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INTRODUCTION OF ANTIBIOTIC RESISTANT MICROORGANISMS FROM CHICKENS TO MINK

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INTRODUCTION OF ANTIBIOTIC RESISTANT MICROORGANISMS FROM CHICKENS TO MINK

By

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ABSTRACT

INTRODUCTION OF ANTIBIOTIC RESISTANT MICROORGANISMS FROM CHICKENS TO MINK

By

Michael Alan McKinney

The use of antibiotics for growth promotion in food animals has raised serious health concerns about the increase of antibiotic-resistant, pathogenic bacteria due to this practice. An increase in penicillin-resistant bacteria in the human intestinal tract would make it difficult to treat a bacterial infection in the intestinal tract with penicillin.

Approximately one-half of all antibiotics made in the U.S. are used in animal husbandry. Evidence indicates that antibiotic-resistant bacteria increase in an animal's intestinal tract when a growth promotant level of an antibiotic is fed. The concern is that the antibiotic-resistant bacteria will pass this resistance to bacteria found in the intestinal tract of humans when they eat antibiotic-fed animals.

The experiments presented in this dissertation were conducted to study the effects on the intestinal microflora of chickens after penicillin was fed at two different levels,10 ppm or 100 ppm. Penicillin-resistant organisms were observed in the intestinal microflora of the chickens before and after feeding of the penicillin. The viscera of

these chickens were fed to mink that had not been exposed to penicillin in the recent past. Changes in the bacterial counts of the feces of mink on different treatments were noted. Penicillin resistance was monitored in the feces of the mink.

The results indicate that the feeding of penicillin at 10 or 100 ppm to broilers raised in a clean environment did not improve feed conversion or increase the level of penicillin-resistant bacteria in the jejunum or cecum of the broilers. The penicillin-resistant, aerobic bacteria declined within three days after penicillin was withdrawn from the feed of the chickens. The penicillin-resistant, anaerobic bacteria did not decline within three days after the withdrawal of penicillin. of the penicillin from the broiler feed. The bacterial counts made on the feces of the mink fed diets containing the viscera of penicillin-fed chickens indicated that there was a significant increase in the percent of penicillin-resistant organisms as compared to the control group.

Similar plasmids were found in penicillin-resistant bacteria isolated from an experimental mink diet and from the feces of a mink. This indicates that a transfer of this plasmid may have occurred in the intestinal tract of the mink.

TO THE ONLY WISE GOD,

OUR SAVIOR... JUDE 1:25(a)

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INTRODUCTION

Penicillin was discovered in the late nineteenth century, although a practical use for it was not discovered until 1927 (Betina, 1983). From 1927 until the early 1950's, penicillin was used only for treating bacterial infections. At this time, it was discovered that the feeding of low levels of penicillin to chicks caused an increase in their body weight without increasing their feed consumption (Heuser and Norris, 1952).

In 1951, only 110,000 kilograms of antibiotics or 16% of all antibiotics produced were used in animal production. The percentage of antibiotics used in all phases of animal production has steadily increased until 1980 when almost half (approximately 5.58 million kilograms) of all antibiotics produced in this country were used for food animals (Stallones et al., 1980; Levy, 1987). The percentage use of antibiotics for food animals will probably continue to increase. The concern of the public in using antibiotics for livestock is the build up of antibiotic-resistant bacteria in the intestinal tract of animals which then may be spread to humans (Hays, 1981).

The use of antibiotics has lead to an increase of antibiotic-resistant organisms in the intestines of farm

animals (Crawford and Shotts, 1982). If these antibiotic-resistant organisms are passed from farm animals to humans then a situation could arise where a person could not be treated effectively with an antibiotic because the pathogenic bacteria are resistant to the antibiotic. The spread of antibiotic-resistant organisms from chickens to chickens and from chickens to man has been demonstrated (Levy et al., 1976b), although the resistant organisms appeared to be transient in nature in the second species of animal.

The British government ordered a study on the use of antibiotics in animal husbandry (Braude, 1978). Based on this report the use of subtherapeutic levels of antibiotics of human importance was prohibited in animals. The percentage of organisms in animals that were resistant to antibiotics was as great or greater four years after the law had been passed. It has also been noted that the amount of antibiotics used in the United Kingdom did not decline after the passing of the law. This indicates that farm animals may still be given the antibiotics illegally.

The United States, specifically the Food and Drug Administration (FDA), gave notice in 1977 that it intended to recommend to the U.S. legislature to restrict or ban the subtherapeutic use of penicillin and the tetracyclines for growth promotion in animal feeds (Hays, 1981). This notice still has not been presented to the U.S. legislature

because the FDA scientists and other research scientists have not demonstrated that the antibiotics in question are ineffective or unsafe. At this time, a law banning or restricting the use of penicillin or the tetracyclines in this country would be based on emotions and not on scientific facts.

The spread of antibiotic resistance from animals to man has been noted (Cohen and Tauxe, 1986). This transfer seems to happen to people that are in contact with the animals (Levy et al., 1976a). Holmberg et al. (1984) implicated the transfer of antibiotic resistance from animal to man by the consumption of hamburger, but direct transfer of the antibiotic resistance was not proven. Gast and Stephens (1988) demonstrated the transfer of antibiotic-resistant bacteria from turkey to rats fed the turkey only when antibiotics were being administered to the rats.

More research needs to be conducted on the transfer of antibiotic resistance through the food chain. This research study was designed to show if antibiotic-resistant bacteria can be transferred from contaminated meat to the animal which eats the meat in the absence of antibiotics.

OBJECTIVES

1. To determine if penicillin, when fed to broilers at 10 or 100 ppm in the feed, improved feed conversion when the broilers were raised in a clean environment.

2. To determine whether the feeding of penicillin to broilers at 10 ppm and 100 ppm changed the number of total aerobic bacteria, total coliforms, total anaerobes, lactobacilli, or Clostridium perfringens in the jejunum or in the cecum of the broilers.

3. To elucidate the number of total aerobic bacteria, coliforms, total anaerobes, lactobacilli, and Clostridium perfringens found in the feces of mink.

4. To determine if the number of penicillin-resistant bacteria decreased in the jejunum or cecum of broilers within three days after penicillin was removed from the feed.

5. To determine if the percent of penicillin-resistant bacteria present in the feces of mink increased when the mink were fed diets containing viscera of broilers that were fed penicillin.

6. To determine if similar plasmids carrying penicillin-resistant genes could be found in both the diet of mink containing the viscera of chickens that were fed penicillin and in the feces of the mink that ingested this diet.

LITERATURE REVIEW

Intestinal microflora of chickens

Although the intestinal microflora of chickens has been studied for years, the types and numbers of bacteria that compose the microflora have not been completely elucidated (Barnes et al., 1972; Beck, 1978). One of the reasons that the microflora has not been elucidated is the tremendous variation in the results of the research that has been presented. One source of the variation of the types and numbers of the bacteria reported is due to the sampling procedure. For example, some research reported the numbers of bacteria found in a fecal sample. This type of sample gave an inaccurately low count of nonsporing, obligate anaerobes due to the exposure of the bacteria in the feces to air at the time of defecation. Extremely high counts of coliforms were found in fecal samples due to the reproduction of the coliform bacteria after the voiding of the feces (Barnes and Goldberg, 1962). Also, a fecal sample only indicates the bacteria present in the colon and not in the small intestine or in the ceca of the bird. Another problem that is encountered is deciding what the sample to be analyzed should contain. Some studies used only

intestinal contents for enumeration and identification of the bacteria (Salanitro et al., 1974) while other studies used fecal samples (Barnes and Goldberg, 1962). Also, a problem that was encountered is the length of time that a sample is held prior to being plated onto growth media. Reproduction of some of the sample bacteria or death of other sample bacteria may have occurred during this period (Barnes et al., 1972). Finally, the diet an animal was fed and the time from the last meal until the animal was killed influenced the kind and numbers of bacteria present in the intestine (Smith, 1965). Due to these problems only a very rough estimate has been made of the numbers of the different bacteria found in the intestine.

Smith (1965) found that the bacterial population of a fecal sample closely resembled the bacterial population in the large intestine of the animal, although large variations in bacterial population occurred in the stomach or small intestine with no noticeable change in the bacterial population found in the fecal sample. It was also reported by Smith (1965) that Escherichia, Clostridium, Streptococcus, Lactobacillus, and Bacteroides were the most common organisms in the small intestine and ceca. He found that approximately one third of the birds had yeast present throughout their intestine. Levy et al. (1976a) found that Escherichia, Proteus, and enterococci were the predominant, aerobic organisms, although the relative numbers of each

type of bacteria varied greatly. The numbers of the different anaerobic organisms isolated from cecal droppings indicated that Lactobacillus, Clostridium, and Bacteroides were the predominant, anaerobic, bacterial genera (Barnes and Goldberg, 1962; Smith, 1965).

Escherichia coli was found to make up approximately 80 to 90 percent of all the enterobacteria in the chicken intestine (Levy et al., 1976a; Mamber and Katz, 1985). E. coli counts in the anterior region of the small intestine ranged from 10^5 to 10^8 viable organisms per gram of sample and ranged from 10^5 to 10^9 in the posterior region of the small intestine. Clostridium perfringens counts ranged from nondetectable to 10^4 organisms per gram of contents in the anterior small intestine, but only from 10^2 to 10^5 in the posterior portion of the small intestine. Total Clostridium spp. counts ranged from 10^2 to 10^4 . Streptococcus spp. numbers ranged from 10^5 to 10^8 bacteria per gram with the higher number of streptococci being counted during the first three days of life and then decreased to the 10^3 to 10^5 level at two to three weeks. The lactobacilli were found to be a slow colonizer in the small intestine with no organisms being detected for the first two days after hatching and then they increased to 10^6 to 10^8 organisms per gram at two to three weeks after hatching. Bacteroides was not detected at any time in the anterior or posterior portions of the small intestine (Smith, 1965).

The number of bacteria were found to be greater in number in the ceca with 10^8 to 10^{10} E. coli per gram of cecal contents. Streptococci spp. counts ranged from 10^4 to 10^8, and Clostridium perfringens numbers ranged from 10^2 to 10^8. The total Clostridium spp. counts varied from 10^5 to 10^8. The lactobacilli in three week-old birds stabilized between 10^6 to 10^9. The Bacteroides counts ranged from nondetectable to 10^9 organisms per gram of contents. The strict anaerobes appeared to take one to three days for them to colonize in the ceca and then they increased quickly (Barnes and Goldberg, 1962; Smith, 1965; Barnes et al., 1972).

Changes in the intestinal microflora of chickens were seen when the birds were fed rations supplemented with penicillin. Mamber and Katz (1985) found that there was a slight decrease in the number of E. coli with an increase of Klebsiella when broiler chicks were fed a ration containing 50 grams per ton of penicillin. When mice were fed penicillin, at any level, there was a large decline of lactobacilli accompanied by an increase in both Gram-negative rods and enterococci (Dubos et al., 1963). However, Lev et al. (1957) found that no changes occurred in the number of lactobacilli, streptococci, or coliforms when broiler chicks were fed a diet containing penicillin at a level of 45.5 mg per kg of broiler feed. It was noted that Clostridium perfringens was reduced to nondetectable

levels in birds when fed this diet. Anderson et al. (1951) found that penicillin increased the number of coliforms and lactobacilli while it reduced the number of enterococci. Penicillin, when fed at 100 ppm to turkeys, was found to increase the total number of anaerobic bacteria while it significantly reduced the number of Clostridium spp. This study also found that the coliform numbers were significantly reduced (Sieburth et al., 1951). Chang and Murphy (1975) reported that chlortetracycline in the feed at 50, 100, or 200 ppm had no significant effect on anaerobe, lactobacilli, coliform, or enterococci numbers in the chicken intestinal microflora. It was also found that bacitracin methylene disalisylate significantly reduced the numbers of anaerobes when it was added to the feed at high levels (100 or 200 ppm) and also reduced the lactobacilli and enterococci counts when added at all levels used in the study.

Digestive tract of the mink

The mink (Mustela vison) is a monogastric carnivore. The digestive system is similar to that of a human in that it consists of an esophagus, a large stomach, and a small intestine that is divided into the duodenum, jejunum, and the ileum. The ileum makes up the last half of the small intestine. The colon is wider than the rest of the

intestine and is approximately five centimeters long. The mink has no appendix (Smith and Krasulak, 1979).

The basal diet of the mink on a dry weight basis contains 45% protein. The food passage time of a mink is approximately three hours (Bleavins and Aulerich, 1980).

The numbers and kinds of bacteria in the intestine of the mink has not been reported in the literature. Also, the effect of penicillin on the intestinal microflora of the mink has not been studied (Aulerich, 1987).

The function and use of penicillin

The antibacterial substance produced by the mold, Penicillium had been observed and used for treatment of wounds before 1900. Fleming, who wrote his paper on the observations of penicillin in 1929, is credited with the discovery of a functional use for penicillin (Betina, 1983). He used it primarily in making selective media for the isolation of Haemophilus, although he also tested it as a topical powder for inhibiting infections in external wounds on people. His observations indicated that penicillin was effective in killing both Gram-positive and Gram-negative cocci. It was not as effective in killing Gram-negative rods due to the inability of Fleming to purify and stabilize the penicillin. The drug was not used routinely for chemotherapy until 1939 (Stewart, 1965;

Betina, 1983). By 1950, enough evidence had been gathered that showed penicillin increased body weight in chickens that farmers started using it commercially (Stallones et al ., 1980).

Over 11 million kilograms of antibiotics were produced in the United States in 1979. Approximately one half of these antibiotics were used in animals, particularly in subtherapeutic amounts (Stallones et al., 1980). Nearly 80% of all the poultry in this country are given antibiotics at some point in their life, while only 60 to 75% of all the other meat animals are fed antibiotics (Hays, 1981).

When a healthy animal is fed a low level of an antibiotic, such as penicillin, the animal will gain more weight on the same amount of feed than an animal not fed an antibiotic (Nelson et al., 1963; Combs and Bossard, 1963; Bird, 1969). There are three theories as to why antibiotics increase feed conversion. They are:

1. Antibiotics cause a nutrient sparing effect. This effect may be caused by the antibiotic killing or inhibiting bacteria that metabolize essential nutrients, such as vitamins or proteins, in the intestinal tract before the animal can absorb them. The antibiotic may also enhance the growth of bacteria that synthesize essential nutrients needed by the animal. Many researchers doubt that sparing vitamins would change the growth of poultry because the known vitamins are provided in excess of dietary needs

for poultry, although eliminating bacteria that compete with the bird for protein may reduce the protein requirement for maximum growth rate (Bird, 1969).

2. Antibiotics, in low levels, control subclinical infections by reducing the numbers of infectious or toxin-producing bacteria. Research has been done that shows a parallel relationship between improved weight gain in poultry and the reduction of clostridia numbers in the intestine by the use of penicillin. Also, ammonia levels are reduced in animals being fed penicillin. When these toxic compounds are reduced by the use of antibiotics, the intestinal lining becomes thinner, similar to the intestinal lining of gnotobiotic animals. The thinner, intestinal lining permits an easier and more rapid absorption of nutrients (Bird, 1969).

3. Antibiotics cause a metabolic change in an animal (Hays, 1969; Wallace, 1970). The metabolic effect may be explained by the control of subclinical disease, but research shows that there is a change in water and nitrogen excretion in animals being fed antibiotics which may affect an animal's metabolic rate (Braude and Johnson, 1953).

Many scientists think that the most plausible theory of the three listed is the elimination of subclinical disease. The problem with this theory is that if the intestinal microflora becomes resistant to antibiotics permanently then the antibiotics should lose their effectiveness as

growth promotants. This has not been demonstrated even after 30 years of antibiotic use in farm animals. The average improvement in feed efficiency attributed to antibiotics in 1952 was three percent. In 1963, the feed efficiency was still improved by three percent when penicillin was fed to broilers (Bird, 1969). Other researchers also indicate that there is still a significant increase in feed efficiency when penicillin is fed to animals (Menge, 1973; Dafwang et al., 1984).

Penicillin is used in both humans and animals to treat bacterial infections. It is given both orally and by injection. Even though penicillin is considered to be a broad spectrum antibiotic, it is most effective against the Gram-positive organisms. Penicillin is the antibiotic most commonly used to treat bacterial infections in animals, tetracyclines being the second choice (Stallones et al., 1980).

Resistance to antibiotics

Gram-negative organisms are normally more resistant to penicillin than the Gram-positive bacteria due to differences in the cell wall. Penicillin exerts its bactericidal effects by inhibiting a transpeptidase or a carboxypeptidase in the bacterium, both of which are needed for the incorporation of peptidoglycan oligomers into the

cell wall. Inhibition of the transpeptidase or the carboxypeptidase triggers the release of a peptidoglycan hydrolase which hydrolyzes the covalent bonds between the peptidoglycans in the cell wall and eventually causes lysis of the bacterial cell (Tomasz, 1979). Peptidoglycan molecules are present on both the Gram-positive and Gram-negative bacteria. The peptidoglycan molecules are randomly cross-linked in the cell wall of Gram-positive bacteria while the Gram-negative bacteria have uniform cross-links between the peptidoglycan molecules in their cell wall. The Gram-negative bacteria have a highly complex, outer membrane which retards the penicillin from reaching the cell wall. They also have beta lactamases located in the periplasmic space (the space between the outer wall layer and the peptidoglycan layer). These enzymes occur naturally in the bacteria and hydrolyze the penicillin before it binds to its binding site. Even with all these protective devices, Gram-negative bacteria can be inhibited in growth by high concentrations of hydrolyzed penicillin in the cell which causes an inhibition of the binding sites for the peptidoglycan (Costerton and Cheng, 1975; Neu, 1986).

Besides having natural resistance, bacteria may develop or acquire a resistance to antibiotics. This resistance may come from a random mutation or from gene transfer from another organism and involve changes either in the

penicillin, binding sites or in the production of beta lactamases. Gene transfer may occur by transformation, transduction, or by conjugation (Maas, 1986).

Transformation happens when a bacterium incorporates, at special sites, free DNA that is present around the bacterium into its own genome. The exogenous DNA must have a homologous segment with some portion of the bacterium's DNA to be inserted into the genome (Davis and Dulbecco, 1973). Transformation can be induced to happen in vitro by treating bacteria with calcium chloride, adding the plasmid DNA and then heat shocking the mixture. The transformation is successful with one DNA molecule out of 10,000 (Maniatis et al., 1982).

Transduction describes the transfer of genetic material from one bacterium to another bacterium by a bacteriophage. This occurs when a temperate phage infects an antibiotic resistant bacterium. In most cases, this temperate phage does not cause lysis of the cell, but if it does occur, occasionally a piece of the bacterial genome is enclosed by a phage coat. This is an infective phage and may recombine with an antibiotic sensitive bacterium (Davis and Dulbecco, 1973).

Conjugation is the third and probably the most important means of antibiotic resistance transfer. For conjugation to occur, a donor bacterium comes into close proximity to a recipient bacterium. A tube, called a sex pilus, which is

located on the donor bacterium, is connected to the recipient bacterium. Once the pilus connects the two cells, one strand of plasmid DNA migrates from the donor cell to the recipient cell. The two cells then separate with both cells carrying the plasmid DNA.

Plasmids usually control the conjugation process. Plasmids are extrachromosomal DNA that are double stranded and circular. They usually weigh from 40×10^6 to 200×10^5 daltons (Willetts, 1972). This is a small segment of DNA when compared to the average bacterial chromosome that has a molecular weight of 2×10^9 daltons. Approximately 33% of the plasmid's genes are used for the transfer of the plasmid, 20% of the genes are used for the replication of the plasmid while the rest of the genes are used for nonessential functions, such as antibiotic resistance (Willetts, 1972).

There are two types of plasmids, conjugative and nonconjugative plasmids. The conjugative plasmid can carry up to ten antibiotic resistant genes while the nonconjugative plasmid is very small and carries no more than one or two antibiotic resistant genes. Both type of plasmids can be transferred to other bacteria by transduction or transformation, but the nonconjugative plasmid cannot be transferred to another bacterium by conjugation without the involvement of a conjugative plasmid (Elwell and Falkow, 1986).

Resistance to antibiotics, by the intestinal microflora, developed when animals were fed antibiotics (Kiser et al., 1970; Levy et al., 1976b; Dawson et al., 1984). This resistance developed whether an animal was fed a therapeutic level or a subtherapeutic level of antibiotic. The evidence seems to indicate that resistance increased to a high level faster (one to two days) when using a therapeutic level of an antibiotic than when using a subtherapeutic level of an antibiotic. A study performed by Langlois et al. (1984) found that 90% of the intestinal coliforms of pigs became resistant to antibiotics within 35 days of being fed antibiotics, independent of the level of the antibiotic in the diet. Only 58% of the fecal coliforms in the control pigs were resistant to antibiotics.

Results vary concerning the length of time that resistant organisms remain at high levels in the intestine of animals after all antibiotics have been withdrawn from the feed. Levy et al. (1976b) indicated that high levels of antibiotic-resistant organisms remained in the intestinal tract over ten weeks after antibiotics had been withdrawn. Langlois et al. (1984) showed that there was a decline in antibiotic-resistant organisms immediately after antibiotics were withdrawn from an animal. This decline continued until the percent of antibiotic resistant organisms reached pretrial levels.

Recent research indicates that antibiotic-resistant

bacteria do not survive in nature as well as antibiotic-sensitive bacteria. This is seen by a return to an antibiotic sensitive state usually within two weeks after the antibiotic treatment is discontinued in the animal (Richmond, 1977; Langlois et al., 1984). The inability of the antibiotic-resistant bacteria to survive as well as the antibiotic-sensitive bacteria may be due to the size of the resistant bacteria being larger than the antibiotic-sensitive bacteria. The larger size may lengthen the reproductive time of the bacteria which would give the antibiotic-sensitive bacteria an advantage. Also, a change in the cell wall that reduces the ability of the antibiotic-resistant bacteria to adhere to the intestinal lining of an animal may be involved (Nordstrom et al., 1977).

Spread of resistance factors

There have been numerous studies done indicating that antibiotic-resistant bacteria are spread from animals that have been fed antibiotics or carry antibiotic-resistant bacteria to animals that have not been fed antibiotics (Levy et al., 1976b; Levy, 1978; Cherubin et al.,1980; Holmberg et al., 1984).

Much of the older research that was done indicated that antibiotic-resistant transfer did occur in animals

naturally, but the research was performed in a manner that the animal was just a living test tube (Jukes, 1971). After that statement was made, Levy et al. (1976b) showed that antibiotic-resistant transfer was possible from one group of animals to another group of animals if both groups of animals were being fed antibiotics. The spread of the resistance was facilitated by an animal caretaker. Cherubin et al. (1980) reported that resistance to ampicillin in 1976-1978 dropped to 1965 levels in New York City, in people, while the ampicillin resistance level increased in cows in the northern part of New York State during the same period. Cherubin suggested that this occurred because of the lack of contact between these two areas. Holmberg et al. (1984) gave the best evidence that antibiotic-resistant bacteria were transferred between animals. This study showed that people living 800 miles away from the beef that they ate had the same antibiotic-resistant plasmid in their intestinal microflora as the beef cattle raised in the same area as the cattle that the people ate. The plasmid was noted only in humans that were on antibiotic therapy. A similar study was reported by Spika et al. (1987). A strain of antibiotic-resistant Salmonella newport reached epidemic proportions in California. A plasmid carrying the antibiotic-resistant gene was isolated from this strain. Examination of hamburger being sold in the area revealed the presence of the same plasmid in bacteria isolated from

the hamburger. When the farms where the cows had been purchased were inspected, the same plasmid was found in other Salmonella spp. This study indicated that antibiotic resistance can be spread from animals to man, but the authors never stated how long the resistant organisms remained in the human intestine. It was noted that over 50 percent of the humans that had to be treated for the Salmonella infection had ingested either penicillin or tetracycline within a month of becoming ill.

Hummel et al. (1986) found that only when people came into direct contact with animals being fed antibiotics did they become resistant to the antibiotic in question. In this study, a new antibiotic, nourseothricin, was introduced into a new geographical area. The antibiotic was used only for growth promotion purposes in pigs. Before the use of nourseothricin in this area, no plasmid-mediated resistance to this drug had been found in this locality. After two years of use of this antibiotic in pigs, over 100 strains of bacteria isolated from humans were found to be resistant to nourseothricin due to plasmids. No plasmid-mediated resistance to this antibiotic was found in areas not using nourseothricin during this same period of time. The authors concluded that the feeding of antibiotics in animals was not important for the increase of antibiotic-resistant bacteria of importance to humans.

Two different conclusions have been presented by the

research reviewed in this thesis. They are that resistance can be transferred from animals to man by plasmids, Spika et al. (1987), or that little danger exists for the public when animals are fed antibiotics, Kiser et al. (1970). These two different conclusions may be explained by the unknown condition of the intestinal microflora in the group of animals to which the plasmid-mediated resistance is passed. Any variance from the normal microflora in the digestive tract can result in an increase of the transfer of plasmid-mediated antibiotic resistance. Stress or antibiotic therapy are just two examples of factors that may cause a change in the intestinal microflora which may allow the transfer of antibiotic resistance to occur (Falkow, 1975; Elwell and Falkow, 1986).

Studies on bacterial plasmids

The study of the transfer of bacterial plasmids should become easier in the future. Many rapid techniques are being developed which will enable a researcher to screen many bacteria for similar plasmids, quickly and accurately. The ability to isolate plasmid DNA in small amounts in three hours has been developed (Maniatis et al., 1982). The use of simple agarose gels to separate the plasmid DNA based on size can then be used. If two plasmids are found to be of similar size then they can be cut with a

restriction endonuclease. If this procedure is done using two different endonucleases and the fragments of the plasmids resulting from the cuttings are similar, then it can be concluded that the two plasmids are identical (Farrar, 1983; Takahashi and Nagano, 1984).

MATERIALS AND METHODS

This research study was conducted in four separate experiments. The first experiment involved the feeding of penicillin to broiler chicks. The second experiment was the feeding of poultry offal, that may have contained penicillin-resistant bacteria, to mink. The microbiological analysis of the intestinal microflora of both the mink and the chickens comprised the third experiment. This experiment was performed to determine any changes in the intestinal microflora, such as increases or decreases in bacterial families, and any change in penicillin-resistant bacteria due to the feeding of penicillin. Identification, isolation, and comparison of the plasmids obtained from penicillin-resistant bacteria of the mink feces and the chicken digestive tract made up the fourth experiment.

Birds

The 156 birds used in the first experiment were Hubbard strain male broiler chicks that were purchased from Fairview Farms, Remington, Indiana at one day of age. The birds were shipped by mail to Michigan State University. The chicks were taken to the M.S.U. Poultry Science

Research and Teaching Center and placed into previously prepared pens. The birds were raised to seven weeks of age (except the birds that were killed for intestinal microflora sampling during the experiment) when they were killed and processed. This experiment was conducted from mid-August to the end of September.

Three groups of birds were used in this experiment. Group one was fed diet one which contained no penicillin. Group two was fed diet two which contained penicillin at a level of 10 ppm. Group three was fed diet three which contained 100 ppm of penicillin. Each group was divided into two replicates. Each replicate contained 26 birds. The two replicates of the same group were housed in the same room with a wire partition to keep them apart. Each replicate was called a pen.

A total of six birds were killed in each pen at timed intervals during the experiment. The birds killed before the seventh week were not included in the determination of weight gain for the pen, but they were weighed so that feed conversion for the pen could be calculated.

The birds were raised on solid cement floors with white pine shavings for bedding. Each bird had 0.33 square meters of floor space. Heat was supplied by one 1500 watt, red, heat lamp placed in the center of each pen two feet above the floor. The temperature was maintained at 32.2C throughout the first week and then the temperature was dropped 2.8C weekly until the birds were four weeks of age.

At this time, the heat lamp was removed. Ventilation was supplied by a fan for each group. The fan was controlled by both a thermostat and a timer. The air was drawn into the pen from a center hallway through a blackout hood and exhausted to the outside. One incandescent light of 60 watts was used in each room. The light schedule was 23 hours of light and one hour of darkness.

The three rooms used in this study were adjacent to one another with a separate door leading from the central hallway into each room. Strict isolation procedures were maintained for all the rooms. Only the principal investigator entered the rooms. Before entering any of the rooms, boots, gloves, and a lab coat, that were kept by the door, were put on by the investigator. The investigator then washed his hands and boots in disinfectant when he entered the room. The hands of the investigator and his boots were also washed when the he left the room.

The diets used in this study were prepared by the M.S.U. Feed Mill. A broiler starter (Appendix A) and a broiler finisher (Appendix A) were prepared for each group. All of the treatment groups were fed the nonmedicated starter diet for three days and then were fed the experimental diets until the birds were four weeks of age. At that time, the starter diets were removed from all of the pens and the finisher diets were placed into the feeders. The finisher diets were fed for the last three weeks. The starter diets contained 24% protein and the finisher diets contained 21%

protein. Diet one was the control diet, which was mixed first, and contained no penicillin or other antibiotics. Diet two had a level of 10 ppm of procaine penicillin (Procaine Penicillin 100, Carl S. Akey Co., Lewisburg, Ohio) mixed into it and diet three contained a procaine penicillin level of 100 ppm. The procaine penicillin was purchased in the form of a premix containing 132 gm procaine penicillin per kg of premix and was added directly to the diet.

The feed for each replicate was placed in a separate container outside of the group's door. A separate scoop was used for each diet. The feed was weighed when it was placed in the container and weighed back on the days the chicks were weighed to determine feed conversion. Feed and water were available ad libitum.

Mink

Three treatment groups of mink were used in the second experiment. Each group of mink was comprised of two replicates. Each replicate contained two mink. All of the mink used in this study were pastel females that were approximately six months of age.

Each group was housed in a separate building at the M.S.U. Mink Ranch. The buildings were open sided pole barns with a space of 20 feet between the barns. The lighting and temperature were both ambient. The mink were housed in

individual cages (30 x 75 x 45 cm) which were made of galvanized hardware screen and had a wooden nest box (32 x 20 x 22 cm) connected on the outside that was accessible from the cage.

The mink were fed a wet feed containing two thirds viscera obtained from the chickens that were processed in the first experiment and one third of a commercial pelleted mink cereal (XK-40 Grower, XK Mink Foods, Thiensville, Wi.). Water was added to the diet to make a workable consistency. After mixing, the feed was placed into one kg bags and frozen at -20C. The feed was kept frozen until two days before the feed was needed. At that time, the feed was placed in a 4C refrigerator to thaw and kept there until the feed was used.

The experimental diets that were fed to the mink were tested for the presence of penicillin using the A.O.A.C. method of analysis for the determination of penicillin in animal feeds (A.O.A.C., 1984). Brain Heart Infusion (BHI) Agar (Difco, Detroit, Mi.) plates were poured and allowed to harden. Five ml of a Staphylococcus aureus culture (ATCC 25923) were added to 45 ml of BHI Agar that was preheated to 48C. The S. aureus culture was grown overnight at 37C in five ml of BHI broth (Difco, Detroit, Mi.). Four ml of the BHI Agar that contained the S. aureus culture were poured onto the BHI Agar plates that had been poured previously.

Once the top layer hardened, five, 10 mm, stainless steel, penicylinders (Fisher Scientific, Pittsburgh, Pa.)

were placed on top of the agar in each plate. Into cylinder one, 250 ul of a 0.05 units/ml of penicillin (Gibco Labs. Grand Island, N.Y.) was added. Cylinder two had 250 ul of a 0.20 units/ml of penicillin added. Into cylinder three, 250 ul of a 0.80 units/ml penicillin solution was added. Cylinders four and five had 250 ul of a feed sample solution added. All of the penicillin standards and the feed samples were prepared according to the A.O.A.C. (1984) method.

The mink were fed by placing the feed in a small can on the floor of the mink's cage. The can was held in place by a wire ring so the mink could not spill the feed. The mink were fed daily and any feed remaining from the last feeding was discarded. An acclimation period of one week was given to the mink for them to become accustomed to the new feed and the different style of feeding. Any of the animals that did not adapt to the new feed were replaced with different animals. Four days after the acclimation period was over, feed consumption was measured for two consecutive days.

Fecal samples were collected from the mink before the experimental feed was given, seven days after the acclimation period ended, and seven days after the experimental diets were withdrawn.

Sampling

Intestinal, microflora samples were taken from each treatment group of chickens at 14, 28, and 46 days after the birds were started on the experimental diet. One treatment group of chickens was sampled at a time. Four birds from each group (two birds from each replicate in the group) were taken to the laboratory. One bird was killed by cervical dislocation while the other birds were kept in a cage in another room. The abdomen was opened and the jejunum, which is the section of the small intestine from the distal end of the duodenum to half the distance to Meckel's diverticulum, was aseptically removed and placed in a sterile petri plate and weighed. The ceca were then removed aseptically and also placed in a sterile petri plate and weighed. The organ was then cut medially and the contents were removed by scraping the wall of the organ once with the long side of a pair of forceps. The organ was placed in a Waring blender while the petri plate and the contents were weighed. The difference in the two weights for each organ was multiplied by nine and this amount of anaerobic, dilution salts (V.P.I., 1973) was added to the Waring blender. The sample was mixed in the blender for one minute. After the sample was mixed, one ml was pipetted into nine ml of anaerobic dilution salts and immediately placed into the anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mi.). Another one ml of sample was removed from

the blender and placed into nine ml of phosphate buffered saline solution (Appendix B) to be used for the aerobic dilutions.

Samples of fresh mink feces were collected from underneath the mink's cages by having the investigator wait by the cage until the mink defecated. A fecal sample was picked up with sterile forceps and placed in a sterile petri plate. The feces were transported to the laboratory, within 15 minutes after they had been collected, where they were immediately prepared for plating. The samples were weighed and put into a sterile 50 ml flask. One ml of the sample was diluted one to ten with anaerobic dilution salts and placed in the anaerobic glove box and another one ml of the sample was diluted one to ten with phosphate buffered saline to be used for the aerobic dilutions.

Microbiological methods

Aerobic dilutions of the feces and gut contents were made using a one to ten serial dilution. The dilution tubes contained phosphate buffered saline solution. The serial dilutions were made with a pipette bulb. Anaerobic dilutions were also a serial one to ten dilution. The dilution blanks contained nine ml of anaerobic dilution salts. The dilution blanks were kept in the anaerobic glove box for 24 hours before they were used so that the anaerobic dilution salt solution would be reduced. The

dilutions were accomplished by using a pipette pump in the glove box.

The first set of samples, that was taken from the chickens, was spread plated onto different agars. This procedure involved pipetting 0.1 ml of a dilution on an agar plate. The sample was then spread across the plate with the use of a sterile bent Pasteur pipette. This method used one agar plate for each dilution. Each sample was plated using three to six dilutions. The drop plate technique of Miles and Misra (1938), which used fewer petri plates and less agar, was employed for the remaining samplings. The drop plate method used one drop of a bacterial dilution placed on an agar plate. The drop was not spread on the agar plate. The method allowed five or six dilutions to be placed on one plate (Davis, 1971). The bacterial counts were similar whether the spread plate or the drop plate method was used (Richmond and Chang, 1976).

The anaerobic glove box used in these experiments was filled with a gas mixture that contained 10% carbon dioxide, 10% hydrogen and 80% nitrogen. Anaerobic conditions were maintained by using palladium pellets which formed water from the oxygen in the glove box. The palladium pellets had to be regenerated every three to four days by heating them to 160C for two hours. The temperature in the glove box was maintained at 37C and the relative humidity was kept at 45% by the use of calcium chloride granules. Hydrogen sulfide was eliminated by using a silver

sulfate solution (Appendix B). Once the sample was placed into the glove box, all further anaerobic work was performed in it.

Growth media used

Five different media were used for the enumeration of bacteria from the intestinal tract of the chickens and the feces of the mink. The media used were Brain Heart Infusion Agar (BHI Agar), Eosin Methylene Blue Agar (EMB Agar), Sulfite Polymixin Sulfadiazine Agar (SPS Agar), Rogosa SL Agar (all from Difco, Detroit, Mi.) and Anaerobic Agar (BBL, Cockeysville, Md.).

BHI Agar is a general purpose agar used for the cultivation of aerobic bacteria. It is a good growth medium even for fastidious organisms. This medium was used to determine the total number of viable bacteria in the samples.

EMB Agar is a differential medium that is recommended for the isolation and detection of Gram-negative intestinal bacteria. The lactose fermenters, which include the coli-aerogenes group, appeared as colonies with dark centers. The non-lactose fermenters appeared as colorless colonies. The total number of coliforms in the samples were determined using this medium (Murphy, 1975).

The determination of total anaerobic bacteria in the samples was accomplished by using Anaerobic Agar. This

medium is a non-selective, non-differential medium that is useful for general purpose anaerobic bacteria growth (Difco, 1953).

The number of Clostridium perfringens was enumerated with the use of SPS Agar. This medium is selective for the growth of Clostridium spps. and differential for Clostridium perfringens. The Clostridium perfringens colonies appeared black while the other organisms were white.

Rogosa SL Agar is a selective medium for the enumeration of lactobacilli. The medium is very acidic which inhibits the growth of most bacteria except for the lactobacilli (Difco, 1968). The lactobacilli were grown in the anaerobic glove box.

The anaerobic plates were placed in the anaerobic glove box 24 hours prior to use to reduce the media and also to check for sterility. The aerobic plates were placed in an incubator for 24 hours to check for sterility. The placing of the agar plates in the incubator or glove box for 24 hours also dried the media and reduced the smearing of the sample drops when they were placed on the media. After the aerobic plates were inoculated, they were incubated for 18 hours and then counted. The anaerobic plates were incubated for 48 hours before they were counted. Any drop that contained less than 50 colonies was counted.

One BHI Agar plate from each sample was replica plated onto the same media containing 400 ug penicillin per ml of media. One Anaerobic Agar plate from each sample was also

replica plated onto the same media containing 100 ug penicillin per ml of media. The amount of penicillin that was added to the media was determined by using a gradient plate described by Carlton and Brown (1981). An aerobic penicillin-resistant microorganism was streaked across a BHI agar plate that had a penicillin-gradient level from 0.0ug/ml to 400 ug/ml. The highest level of penicillin on which the penicillin-resistant organism grew was selected to determine the resistance to penicillin in the rest of the study. A lower penicillin level was selected for the anaerobic microorganisms since the penicillin-resistant anaerobes did not grow on media containing 400 ul/ml of penicillin. The penicillin was added to the media after the media was autoclaved and cooled to 50C to avoid destruction of the penicillin. The BHI-penicillin Agar plates were incubated for 24 hours after inoculation and the Anaerobic-penicillin Agar plates were incubated for 48 hours in the anaerobic glove box before they were counted.

The BHI Agar plates and the Anaerobic Agar plates were replicated using the method of Lederberg and Lederberg (1951). The original plate was placed with the medium exposed to the air. A sterile piece of velveteen wrapped around a die was lightly touched to the surface of the medium of the original plate. The velveteen was then removed from the original plate and touched lightly to the penicillin-containing plate. Only one transfer of the colonies was needed to the velveteen for numerous replicas

to other plates.

Plasmid identification

The bacterial colonies used for plasmid identification were isolated from the BHI-penicillin Agar plates. The purpose of the plasmid identification was to determine if any penicillin resistance was transferred by extrachromosomal DNA from the bacteria in the chicken viscera to the bacteria found in the feces of the mink that ate the chicken viscera.

The purification of plasmid DNA was performed using a modified procedure found in Maniatis et al. (1982). A pure isolated culture was grown in five ml of BHI Broth containing 50 ul of ampicillin (Gibco Labs. Grand Island, N.Y.) per ml for 18 hours. The tube was then centrifuged at 2800 RPM in a microcentrifuge model 235C (Fisher Scientific, Pittsburgh, Pa.) for 10 minutes. The supernatant was poured off and the pellet was resuspended in 350 ul of a Sucrose, Tris, EDTA, Triton 100 solution (STET, Appendix C). This solution was transferred to an Eppendorf tube and 12.5 ul of a lysozyme (Sigma, St. Louis, Mo.) solution (Appendix C) was added. Heat was added to facilitate the action of the lysozyme and then the tube was centrifuged in the microcentrifuge at 12,000 RPM for 10 minutes. The pellet was removed and discarded and 500 ul of isopropanol (analytical grade) was added to the

supernatant. This mixture was placed in -20C freezer for 10 minutes to facilitate the precipitation of the DNA. The tube was then centrifuged in the microcentrifuge at 12,000 RPM at 4C for 10 minutes. The supernatant was poured off and the pellet was washed once with one ml of cold absolute ethanol (EtOH). The sample was again centrifuged in the microcentrifuge at 12,000 RPM at 4C for five minutes and the supernatant was discarded. The precipitate was dried in a dessicator. The dried pellet was resuspended in 200 ul of a Tris EDTA (TE) solution, pH of 8.0 (Appendix C). The sample was extracted using 200 ul of phenol (molecular biology grade), then with 200 ul of a phenol and formaldehyde solution (Appendix C) and finally, with 200 ul of a formaldehyde solution (Appendix C). Each extraction was shaken gently for 10 seconds, and then centrifuged in the microcentrifuge at 12,000 RPM for three minutes. The supernatant was kept and the pellet was discarded. Twenty ul of a three molar sodium acetate solution was added to the supernatant from the extraction process and the solution was precipitated with one ml of 95% cold EtOH. The sample was placed in a -20C freezer for 15 minutes to facilitate the precipitation. The tube was centrifuged in the microcentrifuge at 12,000 RPM at 4C for 15 minute. The supernatant was poured off and discarded. One ml of cold 95% EtOH was added to the pellet and centrifuged in the microcentrifuge at 12,000 RPM at 4C for five minutes. The supernatant was poured off and the pellet was placed in a

dessicator. The dried pellet was resuspended in 50 ul of TE pH 8.0 and stored in a -20C freezer until needed.

The first test done with the sample was to determine by gel electrophoresis if any plasmid DNA was present. The liquid measurements were done using a 20 ul pipetman or a 200 ul pipetman (Gilson Co., France). Eight ul of sample was mixed with one ul of a ribonuclease A (Sigma, ST. Louis, Mo.) solution prepared according to Maniatis et al. (1982). This mixture was heated at 37C for five minutes to destroy any RNA that was present in the sample. At that time 2.5 ul of a five-x loading buffer (Appendix C) was added to the sample and heated at 65C for five minutes. The sample was placed in a well in 0.7% agarose gel that contained 0.01 mg/ml of ethidium bromide. A 0.7% agarose gel was used because it gives an efficient separation of DNA from 10,000 base pairs to 800 base pairs Maniatis et al . (1982). The ethidium bromide was added as a stain. The ethidium bromide intercalates with the DNA which causes the bound ethidium bromide to fluoresce when exposed to ultraviolet light.

The power for running the gel electrophoresis was set at a constant 50 volts DC and run for one hour. After one hour the gel was placed over a ultraviolet light and a picture was taken and the bands of the different plasmids were compared for similar weight or size by the distance that the bands migrated through the gel.

The same plasmid samples were used for cutting by the

restriction endonucleases. Ten ul of sample were added to seven ul of deionized water and two ul of a 10-x react buffer three (BRL, Gaithersburg, Md.). One ml of the restriction endonuclease was added to this mixture and heated for two hours at 37C. One ml of the Ribonuclease A solution was added at this time and kept at 37C for another five minutes. Finally, five ul of the five-x sample buffer was added and heated at 60C for five minutes. This sample was then electrophoresed on a similar gel as the uncut DNA.

EcoRl (BRL, Gaithersburg, Md.) was the first restriction endonuclease used to cut the plasmid DNA. Bgl II (BRL, Gaithersburg, Md.) was the other restriction endonuclease used to cut the plasmid DNA. EcoRl recognizes a five base pair sequence and Bgl II recognizes a six base pair sequence. EcoRl cuts at:

5' G↓AATTC 3'
3' CTTAA↑G 5'

Bgl II cuts at:

5' A↓GATCT 3'
3' TCTAG↑A 5'

Attempts were made to transform the isolated plasmids into an E. coli strain number HB101. The E. coli had previously been made competent by using the calcium chloride method as described by Maniatis et al. (1982).

The data obtained from the first three experiments of this study were analyzed using a split plot repeat measurement. The analysis of variance was performed using Bonferroni-t statistics (Gill, 1978). A level of $P<0.05$ was

used as the level of statistical significant difference unless indicated differently.

A coefficient of variation was calculated for each microbiological sample on each type of media. This is listed in each table in the results. The coefficient of variation is defined as the standard deviation divided by the mean. The number of replicates used for determining the coefficient of variation was ten. A coefficient of variation less than 0.5 is good when working with animals (Gill, 1978).

RESULTS

Experiment one

Summarized in Tables 1 and 2 are the effects of feeding penicillin at 10 ppm or 100 ppm in a broiler diet as compared to a diet containing no penicillin on the body weights of broilers fed these diets. As noted in Table 2, there was no significant difference in body weight between any of the treatments by date. The average body weights of the birds were within the expected range of body weights for broilers of that age. The variation between treatments was smaller than the variation between the birds within a treatment.

The feed conversions for the birds in each pen are listed in Table 3. The final feed conversions ranged from 1.77 grams of feed eaten for each gram of weight gained to 1.82 grams of feed eaten for each gram of weight gained. These final feed conversions were better than expected for commercial broilers. An usual feed conversion for a commercial broiler flock would range from 1.85 to 1.95 grams of feed ingested for each gram of weight gained. The analysis of variance, shown in Table 4, indicates that there was no significant increase or decrease in feed

Table 1. Average body weight of broilers fed different levels of penicillin#.

Age (days)	Pen	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0	1	39±0.61	39±0.56	40±0.58
	2	40±0.72	40±0.62	39±0.40
14	1	421±5.42	448±6.53	448±6.65
	2	421±6.02	459±7.83	440±10.58
28	1	1322±18.28	1214±17.46	1247±17.52
	2	1320±20.30	1351±17.28	1285±23.18
42	1	2356±33.95	2285±29.86	2380±44.31
	2	2349±29.55	2400±36.21	2340±33.03

# Weight shown in gm ± standard error.
n = 20.

Table 2. Analysis of variance of the body weight of broilers fed different levels of penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	79.65	0.093
Error 1	3	849.45	
Time x Trt.	6	746.35	0.443
Error 2	9	1684.94	

Table 3. Feed conversion of broilers fed different levels of penicillin#.

Age (days)	Pen	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
14	1	1.31	1.21	1.22
	2	1.30	1.20	1.24
28	1	1.63	1.73	1.77
	2	1.59	1.69	1.64
42	1	2.20	2.16	2.11
	2	2.15	2.15	2.14
final feed conv.	1	1.81	1.81	1.82
	2	1.77	1.78	1.78

# gm feed per gm body weight gain.
n = 20 broilers in each pen.

Table 4. Analysis of variance of the feed conversions of broilers fed different levels of penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	0.00	0.000
Error 1	3	0.00	
Time x Trt.	4	0.0050	1.515
Error 2	6	0.0033	

conversion between the three treatments on any weigh date.

Experiment two

Summarized in Tables 5 to 14 are treatment responses over time and analysis of variance for total aerobes, total coliforms, total anaerobes, lactobacilli, and Clostridium perfringens in two locations of the intestinal tract of chickens, the jejunum and the cecum. The bacterial counts are displayed as log base 10 transformations based on per gram of intestine (wet weight).

The total, aerobic, bacterial counts in the jejunum (Table 5) fall within the expected range of 10^3 to 10^9. Shown in Table 6 is the analysis of variance for the total aerobes. As indicated, no significant difference was observed between the treatments on the numbers of total aerobes in the jejunum.

The bacterial counts of the microflora of the cecum were much more consistent than the jejunum bacterial counts, as seen in Table 5. The analysis of variance for total aerobes in the cecum shows that there was no significant difference in the total number of aerobic bacteria in the cecum due to the feeding of penicillin at either level in this experiment.

The coliforms that should make up approximately 90% of the aerobic bacteria in the jejunum varied from 80% to 100%

Table 5. The effect of feeding penicillin to broilers on total, aerobic, bacterial counts at two sites in the broiler intestinal tract#.

Days after experimental feed started	JEJUNUM			CECUM		
	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin
14	4.09±0.10	4.21±0.33	4.46±0.37	9.25±0.88	8.71±0.25	10.56±0.48
28	4.57±0.22	5.63±0.99	5.69±0.55	9.41±0.46	10.91±0.59	9.52±0.30
46	4.90±0.39	6.21±0.99	4.18±0.18	10.47±0.38	10.92±0.75	8.75±0.78

# All counts expressed as base 10 logs of bacteria per gram wet weight of the intestinal tissue ± standard error.
n = 4 broilers.
coefficient of variation for the jejunum = 0.66.
coefficient of variation for the cecum = 0.17.

Table 6. Analysis of variance for total, aerobic bacteria at two sites in the intestinal tract of broilers fed penicillin.

Source of var.	d.f.	JEJUNUM		CECUM	
		M.S.	f value	M.S.	f value
Treatment	2	2.18	1.27	1.12	0.88
Error 1	3	1.72		1.28	

of the observed aerobic bacteria, which was close to what was expected, see Table 7. No significant change was observed in the coliform count for the jejunum, as indicated in Table 8.

The coliforms made up 90 to 100 percent of the total, aerobic, bacterial count of the cecum as shown in Table 7. The number of coliforms found in the cecum agree with the findings of Smith (1965) who found that coliform counts vary from 10^2 to 10^8 . No significant change was observed in the coliform counts in the cecum due to the feeding of penicillin at 10 ppm or 100 ppm, as summarized in Table 8.

Shown in Table 9 are the total, anaerobic bacteria counts obtained in this experiment for the broilers. The anaerobic, bacteria counts in the jejunum were quite variable between each animal but the average was consistent at approximately 10^6 for any treatment or time. Barnes et al . (1972) found 10^8 anaerobic bacteria in the small intestine, which was 100 times greater than found in this study, but intestinal contents were also included in those counts which may have increased the counts. As indicated in Table 10, no significant difference was found between the three treatments on the anaerobic, bacterial counts in the jejunum.

The total, anaerobic count in the cecum of the broilers was significantly higher at days 14 and 28 in the birds that were fed the diets containing penicillin than in the

Table 7. The effect of feeding penicillin to broilers on total, coliform counts at two sites in the broiler intestinal tract#.

Days after experimental feed started	JEJUNUM			CECUM		
	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin
14	<3.94±0.00	4.24±0.20	4.14±0.20	8.59±0.65	8.61±0.32	9.72±0.52
28	4.08±0.14	5.62±0.98	5.10±0.68	8.06±0.29	10.93±0.58	9.14±0.21
46	4.68±0.44	5.17±0.50	<3.94±0.00	10.63±0.25	10.98±0.57	8.60±0.57

# All counts expressed as base 10 logs of bacteria per gram wet weight of the intestinal tissue ± standard error.
n = 4 broilers.
coefficient of variation for the jejunum = 0.56.
coefficient of variation for the cecum = 0.30.

Table 8. Analysis of variance for total coliforms at two sites in the intestinal tract of broilers fed penicillin.

Source of var.	d.f.	JEJUNUM		CECUM	
		M.S.	f value	M.S.	f value
Treatment	2	2.12	1.58	4.45	3.40
Error 1	3	1.34		1.31	

birds that were fed a diet that contained no penicillin. The results of the total anaerobic bacteria counts are summarized in Tables 9 and 10.

The lactobacilli counted in the jejunum were slightly lower than expected when compared to the work of Barnes et al. (1972) who found 10^7 or Smith (1965) who found 10^8 lactobacilli per gram of intestine when compared to this study's finding of 10^5, see Table 11. The analysis of variance presented in Table 12 indicates that there was no significant difference seen between treatments on the lactobacilli counts in the jejunum.

The lactobacilli counted in the cecum from any of the treatments were within the expected range of numbers of 10^7 to 10^9 (Smith, 1965). The counts are summarized in Table 11. The lactobacilli counts were not significantly changed by the feeding of penicillin at 10 ppm or 100 ppm in this study.

The Clostridium perfringens counts in the jejunum were not detectable at 10^4, as shown in Table 13. No significant change in the Clostridium perfringens numbers was detectable between the three treatments in the jejunum, see Table 14.

The Clostridium perfringens counts obtained from the cecum in this experiment, as summarized in Table 13, are much higher than those of Barnes et al. (1972) found in chickens of the same age. The feeding of penicillin did not

Table 9. The effect of feeding penicillin to broilers on total, anaerobic, bacterial counts at two sites in the broiler intestinal tract#.

Days after experimental feed started	JEJUNUM			CECUM		
	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin
14	4.11±0.10	5.29±0.56	4.96±0.47	8.89±0.83	11.19±0.28*	10.36±0.48
28	6.79±0.95	6.81±0.64	6.69±0.54	9.50±0.75	11.82±0.70*	11.32±0.16*
46	6.06±0.37	6.63±0.64	5.19±0.49	>12.00±0.00	>12.00±0.00	>12.00±0.00

# All counts expressed as base 10 logs of bacteria per gram wet weight of the intestinal tissue ± standard error.
n = 4 broilers.
coefficient of variation for the jejunum = 0.26.
coefficient of variation for the cecum = 0.22.
* Significantly different from the mean at P< 0.05.

Table 10. Analysis of variance for total, anaerobic bacteria at two sites in the intestinal tract of broilers fed penicillin.

Source of var.	d.f.	JEJUNUM		CECUM	
		M.S.	f value	M.S.	f value
Treatment	2	1.52	0.45	7.51	12.52*
Error 1	3	3.39		0.60	

* Significant at P< 0.05.

Table 11. The effect of feeding penicillin to broilers on lactobacilli counts at two sites in the broiler intestinal tract#.

Days after experimental feed started	JEJUNUM			CECUM		
	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin
14	<3.94±0.00	4.92±0.50	4.44±0.36	7.06±1.06	6.69±0.30	9.60±0.41
28	4.35±0.20	6.26±1.04	6.28±0.65	8.18±0.39	8.26±0.54	8.05±0.25
46	5.72±0.47	6.51±0.52	5.13±0.44	9.52±0.71	11.27±0.28	7.90±0.21

# All counts expressed as base 10 logs of bacteria per gram wet weight of the intestinal tissue ± standard error.
n = 4 broilers.
coefficient of variation for the jejunum = 0.23.
coefficient of variation for the cecum = 0.13.

Table 12. Analysis of variance for lactobacilli at two sites in the intestinal tract of broilers fed penicillin.

Source of var.	d.f.	JEJUNUM		CECUM	
		M.S.	f value	M.S.	f value
Treatment	2	4.45	1.37	2.03	0.80
Error 1	3	3.25		2.53	

Table 13. The effect of feeding penicillin to broilers on Clostridium perfringens counts at two sites in the broiler intestinal tract#.

Days after experimental feed started	JEJUNUM			CECUM		
	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin	0 ppm penicillin	10 ppm penicillin	100 ppm penicillin
14	<3.94±0.00	<3.94±0.00	<3.94±0.00	5.17±0.48	5.61±0.31	5.75±0.25
28	<3.94±0.00	<3.94±0.00	<3.94±0.00	7.39±0.28	8.88±0.97	10.76±0.18
46	<3.94±0.00	<3.94±0.00	<3.94±0.00	8.75±0.61	8.36±0.80	7.49±0.07

# All counts expressed as base 10 logs of bacteria per gram wet weight of the intestinal tissue ± standard error.
n = 4 broilers.
coefficient of variation for the cecum = 0.27.

Table 14. Analysis of variance for Clostridium perfringens at two sites in the intestinal tract of broilers fed penicillin.

Source of var.	d.f.	JEJUNUM		CECUM	
		M.S.	f value	M.S.	f value
Treatment	2	0.00	0.00	2.03	0.80
Error 1	3	0.00		2.53	

significantly effect the number of the Clostridium perfringens in the cecum of the broilers.

Summarized in Table 15 are the total, aerobic, bacteria counts found in the mink feces. The average number of total aerobes found in the mink feces was approximately 10^8, regardless of the treatment group to which the mink belonged. The analysis of variance in Table 16 indicates that there was no significant difference between different treatments.

The coliform counts obtained from the mink feces are summarized in Table 17. The number of coliforms compares with the number of coliforms found in the feces of cats (Smith, 1961). The cat was the most similar animal to mink that information could be found pertaining to bacterial counts in the feces. The total aerobic population of the mink feces was composed of approximately 75 % coliforms. The analysis of variance for the coliform counts in the mink feces is summarized in Table 18. As indicated, no significant difference was found between treatments.

The total, anaerobic, bacteria counts shown in Table 19 were not as variable between treatments as the aerobic, bacteria counts in the feces of the mink. The total anaerobes varied from 10^7 to 10^9. The analysis of variance that is summarized in Table 20 shows that no significant difference was found between treatments.

Summarized in Table 21 are the lactobacilli counts found

Table 15. The effect of feeding a diet containing the viscera of penicillin-fed chickens to mink on total, aerobic, bacterial counts found in the feces of mink#.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0*	8.78±0.72	----	----
7	7.58±0.53	7.90±0.50	8.81±0.83
7 days after put back on normal feed	9.70±0.82	8.46±0.21	8.62±0.89

All counts expressed as base 10 logs of bacteria per gram wet weight of mink feces ± standard error.
* n = 2 for day 0; n = 4 for the other two sampling dates.
coefficient of variation = 0.16.

Table 16. Analysis of variance for total, aerobic bacteria found in the feces of mink fed a diet containing the viscera of chickens that were fed penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	0.67	1.03
Error 1	3	0.65	

Table 17. The effect of feeding a diet containing the viscera of penicillin-fed chickens to mink on total, coliform counts found in the feces of mink#.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0*	8.22±0.61	----	----
7	6.65±0.50	6.33±0.40	7.40±1.09
7 days after put back on normal feed	8.21±1.61	6.02±0.90	6.23±0.33

All counts expressed as base 10 logs of bacteria per gram wet weight of mink feces ± standard error.
* n = 2 for day 0; n = 4 for the other two sampling dates.
coefficient of variation = 0.22.

Table 18. Analysis of variance for total coliforms found in the feces of mink fed a diet containing the viscera of chickens that were fed penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	3.16	0.48
Error 1	3	6.63	

Table 19. The effect of feeding a diet containing the viscera of penicillin-fed chickens to mink on total, anaerobic, bacterial counts found in the feces of mink#.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0	8.56±0.38	----	----
7	7.25±0.58	7.52±0.54	8.29±0.77
7 days after put back on normal feed	8.09±0.60	8.66±0.40	7.67±0.81

All counts expressed as base 10 logs of bacteria per gram wet weight of mink feces ± standard error.
* n = 2 for day 0; n = 4 for the other two sampling dates.
coefficient of variation = 0.49.

Table 20. Analysis of variance for total, anaerobic bacteria found in the feces of mink fed a diet containing the viscera of chickens that were fed penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	0.38	0.48
Error 1	3	0.79	

in the feces of the mink. The counts varied from 10^6 to 10^9. This number relates closely to the number of lactobacilli found in the feces of cats (Smith, 1961). The analysis of variance between treatments for lactobacilli is shown in Table 22. This table indicates that no significant difference was found between the treatments.

The Clostridium perfringens counts are summarized in Table 23. The Clostridium perfringens counts in treatment one compare with the Clostridium perfringens counts Smith (1961) found in the feces of cats. Treatment two and treatment three had higher counts of Clostridium perfringens than Smith (1961) found in the feces of cats. Although a variation was seen between treatments, no significant effect was observed, as indicated in Table 24.

Experiment three

In this study, an increase in resistance to penicillin is defined as an increase in the percent of bacteria that are able to grow in the presence of penicillin.

The effect of feeding penicillin on the resistance of the aerobic, bacterial microflora in the chicken jejunum is summarized in Table 25. The percent of penicillin-resistant, aerobic bacteria found in the jejunum was quite variable between the treatments and time, varying from 0 to 8.2%. The variation was not related to treatment

Table 21. The effect of feeding a diet containing the viscera of penicillin-fed chickens to mink on lactobacilli counts found in the feces of mink#.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0	6.15±0.63	----	----
7	7.47±0.45	7.52±0.58	7.94±0.61
7 days after put back on normal feed	6.07±0.41	8.66±0.40	7.15±1.04

All counts expressed as base 10 logs of bacteria per gram wet weight of mink feces ± standard error.
* n = 2 for day 0; n = 4 for the other two sampling dates.
coefficient of variation = 0.37.

Table 22. Analysis of variance for lactobacilli found in the feces of mink fed a diet containing the viscera of chickens that were fed penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	1.68	1.12
Error 1	3	1.50	

Table 23. The effect of feeding a diet containing the viscera of penicillin-fed chickens to mink on Clostridium perfringens counts found in the feces of mink#.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0	<3.94±0.00	----	----
7	<3.94±0.00	<3.94±0.00	5.44±0.57
7 days after put back on normal feed	<3.94±0.00	5.61±1.11	4.39±0.37

All counts expressed as base 10 logs of bacteria per gram wet weight of mink feces ± standard error.
* n = 2 for day 0; n = 4 for the other two sampling dates.
coefficient of variation = 0.25.

Table 24. Analysis of variance for Clostridium perfringens found in the feces of mink fed a diet containing the viscera of chickens that were fed penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	3.26	0.23
Error 1	3	13.91	

or to time effects, as indicated in Table 26. These data show that no significant difference was found between the treatments.

The percent of resistant, aerobic bacteria found in the cecum of the birds did increase from approximately 3.0% after 14 days of feeding the penicillin to over 10% after 28 days of feeding the penicillin in the two treatment groups (Table 27). The control group showed no increase in penicillin-resistant bacteria in the cecum during the same period of time. The high percent of penicillin-resistant microorganisms indicated for treatment one at day zero was composed of yeast cultures that were present in the birds sampled at the start of the experiment. The increase of penicillin-resistant bacteria in the cecum of the two treatment groups being fed penicillin was not significant (Table 28). The sampling taken at 46 days showed a large drop in penicillin-resistant organisms when compared to the 28 day sample. This may reflect the withdrawal of penicillin from the feed three days prior to the sampling.

The anaerobic bacteria found in the jejunum of the chickens did not show a significant increase in the percentage of penicillin-resistant bacteria, as summarized in Table 29. The control group of birds had a variation from 0.0 to 21.4% penicillin-resistant organisms at any sampling time, while the treatment group fed penicillin at 10 ppm penicillin only had a variation of 5.3 to 10.0%

Table 25. The percent of aerobic microorganisms resistant to penicillin isolated from the jejunum of chickens fed different levels of penicillin.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0#	13.10±10.20	----	----
14	5.00±5.00	0.00±0.00	8.25±8.25
28	0.00±0.00	4.18±4.17	4.28±2.54
46	0.00±0.00	0.88±0.55	0.00±0.00

All counts expressed as percent of total microorganisms ± standard error.
Resistance expressed as growth on media containing 400 ug/ml penicillin.
n = 2 for day 0; n = 4 for the other sampling dates.

Table 26. Analysis of variance for the penicillin-resistant, aerobic microorganisms isolated from the jejunum of chickens fed different levels of penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	37.51	1.02
Error 1	3	36.83	

Table 27. The percent of aerobic microorganisms resistant to penicillin isolated from the cecum of chickens fed different levels of penicillin.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0#	25.00±25.00	----	----
14	22.25±19.37	3.08±0.22	2.90±1.16
28	0.42±0.42	10.53±4.68	16.98±5.61
46	0.00±0.00	0.33±0.32	4.30±3.52

All counts expressed as percent of total microorganisms ± standard error.
Resistance expressed as growth on media containing 400 ug/ml penicillin.
n = 2 for day 0; n = 4 for the other sampling dates.

Table 28. Analysis of variance for the penicillin-resistant, aerobic microorganisms isolated from the cecum of chickens fed different levels of penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	502.29	1.51
Error 1	3	332.61	

penicillin-resistant organisms. The treatment group being fed 100 ppm of penicillin in the feed had a variation from 16.2 to 35.2% of penicillin-resistant organisms. The analysis of variance for these data is shown in Table 30. The increase of penicillin-resistant organisms in the jejunum was not statistically significant between treatments.

The penicillin-resistant anaerobes isolated from the chicken cecum did increase slightly in the birds being fed penicillin as shown in Table 31. The slight increase in penicillin-resistant organisms from the control group to the treatment group fed 100 ppm of penicillin was not significant (Table 32).

The percent of aerobic bacteria found in the feces of mink that were resistant to penicillin at 400 ug/ml did increase in the mink fed the viscera of the broilers that had been fed penicillin. The control group of mink showed a variation of 1 to 5% of resistant organisms while the groups of mink fed the other two groups of broiler viscera had a variation of resistant bacteria from 6.2 to 29%, as summarized in Table 33. The increase in resistance in treatment three as compared to treatments one and two was significant at $P< 0.05$, as shown in Table 34.

The percent of anaerobic bacteria in the feces of mink that were resistant to penicillin at 100 ug/ml was high before any of the experimental chicken viscera was

Table 29. The percent of anaerobic microorganisms resistant to penicillin isolated from the jejunum of chickens fed different levels of penicillin.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0#	0.00±0.00	----	----
14	21.43±21.43	8.00±3.63	35.25±22.35
28	8.52±8.16	10.03±5.90	16.25±6.25
46	0.25±0.25	5.38±2.17	30.00±11.97

All counts expressed as percent of total microorganisms ± standard error.
Resistance expressed as growth on media containing 100 ug/ml penicillin.
n = 2 for day 0; n = 4 for the other sampling dates.

Table 30. Analysis of variance for the penicillin-resistant, anaerobic microorganisms isolated from the jejunum of chickens fed different levels of penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	383.51	0.76
Error 1	3	507.07	

Table 31. The percent of anaerobic microorganisms resistant to penicillin isolated from the cecum of chickens fed different levels of penicillin.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0#	0.00±0.00	----	----
14	6.75±3.12	0.00±0.00	0.00±0.00
28	7.00±6.67	2.50±0.65	2.50±0.65
46	1.00±1.00	2.08±0.67	3.75±2.17

All counts expressed as percent of total microorganisms ± standard error.
Resistance expressed as growth on media containing 100 ug/ml penicillin.
n = 2 for day 0; n = 4 for the other sampling dates.

Table 32. Analysis of variance for the penicillin-resistant, anaerobic microorganisms isolated from the cecum of chickens fed different levels of penicillin.

Source of var.	d.f.	M.S.	f value
Treatment	2	37.10	1.41
Error 1	3	26.33	

Table 33. The percent of aerobic microorganisms resistant to penicillin isolated from the feces of mink that were fed the viscera of penicillin-fed chickens.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0#	5.00±5.00	----	----
7	1.00±0.41	7.00±4.74	10.00±6.47
7 days after put back on normal feed	4.03±2.64	6.25±2.36	29.00±12.29*

All counts expressed as percent of total microorganisms ± standard error.
Resistance expressed as growth on media containing 400 ug/ml penicillin.
n = 2 for day 0; n = 4 for the other sampling dates.
* Significantly different from mean and treatment two at P< 0.05.

Table 34. Analysis of variance for the penicillin-resistant, aerobic microorganisms isolated from the feces of mink that were fed the viscera of penicillin-fed chickens.

Source of var.	d.f.	M.S.	f value
Treatment	2	662.68	10.11*
Error 1	3	65.58	

* Significant at P< 0.05.

fed. The percent of resistant bacteria in the feces in the control group ranged from 11.5 to 23.9%. The mink in treatments two and three fed showed an increase in penicillin-resistant organisms with a range from 22.2 to 47.2% (Table 35). A significant increase in penicillin-resistant microorganisms was found in treatment three seven days after the experimental diet was withdrawn. The data presented in Table 36 indicate that the increase in resistant organisms was not significant at $P< 0.05$, but the increase was significant at $P< 0.10$.

The experimental diets that were fed to the mink were tested for the presence of penicillin. The data summarized in Table 37 indicate that no penicillin was found in detectable amounts in the diets.

Experiment four

A sample of 19, penicillin-resistant, bacterial colonies from the aerobic, BHI-penicillin agar plates were randomly selected for plasmid testing and comparison. Three of the colonies were from the chicken cecum, five of the colonies came from the diets prepared from the chicken viscera, and 11 of the samples were found in the feces of the mink fed the experimental diets. Summarized in Table 38 are the percent of different genera isolated from the plates. The colonies were picked from isolated colonies and maintained

Table 35. The percent of anaerobic microorganisms resistant to penicillin isolated from the feces of mink that were fed the viscera of penicillin-fed chickens.

Days on experimental feed	Treatment 1 0 ppm penicillin	Treatment 2 10 ppm penicillin	Treatment 3 100 ppm penicillin
0#	23.91±10.57	----	----
7	11.50±2.90	22.25±4.31	22.50±7.97
7 days after put back on normal feed	18.00±1.78	29.75±3.01	47.25±11.92*

All counts expressed as percent of total microorganisms ± standard error.
Resistance expressed as growth on media containing 100 ug/ml penicillin.
n = 2 for day 0; n = 4 for the other sampling dates.
* Significantly different from the mean P< 0.05.

Table 36. Analysis of variance for the penicillin-resistant, anaerobic microorganisms isolated from the feces of mink that were fed viscera from penicillin-fed chickens.

Source of var.	d.f.	M.S.	f value
Treatment	2	813.80	7.24#
Error 1	3	112.37	

Significant at P< 0.10.

Table 37. The amount of penicillin found in mink diets containing viscera of penicillin-fed chickens.

sample	mm inhibition	mg penicillin/kg sample
standard one#	1.08±0.35	0.12
standard two	4.23±0.28	0.50
diet 1, 0 ppm penicillin	<0.50±0.00	<0.10
diet 2, 0 ppm penicillin	<0.50±0.00	<0.10
diet 3, 0 ppm penicillin	<0.50±0.00	<0.10
diet 4, 0 ppm penicillin	<0.50±0.00	<0.10
diet 5, 0 ppm penicillin	<0.50±0.00	<0.10
diet 6, 0 ppm penicillin	<0.50±0.00	<0.10

n = 6 for standards one and two; n = 2 for diets one to six.
Numbers expressed as mean ± standard error.

Table 38. The percent of different aerobic, penicillin-resistant, bacterial genera randomly selected from the total, penicillin-resistant, bacterial population.

GENUS	PERCENT
Escherichia	58.0
Klebsiella	5.0
Enterobacter	16.0
Edwardsiella	11.0
Proteus	5.0
Unknown	5.0

on BHI slants containing 50 ul/ml ampicillin (Gibco Labs., Grand Island, N.Y.). Thirteen of the 15 penicillin-resistant colonies tested by gel electrophoresis showed evidence of having a plasmid.

Only two of the plasmids, that were isolated from different colonies, were of the same size with each plasmid containing approximately 6500 DNA base pairs as determined by electrophoresis of the plasmid preparation in a 0.7% agarose gel, see Figure 1. The size of the plasmid was determined by the comparison of the plasmid to a known standard of lambda DNA cut with the restriction endonuclease Hind III, (BRL, Gaithersburg, Md.).

One of the plasmids was isolated from an E. coli found in the mink diet prepared from the viscera of the chickens fed 100 ppm penicillin. The other plasmid was isolated from an E. coli found in the feces of a mink fed a diet containing viscera of broilers that were fed 100 ppm penicillin.

Once the size of the two plasmids was determined to be approximately equal, they were cut with two restriction endonucleases. The two endonucleases used were EcoRl and BglII. The base sequences where these two enzymes cut were described in the Material and Methods. A picture of the plasmids after cutting with EcoRl is shown in Figure 2. The band sizes of the plasmids after cutting with EcoRl are listed in Table 39. Plasmid one (isolated from the feces of

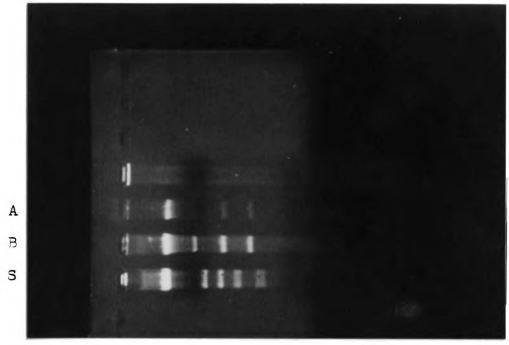

Figure 1. Photograph of an agarose gel electrophoresis of two different plasmids and a standard plasmid.

Plasmid A isolated from mink feed containing viscera from chickens fed 100 ppm penicillin.
Plasmid B isolated from mink fed a diet containing viscera from chickens fed 100 ppm penicillin.
Plasmid S is standard lambda cut with Hind 3.
Gel is a 0.7% agarose gel run at 50 V for 60 minutes.

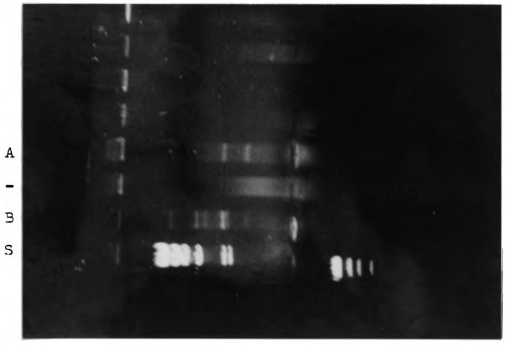

Figure 2. Photograph of an agarose gel electrophoresis of two plasmids cut with EcoRl.

Plasmid A isolated from mink feed containing viscera from chickens fed 100 ppm penicillin.
Plasmid B isolated from mink fed a diet containing viscera from chickens fed 100 ppm penicillin.
Plasmid S is standard lambda cut with Hind 3.
Gel is a 0.7% agarose gel run at 50 V for 75 minutes.

a mink) has three more bands than plasmid two (isolated from the mink diet). The first band in plasmid one is not plasmid DNA since it is larger than the plasmid DNA that was isolated in the first run. The 4350 base pair band and the 2500 base pair band found in plasmid one may just be an artifact from an incomplete cut by the enzyme.

Listed in Table 40 are the band sizes found after cutting the two plasmids with BglII. A picture of the plasmids after cutting with BglII is shown in Figure 3. The first band found in both runs is too large to be plasmid DNA. The next three bands match both plasmids. The last band seen in plasmid one is not seen in plasmid two although this may be due to a quantity of DNA too small to fluoresce enough to be seen.

The transformation experiments performed in this study were inconclusive.

Table 39. Band sizes of two plasmids cut with EcoR1.

Band number	Plasmid 1* size in base pairs	Plasmid 2** size in base pairs
1	23000	6500
2	6500	2300
3	4350	1450
4	2500	
5	2300	
6	1500	

* Plasmid 1 isolated from mink fed a diet containing viscera from chickens fed 100 ppm penicillin.
** Plasmid 2 isolated from mink feed containing viscera from chickens fed 100 ppm penicillin.

Table 40. Band sizes of two plasmids cut with BglII.

band number	Plasmid 1* size in base pairs	Plasmid 2** size in base pairs
1	7500	8000
2	6500	6500
3	5500	5500
4	3100	3100
5	1500	

* Plasmid 1 isolated from mink fed a diet containing viscera of chickens fed 100 ppm penicillin.
** Plasmid 2 isolated from mink feed containing viscera of chickens fed 100 ppm penicillin.

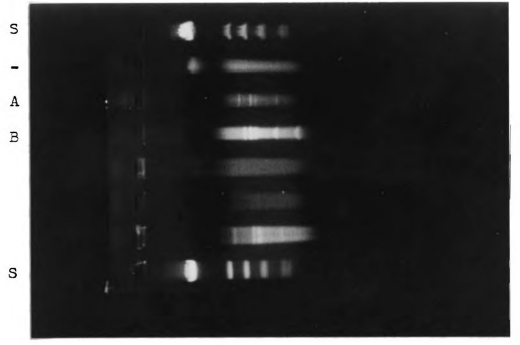

Figure 3. Photograph of an agarose gel electrophoresis of two plasmids cut with BglII.

Plasmid A isolated from mink feed containing viscera from chickens fed 100 ppm penicillin.
Plasmid B isolated from mink fed a diet containing viscera from chickens fed 100 ppm penicillin.
Plasmid S is standard lambda cut with Hind 3.
Gel is a 0.7% agarose gel run at 50 V for 75 minutes.

DISCUSSION

Experiment one

The use of penicillin for growth promotion has been studied extensively. Most results indicate that broilers will gain the same amount of weight on less feed when the feed contains penicillin. The reason that this occurs remains unclear. Three theories have been developed. One theory suggests that the use of penicillin in the feed causes a nutrient-sparing effect by killing bacteria in the intestine that use essential nutrients needed by the animal. Another theory suggests that penicillin causes a metabolic change in an animal which allows a greater percentage of nutrients to be used for growth. The third theory suggests that the use of penicillin in low levels kills or inhibits the growth of bacteria in the intestine that cause subclinical infections. When these bacteria are inhibited, the intestinal wall becomes thinner and the nutrients are more easily absorbed (Murphy, 1975).

The lack of response of weight gain or feed conversion in the broiler chicks fed penicillin in the first experiment of this study supports the theory that penicillin in the feed reduces subclinical infections by

killing undesirable bacteria in the alimentary tract. The birds in the first experiment of the study were raised in facilities that were cleaner than commercial facilities. Also, strict quarantine procedures were enforced while the birds were being raised. Finally, the birds were allowed more floor space than in commercial facilities. This kept the bedding dryer than normal. All of the above conditions reduced stress in the birds and reduced the chance that a subclinical infection would develop. These husbandry methods support the findings of Lev et al. (1957) that birds raised in "clean" facilities showed no growth improvement when fed low levels of penicillin as compared to birds not fed penicillin.

Experiment two

The results from experiment two showed that no significant change in the numbers of aerobic bacteria occurred in the jejunum of broilers which had been fed penicillin at 10 ppm or 100 ppm. The variation in numbers of aerobic bacteria in the jejunum may have masked any changes in the number of bacteria due to the feeding of penicillin. Marked variation in numbers of bacteria is commonly seen when working with intestinal bacteria. In this experiment, an attempt was made to decrease the variation in the numbers of bacteria by discarding the intestinal contents and using the intestinal lining to

obtain the bacterial counts. When using this method, only the bacteria that are closely associated with the lining of the intestinal tract are counted.

The total, aerobic, bacterial counts in the jejunum agree with the findings of Smith (1965). The coliform counts were found to make up approximately 90% of the total, aerobic, bacterial counts as expected.

The anaerobic bacteria in the jejunum increased through the first four weeks of the bird's life and then leveled off at approximately 10^6. No significant change in total, anaerobic, bacterial numbers occurred when the birds where fed penicillin.

The lactobacilli numbers did not change significantly with the feeding of penicillin as found by Anderson et al. (1951), but the lactobacilli reached high concentrations faster in the treatments where penicillin was fed.

The Clostridium perfringens counts were below detectable levels in the jejunum.

The bacterial counts were much higher in the cecum than in the jejunum as expected. The total, aerobic, bacterial counts in the cecum varied little between treatments or time. The number of aerobic bacteria remained constant at 10^8 to 10^{10}. The coliform counts in the cecum of the chicken made up between 80 to 100% of the total, aerobic, bacterial counts. The research that has been published on the effect of feeding penicillin to birds and the effect of penicillin on coliform counts has indicated that the

coliform counts increased when penicillin is fed (Dubos et al., 1963). Mamber and Katz (1985) saw no change in numbers of enteric Gram-negative bacilli in chickens fed antibiotics. A reason that this experiment did not show an increase in coliform numbers may be due to the rearing conditions discussed under experiment one.

The total, anaerobic, bacterial count in the cecum was increased by the addition of penicillin to the feed at 10 or 100 ppm. This increase in anaerobic bacteria has been noted before by Sieburth et al. (1951).

The sporadic and extremely variable counts of the lactobacilli are typical of this genus (Dubos et al., 1963). This variation makes a determination of a possible differences in lactobacilli numbers between treatment groups fed different levels of penicillin difficult.

The Clostridium perfringens numbers in the cecum did not change significantly due to the feeding of penicillin. Lev et al. (1957) reported that clostridia numbers may or may not change when penicillin is fed to chickens.

The feeding of the broiler viscera from the different treatments to the mink did not significantly change the bacterial counts in the mink feces. This is not surprising since the penicillin had been withdrawn from the chickens three days prior to the processing of the birds. The mink diets made with the broiler viscera showed no penicillin present in detectable levels when tested.

Little information is available on the bacteria found in

the mink feces, but when compared to a cat, which is also a carnivore, some similarities can be found. The number of coliforms found in the mink feces was similar to the number of coliforms found in cat feces. Both animals have approximately 10^7 coliforms per gram of feces (Smith, 1961). The total, anaerobic population found in the mink feces was only one percent of the total anaerobes found in the cat feces. This difference may be due to inherent differences in the species, but it may also indicate that the method of collecting the mink feces may need to be done in a shorter period of time so that fewer obligate anaerobes are killed due to the exposure to oxygen.

The lactobacilli counts made on the mink feces compares closely with the number reported in the feces of cats. The Clostridium perfringens counts in the mink feces were much lower than what was reported for cat feces by Smith (1961). The low counts may have been caused by exposure of the bacteria to oxygen, but it may also have been caused by keeping the fecal samples at 4C while the samples were brought into the laboratory. Clostridium perfringens is cold sensitive and a large number of them may have been killed due to the temperature.

Experiment three

The results showed that no significant increase of penicillin-resistant, aerobic bacteria occurred in the

jejunum of broilers that were fed either 10 or 100 ppm penicillin in their diets. A significant difference in resistant bacteria would be difficult to see due to the large fluctuation of aerobic bacteria in the jejunum. Also, the standard error was quite large. The large percentage of resistant organisms seen at day 0 were found to be yeasts.

No significant increase in aerobic bacteria that were resistant to penicillin at 400 ug/ml of media was seen in the cecum of the chickens being fed penicillin at either 10 or 100 ppm. The penicillin-resistant, aerobic bacteria decreased within three days after the penicillin was withdrawn from the feed, as seen from the 46 day sampling. This indicates that the penicillin-resistant bacteria do not survive as well as the wild-type bacteria. This may be due to the inability of the resistant bacteria to adhere to the intestinal lining as well as the wild-type bacteria (Nordstrom et al., 1977). These results agree with the results published by Langlois et al. (1984).

No significant differences in the percent of anaerobic bacteria in the jejunum that were resistant to penicillin were found.

The number of anaerobic bacteria resistant to penicillin found in the cecum of the broilers was not significantly different in the penicillin-fed treatment groups. The most interesting feature of the resistant, anaerobic bacteria is that they did not decrease within three days after the penicillin was withdrawn from the broiler feed as did the

penicillin-resistant, aerobic bacteria. This may be explained by the longer generation time of the anaerobic bacteria.

The percent of aerobic bacteria resistant to penicillin found in the feces of mink fed diets containing viscera of chickens that had been fed penicillin increased over the percent of the penicillin-resistant, aerobic bacteria found in the feces of the mink in treatments one and two. The percent of anaerobic bacteria resistant to penicillin found in the feces of the mink increased significantly at $P< 0.10$ when the animals were fed diets containing the viscera of chickens that had been fed penicillin. This level of significance is important due to the variation in the bacterial counts from the collection and handling methods of the mink feces. The penicillin-resistant bacteria did not decrease after the experimental diets had been removed. The experimental feed was tested and no penicillin residue was seen. The control diet of the mink was also tested and no penicillin-resistant bacteria were found. These tests show that any increase in penicillin-resistant bacteria must be due to the presence of bacterial DNA from the chickens.

Experiment four

The penicillin-resistant, aerobic bacteria isolated were primarily Escherichia coli (58%), with a few Klebsiella

(5%), *Enterobacter* (16%), *Edwardsiella* (11%), and *Proteus* (5%). Five percent of the isolated organisms could not be identified. The plasmids isolated from the different groups of birds, mink diets and the mink feces were all different except for a plasmid isolated from a diet made of viscera of chickens fed 100 ppm of penicillin and a plasmid isolated from the feces of a mink that was fed a diet made from viscera of chickens fed 100 ppm of penicillin. The weights of these two plasmids were identical at 6500 base pairs. When the plasmids were cut with the two restriction endonucleases some differences were noted, but the patterns of the cuts were similar for both restriction endonucleases. This indicates that the plasmids may be identical. The identical weight obtained with the first electrophoretic analysis would have been used as conclusive evidence that the plasmids were identical in the mid 1970's as noted in the article by Meyers *et al*. (1976). The use of two restriction endonucleases to further identify the plasmids was suggested by Farrar (1983). The tests performed in this study meet these two qualifications, but further testing for homologous base pair sequences by tests such as the Southern blot technique (Coussens, 1988) is needed before a positive determination can be made.

The presence of a similar plasmid in the two different samples does not indicate that a transfer of the plasmid between two bacteria occurred. The bacteria in which the plasmids were isolated may be the same bacterium but found in two different locations.

CONCLUSIONS

1. The feeding of penicillin at 10 ppm or 100 ppm to broilers does not improve feed efficiency if the birds are raised in clean and stress-free facilities.

2. The feeding of penicillin to broilers at 10 ppm and 100 ppm does not significantly change the number of total aerobic bacteria, total coliforms, total anaerobes, lactobacilli, or Clostridium perfringens in the jejunum or in the cecum of the broilers.

3. The coliform, lactobacilli, and Clostridium perfringens counts in mink feces are similar to the coliform, lactobacilli, and Clostridium perfringens counts taken in the feces of cats.

4. The number of penicillin-resistant, aerobic bacteria dropped in both the jejunum and in the cecum of chickens fed penicillin within three days after the penicillin was removed from the feed. The penicillin-resistant, aerobic bacteria do not survive as well as the wild-type, penicillin-sensitive bacteria without selective pressure. The anaerobic bacteria resistant to penicillin in the

constant for at least three days after the penicillin is removed from the feed. A longer withdrawal period needs to be tested to see if the number of penicillin-resistant, anaerobic bacteria will also decrease similar to the aerobic bacteria.

5. The percent of penicillin-resistant, aerobic bacteria increased significantly ($P < 0.05$) in mink feces when the mink were fed the viscera of broilers that had been fed penicillin. The percent of anaerobic bacteria resistant to penicillin found in the feces of mink also increased significantly ($P < 0.10$). The increase in resistance in the mink feces was not dependent on the mink being exposed to penicillin in this study.

6. Similar plasmids were found in both mink feed and in mink feces. The plasmids from the two different samples had a similar weight and were cut in a similar pattern by two different restriction endonucleases. More testing needs to be performed on the plasmids to determine if they are identical.

APPENDICES

Appendix A

Formulation of MSU broiler feeds.

1. MSU broiler starter.

Corn	951.8 lbs
Soybean meal 44%	855.0 lbs
Calcium carbonate	23.0 lbs
Dicalcium phosphate	36.2 lbs
Salt	10.0 lbs
Vitamin-Mineral premix	10.0 lbs
Corn oil	110.0 lbs
Methionine	4.0 lbs

2. MSU broiler grower.

Corn	1066.8 lbs
Soybean meal 44%	741.6 lbs
Calcium carbonate	22.4 lbs
Dicalcium phosphate	36.2 lbs
Salt	10.0 lbs
Vitamin-Mineral premix	10.0 lbs
Corn oil	110.0 lbs
Methionine	2.0 lbs

Appendix B

Solutions used for microbiological methods.

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1. Siver sulfafe solution

$AgSO_4$	0.5 gm
H_2SO_4 1.0M	1.0 ml
glycerol	100.0 ml
d H_2O	100.0 ml

2. Phosphate buffered saline

Na_2HPO_4	1.236 gm
NaH_2PO_4	0.180 gm
NaCl	8.500 gm
H_2O	1000.000 ml

Appendix C

Solutions used for plasmid isolation.

1. STET	
Tris 1.0M pH. 8.0	25 ml
EDTA 0.5M	50 ml
Triton X-100	25 ml
Sucrose	40 gm
dd. H O	400 ml
2. EDTA	
Disodium ethylene diamine tetraacetate : 2H O	186.1 gm
dd. H_2O	800.0 ml
NaOH	20.0 gm
3. Lysozyme solution	
lysozyme	10.0 mg
Tris 250mM pH 8.0	1.0 ml
4. TE	
Tris 1.0M pH 8.0	1.0 ml
EDTA 0.5M pH 8.0	0.2 ml
dd. H_2O	98.8 ml
5. Phenol:formaldehyde solution	
Phenol	25 ml
$CHCl_3$	24 ml
Isoamyl alcohol	1 ml
6. Five-x loading buffer	
Bromophenol blue	0.25%
Xylene cyanol	0.25%
Ficoll(type 400) in H_2O	12.50%
7. Formaldehyde solution	
$CHCl_3$	24 ml
Isoamyl alcohol	1 ml

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