INTESTINAL CYTOKINE mRNA EXPRESSION IN BROILER CHICKENS CHALLENGED WITH *SALMONELLA* HEIDELBERG IN THE PRESENCE OF SUBTHERAPEUTIC AND THERAPEUTIC ANTIBIOTICS

by

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(Under the Direction of Dr. Richard Barb)

Abstract

Salmonella remains an important pathogen in recent years effecting both humans and animals. Poultry is the main source of *Salmonella* infection in humans. Many *Salmonella* strains are becoming resistant to antibiotics, which makes it hard to eliminate the infection. Much needs to be learned about how chicken immune systems react to *Salmonella* infections. In this study, the chicken immune response to *Salmonella* infection in the presence of subtherapeutic and therapeutic antibiotics was analyzed. Understanding these effects will provide knowledge about how the immune system works under these conditions. These data indicated that gut cytokine mRNA expression was affected by *Salmonella* infection and by administration of subtherapeutic and therapeutic antibiotics, while hormones such as IGF-I, T3, and T4 did not appear to be affected. Expression of *Salmonella* to subtherapeutic doses of antibiotics resulted in increased resistance to antimicrobials.

INDEX WORDS: Immune response, *Salmonella* Heidelberg, cytokine, antimicrobial resistance, IGF-I, T3, T4

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CHAPTER 1

INTRODUCTION

Salmonella is a major etiologic agent of food-borne infection in humans. The infection may manifest itself as mild to severe gastroenteritis causing symptoms of diarrhea, nausea, and vomiting. Over 2400 serotypes have been recovered from humans and animals. One of the biggest problems facing mankind today is the development of antimicrobial resistance, particularly multiple resistance, among salmonellae. Many factors may contribute to the development of antimicrobial resistance including antimicrobials in animal production, unsanitary housing contributing to the maintenance of resistant bacteria, and exchange of genetic material between both related and unrelated bacterial populations. Additionally, resistance may be evident in the absence of antimicrobial use on the farm or on final product through cross contamination.

The immune system plays a significant role in resolving infections. Studies have reported on the effects of S*almonella* Typhimurium, Enteriditis, and Gallinarum by looking at the role of several cytokines (Withanage *et al.* 2004). There is a paucity of studies on the effects of the hormones triiodothyroxine (T3), thyroxine (T4), and insulin growth factor (IGF-I).

In this study, we report on the outcome of infection following challenge of broiler chickens with a nalidixic acid resistant S*almonella* Heidelberg. Both colonization and the cytokine signaling were studied. Subgroups of birds received either subtherapeutic doses of chlortetracycline or therapeutic doses of enrofloxacin, respectively. Antimicrobial resistance patterns were also analyzed to track any developing resistance.

We hypothesized that there would be an effect on cytokine mRNA expression when birds were exposed to the *Salmonella* with or without antimicrobial treatment, but not necessarily to birds receiving treatment in the absence of a bacterial challenge.

CHAPTER 2

LITERATURE REVIEW

Salmonella

Salmonella, named by the veterinarian and bacteriologist Daniel Salmon (Guthrie 1992), is a gram negative, rod shaped, non-lactose forming facultative anaerobe. It is a member of the non-spore forming bacilli in the family of Enterobacteriacae (Gray and Cray 1990;Jay 1998;Quinn *et al.* 2002d). There is a 90% genetic homology between *Salmonella* with *E.coli* (Sadler and Whitt 1994). Most of the time, it is hard to distinguish between *Salmonella* and *E.coli* microscopically and on some media because of their similar morphologies (Jay 1998). Phenotypically, raised colonies of *Salmonella* are about 2-4 mm in diameter with round and smooth edges (Gast 1997).

Salmonella growth requirements include a pH ranging from 4-9 (Gast 1997;Gray and Cray 1990;Jay 1998) and it has been observed to grow in temperatures ranging from 8 to 45°C on various media. The recommended temperature for growth is 37°C (1998;Gray and Cray 1990;Guthrie Rufus 1992b;Jay 1998). *Salmonella* is typically cultured from feces (Difco Laboratories 1998;Gray and Cray 1990;Guthrie Rufus 1992b), but since it is ubiquitous in nature, it has also been recovered from environmental sources including sewage, feed, and water (Difco Laboratories 1998). Recovery from a septicemia via blood occurs less often (Difco Laboratories 1998;Guthrie Rufus 1992b).

Enrichment, used to increase the number of *Salmonella* cells, is widely advocated and various media are available. Selective enrichment is used also to block out unwanted bacterial species (Gast 1997). Typical enrichment broths that are used for salmonellae

include Gram negative-Hajna (GN), tetrathionate (TT), and Rappaport-Vassiliadis (RV) Broths (Difco Laboratories 1998;Gray and Cray 1990;Quinn *et al.* 2002e). GN, RV, and TT are recommended at incubation temperatures of 37°C for 18 to 48 hours (Quinn *et al.* 2002e).

Agar medium, used for growth of *Salmonella,* include Brilliant green (BG), XLT4, Lysine Iron (LIA), and Triple Sugar Iron agar (TSI) (Difco Laboratories 1998;Quinn *et al.* 2002e). The BG agar inhibits gram-positive bacteria, while permitting *Salmonella* to grow. After incubation at 37°C for 18-24 hours, the colonies appear red against a green background (GUINEE and KAMPELMACHER 1962). XLT4 agar is selective for *Salmonella,* producing black colonies after incubation at 37°C for 18-24 hours. Salmonellae typically produce hydrogen sulfide and the black colonies represent hydrogen sulfide production as a result of xylose fermentation. If the *Salmonella* is H_2S negative, the colonies will appear pinkish-yellowish and are more likely to be ignored during colony selection. Therefore, it is becoming more of a routine practice to use two selective agars in concert, such as BG and XLT4.

LIA and TSI are typically used during the biochemical confirmation step. H_2S production can occur in either media after incubation at 37°C for 18-24 hrs. TSI is positive for *Salmonella* with yellow presenting on the slant portion of the tube indicating acid production and red presenting in the butt portion of the tube indicating alkalinization (Difco Laboratories 1998). Other biochemical tests for *Salmonella* include citrate utilization (which is blue if positive for S*almonella),* β-Galactosidase (yellow color if negative), and the indole test which if red is negative for *Salmonella* (Madigan *et al.* 2000).

Salmonella can be destroyed by heating to temperatures above 70°C or with heat irradiation (Gast 1997;Guthrie Rufus 1992c). However, heat tolerance has been reported and may not effectively destroy all microbes (Mattick *et al.* 2001). Disinfectants such as hydrogen peroxide (Mulder *et al.* 1987), acetic acid (Dickens and Whittemore 1994), lactic acid (Izat *et al.* 1990), potassium sorbate, chlorine (Morrison and Fleet 1985), and trisodium phosphate are very effective against *Salmonella*.

Salmonella are classified according to the O antigens that are expressed; groups include A, B, C, etc (Jay 1998). The O antigen, a somatic or cell wall antigen, is a phospholipid polysaccharide complex that resist attack by serum complement (Salyers and Whitt 2001b) . Serotypes are determined using slide agglutination tests and antisera to the O antigens (Cleary 1984). Most serotypes fall in serogroups B, C, and D (Gast 1997). The H or flagellar antigen, classifies *Salmonella* even further. The H antigen is generally determined by tube agglutination test. The H antigen has two phases, phase I which is shared between very few species and phase II which is shared with a variety of species (Jay 1998). Together, these two antigens allow investigators to identify *Salmonella* serotypes which are typically named after the geographic location from where it was recovered (Gast 1997;Jay 1998).

Serotypes can be further broken down into two species, *S*. enterica which is further divided into six subspecies and *S*. bongori (LeMinor and Popoff 1987). Globally, *Salmonella* Typhimurium is the most common serotype recovered from both humans and animals. Some serotypes are host specific. Examples include *S*. Typhi and *S.* Paratyphi in humans, *S*. Pullorum and *S*. Gallinarum in poultry, *S*. Dublin in cattle, and *S.* Cholereasuis in pigs (Barrow *et al.* 1987;Quinn *et al.* 2002b).

Virulence Factors. Virulence is defined as the ability to cause disease. These factors promote colonization and invasion of the host. Moreover, these factors may also allow the bacteria to survive in the intestinal tract longer than avirulent bacteria. Flagella is used as a motility mechanism for *Salmonella*. The pili (Type I fimbrae) are hair-like structures that allow attachment to the host cell rearranging the membrane (Gast 1997). If *Salmonella* survive the low pH of the stomach it will colonize the distal ileum. In order to do this, pili are used to attach and reshuffle the host cells. Then, by pinocytosis the bacterium is engulfed by the host cell invading the ileum. The bacterium destroys the macrophage (M) cells, lymphoid and polymorphonuclear (PMN) cells. This leads to an inflammatory response causing diarrhea (Gray and Cray 1990). *Salmonella* is composed of a cell wall, membrane, cytoplasm, capsule, flagella, and pili. The cell wall is comprised of petidoglycan and lipopolysaccharide (LPS) layers which protect the cell from damage, osmotic lysis, and digestion by the host cell (Gast 1997;Quinn *et al.* 2002b). As mentioned above, the O antigen is part of the LPS used for serotyping (Quinn *et al.* 2002b). The LPS is also composed of lipid A, which is a lipid associated with polysaccharide. The LPS acts as an endotoxin, because it binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in many cell types (Wikipedia 2006).

Diseases. Animals can be a perfect environment for bacteria to survive and facilitate transfer of bacteria from animals to humans, human to human, and animal to animal (Clarke and Gyles 1986). Most human infections result from consuming contaminated foods (Gray and Cray 1990;Guthrie 1992). Infections are known to begin in mucosal surfaces in the mouth, urinary, respiratory, and gastrointestinal tract. Within

the gastrointestinal tract, the stomach harbors the fewest colonies of bacteria due to its low pH, whereas the colon has the greatest amount of bacteria. Factors that can lead to an increased potential for colonization include stress or changes in diet (Line et al. 1997).

Diseases in humans as a result of *Salmonella* infection are typically where gastroenteritis or typhoid/enteric fever (Madigan *et al.* 2000). Symptoms of gastroenteritis include nausea, fever, abdominal pain, diarrhea, depression, anorexia, and pneumonia after 12 to 14 hours of ingestion of *Salmonella* which can last for two to three days (Gray and Cray 1990;Jay 1998;Quinn *et al.* 2002b). The elderly, young and immunocompromised persons are at the highest risk for infection (Jay 1998). A severe case of infection leads to dehydration, loss of electrolytes, and acid-base imbalance which may lead to death (Clarke and Gyles 1986). Typhoid fever is caused by ingestion by humans of contaminated food or water. *S*. Typhi is the pathogen that causes the illness of typhoid fever (Salyers and Whitt 1994). Asymptomatic carriers of *Salmonella* has been observed following an infection (Guthrie 1992).

In poultry, *Salmonella* can be acquired via contaminated feed (Gast 1997), from vertical transmission in the egg, the environment, insects and even personnel (Herenda and Franco 1996). Once ingested, *Salmonella* colonizes the intestines where it demonstrates extraintestinally to organs such as (Gast 1997) the spleen and liver (Barrow *et al.* 1987). In the intestines, the ceca and ileocecal junction are the main site for colonization (Quinn *et al.* 2002b;Turnbull and Snoeyenbos 1974) and young birds are more susceptible to infection than older birds (Sadler *et al.* 1969;Turnbull and Snoeyenbos 1974). Disease caused by *Salmonella* in poultry, are due primarily to *Salmonella* Pullorum and Gallinarum which are host specific for avian species.

Other serotypes often recovered from poultry include *S*. Enteriditis and Heidelberg, which are also responsible for human illness and *S*. Kentucky which, while prevalent in chickens, does not cause significant illness in humans (Fedorka-Cray *et al.* 1998). *Salmonella* recovered from poultry is the main source of infection in humans causing over 1,300,000 infections and 100 deaths each year (Herenda and Franco 1996). Controlling salmonellosis in poultry would include removing all infected animals, disinfecting buildings and cooking utensils, and employing preventative measures for personnel handling infected materials and birds (Quinn *et al.* 2002c).

Antimicrobials. Antimicrobials are low molecular weight chemical substances produced by organisms that inhibit or kill microbes, fungi, and bacteria (Quinn *et al.* 2002a;Salyers and Witte 1994). Alexander Fleming was the first to discover antimicrobials when he described penicillin. Later, Florey and Chain purified penicillin for clinical use (Quinn *et al.* 2002a). Antimicrobials are either manmade or natural. Most human antimicrobials are natural coming from microbes which include penicillins. The manmade antimicrobials would include sulfa drugs, which were discovered in the 1930's, quinolones in the 1960's, and oxazolidinone in the 2000's (Walsh 2003).

Antimicrobials can either kill or inhibit bacteria and they may be classified as broad or narrow spectrum. Broad spectrum antimicrobials work on Gram negative and Gram positive bacteria and are typically more sensitive to Gram positive bacteria. While broad spectrum antimicrobials can kill most pathogens, they can also harm the resident microflora which will result in a change in gut composition. This in turn may lead to gastric upset from overcolonization of other harmful bacteria normally kept in check with

resident microflora. Pre- and probiotics have been developed to restore normal gut composition and patients are often encouraged to use them during or following treatment (Guarner 2003).

Narrow spectrum antimicrobials are typically directed toward a single group of bacteria. Antimicrobials may be bacteriostatic and bacteriocidal. Bacteriostatic antimicrobials inhibit the growth of bacteria, whereas bactericidal antimicrobials kill bacteria (Salyers and Witte 1994).

Antimicrobials are broken down into classes according to the mechanism by which they inhibit or kill bacteria. Classes include β-lactams, glycopeptides, aminoglycosides, tetracyclines, macrolides and lincosamides, quinolones, and trimethropin and sulfonamides (Salyers and Witte 1994). β-Lactams are bacteriocidal antimicrobials that inhibit cell wall synthesis. They consist of penicillins, cephalosporins, carbapenems, and monobactems. All contain a β-Lactam ring and inhibit the last step in peptidoglycan synthesis of microorganisms. They account for approximately one half of all antimicrobials used (Salyers and Witte 1994).

Glycopeptides are antimicrobials that inhibit peptidoglycan synthesis and include vancomycin and teichoplan. Their major targets are Gram positive bacteria (Salyers and Witte 1994).

Aminoglycosides are produced by prokaryotes along with tetracyclines and macrolides. Aminoglycosides account for about three percent of all antimicrobials used. Certain aminoglycosides include kanamycin, gentamicin, streptomycin, and neomycin. These antimicrobials inhibit protein synthesis by preventing the 30S subunit of the bacteria ribosome from binding to the 50S subunit (Quinn *et al.* 2002a;Salyers and Witte

1994). They are effective against Gram negative bacteria and are typically bacteriocidal. Aminoglycosides are usually held in reserve until other antimicrobials fail to affect the infection (Salyers and Witte 1994).

Tetracyclines are broad spectrum antimicrobials that inhibit the 30S subunit of bacteria ribosomes by distorting the tRNA's A site which prevents aligning with the mRNA codon. Certain tetracyclines include chloratetracycline and oxytetracycline, commonly used in animal production to promote growth. Tetracyclines are bacteriostatic and most often used in human medicine to treat acne and lyme disease (Salyers and Witte 1994).

Macrolides include erythromycin, oleandomycin, spiromycin, and tylosin and account for 11% of antimicrobials used. They prevent the elongation of the 50S subunit and translocation of the ribosome. Macrolides are both bacteriostatic and bacteriocidal against Gram positive bacteria. They are used to treat shipping sickness of livestock and patients that are allergic to penicllins. Lincosamides are closely related to macrolides having the same effect as macrolides, but a different chemical structure (Salyers and Witte 1994).

Quinolones are bacteriocidal antimicrobials that include naladixic acid, enrofloxacin and all newer fluoroquinolones. Quinolones inhibits nucleic acid synthesis by inhibiting the β subunit of the DNA gryase from supercoiling during DNA replication (Quinn *et al.* 2002a;Salyers and Witte 1994). They also inhibit topoisomerase IV, which is involved in chromosomal replication (Axelson 2002). They are currently being used empirically in human medicine as a first line defense against gastroenteritis.

Trimethroprim and Sulfonamides act as competitors with bacteria and prevent the production of tetrahydrofolic acid that some microorganisms produce (Quinn *et al.* 2002a;Salyers and Witte 1994). They are used infrequently in animal medicine (Schwarz and Chaslus-Dancla 2001).

For each of the antimicrobial classes, bacteria have developed an effective means of resistance. For example, bacteria carry a β-lactamase enzyme, which cleaves the β-lactam ring causing inactivation of the pencillin antimicrobials (Salyers and Witte 1994). Binding proteins for penicillin can also be altered which do not allow the antimicrobial to bind while permitting peptidoglycan synthesis to continue. Microorganisms possess genes that prevent binding of glycopeptides which in turn allows transpeptidase to continue. Cytoplasm proteins, enzymatic inactivation, and efflux proteins are used against tetracyclines and bacteria can methylate the 23S subunit on the rRNA inhibiting binding of macrolides and lincosamides. Bacteria also have point mutations to alter the affinity of gyrase which confers resistance to quinolones. Point mutations are also common for trimethropin and sulfonamides reducing the effectiveness (Salyers and Witte 1994).

Antimicrobials not only acquire resistance they also typically cause side effects such as an allergic reaction to β-lactams because of the formation of β-lactam serum protein conjugate which causes an inflammatory immune response. In this case monobactams are given as an alternative treatment. Side effects associated with using aminoglycosides include hearing loss, particularly with streptomycin, and kidney impairment. Quinolones can cause mineralization of cartilage in children (Axelson 2002)

and tetracyclines cause discoloration of teeth in pregnant women and children (Salyers and Witte 1994).

As for chickens, antimicrobials used for treatment of *Salmonella* infection include tetracycline, neomycin, gentamicin, polymyxin B, spectinomycin, sulfa drugs, bacitracin, and sulfate and trimethropim. Polymyxin B is usually used to treat *Salmonella* Enteriditis, while gentamicin and spectinomycin treat yolk sac infections (Gast 1997).

Antimicrobial Resistance. Application of good management practices will result in reduced uses of antimicrobials and subsequent reduced development of antimicrobial resistance. Pelleting chicken feed, chlorinating drinking water in a cloud system, maintaining nest cleanliness by frequent egg collection and broken shell removal, and disinfecting shells afterwards will all reduce *Salmonella* incidence and therefore the need for administration of antimicrobials.

Antimicrobial testing methods include disk diffusion (Pfaller and Jones 2006). Antimicrobials are dissolved in a liquid or agar medium and paper disks are soaked with the solution prior to placement on a bacterial lawn. A zone, called the "Zone of Inhibition", will appear following incubation and growth of the bacterial lawn, represents the diffusion and effect of the drug. The zone demonstrates the amount of inhibition that has occurred (Axelson 2002;Guthrie Rufus 1992a); the lower the susceptibility, the larger the zone (Axelson 2002).

Test methods include determination of the minimal inhibitory concentration (MIC), which gives the minimum concentration of antimicrobials needed to inhibit growth (Axelson 2002;Quinn *et al.* 2002a). A novel method to test antimicrobial resistance is by use of custom made 96-well panels with different concentrations of

antimicrobials. First the microorganisms are inoculated in a broth and this broth is inoculated into these panels and incubated for 18-24 hours. Any growth on the panels will determine the MIC level of resistance (Sensititre™ TREK™ diagnostics, Inc., Cleveland, Ohio).

The R plasmid or transposon within the bacterium contains resistant genes to which confers resistance to antimicrobials. For example, R plasmids can phosphorylate or acetylate aminoglycosides and prevent drug activity. For penicillins, R plasmids split the β-lactam ring. Each gene in an R plasmid is specific for a particular antimicrobial (Salyers and Witte 1994).

No one factor in animal production has been identified which can be altered to prevent the development of resistance. However, some practices may reduce the likelihood that resistance will develop or persist. Certain animal husbandry methods lead to resistance, for example, overcrowding and poor hygiene (Altekruse *et al.* 1997;McKellar 1998;Perreten *et al.* 1997). For example, the overcrowding allows the spread of feces on the feathers of other birds and feces in the feed and water supply. Prevention and spread of resistance in some cases may achieved by use of antimicrobials when serious illness has occured. Combining different classes of antimicrobials could aid in prevention and treatment (Salyers and Witte 1994;Walsh 2003). Furthermore, discontinuing antimicrobials use as growth promoters and educating producers on the use of vaccines would also contribute to the prevention of infection (Khachatourians 1998;Witte 1998). Removal of infected animals would reduce stress, therefore preventing infection (Clarke and Gyles 1986). Non-animal practices include

pasteurization, cooking foods properly, and irradiation which prevent infection (Altekruse *et al.* 1997;McKellar 1998;Witte 1998).

Immune Response

Immunity is defined as the ability of the immune system to recognize foreign proteins, for example antigens, and react to them. The immune system can act in a way where the defense mechanisms could be expressed before any exposure to microbes or other foreign molecules, therefore representing an innate immunity. Another way is acquired immunity, in which the foreign molecules induce the defense mechanisms (Abbas *et al.* 1991b). There are two types of immunity: humoral and cell-mediated. Humoral immunity involves B lymphocytes (B cells) which are produced by the bone marrow. Antibodies, the primary of humoral response, are produced by B cells and circulate throughout the body to detect and initiate response to foreign antigens. B cells mature under the activation of T cells and differentiate into plasma cells (Male *et al.* 1987a).

Cell-mediated immunity is mediated via T lymphocytes (T cells) (Abbas *et al.* 1991b). T cells differentiate in the thymus. There are three different types of T cells: T helper cells, T suppressor cells, and cytotoxic T cells (Male *et al.* 1987a). T cells act in a way like phagocytes by engulfing and destroying bacteria (Abbas *et al.* 1991b;Salyers and Whitt 2001a). There are two primary types of phagocytes: polymorphonuclear (PMN's) and macrophages. Macrophages originate from monocytes which are derived from bone marrow stem cells and circulate in the blood prior to differentiation. Once monocytes reach the tissues they become macrophages (Salyers and Whitt 2001a).

In chickens, the defense against microorganisms comes from the skin and mucous membranes. If organisms pass through these defenses, the lymphatic system takes over releasing white blood cells called lymphocytes to neutralize or destroy microorganisms. The lymphocytes respond by producing antibodies to attach to the antigens of the microorganisms and engulf to destroy. The cloacal bursa located behind the cloaca in chickens activates the antibody production and controls the immunity (Damerow 1994).

Cytokines. Cytokines are regulatory proteins secreted by T cells which mediate cell-to-cell interactions that generate an immune response (Tizard 1992). The study of cytokines began in 1950 when macrophage activating factors and anti-viral interferons were discovered. They were further purified in the 1970's and by the 1980's their properties were defined. Many types of cells secrete these proteins which have pleiotropic effects on other cells. Cytokines share similar characteristics to growth factors and hormones. Cytokines have the same mode of action as hormones since their receptors have common structural features (Bazan 1990). For example, the secretion of cytokines is brief and self-limiting, meaning they cease after a definite period. Functions of cytokines have been categorized into several groups including mediators of natural immunity, regulators of lymphocytes, activators of nonspecific inflammatory cells, and stimulators of immature leukocytes (Abbas *et al.* 1991a). Cytokines can be classified by cell type and include lymphokines which are cytokines secreted from lymphocytes, monokines which are cytokines secreted from monocytes, and interleukins which are cytokines secreted from leukocytes (Male *et al.* 1987b;Tizard 1992). Others include interferons, chemokines, and growth factors (Vilcek and Sen 1996). Monocytes were identified in serum following a Bacillus *calmetteguerin* challenge with LPS (Carswell *et*

al. 1975). Lymphokines, now referred to as cytokines to discourage the idea that proteins are produced by lymphocytes alone, is the product of lymphocytes when exsposed to a specific antigen (Cohen *et al.* 1974;Dumonde *et al.* 1969). Interleukins were named by the Second International Lymphokine Workshop in 1979 (Aarden *et al.* 1979) and given the designation IL; currently there are 32 interleukins, IL-1 through IL-32.

Chemokines are small proteins that are structurally related which regulate the trafficking of cells (Zlotnik and Yoshie 2000). Chemokines also have the ability to enhance cell motility (Riott *et al.* 1998). Chemokines are divided into two subfamilies based on their N-terminal cysteine residues. First there is CXC, which is a chemoattractant for PMNs, and CC which are chemoattractants for macrophages and lymphocytes. The C represents the cysteine residues and X for an amino acid (Vilcek and Sen 1996;Wigley and Kaiser 2003;Withanage *et al.* 2004). .

Cytokines have synergistic and antagonist effects on certain cells. Cytokines are synergistic at different intercellular signaling pathways. For example, interferon (IFN)-gamma can potentiate a cytotoxic action on tumor necrosis factor (TNF). Antagonist effects include IFN providing a growth stimulant and IFN providing a growth inhibitor resulting in an antagonistic response. Cytokines can also stimulate and reduce the assembly of other cytokines (Vilcek and Sen 1996). An example of stimulation is the activation of IL-2 synthesis in the presence of IL-1 (Smith *et al.* 1980). Inhibition of cytokines include monocytes inhibiting IL-10 synthesis (Fiorentino *et al.* 1991).

Cytokines work to activate a cell by binding to the receptor which induces dimerization. This activation of intracellular signaling results in production of activation factors that enters the nucleus. Cytokine receptors are type I membrane glycoproteins

with a single membrane domain and an extra- and intra-cellular domain. When the cytokine binds to the receptor, tyrosine phosphorylation is induced and Janus kinases (Jak) are activated. Jak couples ligand binding to tyrosine phosphorylation. The dimer formed translocates to the nucleus and binds to the DNA and transcription is induced. Another kinase is MAP-kinase used by IL-1β and TNFα performing in the same manner as the Janus kinases (Riott *et al.* 1998).

Cytokines are grouped into families according to their genomic structure and receptor sequence. Interferons have about 15 members in its family, whereas chemokines have over 50 members. The largest family is the cytokine receptor superfamily which includes cytokines with a 200 amino acids long homology. Examples include IL-2 to 7, IL-9 and IL-12 (Riott *et al.* 1998).

Proinflammatory cytokines mediate inflammation during injury or disease. Inflammation includes symptoms of redness, pain, and swelling (Salyers and Whitt 2001a). IL-1β, IL-6, IL-8, and MIP-1β are the all considered proinflammatory cytokines (Wigley and Kaiser 2003).

Human IL-1 is produced by a variety of cells including dentritic cells, neutrophils, T and B cells, astrocytes, endothelial cells, macrophages, and others (Kaiser *et al.* 2004;Male *et al.* 1987b;Mire-Sluis and Thorpe 1998;Tizard 1992). IL-1 activates T cells to be more responsive to IL-2 and IL-2R and also enhances lymphokine release. IL-1 acts synergistically with other lymphocytes in the presence of IL-2 to stimulate B cell proliferation (Abbas *et al.* 1991a;Male *et al.* 1987b;Tizard 1992). Other structurally and actively related cytokines to IL-1 include IL-6 and TNF (Tizard 1992). IL-1 is very similar to TNF and it is hard to distinguish between the two. Other functions if IL-1

includes affecting hematopoietic systems and endothelial cells (Bevilacqua *et al.* 1984;Dejana *et al.* 1984). IL-1 begins as a 33 kDa precursor which is cleaved proteolytically to a 17 kDa peptide which has two forms, IL-1α and IL-1β, each of which share 25% homology (Abbas *et al.* 1991a;Tizard 1992). These two forms are distinguished by their isoelectric points of 5.0 and 7.0, respectively. Both forms are synthesized as cytoplasmic proteins and recognize the same cell surface receptor (Abbas *et al.* 1991a). However, there are several differences between the two forms. IL-1β is preferred by T cells and is located in tissue fluid whereas IL-1α is preferred by B cells and is located in the cell membrane (Abbas *et al.* 1991a;Tizard 1992).

IL-1β was first reported in 1984 (Auron *et al.* 1984;Dejana *et al.* 1984). It is not active until is cleaved to the 17 kDa peptide from the 33 kDa precursor (Abbas *et al.* 1991a;Wigley and Kaiser 2003). It is more prevalent than IL-1α and thus there are more reports regarding its function, particularly the primary function of activating an acute phase response. Activating macrophages and lymphocytes leads to stimulation of other cytokines and chemokines. IL-1β acts as an endogenous pyrogen leading to fever and can be inhibited by corticosteroids, and aspirin (Abbas *et al.* 1991a;Male *et al.* 1987b). Chicken IL-1β shares approximately 25-30% homology to human IL-1β (Wigley and Kaiser 2003). The primary difference between the two is that chicken IL-1β lacks the residue of aspartic acid that is cleaved by capase-1 in humans. Capase-1 is an enzyme that cleaves aspartic acid of IL-1β for processing propeptide. Chicken IL-1β has five exons and four introns and is one fourth the size of mammalian IL-1β (Kaiser *et al.* 2004).

Another proinflammatory cytokine is IL-6 (Wigley and Kaiser 2003), a 21 to 28 kDa cytokine with approximately 200 amino acids (Abbas *et al.* 1991a;Kaiser *et al.* 2004;Tizard 1992). Chicken IL-6 shares 35% homology with human IL-6 (Van *et al.* 1986). Chicken IL-6 has four exons whereas mammalian IL-6 has five exons. It is also one third the size of mammalian IL-6 (Kaiser *et al.* 2004). IL-6 was first discovered by Fahres who was comparing inflammation levels between two patients in 1921. IL-6 was the cause of the inflammation in one of the patients. Originally IL-6 was identified as B cell-stimulating factor-2 (BSF-2) and T-cell replacing factor (Peter and Blum 1997;Yoshizaki *et al.* 1982). The name was finalized in 1988 to IL-6 (Kishimoto 1989). IL-6 is produced by fibroblasts, endothelial cells, macrophages, mononuclear phagocytes and T helper cells and these cells are stimulated by LPS, IL-1, and TNF (Abbas *et al.* 1991a;Gauldie *et al.* 1987;May *et al.* 1988;Tizard 1992). Glucocorticoids are inhibitors of IL-6 production (Mire-Sluis and Thorpe 1998;Sehgal *et al.* 1988). IL-6, along with many other cytokines, is responsible for acute-phase responses, haematopoesis, and immune responses (Abbas *et al.* 1991a;Tizard 1992). IL-6, a pleiotropic cytokine, also activates T and B lymphocytes, neuronal cells, hepatocytes, osteoclasts, keratinocytes, and induces macrophage production (Abbas *et al.* 1991a;Riott *et al.* 1998;Wigley and Kaiser 2003) and can stimulate hormonal release of hormones including adrenocorticotrophic hormones and anterior-pituitary hormones which include prolactin, growth hormone and LH (Spangelo *et al.* 1989). It can act in autocrine, paracrine, and endocrine fashion depending on the responding cells (Mire-Sluis and Thorpe 1998). Measurable amounts can be detected in the serum after Gram negative bacterial infection (Abbas *et al.* 1991a). Kaiser and Wigley report that IL-6 played a major role in chickens,

increasing 8-fold after challenge with different serovars of *Salmonella* (Wigley and Kaiser 2003). IL-6 limits *Salmonella* in the gut by inducing an immune response and inflammation thus preventing systemic disease (Wigley and Kaiser 2003).

IL-8 is a chemokine belonging to a family of structurally similar cytokines exhibiting chemotactic activity (Lindley *et al.* 1993;Peter and Blum 1997;Wuyts *et al.* 1998). IL-8 is a 99 amino acid nonglycosylated peptide with a molecular mass of 8 kDa (Abbas *et al.* 1991a;Tizard 1992). It contains four exons and three introns (Mukaida *et al.* 1989). IL-8 was identified in 1986-87 as a soluble factor following monocyte endotoxin stimulation (Mire-Sluis and Thorpe 1998). It was previously called neutrophil-activating peptide (NAP-1), neutrophil-activating factor (NAF), and granulocyte chemotactic factor (GCP) (Lindley *et al.* 1993;Peter and Blum 1997;Van *et al.* 1988;Walz *et al.* 1987). IL-8 is produced by monocytes, macrophages, endothelial cells, T cells, and NK cells and is induced by LPS, IL-1, and TNF (Abbas *et al.* 1991a;Kunkel and Remick 1992;Peter and Blum 1997;Tizard 1992).

IL-8 has been found in high levels associated with an influx of neutrophils for some diseases (Mire-Sluis and Thorpe 1998;Tizard 1992). *Salmonella* appears to induce IL-8 in mammalian gastroenteritis which causes an influx of neutrophils that in turn damages the epithelium and enables entry of the bacteria (Wallis and Galyov 2000). In avian species, IL-8/CAF is the same as human IL-8. CAF stands for chicken chemokine and angiogenic factor (Martins-Green and Feugate 1998). Chicken IL-8 has 51% homology to human IL-8 (Barker *et al.* 1993). Little is known about chicken IL-8, but it has been shown that in the presence of *Salmonella* Typhimuirium, there is an influx of heterophils which is the equivalent of human neutrophils (Henderson *et al.* 1999).

Heterophils are the main PMN's in chickens (Burton and Harrison 1969;Maxwell and Robertson 1998). It has also been observed that *Salmonella* can cause an increase in IL-8/CAF mRNA levels in chickens (Wigley and Kaiser 2003).

As mention before, there are two subfamilies of chemokines in humans; the alpha chemokines such as CXC, that attract neutrophils and the beta chemokines such as CC, that attract lymphocytes, monocytes, basophils, fibroblast, epithelial cells, and eosonophils (Mire-Sluis and Thorpe 1998;Wigley and Kaiser 2003). Very little is known about MIP-1β in chickens. MIP-1β has been reported to belong to the CC subfamily of chemokines (Mantovani 1999) which has over 25 members (Rollins 1997). These chemokines activate primarily the hematopoeitic system and induce inflammation, monocytes, T cells, and eosinophils. The main function of CC chemokines is to mediate the accumulation of leukocytes during infection (Mantovani 1999).

Avian cytokines or their gene expression can be detected and/or quantitated through use of ELISA, bioassays, and real time polymerase chain reaction (RT-PCR). ELISA's are easy to perform and inexpensive, but very few antibodies are available and those that are available may not always provide consistent results. Bioassays assess the biological factors of the cytokine activity, but can be technically difficult and time consuming to perform. RT-PCR allows for the quantification of cytokine gene expression in chickens but can be technically challenging.

Reverse transcriptase PCR detects expression of mRNA. To accomplish this, mRNA is converted to cDNA by reverse transcriptase allowing the cDNA to be analyzed through PCR. RT-PCR improves upon reverse transcriptase PCR. First, reverse transcriptase is applied, followed by PCR. The PCR uses a fluorescent probe to measure

the amount of mRNA during each cycle. Housekeeping genes, for example the 28S rRNA and β-actin, are used to compare levels of mRNA expressed during this process. The mRNA levels are also compared with standard curves of known RNA quantities (Wigley and Kaiser 2003).

Insulin Growth Factor-I in poultry. Insulin Growth Factors (IGF) production is stimulated by growth hormone (GH) in somatotroph cells. Growth hormone is secreted by the anterior pituitary throughout life and is regulated by growth hormone releasing hormone (GHRH) which is synthesized by cell bodies in the arcuate and ventromedial nuclei of the hypothalamus and somatostatin (SS) which in turn is synthesized by neurosecretory neurons. Through the axon terminals they are release into the hypophseal portal circulation. GHRH recognizes the receptor on the somatotroph cell and binds, followed by a coupling with adenylate cyclase (AC) through a stimulated G protein. This increases cAMP, activates phosphokinase A, and prevides for the secretion of GH. There is also an increase in calcium concentration which promotes secretion of GH. Calcium plays a key role in enzyme function. Somatostatin releasing inhibitory factor (SRIF) couples with AC through an inhibitory G protein. AC is decreased causing a decrease in cAMP and calcium concentration also decreases reducing GH secretion. If both GHRH and SRIF are bound, SRIF will have the dominant effect, otherwise GH goes on to stimulate IGF production (Considine 2003a).

Insulin Growth Factor I (IGF-I) is a 70 amino acid peptide that mediates growth hormone as an effector of the endocrine, autocrine and paracrine systems (McMurtry *et al.* 1997;Van Wyk 1984). It has a molecular weight of 7648 and an isoelectric point of 8.8 (Van der Brande J.L. 1992). IGF is synthesize by various tissues including the spleen

(McMurtry *et al.* 1997). IGF was first termed as "Somatomedin" but the terminology changed to differentiate between IGF-I and IGF-II and their modification (Considine 2003a;McMurtry *et al.* 1997). IGF was designated as IGF-I and IGF-II in 1987. Chicken IGF-I differs from human IGF-I by approximately eight amino acids (Ballard *et al.* 1990).

IGF has a negative feedback on GH by acting directly on somatotroph to inhibit the stimulatory GHRH action on GH. GH can also inhibit its own secretion by stimulating IGF secretion. It does this by inhibiting GHRH and stimulating SRIF. This is all apart of the hypothalamic-pituitary growth hormone axis (Considine 2003a).

There is no clear relationship between chicken and mammalian IGF function. In poultry, the levels are lowest in the fetus and remain low through 2-3 weeks of age when it starts to increase with age (McMurtry *et al.* 1997). Around 4-6 weeks there is a IGF level plateau (Ballard *et al.* 1990). There is a correlation of IGF levels with physical size, the larger the animal, the higher the IGF levels (Baserga *et al.* 2003). Poultry IGF-I is highest in the liver, but can be found in other tissues (McMurtry *et al.* 1997) which account for about 1% of total IGF levels (Daughaday 1989).

Two receptors, Type I and Type II, bind IGF proteins. The receptor is synthesize on a ribosome as a polypeptide chain containing one alpha and beta subunit linked by a disulfide bond. Type I and Type II bind together through disulfide bonds to form a tetramer. Type I receptors, or IGF-I receptors, initiate the response to IGF, mediating most of the effects of IGF-I and II (Jones and Clemmons 1995;Moxham C. and Jacobs 1992), while the Type II receptor has a high affinity to IGF-II, there is no evidence of this receptor in chickens (McMurtry *et al.* 1997). IGF-I regulate cell cycle progression,

differentiation and proliferation of cells, inhibition of apoptosis, and survival (Baserga *et al.* 2003).

IGF-I stimulates a mitogenic response in osteoblasts, chondrocytes, keratinocytes, smooth muscle cells, and many other tissues (Cohick and Clemmons 1993;Giudice 1992;Lowe 1991;Macaulay 1992;Sara and Hall 1990). IGF regulates in an endocrine and autocrine/parcrine fashion, and affects cell proliferation and differentiation. Other regulations include hormonal secretion from many cells, glucose and amino acid uptake, and DNA synthesis (Jones and Clemmons 1995;LeRoith *et al.* 2003;McMurtry *et al.* 1997).

Insulin Growth Factor Binding Proteins. Once IGFs are in circulation they are mostly bound to binding proteins (BP). There are six major binding proteins, IGFBP1 through IGFBP6, which bind to IGF and all have a slight affinity to insulin. The main function of BPs is to transport proteins in plasma in order to control the efflux of IGF. BPs control the metabolic clearance of IGF, lengthen the half-lives and provide cell and tissue localization. Lastly, they mediate binding IGFs with their receptors. They can also alter target cell actions and there are mismatched studies as to whether IGFBPs have inhibitory or stimulatory effects (Jones and Clemmons 1995). About 75% of complexes in circulation are made up of either IGF-I or IGF-II, BP-3 and an acid labile subunit which stabilizes the complex (Jones and Clemmons 1995;McMurtry *et al.* 1997;Rosselot *et al.* 1995). Binding proteins can bind labeled and unlabeled IGFs. When bound they act as transport proteins and modulate the bioavailability of IGF-I and II.

T3 and T4 hormones. Thyroid hormones (TH), thyroxine (T4), and triiodothyroxine (T3), play a key role by maintaining the development and rate of metabolism. The body will not be able to develop in a timely manner without adequate levels of thyroid hormones. The thyroid gland has two lobes attached to the trachea and weighs an average of 20 grams. Each lobe is made up of follicles lined by epithelial cells. The follicular cells are where thyroid hormones are produced and released into circulation.

Thyroid hormones (TH) can have a negative feedback on the hypothalamus. They can feedback on the TRH to stimulate the release of TSH, while somatostatin (SRIF) inhibits the release of TSH. When T4 is converted into T3, the T3 enters nucleus binding to receptors. This changes the genetic expression which decreases the ability of the cells to produce and secrete TSH (Considine 2003b).

In avian species, the thyroid and adrenal axis have interaction with each other. Corticotropin releasing factor (CRF) can stimulate TSH and T4, while corticosterone exerts a negative feedback on T4 (Geris *et al.* 1996;Geris *et al.* 1999). It also has been said that after injection of GH in newly hatched and adult chickens the T3 plasma levels increase and the T4 plasma levels decrease, whereas TRH injections increases both (Kuhn *et al.* 2005).

Thyroid hormones have many functions including promoting the development of the central nervous system, body growth, and basal metabolic rate (BMR). They inhibit cell replication in the brain and stimulate nerve cell body growth. They also stimulate the rate of mylenization of axons and branching of dendrites. Regulation of the rate of oxidative phosphorylation is also seen.

Abnormalities in thyroid hormones can be devastating in humans as observed with T4 alterations which can lead to hyper- or hypothyroidism. Toxicosis begins when there is a stable amount of T4 and elevated levels of T3 (per manufactors directions).

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CHAPTER 3

THE EFFECTS OF SUBTHERAPEUTIC AND THERAPEUTIC ANTIBIOTICS IN BROILER CHICKENS WITH THE PRESENCE OF *SALMONELLA* HEIDELBERG

Introduction

Salmonella is a global problem. Over 2400 serotypes of *Salmonella* are capable of causing disease in humans and animals. The most high risk individuals include children, the elderly, and those who are immunocompromised (Herenda and Franco 1996). Symptoms usually include nausea, diarrhea, fever, and vomiting (Jay 1998). Poultry is a source of *Salmonella* and flocks may become infected through egg transmission, litter, overuse of antimicrobials, and feed (Herenda and Franco 1996).

Subtherapuetic levels of antimicrobials are used in animal feeds at low levels to promote growth and prevent illness. Chlortetracycline is a broad spectrum antimicrobial which can be bacteriocidal when administered in high concentrations. The tetracyclines work against Gram positive and negative bacteria and in chickens, they are used in feed to treat respiratory infections and control enteric infections. CTC is included on a list of antimicrobials of concern as they are still seen as important in human medicine (Quinn *et al.* 2002a).

Therapeutic antimicrobials are typically used at concentrations to effectively kill all target bacteria. Enrofloxacin is a therapeutic antimicrobial belonging to the quinolone class of antimicrobials which are broad spectrum antimicrobials that act on Gram

negative and positive bacteria by inhibiting nucleic acid synthesis through alteration of the DNA gyrase (Quinn *et al.* 2002a).

In this study, broiler chickens at day of hatch were exposed to seeder birds challenged with *S*. Heildeberg and exposed to combinations of CTC and/or enrofloxacin. Ceca were cultured to assess colonization and development of resistance in recovered Heidelberg. Pulsed field gel electrophoresis and plasmid analysis was also conducted to determine relatedness of isolates.

Materials and Methods

Bacterial Strain. A strain of S*almonella* Heidelberg was obtained from the National Antimicrobial Monitoring System (NARMS) bank of isolates (Tankson *et al.* 2006). It was susceptible to all antimicrobials tested with the exception of nalidixic acid. Nalidixic acid resistance was selected as a marker for recovery during bacterial culture.

Experimental Design. Three hundred fifty *Salmonella*-free birds were transported to the laboratory on the day of hatch. Paper pads and the ceca from ten birds were cultured as described (Appendix A) to check for pre-existing *Salmonella*. Two hundred forty birds were divided into eight rooms and rooms were divided into two replicate pens each containing 15 birds. Each room served as a group and treatments were designated as follows: negative control (C), chlortetracyline (T)/Heidelberg infection (B)/enrofloxacin (E), BE, TB, B, E, T, TE

For the *S*. Heidelberg infection, three seeder birds per group were also placed into each pen. Seeders birds were inoculated with 1 ml of 10^9 CFU *S.* Heidelberg and immediately placed into the appropriate study group of 15 birds. Three seeder birds

sham inoculated with 1ml of 1X PBS were also placed into non-HI groups. Seeder birds were ink marked at placement and necropsied at week one.

CTC-free and CTC (15 mg/lb) feed was obtained from a local feed mill and distributed accordingly per group. Feed and water were given ad-libitum at placement. CTC feed was fed throughout the study. Enrofloxacin (10mg/kg), was administered in the water starting on Day 21 (week 3) for 3 days as per manufacturer's instructions.

Ten birds per group (five per pen) were necropsied on days 7 (week 1), 21 (week 3), and 35 (week 5), and the ceca was cultured for *Salmonella* as previously described (Appendix A). Necropsied birds and the ileum from each bird were also weighed to assess differences between treatment groups. All presumptive positive isolates were serogrouped and sent to the National Veterinary Services Laboratories (Ames, IA) for serotyping.

Susceptibility Testing. Susceptibility testing was conducted according to manufacturer's directions using a semi-automated broth microdilution system (Sensititre™ TREK™ diagnostics, Inc., Cleveland, Ohio). The antimicrobials and concentrations that were used are listed in Table 1. The breakpoints for enterobacteriaceae were determined using the CSLI interpretive criteria (Clinical and Laboratory Standards Institute 2003). *Escherichia coli* ATCC 25922, *S*. auereus 29213, and *E*. facealis 29212 were used as quality control strains.

Pulsed field gel electrophoresis and plasmid analysis. Twelve isolates from various groups that were only resistant to nalidixic acid and all 24 isolates with additional resistance were subjected to pulsed field gel electrophoresis (Centers For Disease Control

and Prevention 2002;Zhao *et al.* 2006) and DNA plasmid analysis (Qiagen Inc. 2003) to assess relatedness between isolates.

Detailed procedures for culturing the *Salmonella*, susceptibility testing, PFGE, and plasmid preparation are located in appendices A-D.

Results

Bacterial culture and weight. All paper pads and ceca from day 0 birds were negative for *Salmonella*. No *Salmonella* were recovered from non-challenged groups at necropsy. At week one, all seeder birds were positive for Heidelberg. Of the ten birds necropsied per room, Heidelberg was recovered from seven given bacteria alone, six from the TB group, seven from the TEB group and 4 from the EB group (E had not been administered in the water). By weeks 3 and 5 all birds were positive for Heidelberg.

There were no differences among groups were observed after birds were weighed (Figure 3.1). Ileum weights were recorded for use in subsequent experiments and are not recorded in this paper.

Antimicrobial Resistance. All recovered isolates were tested for susceptibility. At week one, isolates recovered from the B (n=7), TB (n=6) and EB (n=4) groups were only resistant to nalidixic acid as observed for the challenge strain. However, for the TEB group, additional resistance was observed to gentamicin, streptomycin, sulfamethoxazole, and tetracycline Table 3.2. A graphic depiction by percent resistance is shown in Figure 3.2.

By week three, all ten birds necropsied from the B, TB, TEB and EB groups were positive for *Salmonella* and additional resistance had developed in the B and TB rooms

(Table 3.3). Resistance to streptomycin was not evident in the TEB group and with the exception of nalidixic acid, no other resistance had developed in the EB group. A graphic depiction by percent resistance is shown in Figure 3.3.

By week five, treatment with enrofloxacin had been administered to the TEB, EB and E groups. Only resistance to nalidixic acid was observed in the B and EB groups. In the TEB group, resistance to streptomycin was observed again as resistance to gentamicin, sulfamethoxazole and tetracycline among a number of isolates persisted through to week 5. Resistance to multiple antimicrobials was observed in the TB group although isolates were no longer resistant to gentamicin or kanamycin as observed in week three (Table 3.4). A graphic depiction by percent resistance is shown in Figure 4. infection room, all resistance had been lost.

Cumulative resistance by week regardless of treatment is shown in Table and Figure 3.5. All groups were susceptible to ciprofloxacin, amikacin, trimethroprim/sulfamethoxazole, and ceftriaxone.

Pulsed Field Gel Electrophoresis. A total of 24 isolates were subjected to PFGE and are shown in Figure 3.6. Samples that were analyzed included 12 samples that had the same resistance pattern of the original inoculum. These 12 isolates came from each positive treatment group representing the various weeks. There were 24 isolates that had resistance patterns different from the original that were also analyzed.

All 12 isolates with the same resistance pattern were homologous with the original isolate and clustered in one large group. Of the 24 isolates that had different resistance patterns, 19 were also homologous with the original isolates. The remaining five isolates clustered in three separate groups. Pulsotype pattern comparison with the

USDA VetNet database (Centers For Disease Control and Prevention 2002;Hunter *et al.* 2005) indicated that isolate 20 was a new pattern that was yet assigned a pattern name.

Large plasmids were identified following plasmid analysis among the resistant isolates (data not shown).

Discussion

Colonization of broilers by *Salmonella* is frequently observed particularly within the first two weeks of placement (Doyle and Erickson 2006). While *Salmonella* levels are noted to decline throughout grow-out, *S*. Heidelberg is frequently isolated at slaughter (NARMS). Although colonization by *Salmonella* occurs, illness is not typically observed. In a sense, this makes *Salmonella* an ideal food borne pathogen since it is unlikely that producers and slaughter plants would know that *Salmonella* is present unless they conduct some type of bacteriologic culture.

In this study, we demonstrated that a small number of seeder birds can spread salmonellae throughout a pen. No overt clinical signs were observed. At one week, colonization of the entire pen was not evident as only a portion of birds per treatment were positive at necropsy. Colonization of the entire pen was evident by weeks three and five. The colonization potential of different serotypes of *Salmonella* differs and reports have noted differences in shedding and colonization between pan-susceptible and multi-resistant challenged birds (Dargatz *et al.* 2000). However, these data suggested that this strain of *S*. Heidelberg colonized well even though it was only resistant to nalidixic acid.

Antimicrobials have been used to promote growth and enhance feed efficiency for decades (Gast 1997;Guthrie Rufus 1992a). Although a production effect is often noted, the exact mechanism(s) supporting enhanced growth and feed efficiency are not known

(McEwen and Fedorka-Cray 2002). No differences in weight were noted between treatment groups. This is not surprising as the numbers per group at necropsy are small. However, it is of interest that the groups receiving enrofloxacin or enrofloxacin plus Heidelberg had the lowest recorded weights at week five.

Enrofloxacin is a fluoroquinolone within one of the newest classes of antimicrobials, the quinolones. Enrofloxacin was licensed for use in poultry as a water soluble treatment for air sacculitis prior to its removal from the market in 2005 (Food Drug Administration). Resistance to fluoroquinolones develops in a two step process (Giraud *et al.* 2006;Hansen and Heisig 2003), requiring resistance to nalidixic acid first, then exposure to a fluoroquinolone. Selection of a nalidixic acid (a first generation quinolone) resistant Heidelberg and use of enrofloxacin as a treatment antimicrobial was postulated as one means to study the effects of both quinolone resistance, and with the addition of tetracycline, the acquisition of additional resistances. Tetracycline use, particularly at subtherapeutic levels, has been implicated as a means by which resistance is conferred to other antimicrobials since it is often located on a transposon (Roberts 2005).

Resistance to Ciprofloxacin, the equivalent antimicrobial used in human medicine was not observed in any treated group at five weeks. Interestingly, in the EB group, no other resistance to any other antimicrobial was observed, nor did there appear to be any effect on colonization of *S*. Heidelberg as all birds in the enrofloxacin treated groups were positive at necropsy.

The most interesting observation throughout this study was the acquisition of additional resistance attributes among recovered Heidelberg isolates. In the present

study, only one isolate from the TEB group was resistant to tetracycline after one week of exposure to subtherapeutic concentrations of tetracycline. At no time during the five week feeding of subtherapeutic CTC did birds develop resistance to tetracycline. The highest number developing resistance to tetracycline was observed by week five in the TEB group where 6 isolates were tetracycline resistant. It is of interest to note that at no time did any isolate in the EB group express resistance to tetracycline while two isolates from the Heidelberg alone group were resistant to tetracycline by week three. It is not unusual for bacteria to develop resistance in the absence of an antimicrobial. Resistance in these two isolates may have occurred through cross contamination during necropsy or the transfer of resistant genes from one organism to another through various mechanisms such as conjugation (Salyers and Witte 1994;Walsh 2003).

Since it was unlikely that cross-contamination occurred at necropsy due to use of aseptic techniques, a more likely scenario involving the transfer of genes was speculated. We tested several *E.coli* isolates recovered from the same birds from which we recovered resistant *Salmonella* and none of the same resistances were observed (data not shown). This suggests that the resistance genes did not come from *E. coli* and that other unknown bacteria may have transferred the genetic material, particularly since we observed the presence of large plasmids among the resistant Heidelberg.

Subtherapeutic use of tetracycline has been reported to promote resistance in the normal flora of animals (Gast 1997;Guthrie Rufus 1992a). The greatest increase in resistance was observed between the TB and TEB groups. It is possible that tetracycline played a role in the acquisition/expression of additional antimicrobial resistance, the

numbers per group are too small to draw any definitive conclusion. Additional studies in this area are warranted.

Pulsed field gel electrophoresis results, support the conclusion that the majority of birds were colonized with the original challenge strain. The band differences noted in the five outlier isolates are suggestive of the presence of a plasmid, which would also account for the differences in observed resistance patterns.

Although this strain of *S*. Heidelberg did not cause any morbidity or mortality among challenged birds, it does appear to have an affinity for acquisition of additional resistance genes. Further analysis of the plasmids, conjugation experiments, and identification of other bacterial populations that harbor these resistance genes is warranted. The natural transfer of resistance genes increases the difficulty in the control/amelioration of resistance among *S*. Heidelberg, a common serotype recovered from broilers.

Table 3.1. Antimicrobials tested and breakpoint ranges.

Drug	Susceptible (µg/ml)	Intermediate (µg/ml)	Resistant (µg/ml)
Amikacin	≤ 16	32	≥ 64
Amoxicillin/Clavulanic acid	≤ 8/4	16/8	≥ 32/16
Ampicillin	≤ 8	16	≥ 32
Cefoxitin	≤ 8	16	≥ 32
Ceftiofur	≤ 2	4	≥ 8
Ceftriaxone	≤ 8	16,32	≥ 64
Cephalothin	≤ 8	16	≥ 32
Chloramphenicol	≤ 8	16	≥ 32
Ciprofloxacin	≤ 1	2	≥ 4
Gentamicin	≤ 4	8	≥ 16
Kanamycin	≤ 16	32	≥ 64
Nalidixic acid	≤ 16		≥ 32
Streptomycin*	≤ 32		≥ 64
Sulfamethoxazole	≤ 256		≥ 512
Tetracycline	≤ 4	8	≥ 16
Trimethoprim/sulfamethoxazole	≤ 2/38		≥ 4/76

* No CLSI interpretive criteria for the bacteria/antimicrobial currently available

Table 3.2. Percent resistance by treatment (B=S.Heidelberg, T=Chlortetracycline, E=Enrofloxacin) at week 1

	WEEK 1 PERCENT RESISTANCE							
	B		TB		TEB		EB	
	COUNT	PERCENT	COUNT	PERCENT	COUNT	PERCENT	COUNT	PERCENT
Amikacin	0	0	0	0	0	0	0	0
Amoxocillin/Clavulanic Acid	0	0	0	0	0	0	0	0
Ampicillin	0	0	0	0	0	0	0	0
Cefoxitin	0	0	0	0	0	0	0	0
Ceftiofur	0	0	0	0	0	0	0	0
Ceftriaxone	0	0	0	0	0	0	0	0
Cephalothin	0	0	0	0	0	0	0	0
Chloramphenical	0	0	0	0	0	0	0	0
Ciprofloxacin	0	0	0	0	0	0	0	0
Gentamicin	0	0	0	0	1	14.3	0	0
Kanamycin	0	0	0	0	0	0	0	0
Nalidixic Acid	7	100	6	100	7	100	4	100
Streptomycin	0	0	0	0	1	14.3	0	0
Sulphamethoxazole	0	0	0	0	2	28.6	0	0
Tetracycline	0	0	0	0	1	14.3	0	0
Trimethoprim/Sulphamethoxazole	0	0	0	0	0	0	0	0

Table 3.3. Percent resistance by treatment (B=S.Heidelberg, T=Chlortetracycline, E=Enrofloxacin) at week 3

	WEEK 3 PERCENT RESISTANCE							
	B		TB		TEB		EB	
	COUNT	PERCENT	COUNT	PERCENT	COUNT	PERCENT	COUNT	PERCENT
Amikacin	0	0	0	0	0	0	0	0
Amoxocillin/Clavulanic Acid	2	20	4	40	0	0	0	0
Ampicillin	1	10	4	40	0	0	0	0
Cefoxitin	1	10	4	40	0	0	0	0
Ceftiofur	1	10	4	40	0	0	0	0
Ceftriaxone	0	0	0	0	0	0	0	0
Cephalothin	2	20	4	40	0	0	0	0
Chloramphenical	2	20	4	40	0	0	0	0
Ciprofloxacin	0	0	0	0	0	0	0	0
Gentamicin	0	0	1	10	4	40	0	0
Kanamycin	0	0	2	20	0	0	0	0
Naladixic Acid	10	100	10	100	10	100	10	100
Streptomycin	1	10	4	40	0	0	0	0
Sulphamethoxazole	1	10	4	40	6	60	0	0
Tetracycline	2	20	4	40	3	30	0	0
Trimethoprim/Sulphamethoxazole	0	0	0	0	0	0	0	0

Table 3.4. Percent resistance by treatment (B=*S.*Heidelberg, T=Chlortetracycline, E=Enrofloxacin) at week 5

	WEEK 5 PERCENT RESISTANCE							
	B		TB		TEB		EB	
	COUNT	PERCENT	COUNT	PERCENT	COUNT	PERCENT	COUNT	PERCENT
Amikacin	0	0	0	0	0	0	0	0
Amoxocillin/Clavulanic Acid	0	0	1	10	0	0	0	0
Ampicillin	0	0	2	20	0	0	0	0
Cefoxitin	0	0	1	10	0	0	0	0
Ceftiofur	0	0	1	10	0	0	0	0
Ceftriaxone	0	0	0	0	0	0	0	0
Cephalothin	0	0	1	10	0	0	0	0
Chloramphenical	0	0	1	10	0	0	0	0
Ciprofloxacin	0	0	0	0	0	0	0	0
Gentamicin	0	0	0	0	5	50	0	0
Kanamycin	0	0	0	0	0	0	0	0
Naladixic Acid	10	100	10	100	10	100	10	100
Streptomycin	0	0	1	10	1	10	0	0
Sulphamethoxazole	0	0	1	10	9	90	0	0
Tetracycline	0	0	1	10	6	60	0	0
Trimethoprim/Sulphamethoxazole	0	0	0	0	0	0	0	0

Table 3.5. Percent resistance by week for all resistance isolates

	PERCENT RESISTANCE					
	WEEK 1		WEEK 3		WEEK 5	
	COUNT	PERCENT	COUNT	PERCENT	COUNT	PERCENT
Amikacin	0	0	0	0	0	0
Amoxocillin/Clavulanic Acid	0	0	6	15	1	2.5
Ampicillin	0	0	5	12.5	2	5
Cefoxitin	0	0	5	12.5	1	2.5
Ceftiofur	0	0	5	12.5	1	2.5
Ceftriaxone	0	0	0	0	0	0
Cephalothin	0	0	6	15	1	2.5
Chloramphenical	0	0	6	15	1	2.5
Ciprofloxacin	0	0	0	0	0	0
Gentamicin	1	4.2	5	12.5	5	12.5
Kanamycin	0	0	2	5	0	0
Naladixic Acid	24	100	40	100	40	100
Streptomycin	1	4.2	5	12.5	2	5
Sulphamethoxazole	2	8.3	11	27.5	10	25
Tetracycline	1	4.2	9	22.5	7	17.5
Trimethoprim/Sulphamethoxazole	0	0	0	0	0	0

Figure 3.1. Average body weight by treatment (B=*S.*Heidelberg, T=Chlortetracycline, E=Enrofloxacin)

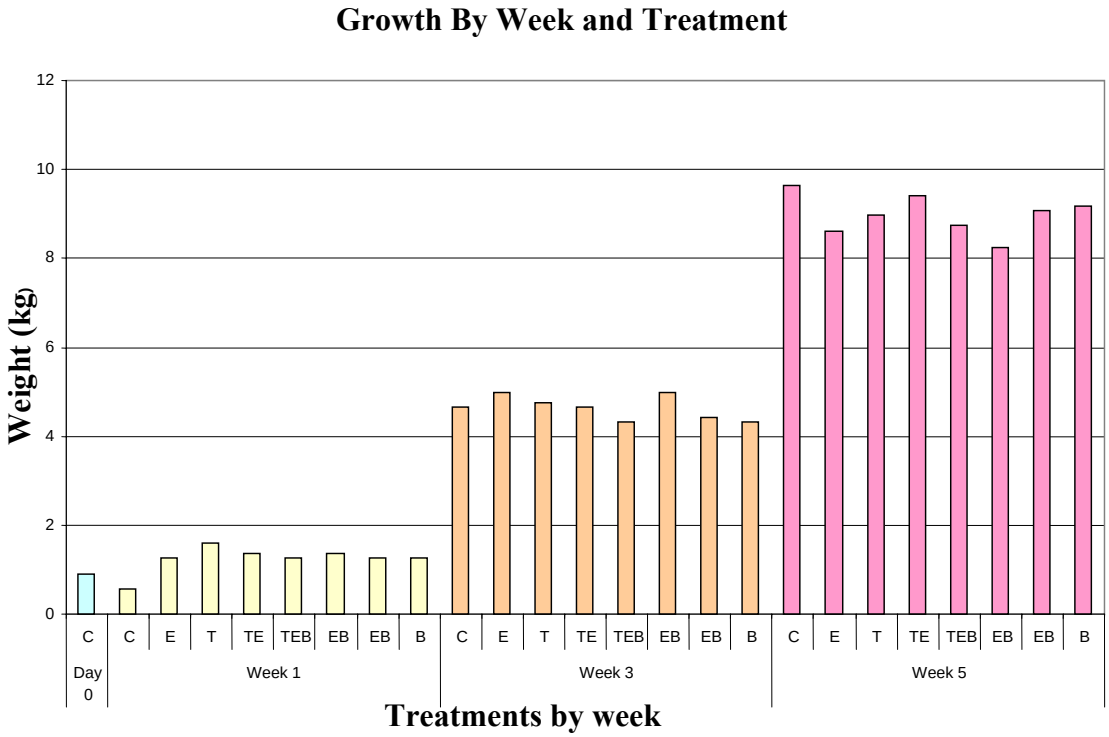


Figure 3.2 Percent Resistance by treatement (B=S.Heidelberg, T=Chlortetracycline, E=Enrofloxacin) at week 1

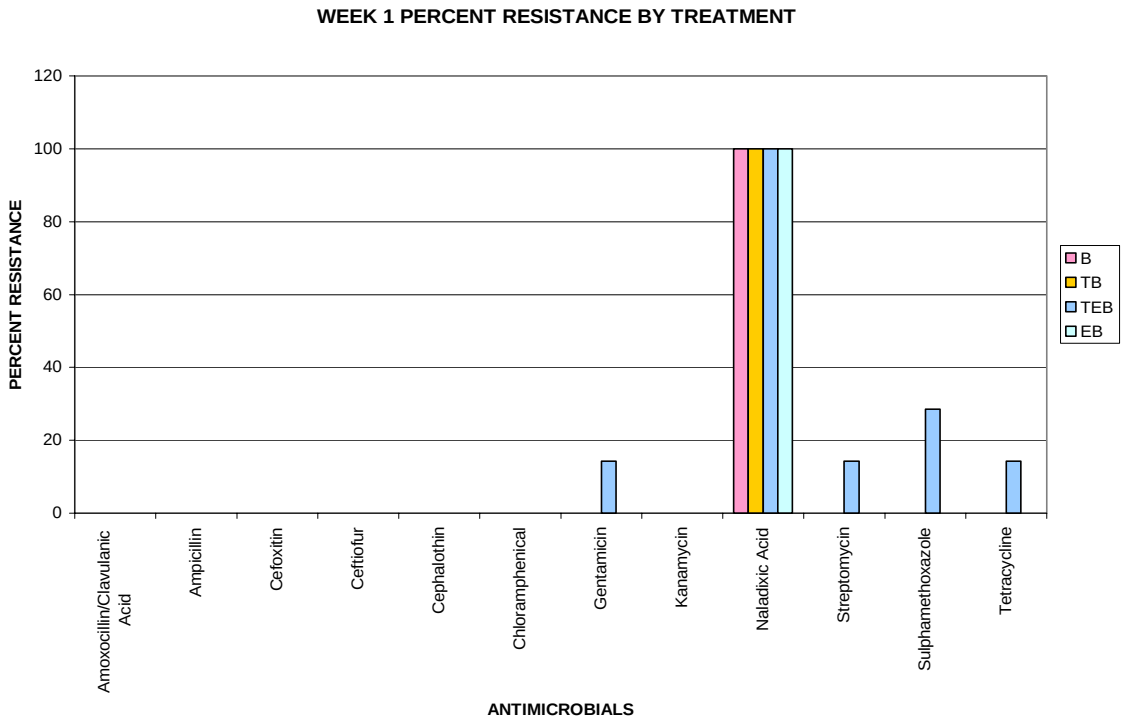


Figure 3.3 Percent Resistance by treatement (B=*S.*Heidelberg, T=Chlortetracycline, E=Enrofloxacin) at week 3

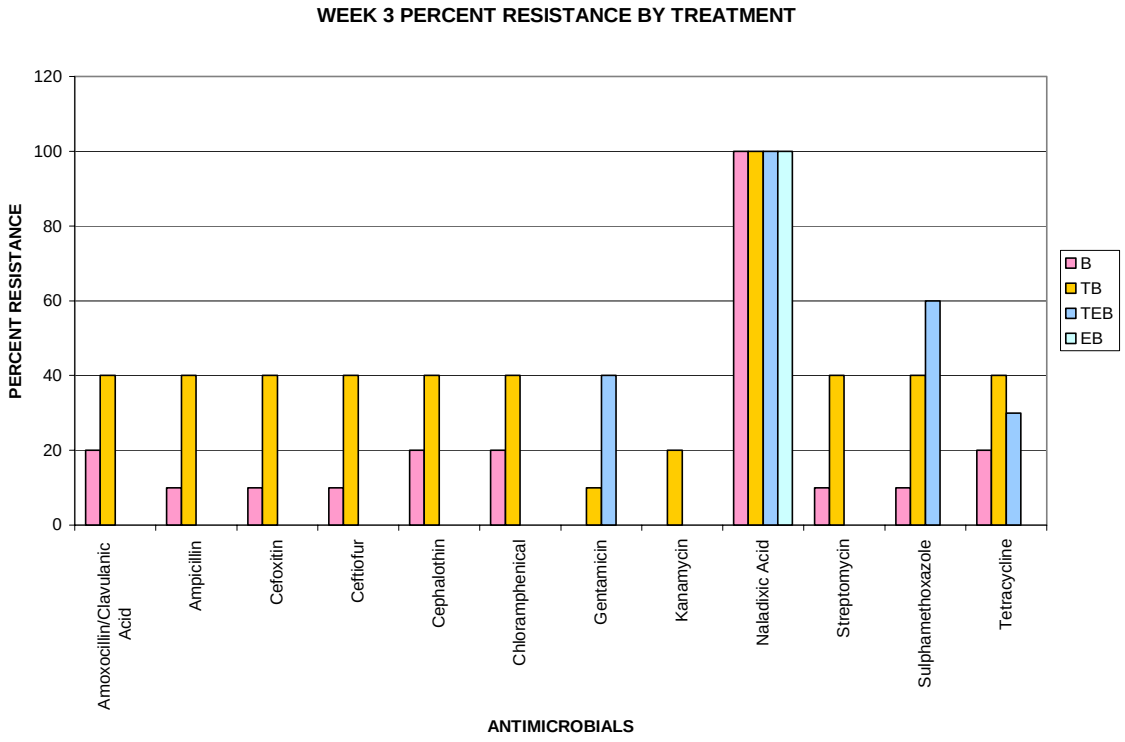


Figure 3.4 Percent Resistance by treatement (B=*S.*Heidelberg, T=Chlortetracycline, E=Enrofloxacin) at week 5

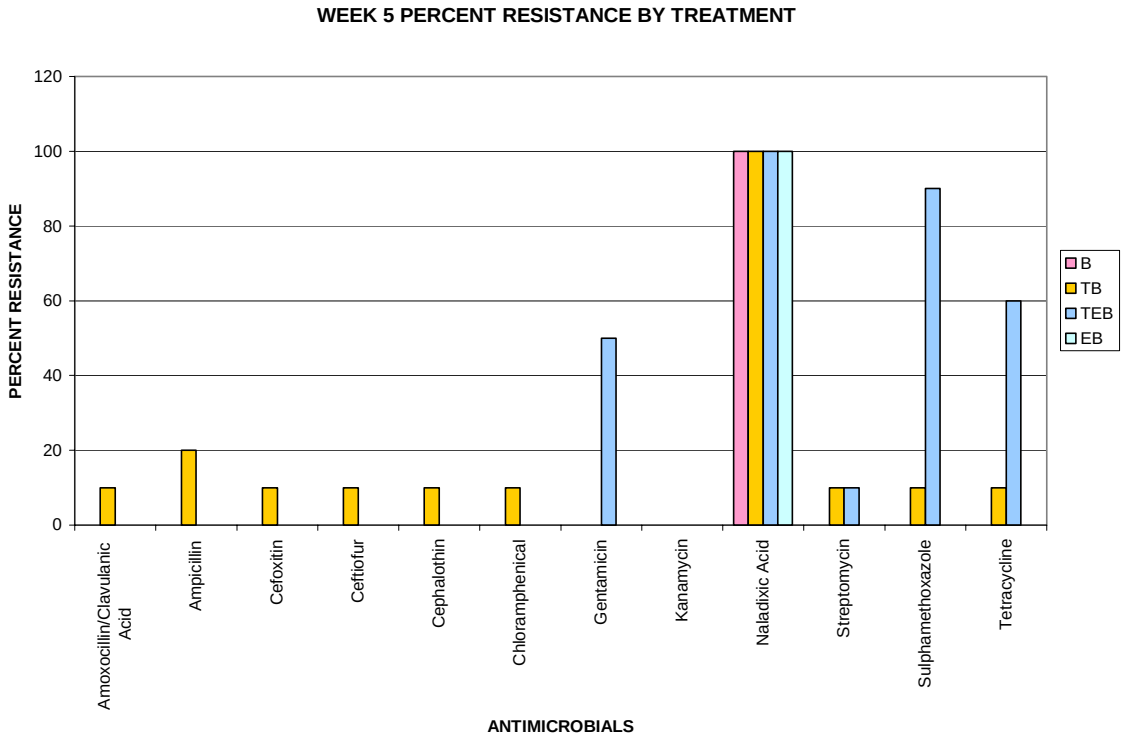


Figure 3.5 Percent Resistance by week

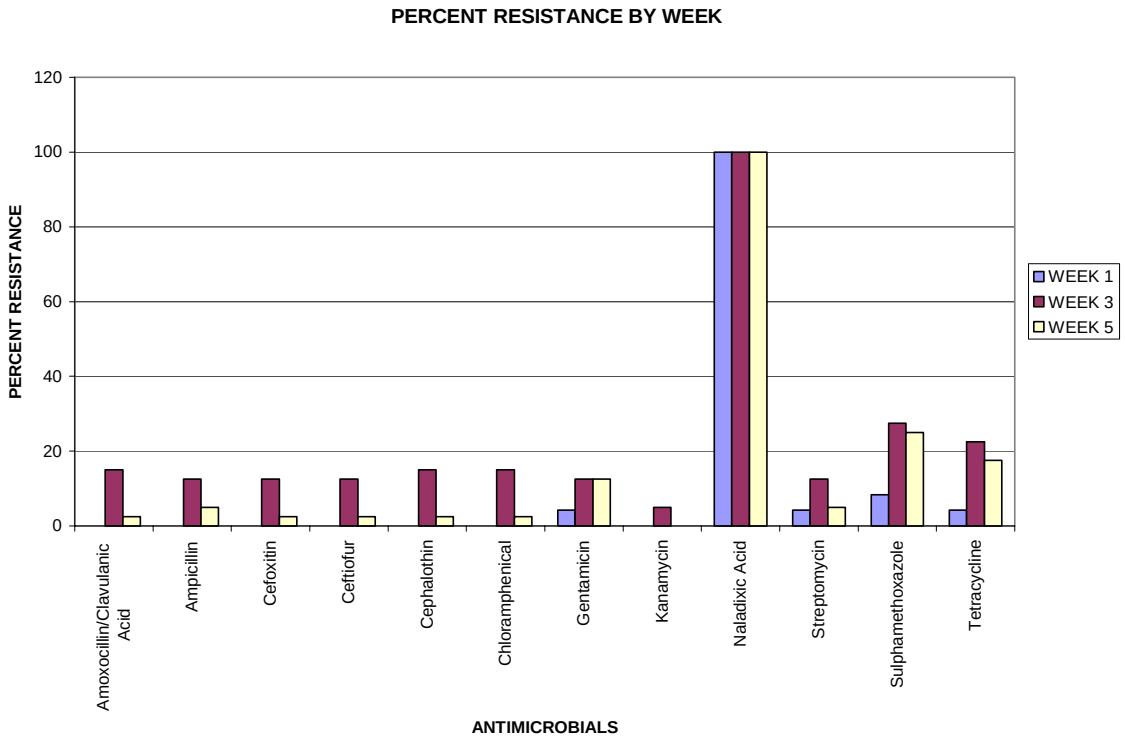
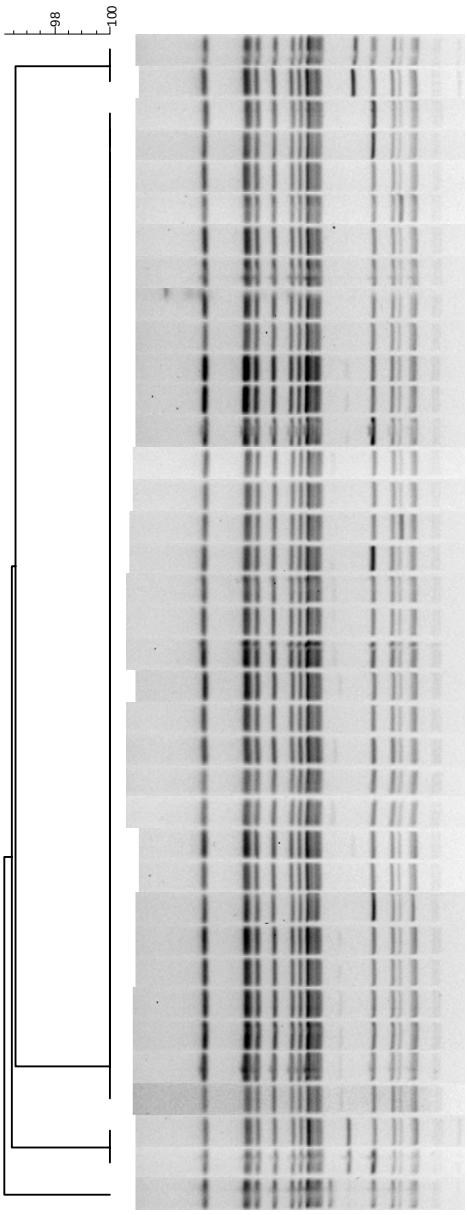


Figure 3.6 Pulsed Field Gel Electrophoresis on susceptible and resistant isolates (1=TEB [T=Chlortetracycline, E= enrofloxacin, B= *S*. Heidelberg] 2=B, 3=EB, 4=TB, R=resistant, S=susceptible)



Isolate No.	Serotype	Pattern No.		Treatment	Status	Week
21	Heidelberg	JF6X01.0081	ARS 4	R		3
34	Heidelberg	JF6X01.0081	ARS 4	R		5
1	Heidelberg	JF6X01.0011	ARS 2	S		3
10	Heidelberg	JF6X01.0011	ARS 4	S		5
11	Heidelberg	JF6X01.0011	ARS 3	S		5
12	Heidelberg	JF6X01.0011	ARS 1	S		3
13	Heidelberg	JF6X01.0011	ARS 1	R		3
14	Heidelberg	JF6X01.0011	ARS 1	R		3
15	Heidelberg	JF6X01.0011	ARS 1	R		3
16	Heidelberg	JF6X01.0011	ARS 1	R		3
17	Heidelberg	JF6X01.0011	ARS 1	R		3
18	Heidelberg	JF6X01.0011	ARS 3	S		3
19	Heidelberg	JF6X01.0011	ARS 4	S		3
23	Heidelberg	JF6X01.0011	ARS 1	S		5
24	Heidelberg	JF6X01.0011	ARS 1	R		5
25	Heidelberg	JF6X01.0011	ARS 1	R		5
26	Heidelberg	JF6X01.0011	ARS 2	S		5
27	Heidelberg	JF6X01.0011	ARS 1	R		5
28	Heidelberg	JF6X01.0011	ARS 1	R		1
29	Heidelberg	JF6X01.0011	ARS 3	S		1
3	Heidelberg	JF6X01.0011	ARS 2	R		3
30	Heidelberg	JF6X01.0011	ARS 1	R		5
31	Heidelberg	JF6X01.0011	ARS 2	S		1
32	Heidelberg	JF6X01.0011	ARS 1	S		1
33	Heidelberg	JF6X01.0011	ARS 4	S		1
35	Heidelberg	JF6X01.0011	ARS 4	R		5
36	Heidelberg	JF6X01.0011	ARS 1	R		5
4	Heidelberg	JF6X01.0011	ARS 2	R		3
5	Heidelberg	JF6X01.0011	ARS 1	R		5
6	Heidelberg	JF6X01.0011	ARS 1	R		1
7	Heidelberg	JF6X01.0011	ARS 1	R		5
8	Heidelberg	JF6X01.0011	ARS 1	R		5
9	Heidelberg	JF6X01.0011	ARS 1	R		5
Original A	Heidelberg	JF6X01.0011	ARS			
2	Heidelberg	JF6X01.0040	ARS 4	R		3
22	Heidelberg	JF6X01.0040	ARS 4	R		3
20	Heidelberg	New Pattern	4	R		3

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CHAPTER 4

IMMUNE RESPONSE IN BROILER CHICKENS WITH THE PRESENCE OF *SALMONELLA* HEIDELBERG AND SUBTHERAPEUTIC AND THERAPEUTIC ANTIBIOTICS

Introduction

Salmonella is a global concern for humans and animals, and one source of zoonotic transfer is poultry (McEwen and Fedorka-Cray 2002). Recently, concern has also emerged regarding the development of antimicrobial resistance of bacteria including multiple antimicrobial resistance (McEwen and Fedorka-Cray 2002). The subtherapeutic use of antimicrobials has been implicated in promoting and maintaining resistant *Salmonella* in animal production. Antimicrobials are used in animal production at subtherapeutic levels to enhance growth and feed efficiency and may also be used as prophylatic. Although many reports document the benefits in animal production, subtherapeutic antimicrobial use is controversial, primarily because of their potential effects on resistant bacteria populations (McEwen and Fedorka-Cray 2002). While the withdrawal of subtherapeutic uses of antimicrobials is widely supported (McEwen and Fedorka-Cray 2002), their removal during production has resulted in an increase in enteritis (Casewell *et al.* 2003) as well as increased uses of therapeutic levels of antimicrobials to treat increasing disease problems (Bager *et al.* 2001).

Despite the controversial use of antimicrobials in animal production, subtherapeutic levels of antimicrobials do appear to enhance immunity, particularly in the gut (McEwen and Fedorka-Cray 2002). Once *Salmonella* established itself in the gut,

many defense mechanisms begin to activate. Immunity is established when the immune system recognizes a foreign molecule, such as *Salmonella*, reacts to it, and produces a defensive response aimed at eliminating the foreign molecule (Male *et al.* 1987).

Cytokines are part of the immune response and that have many functions including mediating natural immunity, stimulating immature leukocytes, regulating lymphocyte activity, growth, and differentiation, and activating nonspecific inflammatory cells. Cytokines originate from different cell types: interleukins are produced by leukocytes, chemokines are produced by cytokines that have chemotactic activity (Abbas *et al.* 1991).

While much is known about human and murine cytokines, avian cytokine information is in short supply due to the lack of antibodies and reliable assays. Recently, avian cytokines have been cloned and a number of cytokines and chemokines can now be analyzed (Wigley and Kaiser 2003). The role of cytokines and chemokines in pathogenesis is critical for the development of immune control measures that can be used in poultry to prevent colonization.

In this study, the immune response is analyzed in broiler chickens exposed to *Salmonella* Heidelberg while under subtherapeutic and therapeutic antibiotic treatment. Both chlorotetracyline (T) and enrofloxacin (E) were used as subtherapeutic and therapeutic treatments, respectively. Our hypothesis was that there will be a negative cytokine expression with no treatment and a positive cytokine expression in groups exposed to Heidelberg. A weak, but demonstrable cytokine expression is expected with antimicrobials alone.

Materials and Methods

Bacterial strains and study design. The bacteria strains and study design are described in chapter 3 of this thesis. Briefly, Three hundred fifty *Salmonella*-free birds were transported to the laboratory on the day of hatch, cultured to check for pre-existing *Salmonella*, and divided into eight rooms with two replicate pens each containing 15 birds. Each room served as a group and treatments were designated as follows: negative control (C), chlortetracyline (T)/Heidelberg infection (B)/enrofloxacin (E), EB, TB, B, E, T, TE. Seeder birds either challenged with *S*. Heidelberg infection or sham inoculated were placed into appropriate pens and birds were fed either chlortetracycline-free or chlortetracycline (15 mg/lb) feed. Enrofloxacin (10mg/kg), was administered in the water starting on Day 21 (week 3) for 3 days as per manufacturer's instructions. Ten birds per group (five per pen) were necropsied on days 7 (week 1), 21 (week 3) and 35 (week 5). Necropsied birds and the ileum from each bird were also weighed.

Tissue Samples. The ceca were cultured for *Salmonella* as previously described (Appendix A). At necropsy the cecum was collected for culture to detect *Salmonella* and the ileum was weighed to obtain between 180-250 mg and stored in 2.5 mls of RNA-later which keeps RNA from degrading. The ileum samples were stored in 4^{o}C for later use.

Real-Time PCR for detection of cyctokines. Ileal samples were removed from the RNA-later solution and homogenized. Once homogenized, the sample was purified by using the Qiagen mini-kit as per manufacturer's direction (QIAGEN,Valencia, CA). Detailed procedures for purifying the RNA can be found in appendix H. Briefly, a 1:10 dilution is made of the 100 µls of sample. A master mix

containing RNase free water, 5X EZ buffer, manganese acetate, dNTPs, reverse transcriptase DNA polymerase, Amp Erase UNG, and the primers and probes of choice is added to the sample for amplification of the RNA. Primers and probes sequences were obtained from previous reports (Withanage *et al.* 2004) and included IL-6, IL-8, MIP-1β and IL-1β. The 5' end was tagged with 5'carboxyfluorescence, a fluorescent dye. N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA) was the quencher at the 3' end.

In a 96 well plate, 20 µls of the master mix was added per well along with 5 µls of sample. Each sample was tested in triplicate and a negative control, as well as serial dilution (10-fold) standards were added for every run. Using an Applied Biosystems 7300 (Applied Biosystems, Foster City, CA), the RNA was amplified as follows; stage one 50°C for two minutes, stage two 96°C for five minutes, stage three 60°C for 30 minutes and stage four, 40 reps at 94°C for 20 seconds followed by 59°C for one minute.

Results are expressed as the threshold cycle value (Ct) and once collected, statistical analysis was done to calculate the corrected mean (Appendix I). The 28s rRNA Ct values were used to normalize the RNA levels between all the samples and calculations were done by pooling all sample Ct values per group. A detailed description of the calculations can be found in appendix I.

Results

Bacterial culture. All paper pads and ceca from day 0 birds were negative for *Salmonella*. No *Salmonella* were recovered from non-challenged groups at any necropsy. By week one, 24/40 (60%) birds at necropsy from the challenged rooms were positive for *Salmonella* and by week three, all birds were positive (see Chapter 3). At week one, all seeder birds were positive for Heidelberg. Antimicrobial resistance in the TEB emerged

at week one and by week three in the B, TB, and EB groups. No resistance, other than to nalidixic acid, was observed in the EB group at any time.

Cytokine expression. No significant differences between treatment groups and controls were observed at week one for any group. IL-1β results by week and treatment are shown in Figures 1a and 1b. Significant ($p<0.05$) differences were noted between the TB, B, TEB, EB, T, and TE groups when compared to the control and E groups (Figure 4.1a). However, by week 5, only the E group was significantly ($p<0.05$) different than all other groups (Figure 4.1b). IL-6 results by week and treatment are shown in Figures 4.2a and 4.2b. Significant ($p<0.05$) differences between all groups and the control was observed at week three (Figure 4.2a). By week five, only the E group was significantly ($p<0.05$) different than all the other groups (Figure 4.2b). IL-8 results by week and treatment are shown in Figure 4.3. Significant ($p<0.05$) differences were observed between all groups and the control during both weeks three and five. MIP-1β results by week and treatment are shown in Figures 4.4a and 4.4b. Significant ($p<0.05$) differences are noted between the TB, B, TEB, EB, T, and TE groups when compared to the control and the E groups (Figure 4.4a). By week five, only the E, TB, and B groups were significantly ($p<0.05$) different than all the other groups.

Discussion

In this study, we demonstrated that by week one, Heidelberg had colonized 60% (24/40) of the birds and that by necropsy at week three, all birds were colonized. No overt clinical signs were present (Chapter 3). Additionally, we were able to demonstrate that resistance to additional antimicrobials appeared as early as week one in the TEB group and week three and five in the TEB and B groups indicating the likely exchange of

genetic material between Heidelberg and other bacteria (Chapter 3). No significant differences of bird weight between treatment groups were noted. This was not surprising as the numbers per group at necropsy are small.

The observation that there was no significant difference between treatment groups and controls regarding cytokine expression at week one, but that one could be observed at weeks three and five for all groups (with the exception of the E group for IL-1β and MIP-1β) suggests that some type of inflammatory response was occurring. Whether this was due to the Heidelberg exposure in the TEB, B, and EB groups or other environmental bacteria is unknown. The exposure of birds to tetracycline itself may have also caused some inflammatory response, especially since the E group, which did not elicit any type of activation until after enrofloxacin was administered as observed by the response in week five, suggests that some level of antimicrobial exposure will also elicict a cytokine response. IL-1β and MIP-1β exert different effects when compared to IL-6 and IL-8. One of the main postulates posed for subtherapeutic use of antimicrobials is that they stimulate a low level inflammatory immune response (McEwen and Fedorka-Cray 2002) and activation of cytokines may be one mechanism by which this is effected.

The long term effect of IL-8 was not sustained for all groups at the same level in the absence of a bacterial infection since the responses for the E, T, and TE groups significantly are lower when compared to the B, TB, TEB, and EB groups at week five. This would be expected since the presence of the bacteria would likely stimulate a greater inflammatory response, particularly if the infection had not cleared. As mentioned in chapter one, *salmonella* induces IL-8 causing an influx of neutrophils that damage the epithelium allowing the entry of the bacteria. Previous reports assessing humoral and

other types of cell-mediated immunity support the notion that infection, particularly when sustained, induces a continued immune response (Stabel *et al.* 2002). In this study, bacterial load as well as sustained infection may play a role in the observed IL-8 response.

The observed response at week five for MIP-1β, a chemokine like IL-8, also supports a role for sustained infection as well as the possibility that therapeutic levels of enrofloxacin also plays a role in eliciting the MIP-1β response. One could postulate that the reason the TEB group is not significantly different is that treatment with enrofloxacin could have killed a sufficient number of Heidelberg which in turn affected the stimulus for cytokine expression. Further, use of both tetracycline and enrofloxacin may have been antagonistic, canceling any effect treatment alone may have had on the immune response.

Some reports have demonstrated altered or enhanced virulence for resistant versus pan-susceptible *Salmonella*e (Bauer-Garland *et al.* 2006). The acquisition of additional resistance in Heidelberg did not appear to alter the immune response in this study.

Collectively, these data demonstrate that cytokine responses are stimulated following infection with *S*. Heidelberg and that use of therapeutic levels of antimicrobials alone may also induce a cytokine response. Additional research is warranted to study the immune response using other *Salmonella* serotypes and antimicrobial combinations.

Figure 4.1a IL-1β cytokine expression for week 1, 3, and 5 (* = P < 0.05)

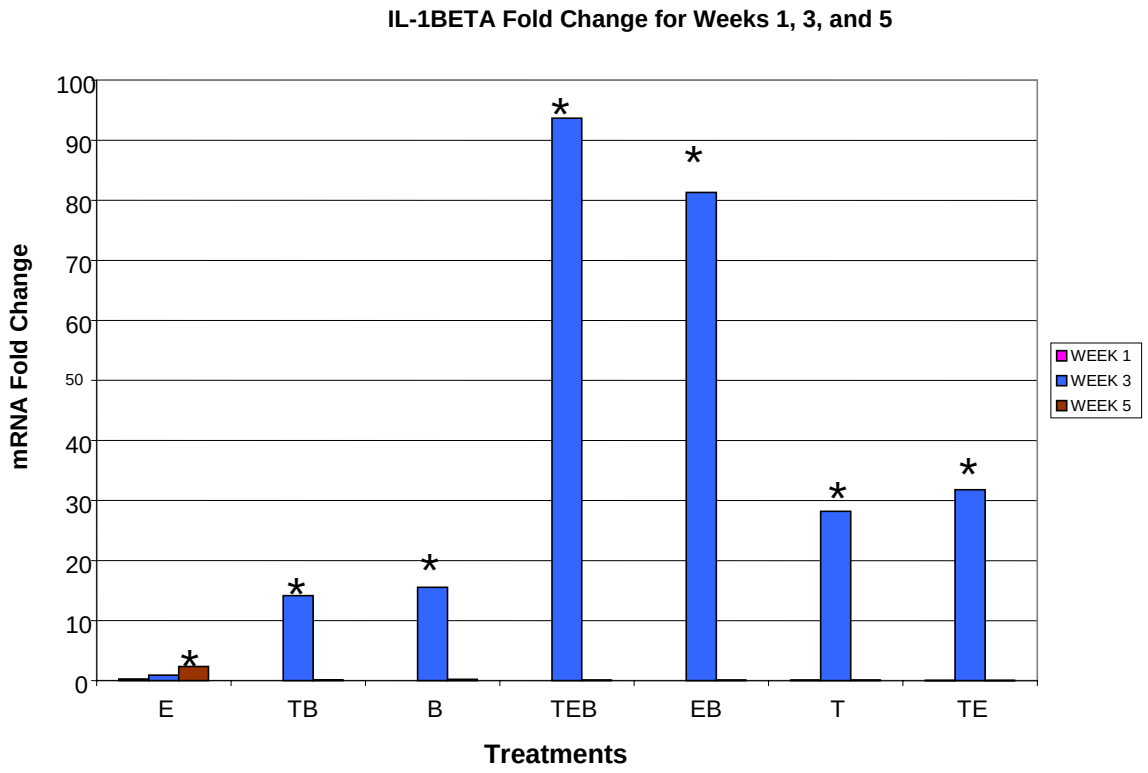


Figure 4.1b IL-1β cytokine expression for week 1 and 5 (* = P < 0.05)

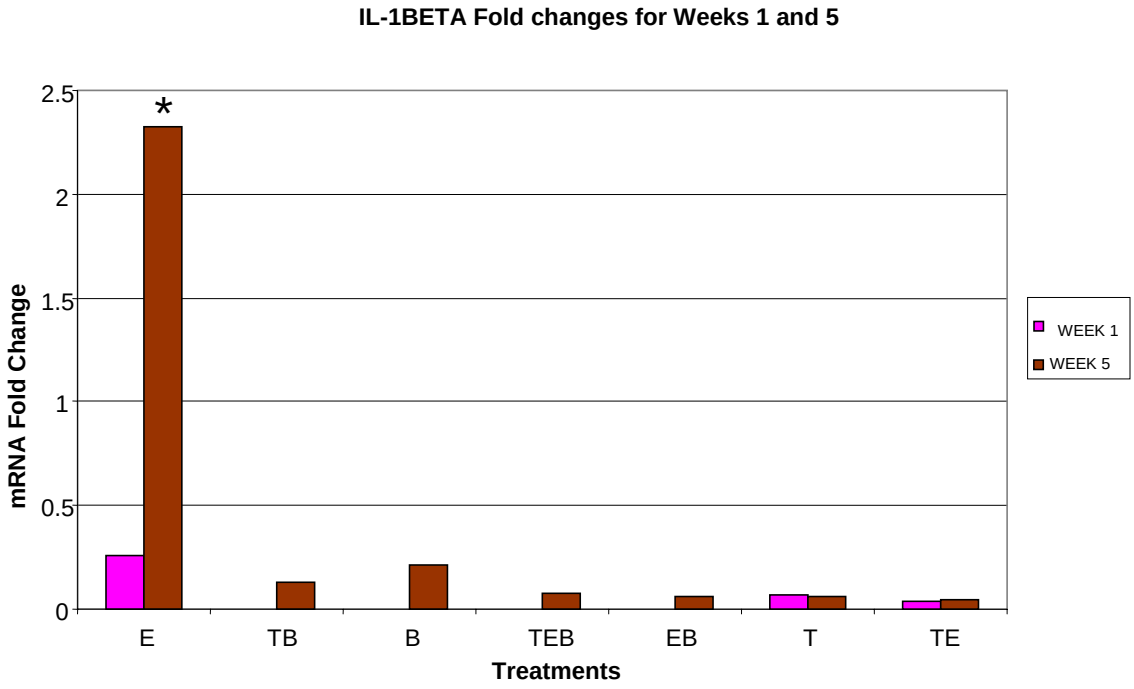


Figure 4.2a IL-6 cytokine expression for week 1, 3, and 5 (* = P < 0.05)

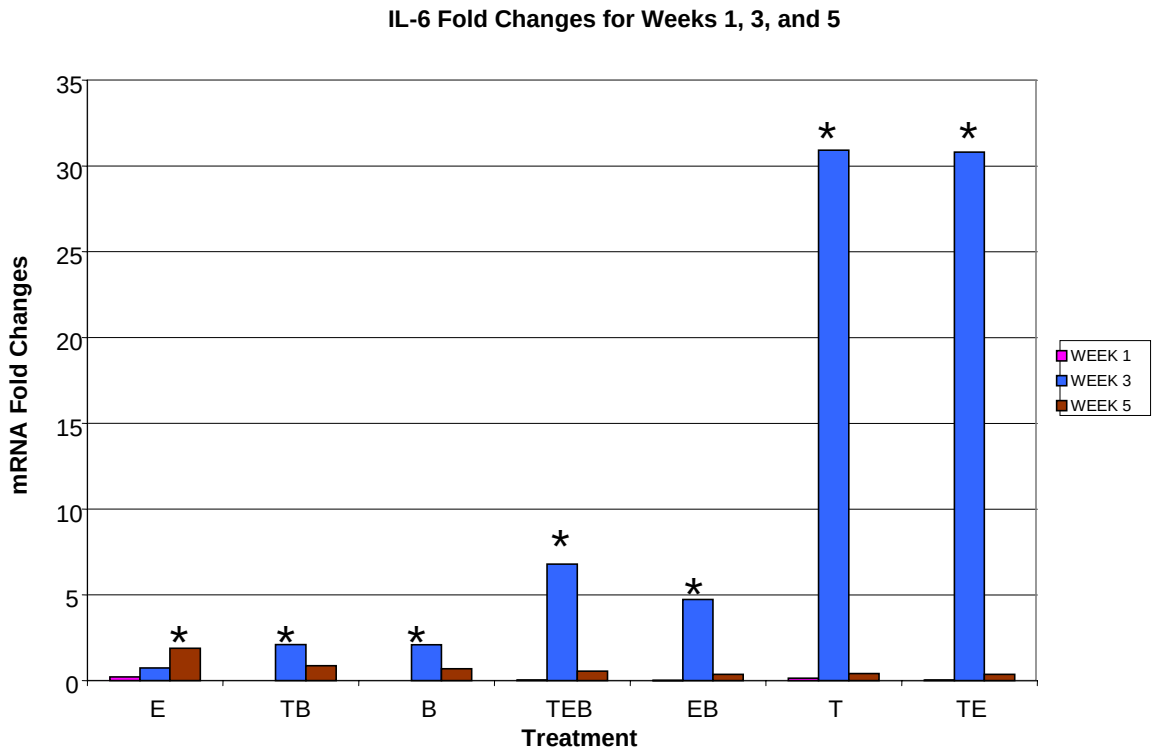


Figure 4.2b IL-6 cytokine expression for week 1 and 5 (* = P < 0.05)

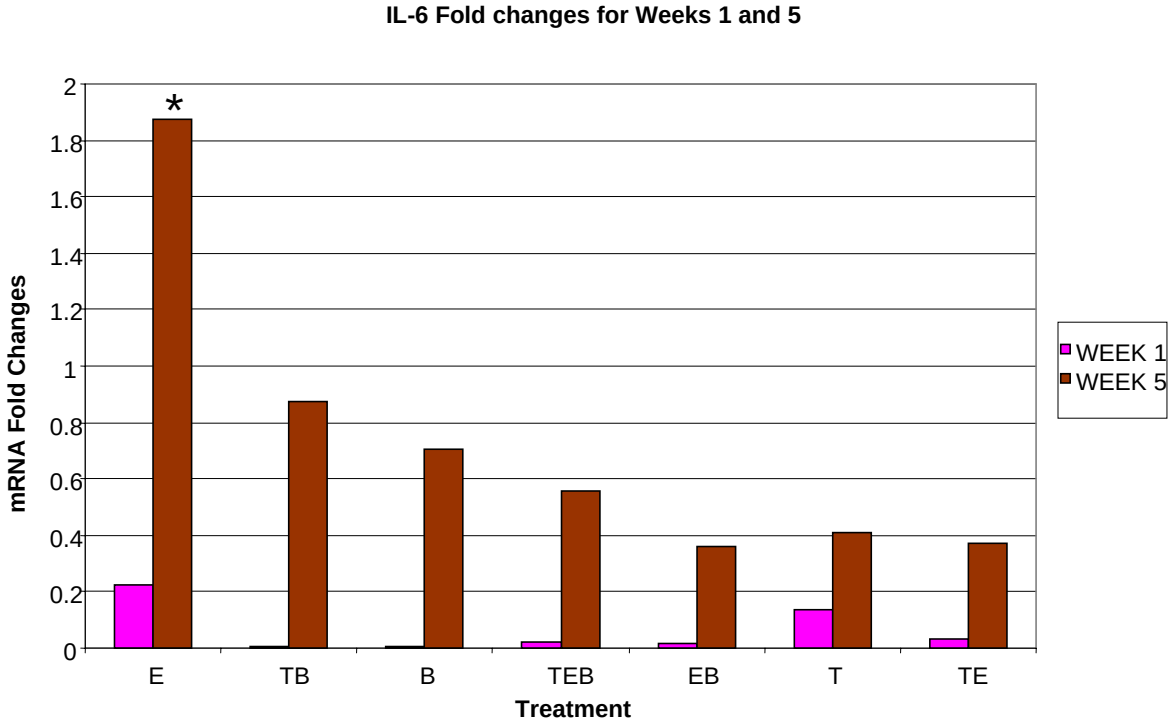


Figure 4.3 IL-8 cytokine expression for week 1, 3, and 5 (* = P < 0.05)

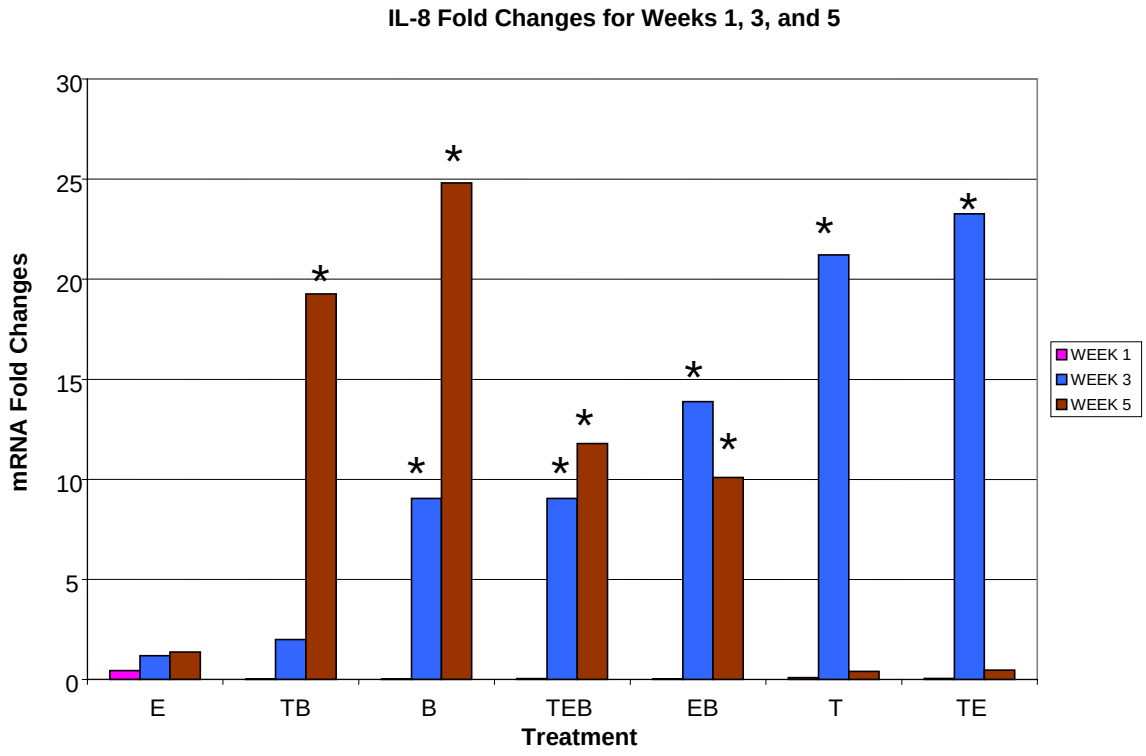


Figure 4.4a MIP-1β cytokine expression for week 1, 3, and 5 (* = s P < 0.05)

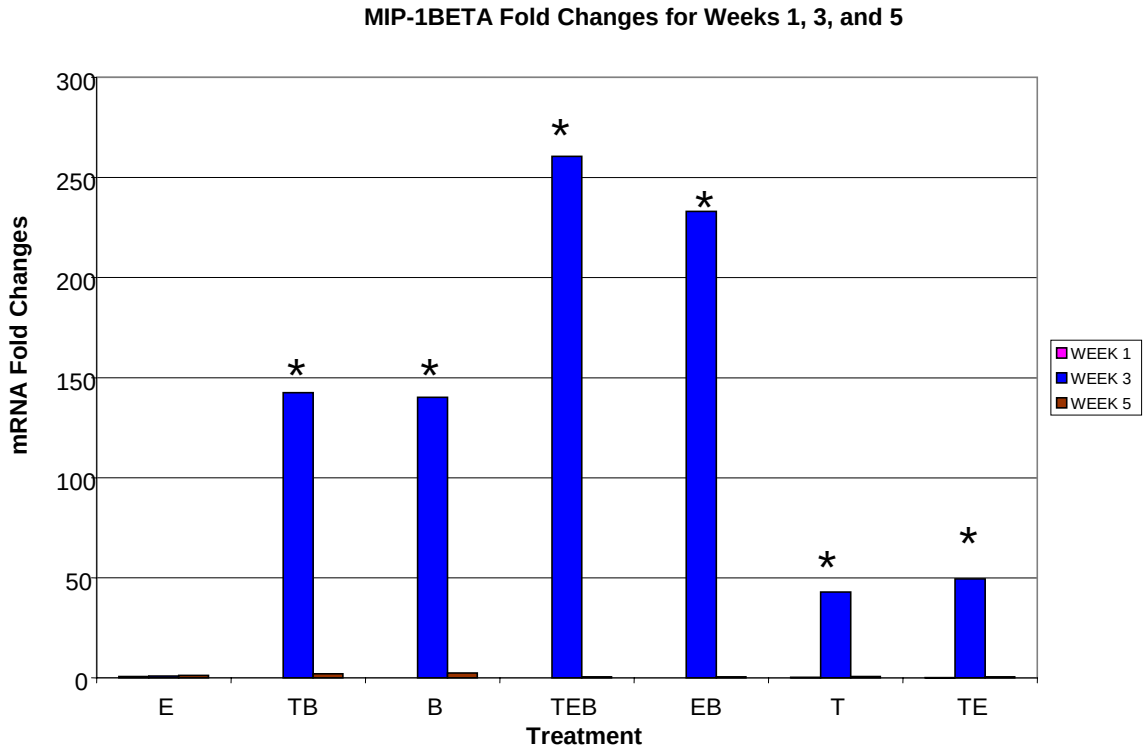
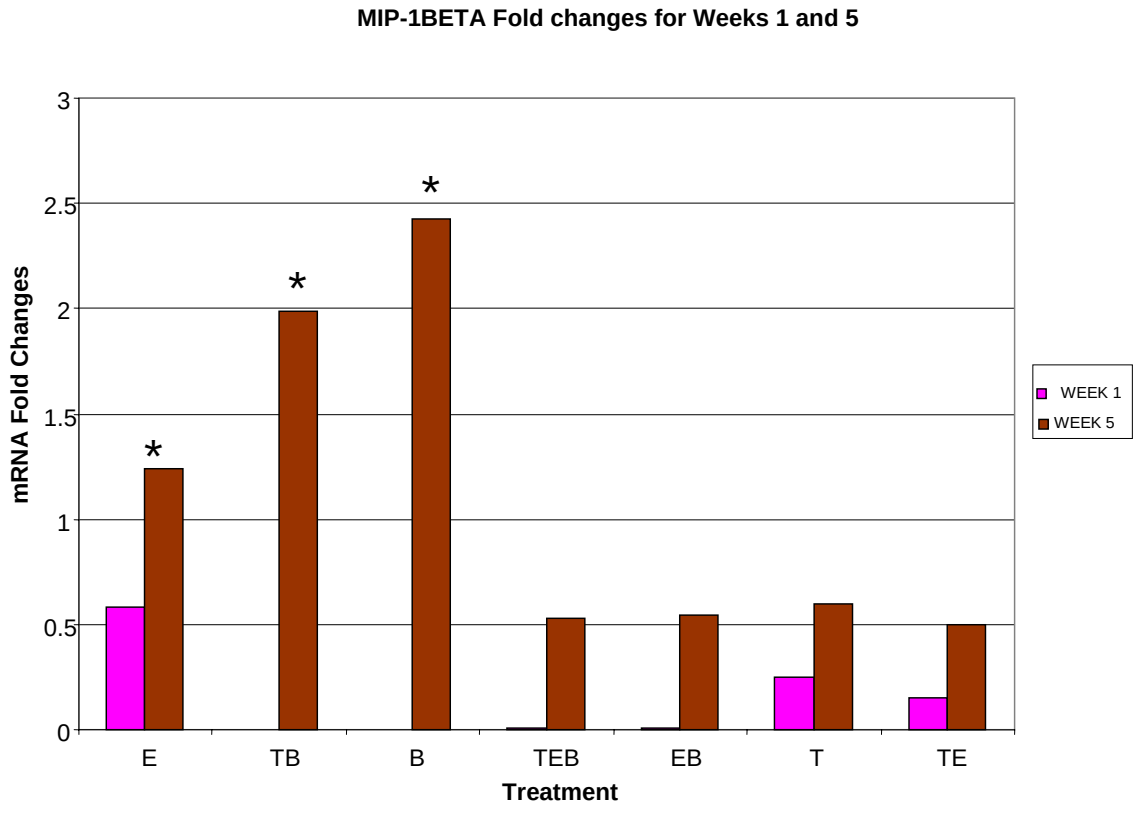


Figure 4.4b MIP-1β cytokine expression for week 1 and 5 (* = P < 0.05)



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CHAPTER 5

SUMMARY

Emerging bacterial infections continue to be a global problem. In addition, the emergence of antimicrobial resistance has compounded the problem in many bacteria, including zoonotic bacteria. *Salmonella* has been reported to be increasing in both prevalence and antimicrobial resistance including multiple antimicrobial resistance. Although many reports have been published on both *Salmonella* pathogenesis and antimicrobial resistance, we have not developed effective means for controlling the transmission of *Salmonella* or the development of resistance in *Salmonella*. Antimicrobials are used in animal production for both therapeutic treatment of disease and at subtherapeutic levels to promote feed efficiency and enhance growth. We do not have a good understanding of the mechanisms by which subtherapeutic antimicrobials work, although the immune system is thought to play a role in this response.

In this study we report on the effect of various treatments on the immune response. Treatments included subtherapeutic use of tetracycline in feed, therapeutic treatment with enrofloxacin, and/or exposure to a strain of *Salmonella* Heidelberg that was only resistant to nalidixic acid. We were able to demonstrate that at one week post exposure to infected seeder birds, 60% of the broiler chickens were positive for Heidelberg at the one week necropsy. By the third and through the fifth week, 100% of the birds were colonized with Heidelberg without clinical signs of disease. Interestingly, in week one, the Heidelberg recovered from the tetracycline/enrofloxacin/Heidelberg group had developed resistance to additional antimicrobials. By weeks three and five

post-challenge, birds from the Heidelberg alone, and tetracycline/Heidelberg group had also developed resistance to additional antimicrobials. This suggests that the Heidelberg strain likely acquired these additional resistance attributes through movement of mobile genetic elements including plasmids from other resident bacteria (Davies 1994;Spratt 1994). The observation that the enrofloxacin/Heidelberg group never acquired additional resistance may result from the therapeutic level of enrofloxacin acting as an inhibitor of DNA uptake, despite the fact that this level of antimicrobial did not eliminate Heidelberg. This warrants further investigation.

No significant hormonal response was elicited for any treatment group suggesting that infection or exposure to chemicals does not affect this response. However, there was an age effect that was observed which is not unexpected as hormonal responses increase as the host matures. Additional research is warranted to assess hormone levels in tissue(s) other than the gut as Guthrie (Guthrie Rufus 1992) describes.

Bacterial exposure and treatment (either therapeutic or subtherapeutic) affected a cytokine response, although variations were observed between treatment groups. Cytokines were not activated until week three suggesting that the infectious dose was not sufficient to elicit an immune response and that continued exposure of tetracycline in excess of one week is required. Other studies show expression of IL-8, IL-1β, and MIP-1β from the ilea at 12 to 48 hours ranging from a 10-100 fold change (Withanage ***et al.*** 2004). Interestingly in this study, significant differences were only observed for all groups for both weeks three and five for IL-8. The cell target for IL-8 is the neutrophil, which is a primary phagocyte for *Salmonella*.

Since these study results were obtained with *S*. Heidelberg, additional research is warranted for other serotypes of *Salmonella*. Additionally, a better understanding of the immune system in broiler chickens and other animals will facilitate the development of better vehicles, vaccines and production practices to stop the spread of disease and development of antimicrobial resistance, ultimately providing more wholesome chickens and other meat animals.

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APPENDIX A

Isolation and Identification of *Salmonella* spp. From Ceca

1. Ceca samples undergo primary enrichment in two separate selective media followed by a secondary selective enrichment. The ceca tissue is macerated with a mallet in a whirlpack bag with 10 ml of PBS. Ceca aliquots of 1 ml are dispenced into 10 ml each of GN Hajna and tetrathionate broth (BD-Difco, Sparks, MD. 21152). The GN Hajna and tetrathionate broth enrichments are incubated 37^{o} C for 24 and 48 hours respectively.
2. Following primary enrichment(s), transfer a 100µl aliquot of the selective enrichment culture into Rappaport R-10 broth (10 ml) (BD-Difco, Sparks, MD. 21152) and incubate 24h at 37^{o} C for a secondary selective enrichment.
3. Isolate *Salmonella* by streaking to selective agar plates, Brilliant Green sulfa (BGS) (BD-Difco, Sparks, MD. 21152) and XLT4 (Hardy Diagnostics, Santa Maria, CA.) and incubate 24h at 37^{o} C. Presumptive positive colonies are lactose negative, forming a pink colony on BGS plates. Presumptive positive colonies are typically yellow with a black center (indicating hydrogen sulfide production) on XLT4 plates.
4. Inoculate presumptive positive colonies in Triple Sugar Iron (TSI) (BD-Difco, Sparks, MD. 21152) and Lysine Iron Agar (LIA) slants (BD-Difco, Sparks, MD. 21152), and incubate for 24h at 37^{o} C. A TSI positive reaction for *Salmonella* spp. = K/A+ (alkaline; red) reaction on the slant surface and an acid (yellow) reaction with hydrogen sulfide production in the stabbed agar butt. A LIA positive reaction for *Salmonella* spp. = K/K+ (alkaline; purple) reaction throughout the slant with

hydrogen sulfide production in the stabbed agar butt. Strains produce variable amounts of H_2S.

5. Presumptive positive colonies are serogrouped by slide agglutination with serogroup specific antisera (BD-Difco, Sparks, MD. 21152) stored on TSA (Tryptic Soy Agar) slants (BD-Difco, Sparks, MD. 21152) and are sent to NVSL for serotyping.

APPENDIX B

Procedure for Susceptibilty testing

Isolation. *Salmonella* isolates were streaked onto 5% sheep blood agar (SBA) plates for isolation. Plates were incubated at 37°C overnight. The following morning one well isolated colony from each plate was picked and streaked on a second SBA plate which was incubated at 37°C overnight.

Screening for resistance. One sterile dd H_2O tube and 1 Mueller Hinton broth (MHB) tube was set in a rack for each isolate. Two to six colonies from the second streak of a SBA plate were collected with a sterile cotton tipped swab and used to inoculate the water tube. The tube was vortexed and the density was adjusted with the nepholometer as per manufacturer's instructions. (Note: the machine is calibrated with a 0.5 McFarland standard prior to starting the procedure). A 10ul disposable loop (Trek Diagnostic Inc.) was used to transfer 10ul from the inoculated water to a MHB tube. The MHB tube was vortexed and placed into the auto inoculator (one isolate per microtiter plate) as per manufacturer's instructions. The microtiter plate was incubated at 37° for 18 hours. At 18 hours the plates were read using the autoreader as per manufacturers instructions.

APPENDIX C

Procedure for Pulsed Field Gel Electrophoresis (PFGE)

Molecular suptyping by pulsed field gel electrophoresis was performed on 36 *S*. Heidelberg isolates. The procedure was performed as described by Pulse Net: The National Molecular Subtyping Network for Foodborne Disease Survaillance (Centers For Disease Control and Prevention 2002;Hunter *et al.* 2005). In brief, bacterial genomic DNA embedded in 1.0% Seakem Gold agarose (BioWhittacker Molecular Applications, Rockland, ME) was digested with 10 U of *Xba*I (Roche Molecular Biochemicals, Indianapolis, IN). DNA standards were prepared from *Salmonella* Braenderup H9812. Digested DNA was separated using the CHEF Mapper® XA Pulsed Field Electrophoresis was carried out at 6 V for 18.5 h with a ramped pulse time of 2.16-63.8s in 0.5X Tris-borate- EDTA (TBE) at 14°C. Gels were stained with ethidium bromide and imaging was done on a Bio-Rad Gel Doc 1000 (Bio-Rad, Hercules, CA). Cluster analysis was determined using BioNumerics software version 3.0 (Applied Maths Scientific Software Development, Sint-Martens-Latem, Belgium) using Dice coefficient and the unweighted pair-group method (UPGMA).

APPENDIX D

Procedure for Plasmid Preparation (QIAGEN Valencia, CA)

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 10 ml LB medium. Each sample has five replicates. Incubate for approximately 8 hours at 37°C with vigorous shaking. (about 300 rpm)
2. Add all five replicates into on 50 ml tube. Harvest the bacterial cells by centrifugation at 4000 rpm for 25 min.
3. Resuspend the bacterial pellet in 4ml of Buffer P1.
4. Add 4 ml Buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate at room temperature for 5 min.
5. Add 4ml of chilled Buffer P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 15 min.
6. Centrifuge at 11,000 rpm for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.
7. Centrifuge the supernatant again at 11,000 rpm for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly.
8. Equilibrate a QIAGEN-tip 100 by apllying 4 ml Buffer QBT, and allow to empty by gravity flow.
9. Apply the supernatant from step 7 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
10. Wash the QIAGEN-tip with 2 x 10 ml Buffer QC.

11. Elute DNA with 5 ml Buffer QF.

12. Precipitate DNA by adding 3.5 ml room temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at 11,000 rpm for 30 min at 4°C. Carefully decant the supernatant without disturbing the pellet.

13. Wash DNA pellet with 2 ml of room-temperature 70% ethanol, and centrifuge at 11,000 rpm for 10 min. Carefully decant the supernatant without disturbing the pellet.

14. Air dry the pellet and redissolve the DNA in 750 µl of DNA hydration solution (Puregene^{TM}, Minneapolis, MN)

APPENDIX E

T3 Radioimmunoassay Procedure

1 Day Assay

Setup:

1. Label all tubes appropriately:

   Standards: S1-S6 x 2

   Samples: 1-end x 2

2. Add 100 µl of T_3 serum standards and samples to every tube.
3. Add 1 ml of T_3 tracer soln. to each tube, then vortex.
4. Incubate all tubes in a water bath @ 37 $\pm$ 1 °C for 1 hr. from the time of the last addition (in step 3). Place all tubes in the bath at the same time.
5. At the end of the required time for incubation, aspirate or decant all tubes. Blot the rims of the tubes after decanting.
6. Add 1 ml or more dH_2O to all tubes, making certain to rinse the sides of the tubes.
7. Reaspirate or decant all tubes.
8. Count the radioactivity in all tubes in sequence for one minute with a gamma counter. The counting time required for an accumulated trace level (Standard A) cout of 10,000 will indicate the time required for all the assays with the user's gamma counter.

	Standards	Samples
T3 Standards	100 µl	
Unkown Samples		50 µl
PBS Buffer		50 µl
I125 Soln.	1 ml	1 ml
Incubate	37 $\pm$ 1°C	37 $\pm$ 1°C
Aspirate/Decant		
dH_2O	1 ml	1 ml
Aspirate/Decant		
Count for 5 min.		

Calculations:

1. Use counts from gamma counter to average the triplicates
2. The averages were loaded into SAS to get the Anova analysis
3. Use the LS means to plotted data by age

APPENDIX F

T4 Assay Procedure

1 Day Assay

Setup:

1. Label all tubes appropriately:

   Standards: S1-S6 x 2

   Samples: 1-end x 2

2. Add 100 µl of T_4 serum standards and samples to every tube.

3. Add 1 ml of T_4 tracer soln. to each tube, then vortex.

4. Incubate all tubes at room temperature for 1 hr.

5. At the end of the required time for incubation, aspirate or decant all tubes. Blot the rims of the tubes after decanting.

6. Count the radioactivity in all tubes in sequence for one minute with a gamma counter. The counting time required for an accumulated trace level (Standard A) cout of 10,000 will indicate the time required for all the assays with the user's gamma counter.

	Standards	Samples
T4 Standards	25 µl	
Unkown Samples		25 µl
I125 Soln.	1 ml	1 ml
Incubate	ambient	ambient
Aspirate/Decant		
Count for .5 min. or longer	Std. 1 (BO) should yield 20,000-60,000	

Calculations:

4. Use counts from gamma counter to average the triplicates
5. The averages were loaded into SAS to get the Anova analysis
6. Use the LS means to plotted data by age

APPENDIX G

Insulin Growth Factor-I (IGF-I) Assay Protocol

Chemicals:

1. EDTA-Sigma (ED2SS)
2. Protamine Sulfate (Sigma P-4020)
3. Sodium Azide (Sigma S-2002)
4. Tris Base (Sigma T-1503)
5. Bovine serum Albumin (Sigma A-7888)
6. Dulbecco's Phosphate Buffered Saline (Sigma D5773)
7. Calcium Chloride
8. Polyethylene Glycol 6,000-(Fluka 81260)
9. Absolute Ethanol
10. 2N HCL
11. Sodium Phosphate, monobasic, monohydrate
12. Sodium Phospate, dibasic, heptahydrate
13. Tween-20-Sigma
14. Human sequence IGF-I (Gropep)
15. Chicken IGF-I (Gropep)

Reagents:

1. IGF Buffer, pH 7.5 and dissolved in 500 mls of distilled water, followed by bring volume up to 1L with distilled water

   A. 0.03M phosphate:

      sodium phosphate, monophasic, monohydrate = 4.14gm

      sodium phosphate, dibasic, heptahydrate = 8.04gm

   B. 0.01 M EDTA = 3.72

   C. protamine sulfate = 200 mg

   D. sodium azide = 200 mg

2. IGF RIA diluent:

   IGF buffer containing 0.05% Tween-20

      -add 50µls of Tween-20 to 100 mls of IGF buffer and stir at low speed.

3. IGF BSA diluent:

   Add 100 mg of BSA to 100 mls of IGF buffer.

4. PBS-Dulbecco's

   Add Dulbecco's Phosphate Buffered Saline (Sigma D-5773) + 0.100 mg of Calcium chloride

5. Acid/Ethanol:

   Add 12.5 mls of 2N HCL (43.10 mls of concentrated HCL into 250 mls of distilled water) + 87.5 mls of absolute ethanol.

6. Tris Buffer: (0.855 M Tris Buffer)

   Dissolve 25.89gm of Tris Base in 250 mls of distilled water

7. Acid/Ethanol Blank (A/E Blank)

Add 40 mls of acid/ethanol + 20 mls of Tris buffer + 10 mls PBS, pH 7.4

8. Chick assay:

   A. Diluent: IGF buffer containing 0.05% Tween-20

   B. First Antibody: (guinea pig anti-chick IGF-I)

   C. Tracer: Chicken I-125 IGF-I, make up to 6000 cpm/100 µl in IGF RIA diluent.

   D. Carrier : Normal guinea pig serum diluted to 1:200 in IGF buffer.

   E. Second antibody: sheep anti-guinea pig IgG (AGPGG) diluted in IGF buffer 1:15.

   F. 5.5% PEG in Dulbecco's PBS at concentration of 5.5 gm per 100 mls of PBS.

Supplies:

Eppendorf 1.5 ml microcentrifuge tubes

12 X 75 mm polypropylene tubes (Sarstedt 55.526)

Tracer:

1. Need Human sequence I-125 IGF-I (Amersham 1M.172)
2. Need chicken I-125 IGF-I
   A. To store frozen at -70°C, dilute in IGF buffer containing 0.1% BSA
   B. For assay, dilute in IGF RIA buffer containing 0.05% Tween-20

Standards: Prepared in IGF buffer containing 0.1% BSA

1. Prepare Chicken IGF-I at 40ng/100µl or 20ng/50 µl
2. Prepare Human IGF-I at 2ng/100 µl or 1ng/50µl
3. Store at -70°C

Sample Extraction:

1. Pippette 50 µl of sample into 1.5 microcentrifuge tube
2. Add 500 µl of acid/ethanol to the extraction tube using a repeater pipet.

   Let it sit for extraction for 30 minutes with caps on at room temperature.

   A. Add 250 µl of Tris base solution to each tube using a repeater, vortex and centrifuge at 2500 RPM for 15 minutes at 4°C.

   B. Add 50 µl of the extracted supernatant to the appropriate assay tubes containing 50 µl of IGF RIA diluent.

Dilution of Standards:

1. Standards are set up with 12 tubes in triplicates.
2. Pipette 100 µl of IGF BSA diluent into the 12^{th} tube.
3. Remove 50 µl from that tube and dilute down to tube one.
4. Add 50 µl of A/E blank to each standard tube after dilution.

IGF Protocol:

Day 1: Set up standards, a tube for total counts (TC), a tube for blank (B), and unknowns.

Add 100 µls of first antibody to all tubes except the TC and B.

Vortex, and incubate overnight at 4°C.

Day 2: Add tracer diluted in IGF RIA diluent 6,000 cpm/100 µls.

Vortex and incubate overnight at 4°C.

Day 3: Add 100 µls of carrier + 100 µls of second antibody to all tubes except TC tubes.

Vortex and incubate overnight at 4°C.

Day 4: Add 1.5 mls of cold 5.5% PEG 6000 to all tubes except TC tubes.

Vortex gently and centrifuge at 2500 RPM for 30 minutes.

Aspirate the supernatant and count in a gamma counter.

APPENDIX H

RNA Isolation from tissues and cells (In RNA later)

*Procedure is used from the RNeasy® MINI KIT from QIAGEN. Protocol for animal tissues

1. Add 10 µl β-mercaptoethanol (ME) to every 1 ml of RLT.
2. Add 4 volumes of ethanol (ETOH) to 1 volume of RPE.
3. Take the tissue sample (180-250 mg of tissue in 2.5 ml of RNA later) and cut no more than 30 mg into 600 µl of RLT.
4. Homogenize the tissue for one minute, sit on ice for a minute, and homogenize for one minute again to get uniform homogenization.
5. Centrifuge in microcentrifuge tubes at maximum speed for 3 minutes.
6. Transfer supernatant to fresh centrifuge tube.
7. Add 1 volume of 70% ETOH to the supernatant and mix immediately by pippeting up and down. Then quickly add 700 µl to an RNeasy column with 2 ml collection tube attached. Centrifuge at 10,000 RPM for 15 seconds. Discard flow-through but save the collection tube. If volume exceeds 700 µl from supernatant/ETOH, add the last amounts to column and repeat the spin.
8. Add 700 µl buffer RW1 to the column, close cap and centrifuge 15 seconds at 10,000 RPM. Discard flow-through and collection tube.
9. Transfer RNeasy column to new 2 ml collection tube. Add 500 µl buffer RPE onto column and centrifuge 15 seconds at 10,000 RPM. Discard flow-through but

keep collection tube.

10. Add another 500 µl buffer RPE to column, close tube and centrifuge 2 minutes at 10,000 RPM. Carefully remove collection tube and discard.
11. Transfer RNeasy column to microcentrifuge tube and spin at maximum speed for one minute to dry column.
12. Transfer RNeasy column to new 1.5 ml collection tube. Pippette 50 µl RNase-free water onto column. Centrifuge fro 1 minute at 10,000 RPM.
13. Add a second 50 µl RNase-free water onto column. Centrifuge for 1 minute at 10,000 RPM. Save flow-through and freeze for PCR.

APPENDIX I

Calculations for cytokine data

28s

1. Take 28S data and put Ct results in a column in excel.
2. In a second column, subtract eact Ct result from 40 (The instrument runs 40 cylces so the data will be in reference to the 40 cylcles).
3. Calculate the mean for all your samples (Do not include no-template controls or standards)
4. Calculate the mean of each triplicate in column 2 and put it in column 3.
5. Calculate the degrees of freedom, which is the correction factor to be used in the cytokine calculations.

   DF=sample mean for each triplicate (=column3)/overall mean (= #3)
6. Run standard curve for known standards. Get the slope of the standard curve. (y = mx + b; m is slope). This should be done by the ABI program if the information for the standards is put in correctly

Cytokine calculations.

1. Take cytokine data and put Ct results in a column
2. In a second column, subtract each Ct result from 40.
3. If samples are done in triplicate then calculate the mean for each triplicate in column 2 and put it in column 3.
4. Run your standard curve for your known standards. Get the slope of the standard

curve (y = mx + b; m is the slope). This should be done by the ABI program if the information for the standards was put in correctly

5. Calculate the corrected sample mean

   = (Sample mean X cytokine slope)/(28s slope X DF for each triplicate)

6. Calculate fold change

   $= 2^{(\text{Corrected treated sample mean} - \text{Corrected control mean})}$

   Make sure (Corrected sample mean – Corrected control mean) is the exponent

   To do this in Excel is 2^ (corrected treated mean –treated control mean)

APPENDIX J

PILOT STUDY

Introduction. In addition to cytokines, hormones, such as insulin-like growth factor (IGF-I) and thyroid stimulating hormones (T3 and T4), are also part of the immune response. IGF-I and II are synthesized in somatotroph cells and produced by growth hormone (Considine 2003a). IGF-I regulates through the endocrine and autocrine/paracrine systems causing cell proliferation and differentiation. It also regulates secretion from cells, glucose and amino acid uptake, and DNA synthesis (Jones and Clemmons 1995;McMurtry *et al.* 1997). T3 and T4 are synthesized in thryotroph and produced by thyroid stimulating hormone. Their key role is maintaining the development and rate of metabolism (Considine 2003b). By use of a radioimmunoassay (RIA), a competitive binding assay in which labeled and unlabeled antigens competes for a limited amount of antibody binding (Peavy D 2003), T3, T4, and IGF-I levels are measured.

Prior to running the experiment as described in Chapters 3 and 4, a pilot study was conducted to determine if the broiler chickens elicit a hormonal response.

Study design. It was decided that only two groups would be required for the pilot study, a control group and the tetracycline/enrofloxacin/Heidelberg group. It was hypothesized that if a response was to occur, it would occur in the group receiving all three treatments.

Salmonella-free birds were transported to the laboratory and 120 birds were placed per room and further divided into 60 birds per pen. Twenty birds were necropsied upon arrival and cultured for *Salmonella* as described in Chapter 3. No additional culture was conducted at any subsequent necropsy. Birds were challenged and treated as

described in Chapter 3. Twenty birds, 10 per pen, were necropsied at day 3 post-challenge and at week one then weekly through five weeks.

Serum samples. To collect serum, blood was taken from the jugular and up to 15 mls were collected in 15 ml conical tubes. The blood samples were incubated for 2 hours at room temperature then refrigerated overnight. The next day, the samples were centrifuged at 10,000 x g for 20 minutes, and the serum layer was aliquoted and frozen for use in the RIA.

RIA testing for hormones. T3 and T4 were measured by use of RIA kit (MP Biomedicals Diagnostic Division, Orangeburg, NY) as per manufacturer's direction. Briefly, I^{125} tracer was added and the samples were incubated at 37^{o}C (T3) or room temperature (T4) to allow binding. For T3 distilled water (1ml) was added to the tube, then the entire supernatant was aspirated. For T4 the supernatant was aspirated without the addition of water. The samples were then counted on a gamma counter. Once the counts are collected from the gamma counter, the results were loaded into a statistical program (SAS, Cary, NC) to calculate the least square means. See appendices E and F for T3 and T4 procedures, respectively.

IGF-I is bound to serum protein and must be purified prior to use in the RIA. Once purified, they must be coupled to the antibody for analysis in a gamma counter. Binding counts are calculated by nanograms/ml for one minute ((McMurtry *et al.* 1994); also see appendices E-G).

Briefly, serum samples plus 100 µls of the first antibody were mixed and incubated overnight at 4^{o}C. Tracer diluted in IGF RIA diluent was added followed by an additional overnight incubation at 4^{o}C. On day three, 100 µls of carrier and second

antibody were added and incubated overnight at 4°C. On day four, 1.5 mls of 5.5% of PEG 6,000 (Sigma Chemical Co., St. Louis, MO) was added to the tubes, mixed, and centrifuge at 2,500 rpm for 30 min. The supernatant was aspirated and counts were collected in the gamma counter. The probability and least square means for each group were calculated in SAS to access significance between treatments and age.

Results

Hormone expression. No treatment effect was observed for IGF-I (Figures J.1, J.2, and J.3, respectively) between treatment and control. However, there is a significant ($p<0.05$) difference between age (week) and control and for each of the hormones.

Discussion

The IGF-I, T3, and T4, are hormones that can be considered to give off an immunological response. In healthy chick, the levels are not expected to be high due to low production at a young age. McMurtry et al. mentions that IGF-I is usually low at weeks two and three and increases to a plateau at about five weeks (McMurtry *et al.* 1997). Not much is known about the T3 and T4 levels in chickens, but is expected to act in the same fashion. After the means of the hormone counts were compared and analyzed, there was a negative effect to all the hormones when *Salmonella* infection and antibiotic treatments were presented. There was no interaction with the treated and the controls. Age did have a positive response on the hormones. As the birds got older the hormones became more active. With the negative effects, it could be said that this infection and antibiotics did not play a role in metabolism. The growth rate maintained consistency throughout he lifespan of the birds.

Figure J.1 Serum IGF-I concentration (mean +/- Standard Error (SE)) by week

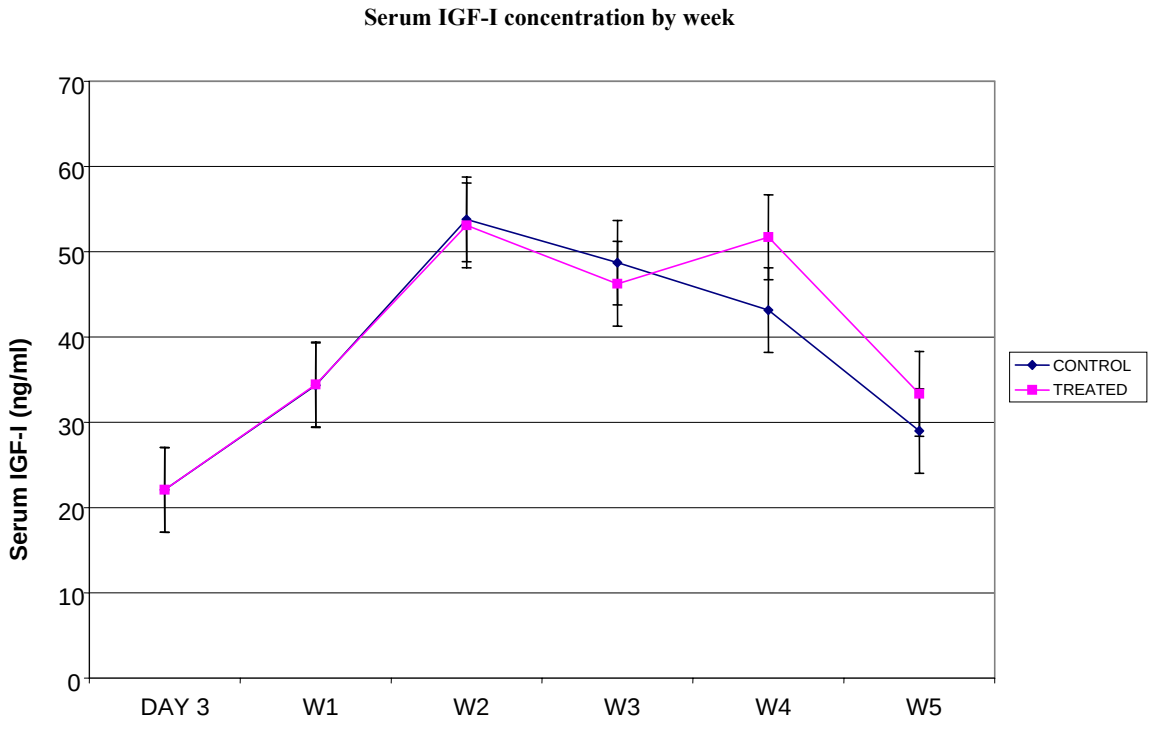


Figure J.2 Serum T3 concentration (mean +/- Standard Error (SE)) by week

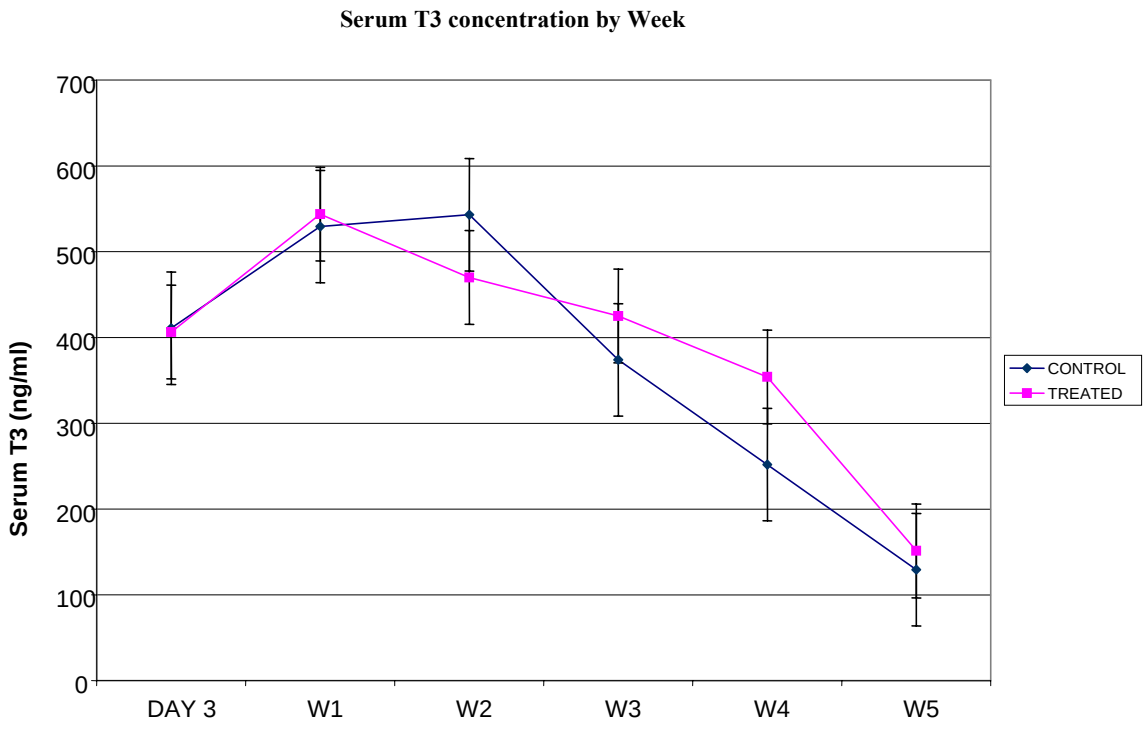
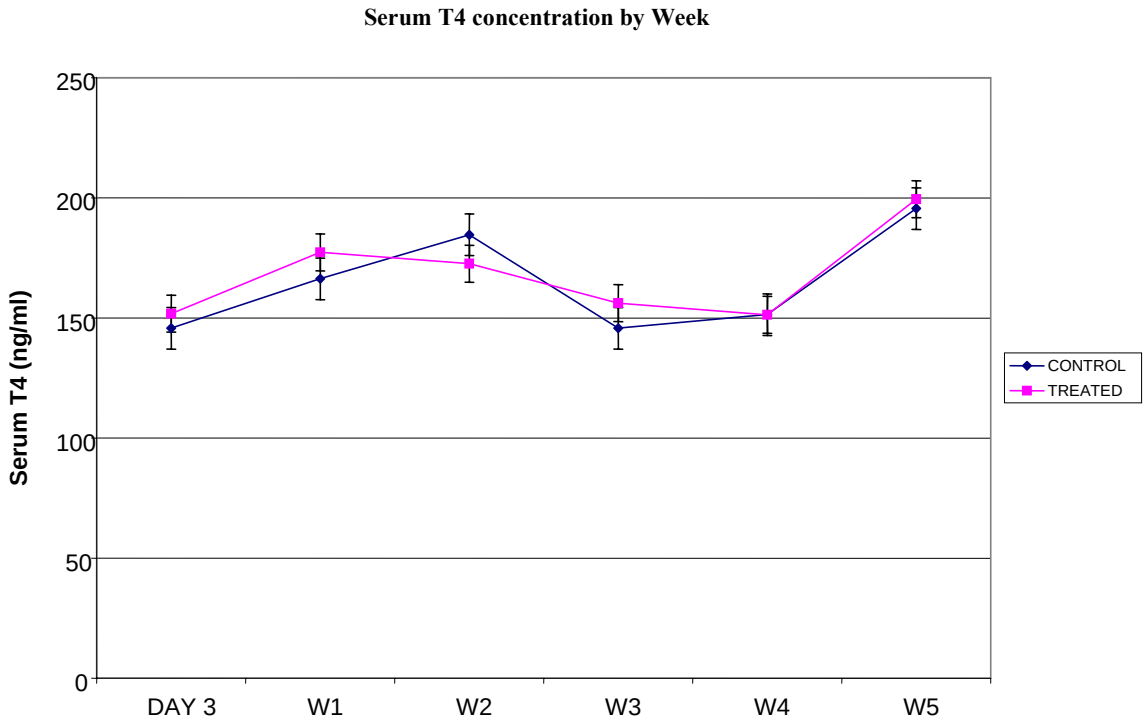


Figure J.3 Serum T4 concentration (mean +/- Standard Error (SE)) by week



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