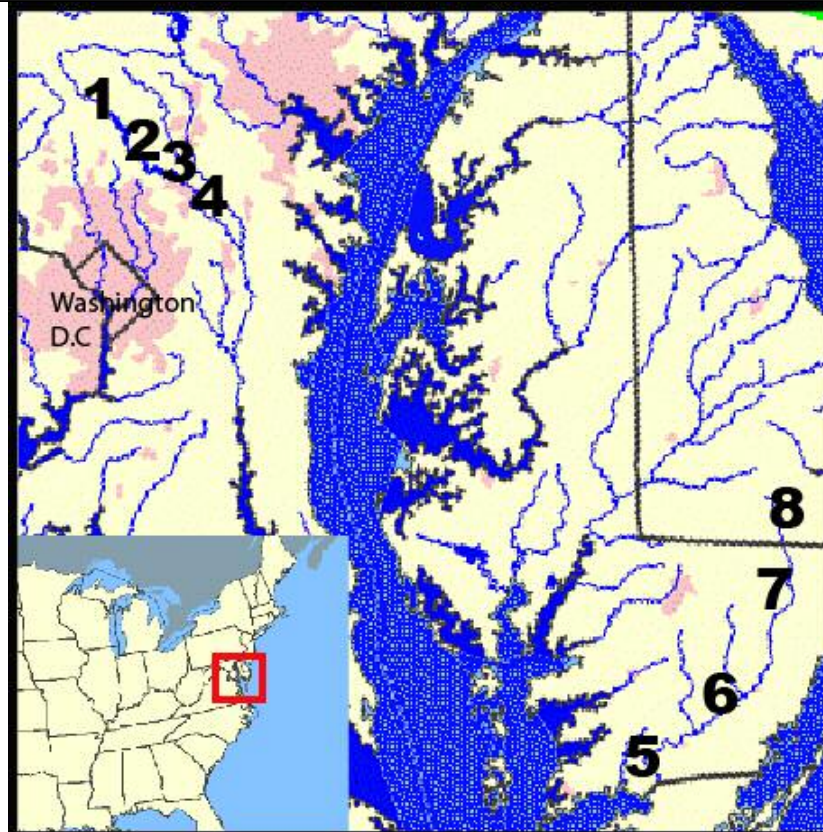
Sites 1-4 are located along the Patuxent river

Sites 5-8 are along the Pocomoke river
Map courtesy of the National Atlas of the United States,
http://nationalatlas.gov

In November 2002, water temperatures ranged from 9º C to 12º C and in

May 2003 from 16º C to 17º C. At each site and within each temperature, R2A

yielded significantly higher counts than either PCA or HPC (p<0.01). As shown

in figures 3 and 4, in November, plates incubated at 15º C yielded higher numbers

of bacterial colonies than plates at 30º C. However, in May, this pattern was

reversed (figures 5 and 6). In both months, within each incubation temperature,

neither PCA nor HPC counts were significantly higher than the other. Pocomoke river water samples generally yielded higher bacterial counts than water samples from the Patuxent. Bacterial counts for water samples collected in November ranged from 1.6×10^2 to 2.89×10^3 cells/ml for Patuxent samples and from 2.7×10^2 to 8.6×10^4 cells/ml for the Pocomoke. Bacterial counts in May ranged from 7.1×10^2 to 3.7×10^5 cells/ml for Patuxent water samples and from 6.3×10^3 to 3.14×10^5 cells/ml in Pocomoke samples.

There were no significant differences between the sites sampled. Temperature and salinity measurements were similar and the numbers of bacteria in water samples collected from each river were similar. Higher bacterial numbers in samples from the Pocomoke river may be explained by higher nutrient concentration from the runoff from agricultural areas on Maryland's Eastern Shore. R2A agar consistently supported greater numbers of bacteria.

Of the media available for obtaining total viable, aerobic, heterotrophic bacterial counts, results of the study by Gangle *et al.* (88) showed that, for the two rivers studied, the two seasons, and different incubation temperatures, R2A agar yielded higher bacterial recovery than either PCA or HPC and can be used to monitor antibiotic resistance patterns of bacteria in the estuarine environment.

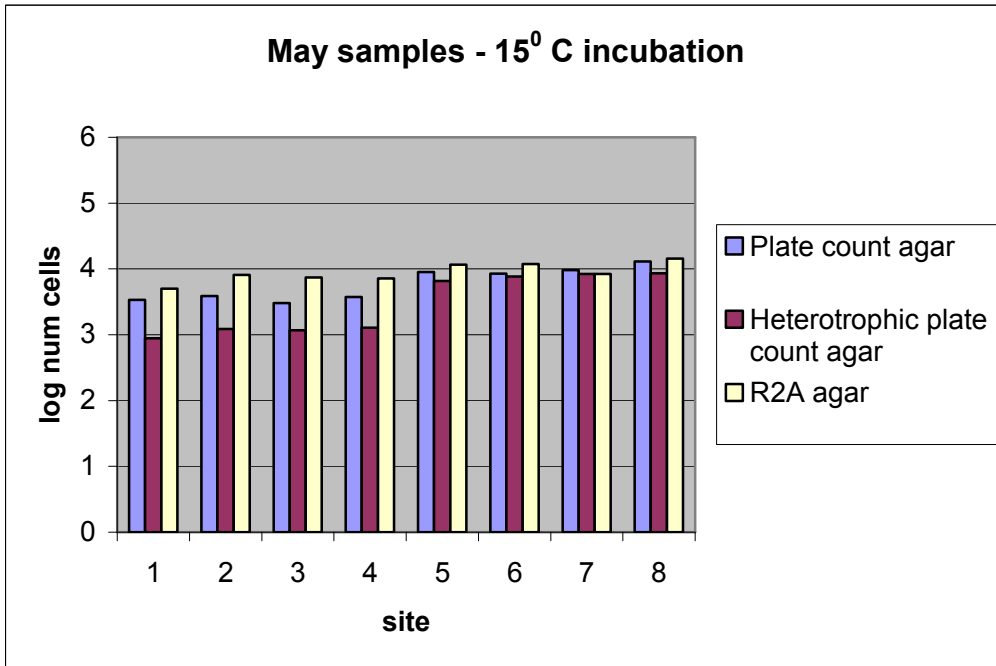**Figure 3. November 15° Incubation Heterotrophic Plate Counts**
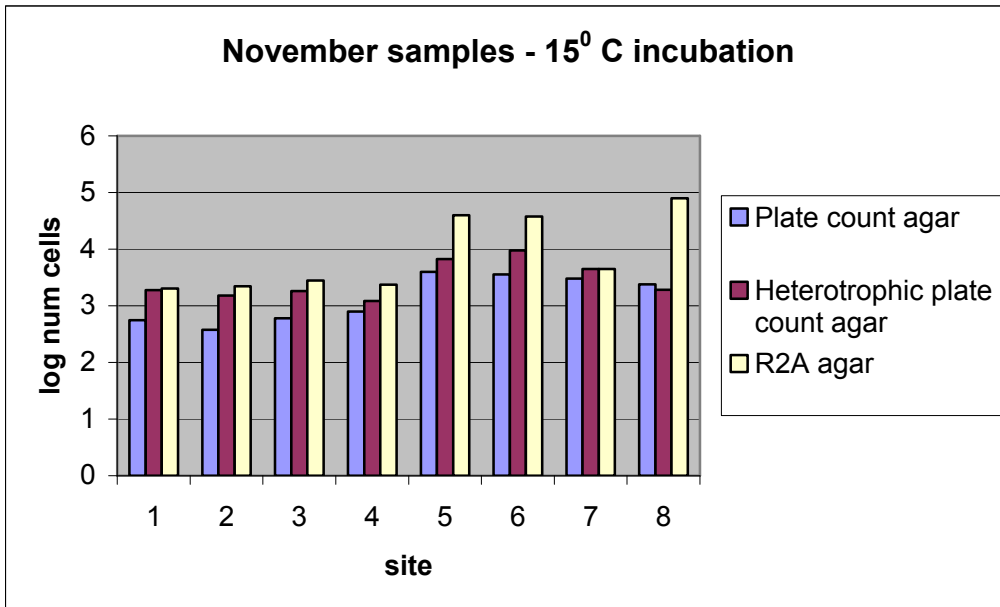
Data shown are means of two replicates

Figure 4. November 30° Incubation Heterotrophic Plate Counts
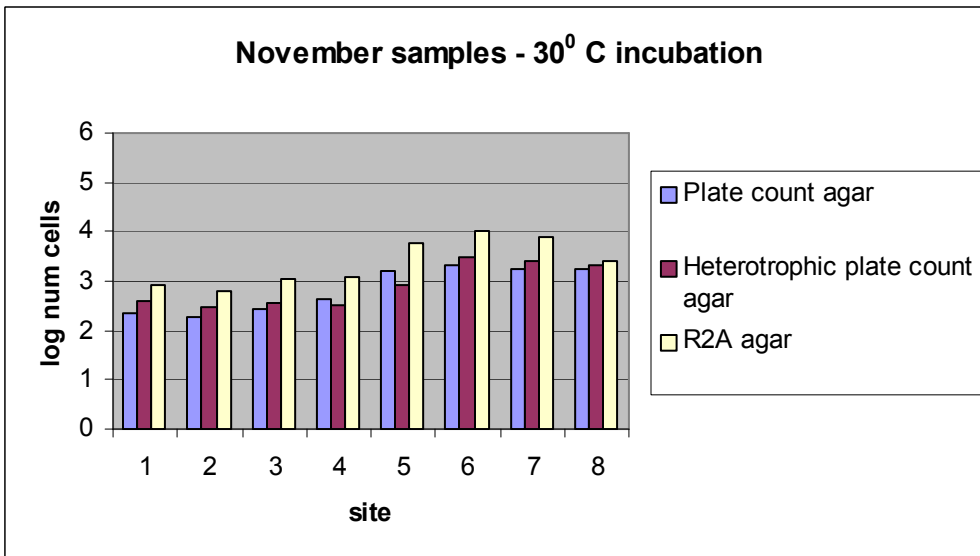
Data shown are means of two replicates

Figure 5. May 15° Incubation Heterotrophic Plate Counts
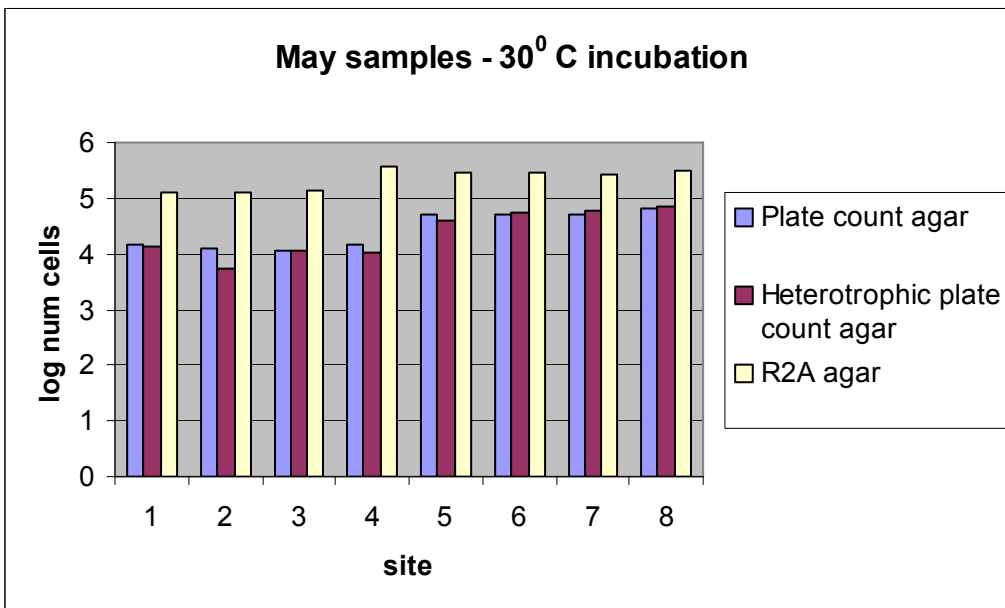

May samples - 15^0 C incubation

Data shown are means of two replicates

Figure 6. May 30° Incubation Heterotrophic Plate Counts

May samples - 30^0 C incubation

Data shown are means of two replicates

III. Conclusion

Since the discovery and introduction of antibiotics, microbial resistance to antibiotics has steadily increased. Humans have significant influence on bacterial populations in the natural environment via large scale selective pressure through waste management, agricultural, and other practices, notably, in antibiotic resistance. In past years, development of resistance to antibiotics was not considered a serious problem because of the notion that there would always be a new antibiotic discovered. The reality is, whether for economic, political, or scientific reasons, no novel antibiotics have been produced for several years, and few are on the horizon. While humans have been idle in this biological arms race, bacteria continue to develop resistance against each new drug we create. Infections once easily curable are now regarded as a growing threat from the drug-resistant microbial agents of these diseases.

While the seriousness of the problem of antibiotic resistance is now recognized, the complex web of resistance linking humans, animals, and the environment is only beginning to be understood. Indiscriminant use of antibiotics for medical purposes has taken the brunt of the blame, namely, use by those physicians who prescribe antibiotics for viral infections to make their patients feel comfortable when antibiotics are known to be useless against viruses. In fact, all antibiotic use, whether medical, agricultural, and necessary or not, leads to increased resistance.

Antimicrobial use in agriculture and aquaculture has been blamed for the increase in microbial resistance to antibiotics. But can this use lead to detrimental

effects in humans? Some argue that no link has yet been proven (208, 216, 217).

Ample evidence presented in this review suggests such a link. Many studies have

shown that application of antibiotics leads to resistance in the guts of animals, and

that humans acquire resistant bacteria via contact with farm animals and the

farming environment. Because slaughter and processing of food animals

contaminates the meat with fecal bacteria, humans can also acquire resistant

organisms through the food chain.

As illustrated in figure 7, agricultural antibiotic use leads to increased

resistance in the environment since resistant organisms and antibiotic residues are

excreted as waste that is frequently spread onto farmland as organic fertilizer.

Fecal bacteria survive long periods in the environment and spread through runoff

into groundwater, rivers, and marine ecosystems.

Some investigators claim that there is danger to humans only if resistant

pathogens spread to humans (46, 215-217). However, horizontal gene transfer

occurs in the guts of humans and animals and in a variety of ecosystems, creating

a pool of resistance in fields and waters. This leads to several dangers for

humans. Even if people do not directly contract resistant disease from food

animals, there is the danger of contact with resistant fecal pathogens from the

environment. Additionally, pathogens that are autochthonous to the environment

can acquire resistance genes from animal fecal bacteria. Aquatic pathogens, such

as *Vibrio vulnificus* would be very dangerous if they did not respond to antibiotic

treatment. There is another level of danger, however. Non-pathogenic bacteria

can also acquire resistance genes and serve as a continuing source of resistance for other bacteria, both in the environment, and in the human gut.

Figure 7. Animal-Environment-Human Web of Resistance Conceptual Diagram
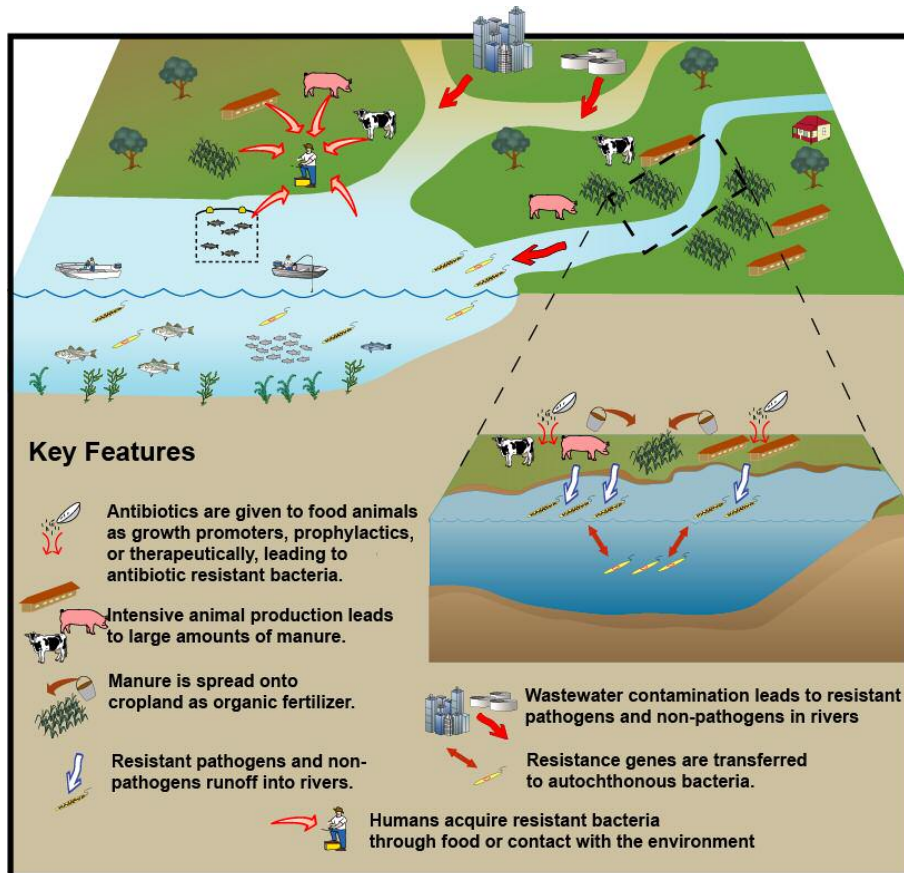
Symbols for diagrams courtesy of the Integration and Application Network (ian.umces.edu/symbols), University of Maryland Center for Environmental Science.

As the effectiveness of antibiotics for medical applications declines, the dilemma of antimicrobial use in non-medical applications must be addressed from a public health perspective. How then, do we approach this problem? Several recommendations can be made. Monitoring of antibiotic resistance is necessary to keep abreast of changes, especially any changes that may result from

management decisions. In the U.S., there is only one governmental organization whose function is to monitor antibiotic resistance: the National Antimicrobial Resistance Monitoring System (NARMS). NARMS is a joint venture of the Centers for Disease Control and Prevention (CDC), the Food and Drug Administration (FDA), and the U.S. Department of Agriculture (USDA) (47). NARMS receives isolates of food-borne pathogens from public health laboratories around the country to test for and monitor antibiotic resistance. Unfortunately, their purview is limited to several food-borne pathogens and indicator organisms isolated from food, food animals and humans. In the European Union, there are resistance monitoring agencies much like NARMS in most countries. However, the testing of these agencies is limited to veterinary pathogens, zoonotic agents, and indicator bacteria, and only from food animals, farms, and humans (279). There are non-governmental organizations that attempt to monitor resistance, one of which is the Alliance for the Prudent Use of Antibiotics (APUA). The APUA is an advocacy group comprised mostly of scientists whose function is to coordinate programs like the Global Advisory on Antibiotic Resistance Data program, which collects data on resistance from global sources, and the Reservoirs of Antibiotic Resistance Network, which provides funding for projects to study antibiotic resistance in non-pathogenic bacteria (12). The most common source of information on resistance in the environment is short-term studies by researchers. Much of this data is compiled by groups like the APUA, but there is no program of on-going monitoring of environmental resistance in the U.S. or European Union.

There are options available to reduce the threat of antibiotic resistance. Since one of the ways that resistant organisms are spread into the environment is through land application of poultry manure, treating the manure may reduce or eliminate the threat. Reducing the use of antibiotics through legal bans or alternative health methods may also be viable.

Many scientists feel that a reduction in agricultural antibiotic use will only happen if there is a ban on that use. Some have suggested banning all "human-use" antibiotics and some have suggested banning only non-therapeutic use, including growth promotion. Human-use antibiotics are those that are medically important to humans. There are problems with banning medically important drugs. First, there are only a few classes of antibiotics and most drugs that are not specifically used in humans are structurally related (and cross-resistant) to those that are. Second, as in the case of virginiamycin and the streptogramins, a drug, or class of drugs, may not be medically important now, but may someday become important.

Banning of growth promoters is the step most often recommended to deal with antibiotic resistance in the environment. Most agricultural use of antibiotics (at least in the U.S.) is for growth promotion. Estimates vary, but surveys suggest that between 15 million and 28 million pounds of antibiotics are used for non-therapeutic uses in the U.S. each year (152, 174). The efficacy of a growth promoter ban is unknown. All European Union countries currently ban the use of growth promoters. After the ban in the E.U., resistance has decreased (in some cases drastically) (217). But, while it is important to reduce the levels of

resistance in our environment, this solution is not perfect. With the overall

decrease in antibiotic usage, therapeutic use has increased to deal with an increase

in disease. Some authors argue that animal disease has increased and the

finishing weights of animals have decreased (46, 217). And, in addition to

finding only a limited decrease in resistance in humans, there has actually been an

increase in food-borne disease (46).

To reduce the effect of bacterial runoff from manure, Perdue Farms Inc.

has joined with a company called AgriRecycle to produce a pelletized fertilizer

called MicroStart60™ from poultry litter. The plant, located in Delaware, was

designed to process 95,000 tons of litter each year and the company will pick up

litter from any farmer, regardless of company affiliation. The process includes

heating to kill any microorganisms and to completely dry the product (69).

Perdue's goal is to remove nutrients from the Delmarva peninsula by shipping

them to the rest of the country. The process should work, however, the economic

feasibility (and therefore long-term sustainability) is unknown.

Long-term storage of manure before land application has also been

suggested as a means to eliminate bacteria from poultry manure. This can be a

simple, low-cost method of treatment that has some utility, however, even after

three to four months, there are reduced, but still significant numbers of multiply

antibiotic resistant bacteria found in poultry manure (137, 196).

Another method to reduce bacterial runoff that is useful is the use of

buffer zones. Using riparian or vegetated buffer zones can reduce sediment loss

and runoff of bacteria. Giddens and Barnett (94) were able to show drastically

reduced bacterial runoff over grassland versus fallow land. Both buffer zones and

manure storage are not effective in completely eliminating bacterial runoff, but

they are useful in that they are already encouraged, at least in the Chesapeake Bay

region, due to nutrient management regulations, and they do lead to some

reduction in runoff (37).

A reduction in the use of antibiotics is generally regarded as the most

effective way to reduce antibiotic resistance, and there are several ways that

animal producers can reduce their need. If vaccines are developed for important

diseases, the animals can be immunized and therefore the need for antibiotics is

reduced. Vaccines are under development for *Salmonella* and coccidiosis in

poultry, and for many bacteria and viral diseases in pigs and cattle (174).

Vaccination has been very successful in aquaculture. In Norway, antibiotic use

has decreased drastically since the development of vaccines against *Aeromonas*

salmonicida and *Vibrio salmonicida* infections (248).

Probiotics are beneficial bacteria, generally lactic acid bacteria, that are

fed to animals (or humans) in an effort to promote greater health. These bacteria

can competitively exclude harmful bacteria by occupying a particular growth

niche and/or producing antagonistic conditions for harmful bacteria. Probiotics

have been shown to have some success, but results are inconsistent (5). In

addition to probiotics, general steps can be taken to reduce disease in animal

production. Good herd management, including disease screening, good

sanitation, and appropriate air and water quality can help to maintain healthy

animals and reduce the need for antibiotics (174). Other potential options include

using immune modulators, such as cytokines, to increase growth (158), or bacteriophages to treat bacterial infections (118).

While some steps are being taken to reduce agricultural antibiotic use, more needs to be done. The evidence that links agricultural use of antibiotics to antibiotic resistance in both the environment and humans is compelling, and because of the tremendous possibilities for horizontal gene transfer and the spread of resistant organisms, we must recognize and deal with the threat posed by overuse of antibiotics.

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