Integrons, Genetic Elements Responsible for Acquisition of Antibiotic Resistance Genes, and their Prevalence Among Gram- Negative Enterics in Poultry Litter

by

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(Under the Direction of Dr. John Maurer)

Integrons are a genetic element, similar to transposons, that mediate multiple drug resistance. This genetic element has the potential for acquiring "new" antibiotic resistance genes through the recombination of these genes into the integron's integration site *attI*. An integrase gene, *intI1*, a signature of class 1 integrons, mediates this recombination event. My hypothesis is that the integron serves as a vehicle for exchange of drug resistance genes and their eventual dissemination within microflora in the farm, including their final disposition into food-born pathogens like *Salmonella* and *Campylobacter*.

This study examines the prevalence of class 1 integrons in gram- negative bacteria in chicken litter from two poultry farms. Litter was collected from two farms participating in this study. Samples were collected at the time of chick placement (time 0), as well as at 2, 4, and 6 weeks following chick placement. Gram- negative enterics were isolated from the poultry litter on MacConkey agar. Isolated colonies were screened by Southern colony blot for *intI*. Those colonies that screened positive in the Southern blot were further analyzed by 5'3' PCR amplifying the integrons and cassettes as well as speciation of the colonies. A second Southern blot was performed after the PCR to screen for *aadA1* (aminoglycoside resistance). The number of gram- negatives with *intI* consistently increased, although varying in the actual percentages. The lowest

percentage was 12% and the maximum found was 68%. The amplified PCR products displayed a vast array of cassette combinations and sizes. Most samples showed 1 band that was 1 Kb. However, as many as 8 bands was found and sizes ranged from 600bp to 4 Kb. The class 1 integrons were present among a diverse number of genera, species, and genetic types. *E. coli* was the most prevalent organism found. It was hypothesized that the 1 Kb band was for *aadA1*. All samples with a 1 Kb band screened positive in the Southern blot for *aadA1*. My results indicate that integrons are not confined to veterinary or human pathogens, but are present among the normal microflora of the poultry farm as well.

INDEX WORDS: Integron, Antibiotic Resistance, Poultry Microflora

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by

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B.S., Valdosta State University, 1999

A Thesis Submitted to the Graduate Faculty

of The University of Georgia

in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA
2002

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May 2002

Acknowledgements

I would like to take this opportunity to give "thanks" to those that supported me in my studies, albeit financially, academically, or most importantly emotionally. Whether I was moving along smoothly or expressing a rash profanity, someone was always there to remind me to make haste slowly and to keep trying. A grand reward almost always entails a lot of hard work and even harder obstacles.

A special "Thank You" goes out to my sister, Kristin Rookey, whose footsteps I have chosen to follow, for her extra guidance in her baby sister's career, to my sister, Austin Mullen, for continuously telling me to keep my chin up, and to my Parents, Tom and Rosann Rookey, for their endless philosophies that never seem to make sense, but always make things better.

My committee members:
John J. Maurer
Margie D. Lee
Charles Hofacre

My co-workers and friends:

Karen Liljiebjielke	Tameka Buffington	Trey Keadle
Yang Hong	Joseph Minicozzi	
Marie Maier	Tongrai Liu	

"Education is a progressive study of our own ignorance."
Will Durant

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Chapter 1

Introduction and Literature Review

Antibiotics were created to help in the fight against infectious diseases such as tuberculosis, meningitis, and plague. Unfortunately, resistance to these drugs has followed each new antibiotic, with varying time and intensity. Many bacteria produce secondary metabolites, antibiotics, in order to kill or inhibit their microbial competitors. While these microbes carry genes to produce antibiotics, they also carry genes to protect themselves against these antibiotics. Bacteria have very sophisticated methods of exchanging genetic information. The threat, therefore, is the spread of these antibiotic resistance genes and thus, the spread of antibiotic resistance to important human and veterinary pathogens.

Mycobacterium tuberculosis, Streptococcus pneumoniae, Neisseria gonorrheae, Plasmodium falciparum, and certain *Salmonella* serotypes have developed resistance(s) to important therapeutic antibiotics. *Escherichia coli* carries a gene which is now present in 40 – 60% of isolates of this species (coding for an enzyme for resistance to β-lactams). Of 114 strains of *Vibrio anguillarum* investigated in Japan between 1989 and 1991, only one was found to be susceptible to all drugs tested.

Since antibiotics were approved for agricultural use, to treat and prevent illness as well as growth promotion, researchers have found resistance to these antibiotics in foodborne pathogens. More importantly, they have noted resistance to multiple classes of antimicrobials. For example, *Salmonella enterica* Typhimurium DT104 has been found

to be resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline.

ANTIBIOTICS

What are antibiotics? Antibiotics are agents that affect bacteria by either killing cells or inhibiting their growth. They are natural by- products of an organism's metabolism, usually bacteria or fungi (12). Antibiotics are used to treat disease and/or infection, as a prophylactic for surgery, as growth promoters in farms, as preventive treatments in agriculture, and for research in microbiology or molecular biology. For therapeutic use, the agent should be harmless to the host and toxic to the pathogen. Some antibiotics are active against a narrow range of species, and some against a broad range. In some cases, a natural antibiotic can be chemically modified in a laboratory to form semi- synthetic antibiotics which may have a significantly different spectrum of activity (12).

There are a number of different classes of antibiotics, each targeting a different part of a bacterial cell's growth system. Antibiotics are bacteriostatic, growth inhibitory, or bacteriocidal. β- lactams and cephalosporin are bacteriocidal antibiotics that inhibit cell wall synthesis (62). They inactivate penicillin binding proteins which are enzymes involved in peptidoglycan crosslinking. These antibiotics kill only growing cells. Tetracyclines are bacteriostatic agents that inhibit protein synthesis by binding to the 30S ribosomal subunit and inhibit binding of aminoacyl tRNA (60). Aminoglycosides, a broad spectrum class of antibiotics, inhibit protein synthesis by binding to the 30S ribosomal subunit and causing misreading of mRNA (45). Quinolones, bacteriocidal antimicrobial agents, inhibit DNA replication through its interaction with DNA gyrase and topoisomerase, inhibiting enzymatic activity and inducing double- stranded DNA

breaks (26). Chloramphenicol inhibits protein synthesis by binding to the 50S ribosomal subunit inhibiting the activity of peptidyltransferase (37).

Antibiotic use in agriculture. Animals raised for food purposes are commonly given antibiotics to treat and prevent disease, but also to improve growth rate and feed conversion (25, 38). The treatment and prevention of disease is achieved by either therapeutic (individual animal), metaphylactic (treatment of all animals in a group or herd or flock to prevent spread of infection from one or two animals already infected), or prophylactic (given for prevention during key periods such as surgery, vaccination, transport and mixing of animals, etc) (53). Subtherapeutic doses may be used when pathogens are known to be present. Subtherapeutic doses are smaller than those required to treat established infections (38).

There are three mechanisms of action through which antibiotics appear to enhance growth production (38). The first is biochemical, nitrogen excretion, efficiency of phosphorylation reaction in cells, etc. The second is through a direct effect on metabolism, including generation of essential vitamins and cofactors. Finally, antibiotics decrease gut mass and increases intestinal absorption (38). For example, broiler chicken producers almost always include a coccidiostat, an arsenical, and an antibiotic to improve feed efficiency and body weight gains and for reduced morbidity and mortality (38). This type of use involves continual, subtherapeutic doses that provide the environment with a selection pressure for resistance to these antimicrobial agents (25).

Some common antibiotics used for growth promotion in chickens include bacitracin, virginiamycin, and flavomycin. Flouroquinolones are currently prescribed for

treatment of colibacillosis and air sacculitis, *E. coli* infections, in chickens and turkeys (28). Antibiotics in hog feed for growth promotion include bacitracin, chlortetracycline, and penicillin. Cattle may receive ampicillin, bacitracin, or oxytetracycline (38). The aminoglycosides, penicillins, cephalosporins, lincosamides, macrolides, polypeptides, quinolones, streptogramins, and tetracyclines have all been used in cattle, fowl, goats, poultry, sheep, and swine for disease treatment and prevention (53). Whether this type antibiotic use in agriculture bears any influence on bacterial resistance remains unanswered.

Antibiotic Resistance. Bacteria have developed methods to resist antibiotics. Most antimicrobials are substances produced by fungi or soil bacteria and provided a selective advantage in the fight for resources and/or ecological niches. Therefore, bacteria have come into contact with these antimicrobial substances a long time, before antibiotics were put into clinical use. This selective pressure has forced organisms to develop mechanisms to escape the inhibitory activity of bacteriocidal effects (52).

Resistance is an example of rapid adaptation of bacteria to selection pressure, antibiotics. Some organisms are resistant to antibiotics because they naturally lack the specific target or process. For example, *Mycoplasma* have no cell wall and are therefore resistant to penicillin or other cell wall active antibiotics. Bacteria also become resistant through the acquisition of resistance genes (5). A single resistant cell in a population can grow and give rise to a population of resistant cells. There are three major mechanisms of resistance: 1) enzymatic inactivation, 2) decreased intracellular accumulation, and 3) alteration of the target (52).

A wide variety of enzymes are known that inactivate antimicrobial agents by transferring chemical groups to the antibiotic, thus destroying its activity. This resistance mechanism allows bacteria to grow in the presence of antibiotics like chloramphenicol and aminoglycosides. For example, the type I CAT (chloramphenicol acetyltransferase) has two activities, in addition to catalyzing the acylation of chloramphenicol, the protein forms tight complex with the antibiotic fusidic acid rendering the antibiotic ineffective (13). Other enzymes directly attack the drug molecule and destroy it, an esterase or β– lactamase for example. *Staphylococcus aureus* produce β– lactamase that inactivate the β– lactam antibiotic, penicillin.

Another mechanism of resistance involves decreasing intracellular accumulation of the drug by blocking its uptake or transport into the cell or actively pumping the antibiotic out of the bacterial cell. The outer membrane of gram- negative bacteria provides a formable permeability barrier to hydrophobic antibiotics, like erythromycin and novobicin. Tetracycline resistance in gram- negatives is generally attributed to efflux pumps. The efflux proteins exchange a proton for a tetracycline- cation complex against a concentration gradient (13).

Alteration of the target site is another way in which microbes counteract antibiotics. This may be achieved a number of different ways. Chemical modification of the target is one method. For example, methylation of ribosomes provides gram-positives resistance to the macrolides (29). Another resistance mechanism provides protection of the target site by specific proteins, like ribosome protective proteins involved in gram- positive tetracycline resistance (60). Finally, a third mechanism involves replacing the target pathway with one immune to the antimicrobial agent, as is

the case for trimethoprim and sulfonamide resistance (27). In order for many microorganisms to become resistant to antibiotics, their acquisition of genetic information is required. The number of such antibiotic resistance genes seems endless.

Genetic transfer of antibiotic resistance. Bacteria not only transfer these genes vertically (cell division), but they also swap them around. There are three major mechanisms for horizontal genetic exchange: transformation, transduction, and conjugation (52). Transformation involves the uptake and incorporation of naked DNA, which may become available in the environment following bacterial lysis (5). However, transformation is limited to a few bacteria (*Actinobacter, Pseudomonas, Bacillus,* and *Neisseria*). Organisms may become competent for transformation under special chemical or physical conditions (5). Transduction utilizes bacteriophages as vehicles for genetic exchange. The phage injects their DNA into a host cell and new phage DNA is replicated and released. However, phage DNA may integrate into the chromosomal DNA and host genes may become "accidentally" incorporated into the phage head in place of the viral DNA, thus spreading these bacterial genes to new host cells upon infection with this phage particle. This type of spread is limited by the amount of DNA that can be packed into a phage head and the distribution of phage receptors among bacteria (5). Conjugation involves physical, cell- to- cell interaction and delivery of genetic information, in the form of plasmid or transposon from donor to recipient cell, requires a set of genes required for transfer (*tra, ori*T), and mobilization. Bacterial conjugation is probably the most frequently used mechanism for horizontal gene transfer (5).

Plasmids are extrachromosomal elements that have been detected in virtually all bacterial genera of medical or veterinary importance. Their size varies from less than 2 Kb to more than 100 Kb. Plasmids are capable of autonomous replication and may co-exist in the same bacterial cell. Plasmids may pass from one organism to another, often within the same species or family, and occasionally between families (narrow vs. broad-host-range plasmids) (41). Plasmid-born properties are often not essential for the survival, but may be beneficial under certain conditions, for example, virulence or antibiotic resistance. Plasmids may form cointegrates with other plasmids, integrate into the bacterial chromosomal, or serve as vectors for transposons and integrons (52).

Transposons do not replicate autonomously and therefore must integrate into bacterial chromosome, plasmids, or phage for their survival. They are capable of moving from one DNA molecule to another, independent of *recA*- dependent recombination (41). They have insertion sequences on either end and may have various genes, including drug-resistance genes, in between these insertion sequences. Transposons vary in size from less than 1 Kb to 60 Kb. Many transposons have little or no target specificity and therefore can insert themselves randomly into DNA (52). There are several classes of transposons, grouped according to their structure, function, size, conserved regions, genetic composition, and organization (31).

INTEGRONS

Genetic structure of integrons. An integron is a genetic element that contains integrase, which is responsible for insertion, excision, and rearrangement of gene cassettes as well as an adjacent integration site, *att*, in which a variety of resistance genes are inserted

(8,10,21,57). Integrons contain a variety of antibiotic resistance genes (57). The antibiotic resistance genes are inserted as cassettes. They not only contain the drug-resistance gene, but also a 59 base pair element (be) that the integrase recognizes and inserts at the attachment site (*attC*) (21,57). Cassette integrations usually involve the *att* site, whereas cassette excisions use the 59- base element (18). Gene cassettes do not have their own promoter and therefore rely on a promoter, P_{ant}, located in the integrase for expression (Fig. 1) (21,57).

Integrons are located in plasmids and transposons, such as the Tn*21* group of transposons (5). Tn*21* is a member of a family of transposable elements widely distributed among bacteria (33). Transposable elements are specific DNA sequences that can insert into unrelated DNA sequences (33). Tn*21* is a subgroup of the Tn*3* class of transposons. All the transposons belonging to this family contain inverted repeats (IRs) and extensive homology in their transposition genes. Within a subgroup, in addition to DNA homology, there is also functional relatedness of sites and gene products. Tn*21* encodes genes and sites required for transposition, including *tnpA, tnpR, tnpM, res,* and Irs, as well as genes primarily responsible for resistance to streptomycin and spectinomycin (*aadA1*), sulfonamides (*sul1*) and inorganic mercury (*merA*) (33). There are several Tn*21*- related transposons; Tn*501*, Tn*3926*, Tn*1721*, Tn*2424*, Tn*2603*, Tn*2410*, Tn*4000*, and Tn*2501*, all with various antibiotic resistance genes. Martinez and de la Cruz (33) showed that these antibiotic resistance genes became integrated in the Tn*21* and Tn*21*- like transposons through a *recA*- independent, site- specific integration system which was later identified as a class 1 integron. The fact that integrons are associated with Tn*21* is important because Tn*21* and its derivatives can transpose or

"hop" and may explain the widespread dissemination of integrons and its drug- resistance genes (5).

ALL known integrons have three key components (Fig. 1): 1) a gene coding for integrase, 2) a primary recombination site, and 3) a promoter (21, 22). The differences between classes of integrons are in the integrase sequence. The integrases are part of a large family, the tyrosine recombinases (14). They rearrange DNA duplexes by a site-specific recombination mechanism. Other family members function in the maintenance of plasmid copy number, the resolution of dimers, and alteration of cell surface components, as well as in the life cycle of temperate phages (14). All tyrosine recombinases have two conserved regions and three shorter conserved regions. The *att* sites of the different integrase classes are also different. In fact, the only common feature is a core 59- be, GTTRRRY (24).

There are currently 8 known classes of integrases, which are part of a large and diverse family of site- specific recombinases (40). Integrases form a tight homology group within the recombinase family, with amino acid sequences from the different integron classes displaying 41- 57% identity (40). Class 1 integrase, *intI1,* is found in the majority of integrons found in clinical isolates (57). This integron class is the most widely studied and defined. Class 2 integrons are present on transposon Tn*7* and related transposons. The class 2 integrase shows 46% homology with *intI1* (44). The 3' end of Tn*7* is composed of genes involved in the transposition (5). Tn*7* contains three integrated gene cassettes (*dhfrI- sat- aadA1)* adjacent to a defective integrase gene (int*I2*). The Tn*7* *attI* site is located between the integrase and the first inserted resistance gene as is for class 1 integrons, however, class 2 integrons do not contain the *sul1* gene (5). Class 3

integrase shows 60.9% homology with *intI1*. This class has only been described in impemenim-resistant *Serratia marcescens*. This integron contains a metallo-β-lactamase gene cassette, bla_{IMP}, which confers beta-lactam and cephalosporin resistance, and the tobramycin-resistance gene, *aacA4* (1). Classes 1, 2, and 3 all have been found to contain antibiotic resistance gene cassettes. Despite differences in the integrase and *attI* site sequences, all three integron classes are thought to acquire the same gene cassettes, as identical gene cassettes have been found in these three classes (1, 59). The class 4 integron is found in the *Vibrio cholerae* genome and called the "super- integron" because it contains hundreds of gene cassettes (6, 15, 35, 48). In addition to many antibiotic resistance genes, two virulence genes of *Vibrio cholerae* are also structured as gene cassettes (48). The activity of class 4 integrase is identical to class 1 integrase. This integrase inserts repeated gene sequences in clusters (6, 15, 35). Class 5 integron is associated with *Vibrio mimicus* and is said to have 75% identity with *intI4* (39). Nield et. al. identified "new" integron classes 6, 7, and 8 from the environmental (39). Integron classes 6, 7, and 8 not only vary in their integrase sequence, but they also have a promoter. However, the promoters for classes 6, 7, and 8 are all at different locations in the integron, and they are not located within *intI* (39).

Recombination. In general, integrase recognizes the 59- be of a gene cassette and inserts it at either the *att* recombination site or the 59 base element (59- be) of a resident cassette. The upstream, common promoter, P_{ant}, expresses the inserted gene cassettes.

Collis et al. and Martinez and de la Cruz showed that the *attC* site is active in recombination of gene cassettes into class 1 integrons (10, 33). The 59- be consists of

inverted repeats and they have a core site at the 3' end and an inverse core site at the 5' end. When the sequence of *attC* was examined, only a short inverted repeat and the core site was found (47). This site has none of the features of the other recombination site, the 59- be, except for the 7 base core site (GTTRRRY) (10, 42). Since both *attC* and the 59- be have the core site with the GTT cross- over point, recombination at these sites were compared by Hansson et al. and Collis et. al. (11, 24). Hansson et al. showed recombination occurring between two *attC* sites (24). Collis et al. compared efficiencies of recombination between two 59- be, *attC* and 59- be, and two *attC* sites. First, they compared recombination using various 59- be and found recombination ranged from 10^{-2} to 10^{-5}, demonstrating that sequence of the 59- be influences recombination. Recombination between 59- be and *attC* occurs at a much higher frequency than between two 59- be. They also saw that recombination between two *attC* sites occurs at a very low frequency (11). Partrtidge et al. studied the differences in lengths of the *attC* sites on recombination (42). They found that recombination between *attC* and a 59- be is 100 fold more efficient than recombination between 2 *attC* sites. A full 65 bp, *attC* site is required for recombination with a 59- be to occur and contains three integrase binding domains (42).

Cameron et al. did a comparison of DNA sequences and demonstrated the site-specific gene insertion into class 1 integrons (4). The sequences revealed that unrelated antibiotic resistance genes (*aadB, aadA,* and *dhfrII*) were flanked by identical DNA sequences consisting of an imperfect inverted repeat sequence of 59 bases located 3' of the inserted genes (4). Site- specific recombination between two DNA molecules require the recombination site to be present on both molecules. Since many versions of the 59-

be associated with gene cassettes had been identified, it was suggested that they may play an important role in recombination (19, 50, 54, 57, 59, 61, 65). When the published sequences were compared, the recombination site of gene cassettes was confined to a consensus sequence of GTTAGGC with GTT always conserved (57). This sequence was located both 5' and 3' of the gene cassettes. This sequence is the last 7 bases of all the 59- be found as well as the sequence of the first 7 bases of the conserved 3' segment of the 59- be. Martinez and de la Cruz were able to demonstrate that integrase is necessary for recombination and to confine the recombination cross- over point to the first 5 bases of this sequence (GTTAG) (33, 34). They also deleted sequences and found that at least one complete 59- be is required for integrase mediated site- specific recombination.

Hall et al. examined the role of the 59- be in recombination (23). They used 59- be with varying sequences and found that regardless of the size and sequence, they can act as recombination sites. They also showed activity between two 59- be. Since the 59- base elements are flanked by unrelated sequences, they concluded that only the 59- be sequence are needed for recombination to occur. This study also confined the cross- over point to the GTT found in the consensus sequence. They saw that recombination occurred only at the 3' sequence of gene cassettes, and not in the inverse sequence, located 5' of gene cassettes as well (23).

In this same study, a model for insertion of genes into integrons was proposed. The last four bases of the 59- be are not conserved and the cassettes are thus flanked by two GTT sequences. However, only one of the GTT triplets is part of the cassette, located 5' of the cassette. Therefore, cassettes consist of the gene, the 5' core site, and all except the last seven bases of the 59- be. The idea is that the cross- over point lies within

or on either side of the conserved GTT triplet. Upon insertion, the circular cassette is split at the 59- be at the cross- over point and part of the 7 base pairs of 59- be to goes 5' of the gene cassette and the other part 3' of the gene cassette. This model also shows that there would be an additional insertion site, the original one from the integron and the new one from the 59- be of the inserted cassette (23).

Collis and Hall later extended the insertion model and showed that cassettes can also be excised by integrase and generate a circular cassette (7). Recombination between core sites flanking an insert results in excision of the gene and generates a circular cassette. This cassette can be re-inserted at a new location. It was already shown that the inserted genes could be deleted, but Collis and Hall showed that excision was integrase dependent and precise (7, 51, 64). Evidence that gene cassettes can be inserted, excised, and rearranged shows that they are mobile functional units (8). The next step in building the integron model was to investigate the transmission of circular gene cassettes from one bacterial cell to another (10). Collis and Hall demonstrated acquisition of these circular cassettes by the integron only occurred when the integrase was present.

A model for the insertion of circular cassettes via site- specific recombination was proposed and verified. Cassettes can exist in two forms, either as free circular molecules (7) or integrated into an integron (57). The genes in a cassette vary as well as the 59- be. A unique 59- be is associated with each gene. Stokes et al. looked at the various 59- be (58). They saw that introducing mutations into the core sites led to a reduction in integration site activity. This 59- be is what is responsible for the recognition of its gene by integrase for insertion.

Collis and Hall investigated expression of the inserted gene cassettes (9). The inserted genes are expressed from a promoter in the 5' conserved sequence of the integron. Cassettes do not contain a promoter and are therefore dependent on the integron for expression (57). They used a variety of integron fragments and genes to compare their expression. They found that the position of the cassette within the integration site had an affect on its expression. The closer the cassette was to the promoter, the more efficient its expression (9).

Integron gene cassettes. The gene cassette is defined as a discrete unit consisting of one complete ORF followed by a recombination site, the 59- be (5). Integron cassettes contain genes that encode for resistances to aminoglycoside, β-lactams, cephalosporins, trimethoprim, chloramphenicol, and quaternary ammonium compounds (5). Single (*catB*, *cmlA*), family (*aadA1*) or families of genes (*dfrA*, *dfrB*) have been described for gene cassette(s) present in class 1 integrons (46). Where two or more members of the same protein family have been found, the level of amino acid identity ranges from 25% to 99% (46). The integron gene cassettes come in many different sizes and confer resistance to many different antibiotics (5). Numerous cassettes have been described. For example, at least 15 different genes encoding aminoglycoside- adenyltransferase (*aad*) or aminoglycoside- acetyltransferase (*aac*), and dihydrofolate reductase (*dfr*) have been identified as part of integron cassettes (32). Levesque et al. mapped integrons via PCR and found several combinations of resistance genes (30). The genes included *sulI, aac6Ia, aac6Ib, aac3Ia, aac3IIa, aac3IVa, ant3Ia, ant3Ib, ant2Ia, oxa2, psc2, tctB,* and *dhfrI.* A review by Fluit and Schmitz lists 63 of these genes and their sizes (16).

Eighteen of the genes encode resistance to β- lactams (*bla, oxa*), 20 to aminoglycosides (*aad, aac*), 7 to trimethoprim (*dfr*), 7 to chloramphenicol (*cat, cml*), 3 to quarternary compounds (*qac*), 1 to rifampin (*arr*), 1 to erythromycin (*ere*), and 6 unidentified open reading frames (orf). A study by Peters et al. (43) added *aadA8* to the aminoglycoside resistance list and separated the erythromycin resistance gene into two related genes (*ereA1, ereA2*).

INTEGRON EPIDEMIOLOGY

Billions of bacteria of many different families, genera, and species are normally present in or on an animal's body, including humans, and usually do not cause harm. They live on the skin, in the respiratory tract, in the urinary tract, and in the digestive tract. These organisms are referred to as "normal flora" and have many functions (56). They synthesize and excrete vitamins, which the host may absorb as nutrients. The normal flora helps to prevent colonization by pathogenic organisms by acting as competitors for nutrients and attachment sites. They also may prevent colonization by other organisms by producing substances that may inhibit or kill non- indigenous species. The normal flora also stimulates the production of cross- reactive antibodies. The resident bacteria act as antigens and therefore induce an immunological response which, in turn, produces low level of antibodies that may cross- react with related pathogenic species (56).

Ruminants have even more uses for bacteria in the rumen. Initial digestion of cellulose is dependent upon bacteria and protozoa, this digestion results in the formation of carbohydrate and volatile fatty acid end products that serve as carbon and energy

sources for the animal. Ruminants also depend on microorganisms for synthesis of vitamin B. Lastly, bacteria provide ruminants with nitrogen and essential amino acids. The most common nitrogen source is protein in forage. These proteins are degraded to peptides, amino acids, and NH_3 by bacteria that utilize these products to synthesize bacterial protein. The bacteria die and are digested by the ruminant. Protein is used by the host in the form of rumen bacteria, which become the major source of nitrogen for the ruminant (56).

Many bacteria are common to different animals. In general, there are more gram-positive bacteria like *Staphylococci* and *Corynebacteria* on the skin of humans compared to the flora of the intestines, which contains various groups of gram-negatives (56). A large part of the intestinal flora is not cultivable and knowledge of this microbial community is minimal. *E. coli, Clostridium, Streptococusi,* and *Lactobacillus* are usually present in the intestine of cattle, sheep, pigs, chickens, dogs, cats, and humans. In addition, *Salmonella, Campylobacter, Yersinia, Shigella, Vibrio,* and *Staphylococcus* are also present as transient or normal flora organisms. The normal flora is of importance when dealing with the spread of antibiotic resistance because they are potential genetic partners in the transfer of drug resistance to pathogens (56).

Over the past few years, the analysis of many antibiotic resistance genes identified in clinical and veterinary isolates, pathogens and normal flora, established the importance of integrons in the dissemination of resistance among bacterial pathogens (5). *E. coli* present in the ECOR collection, a set of 72 *E. coli* reference strains isolated between 1973 and 1983 from healthy humans and animals, contained class 1 integrons (34). Eighteen isolates were resistant to at least one antibiotic and fourteen *E. coli*

isolates in this collection were resistant to two or more antibiotics. Four *E. coli* strains were positive for class 1 integrons, three of which possessed the mercury resistance transposon, Tn*21*. In another study, 63% of multi- drug resistant, avian *E. coli* had class 1 integrons. Widespread distribution of this genetic element among these isolates appeared to be attributed to the dissemination of Tn*21* in this population (2). Sallen et. al. reported that 59% of clinical, gram- negative enterics possessed class 1 integrons. They identified *ant(3"), aac(6'), dhfr,* and *bla* within the integration site of the class 1 integrons (49). In *Salmonella enterica,* from Scottish Salmonella Reference Laboratory, nineteen percent of the isolates contained class 1 integrons (3). All *Salmonella* isolates were resistant to aminoglycosides with seven also resistant to ampicillin. Goldstein et al examined the distribution of class 1 and 2 integrons among clinical and commensal bacteria from livestock, companion animals, and exotics (17). Forty- six percent of these isolates had class 1 integrons. Class 1 integrons have been identified from a number of pathogenic *E. coli* isolates (63, 66). Often these isolates exhibit resistance to two or more antibiotics; most commonly streptomycin and sulfamethoxazole, resistances often associated with class 1 integrons.

Integrons are common in nature. Integrons have been found among clinical and commensal isolates of both humans and animals. Even though more than sixty drug-resistance genes have been identified in class 1 integrons, it is becoming apparent that the common gene cassettes are those associated with aminoglycoside and trimethoprim resistance. More importantly, studies have clearly demonstrated swapping of these antibiotic resistance genes into integrons, therefore presenting integrons as important

tools in the rapid evolution and dissemination of antibiotic resistance among the gram-negatives.

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Chapter 2

Integrons, Genetic Elements Responsible For Acquisition Of Antibiotic Resistance Genes, And Their Prevalence Among Gram-Negative Enterics In Poultry Litter

ABSTRACT

Gram-negative, poultry litter isolates were screened by DNA: DNA hybridization for class 1 integrase, *intI1*. Thirty-six percent of gram-negative litter isolates were positive for *intI1*. In order to characterize these integrons further, PCR, using primers anchored in the 5' and 3' conserved sequences of class 1 integrons, was used to amplify their gene cassette(s). The sizes and number of the PCR amplicons varied with each organism and ranged in size from 0.6 to 4.0 kb. Despite the diversity of PCR amplicon sizes, there were four common PCR amplicons of 0.8, 1.0, 1.5, and 2.0 kb in coliforms, and non-coliforms, with the 1.0 kb being the most common. Eigty-one percent of the 1.0 kb, PCR amplicons contained the antibiotic resistance cassette, *aadA1*. Sixty-six percent of these *aadA1* integron cassettes were mapped to mercury-resistance transposon, Tn*21* in gram-negative litter isolates.

Introduction

Antibiotic resistance is an ongoing issue, not only in medicine, but also in food safety. The *Salmonella* Typhimurium DT104 strain of *Salmonella* is a prime example of

such a food pathogen of concern. The bacterium was originally associated with cattle, but spread to other food animals resulting in many vehicles of infection (Humphrey, 2001). Vehicles include unpasteurized milk, cooked meat, and direct or indirect contact with an infected animal. This pathogen can show resistance to up to nine different antibiotics, although five (ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracyclines) is more common. *Salmonella* Typhimurium DT104 has a chromosomal multi-drug resistant locus whose resistance genes match the phenotyp of these five common resistances (*aadA1, flo, tet, bla,* and *sul*). These resistance genes are flanked on either end by a class 1 integron.

Integrons are natural cloning systems that capture, insert, and mobilize antibiotic resistance genes. An integron is a genetic element that contains an integrase that mediates capture of antibiotic resistance genes into the integration site, *attC*. More than 60 different antibiotic resistance genes have been identified within gene cassette(s) of class 1 integrons (Fluitt & Schmitz, 1999).

Integrons are widely distributed in nature. Fifty-nine percent of human clinical isolates belonging to six different species of *Enterobacteriaceae* possessed class 1 integrons (Sallen et al., 1995). Some of these isolates also carried multiple copies of class 1 integrons. Integrons are widely disseminated among the aminoglycoside resistant isolates of *Enterobacteriaceae* and pseudomonads described by Levesque et al (1994). In addition to human isolates, integrons have a high incidence among veterinary pathogens and commensal, animal isolates. Distribution of this genetic element was highest in *E. coli* and *Salmonella*, although its frequency within these two groups varied depending on the animal source of the isolate (Goldstein et al., 2001). Characterization of antibiotic

susceptibilities of Shiga toxin-producing *E. coli* (STEC) found that most non-O157:H7 isolates were resistant to two or more antimicrobial classes. Multiple resistances to streptomycin, sulfamethoxazole, and tetracycline were most often observed, with class 1 integrons bearing the streptomycin/spectinomycin resistance gene *aadA1* (Zhao et al, 2001).

In seeing such trends, it was logical to ask where do antibiotic resistance genes in foodborne pathogens originate. Our hypothesis is that gram-negative enteric bacteria can serve as a reservoir for antibiotic resistance genes, which can be transferred via integrons to pathogenic bacteria. The objective of this study was to determine the prevalence of class 1 integrons in poultry litter of commercial broiler chickens by a DNA:DNA hybridization probing for class 1 integrase. These integrons were then characterized by PCR as well as identifying the organisms harboring these integrons.

Materials and Methods

Bacterial Strains. Litter was collected from two flock houses from two broiler chicken farms at placement of the chicks (0 weeks), 2 weeks, 4 weeks, and 6 weeks following placement over one grow out. Five litter samples were collected at each sampling time from each house on both farms. 5g of poultry litter was suspended in 45 ml of Phospahte Buffered Saline (PBS) pH 7.4. Samples were shaken using Burrell Scientific Wrist Action Shaker model 75 (Burrell Scientific; Pittsburgh, PA) at maximum setting for five minutes. Debris was removed by a low speed centrifugation (50xg for 15 min). The bacteria were pelleted by a high-speed centrifugation (3,650xg for 15 min), re-suspended in superbroth with 15% glycerol, and stored at –70^{O}C.

The frozen glycerol stocks of poultry litter bacteria were diluted in Buffered Saline Gelatin solution (BSG) pH7.0, ranging from 10^{-4} to 10^{-11}, and dispensed onto MacConkey Agar (MAC) (Becton Dickinson, Sparks, Maryland) and Brain Heart Infusion Agar (BHI) (Becton Dickinson, Sparks, Maryland) and incubated overnight at 37^{o}C. The dilution that yielded 30- 300 isolate colonies was used to inoculate colonies onto MacConkey Agar for isolation. Bacterial identification of litter isolates was done using Enterotube (Beckton Dickinson; Sparks, Maryland).

Screen of poultry litter, gram-negative enterics for *intI1* by DNA: DNA hybridizations. Fifty colonies were isolated from each sample and screened by DNA: DNA hybridizations for *intI1* as described by Bass et al (1). *E. coli* K12 strain SK1592 (pDU202: Tn*21*) and *Salmonella typhimurium* SR11 served as positive and negative controls respectively.

PCR amplification and characterization of antibiotic resistance gene(s) present in the class 1 integron. Integron gene cassettes were amplified by PCR as previously described (Levesque et al., 1994) using whole cell template (Hilton et al., 1997). PCR amplicons of 1 kb were characterized by Southern analysis. DNA was separated on a 0.8% agarose-1X Tris-acetate-EDTA gel and ethidium bromide (5 μg/ml) and transferred to nylon membrane using BIORAD Vacuum Blotter (BIORAD; Hercules, CA). The 1 kb ladder served as the molecular weight (MW) standards for determining the size(s) of PCR product(s). Single-stranded DNA was cross-linked to membranes with ultraviolet light (optimal cross-linking setting; Fisherbiotech UV Crosslinker). Membranes were

hybridized with 3' end blocked, biotin-labeled oligonucleotide probe for *aadA1* (GCAGCGCAATGACATTCTTG) according to procedure of Levesque et al (1994). Bound probe was detected with an anti-biotin Ab-alkaline phosphatase conjugate as specified in the protocol for the Genius 3 kit (Boerhringer Mannheim; Indianapolis, IN).

PCR screen for Tn*21*. Transposon Tn*2* was analyzed using GATAGCACTCCAGCCCGCAGAA and AGGATCTGCTCGGCCATTCC primers anchored in *tnpR* and *intI1*, respectively. This PCR primer pair was identified that amplified a 595 bp PCR product. PCR template was prepared according to procedure of Hilton et al (1997). PCR reaction mix contained 2mM $MgCl_2$, 0.1mM primers, 0.2mM nucleotides, 1.0 units Taq DNA polymerase per 10 μl PCR reaction (Boehringer Mannheim). The PCR program parameters for the Idaho Technology Rapidcycler (Idaho Falls, Idaho) were: 1) 94^{o}C for 0 s; 2) 55^{o}C for 0 s; 3) 72^{o}C for 15 s for 30 cycles. All the DNA products were analyzed by gel electrophoresis on 1.5% agarose, 1 X Tris-acetate- EDTA gel at 80V for one hour. The 100bp ladder (Promega, Madison, Wis.) served as the molecular weight (MW) marker standard.

Results and Discussion

Prevalence of class 1 integrons in gram-negative enterics present in poultry litter. We had previously determined that class 1 integrons were prevalent among *Salmonella* and *E. coli* isolates from companion and food animals (Goldstein et al., 2001). We screened gram-negatives from two poultry farms in Northeast Georgia for class 1 integrase. Prevalence of *intI1* among litter, gram-negative isolates increased during the maturation of commercial broiler chickens on both farms (Fig. 2). On average, Thirty-

seven percent of these litter isolates possessed the class 1 integrase; most prevalent among the coliforms in this environment (Fig. 3). 37% prevalence of *intI1* among these commensal poultry isolates was rather surprising since we previously reported that 66% of clinical poultry isolates possessed class 1 integrons (Bass et al., 2000; Goldstein et al., 2001). This discrepancy may be due to genetic differences between commensal and pathogenic *E. coli* and the physical linkage of antibiotic resistance genes to virulence plasmids or pathogenicity islands (Harnett and Gyles, 1984; 1985).

Gene cassette(s) of class 1 integrons in gram-negative enterics isolated from poultry litter. To characterize the integrons of our poultry isolates further, PCR, using primers anchored in the 5' and 3' conserved sequences of class 1 integrons, was used to amplify their gene cassette(s) (Levesque et al., 1994). The sizes and number of the PCR amplicons varied with each organism and ranged in size from 0.6 to 4.0 kb (Table 1, Fig. 4). Despite the diversity of PCR amplicon sizes, there were four common PCR amplicons of 0.8, 1.0, 1.5, and 2.0 kb in coliforms, and non-coliforms. PCR amplicons of 0.8, 1.0, and 1.5 kb were also common to *Enterobacteriaceae* isolated from different European hospitals (Martinez-Freijo et al., 1999).

Of the four amplicon bands, the 1.0 kb was most common in *E. coli*, *Klebsiella sp., Enterobacter, Citrobacter, and Serratia*, while the 2.0 kb PCR amplicon was more frequently observed in *Proteus sp.* Since the streptomycin/spectinomycin resistance gene, *aadA1* is generally associated with 1.0 kb, PCR amplicon (Bass et al., 1999; Fluit and Schmitz, 1999) and this gene is widespread in nature (Liebert et al., 1999), we determined the identity of this 1.0 kb PCR amplicon as *aadA1* by DNA:DNA

hybridization (Levesque et al., 1994). Eighty-one percent of 1.0 kb PCR amplicons contained the antibiotic resistance cassette, *aadA1*.

As determined by PCR, sixty-six percent of these *aadA1* integron cassettes mapped to mercury-resistance transposon, Tn*21* in our poultry litter isolates, similar to our earlier report concerning distribution of Tn*21* in avian *E. coli* (Bass et al., 2000). Thirty percent of our *Salmonella* isolates also contained Tn*21*-like transposon, as determined by PCR. Tn*21*-like transposons, which carry class 1 integrons, account for the high incidence of this element in nature (Dahlberg and Hermansson, 1995; Wireman et al., 1997; Zuhlsdorf and Wiedmann, 1992).

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Table 1. Number of class 1 integrons in litter, gram- negative enterics as determined by PCR. Primers were designed to amplify gene(s) present in class 1 integron's integration site, *attC*. The forward primer begins in integrase (position 1190-1206 in the published sequence) in the 5' conserved region of the class 1 integron. The reverse primer begins at the 3' conserved region of the class 1 integron preceding *qacΔE* (position 1342- 1326 in the published sequence).

PCR Amplicon(s)	Percent of Isolates (n= 717)
0	20
1	67
2	8
3	3
4	1
5	1

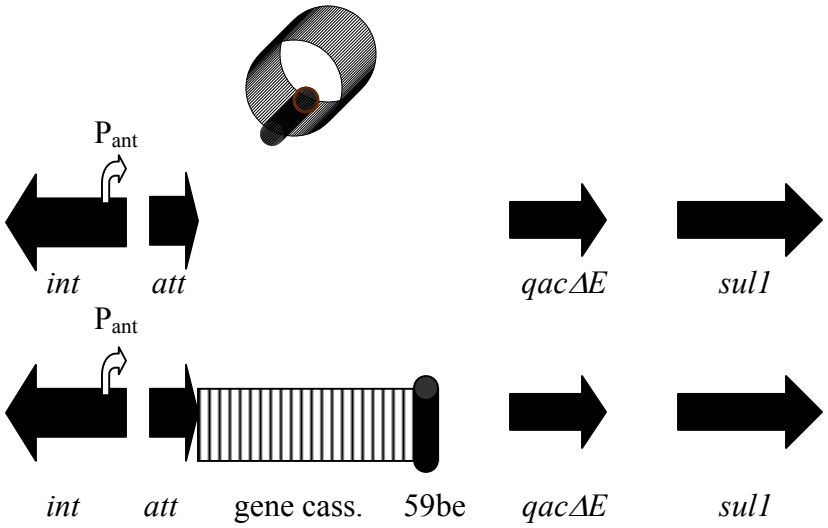
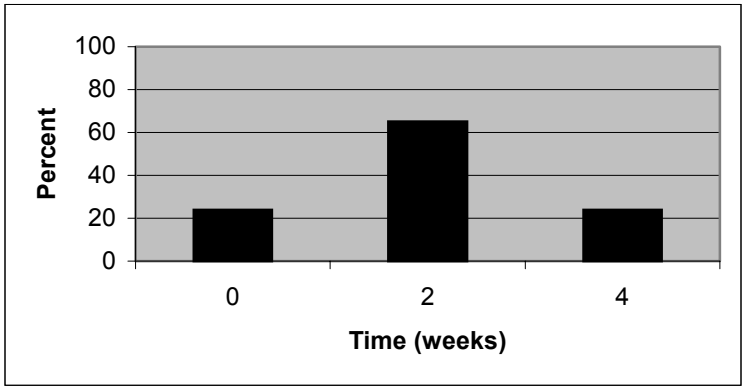


Figure 1. Schematic of a class 1 integron.

Farm 1



Farm 2

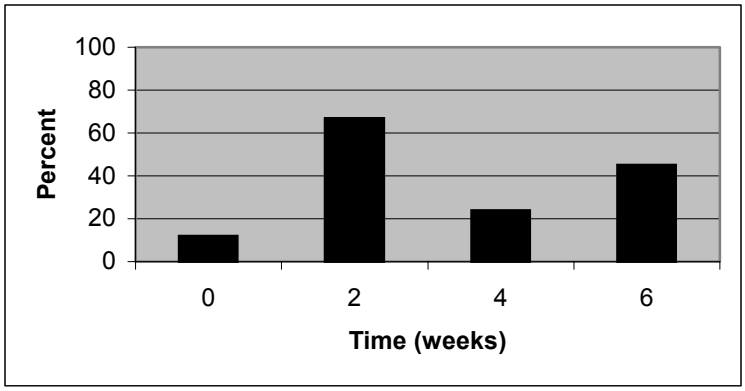


Figure 2. Prevalence of class 1 integrons among litter gram- negative enterics during maturation of commercial broiler chickens. Poultry litter, gram- negative, enterics from two separate farms were screened for *intI1* by DNA: DNA hybridization.

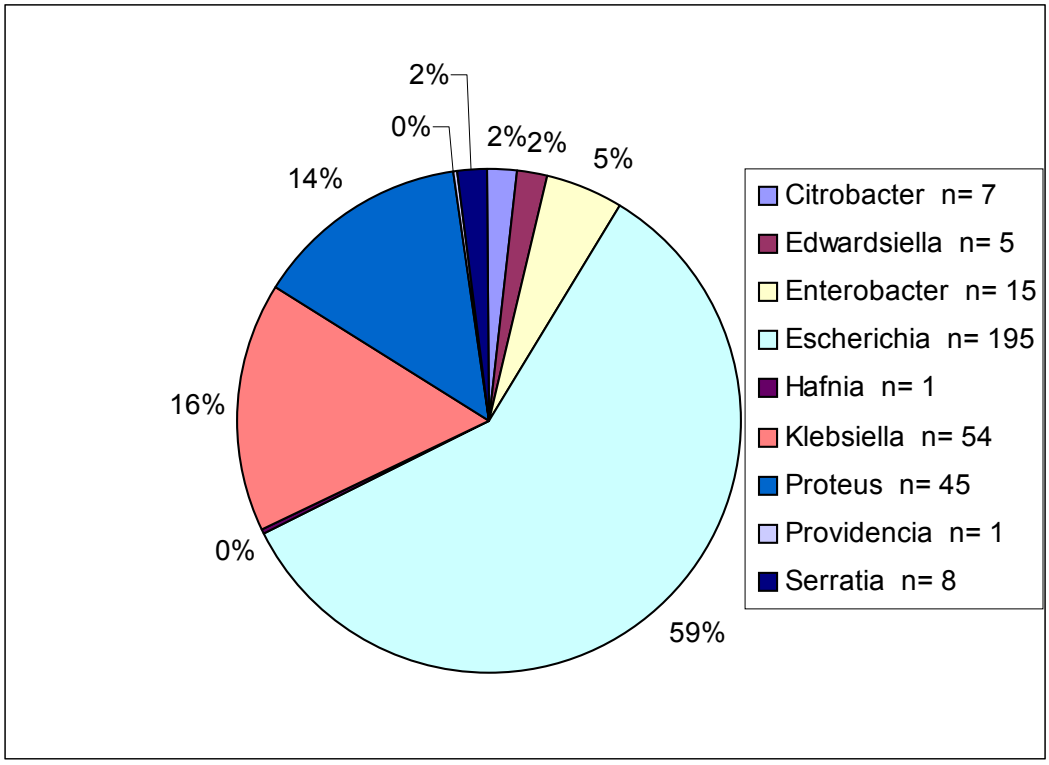


Figure 3. Incidence of class 1 integrons in gram- negative enterics in poultry litter.

Fig. 4A

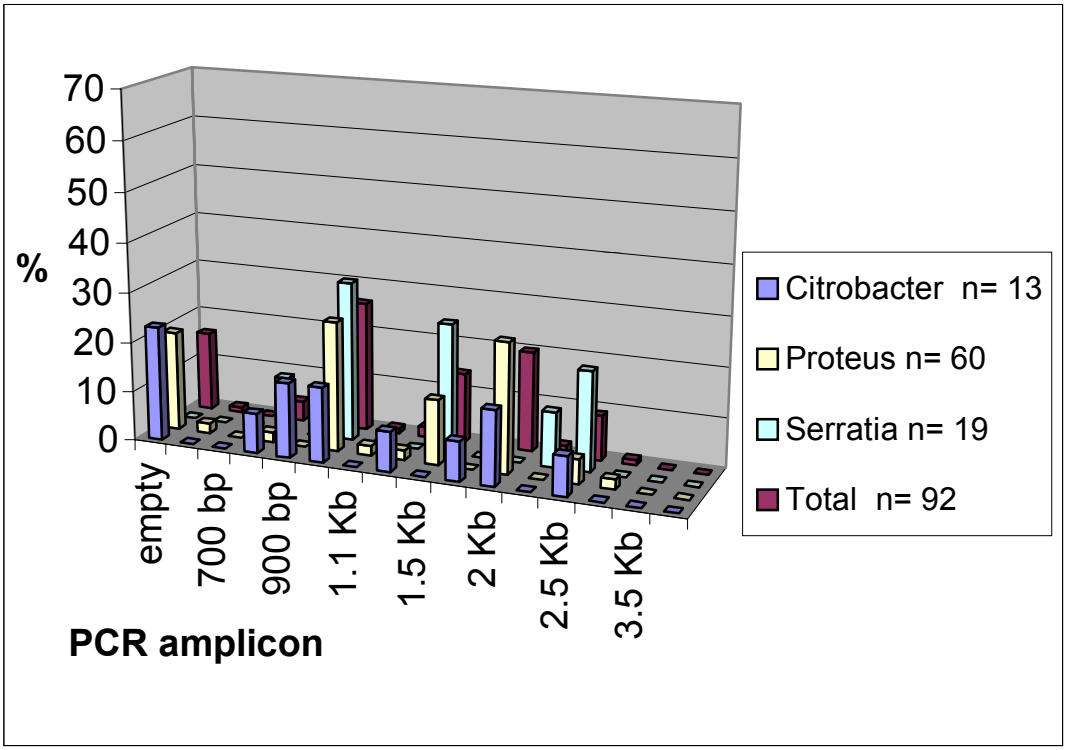

70
60
50
40
30
20
10
0
%
empty
700 bp
900 bp
1.1 Kb
1.5 Kb
2 Kb
2.5 Kb
3.5 Kb
PCR amplicon
Citrobacter n= 13
Proteus n= 60
Serratia n= 19
Total n= 92

Fig. 4B

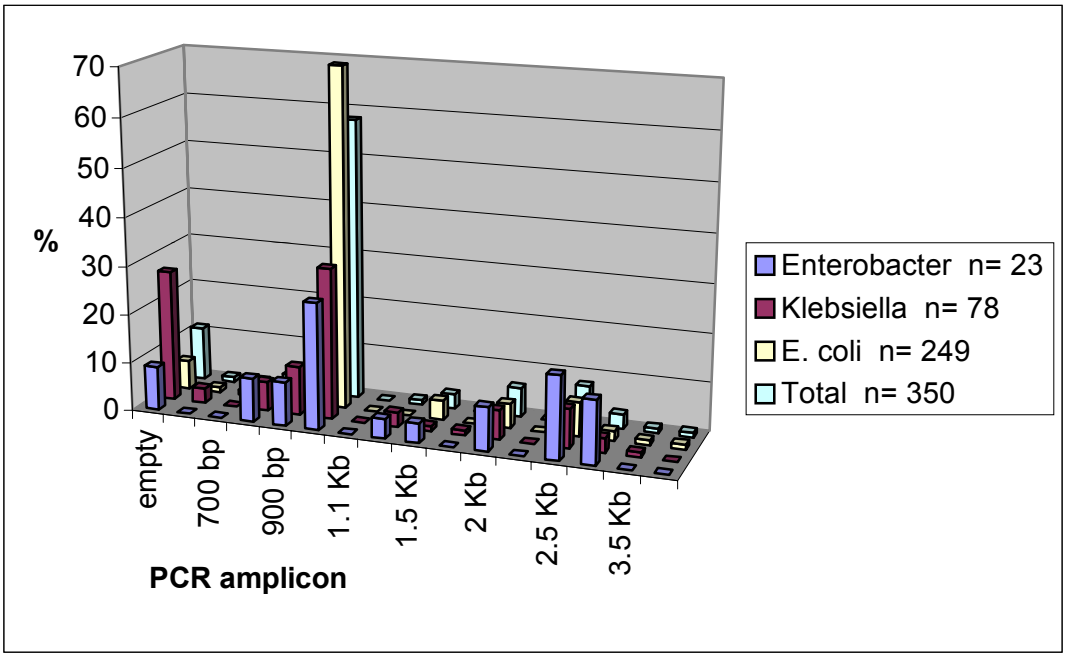

70
60
50
40
30
20
10
0
%
empty
700 bp
900 bp
1.1 Kb
1.5 Kb
2 Kb
2.5 Kb
3.5 Kb
PCR amplicon
Enterobacter n= 23
Klebsiella n= 78
E. coli n= 249
Total n= 350

Fig. 4C

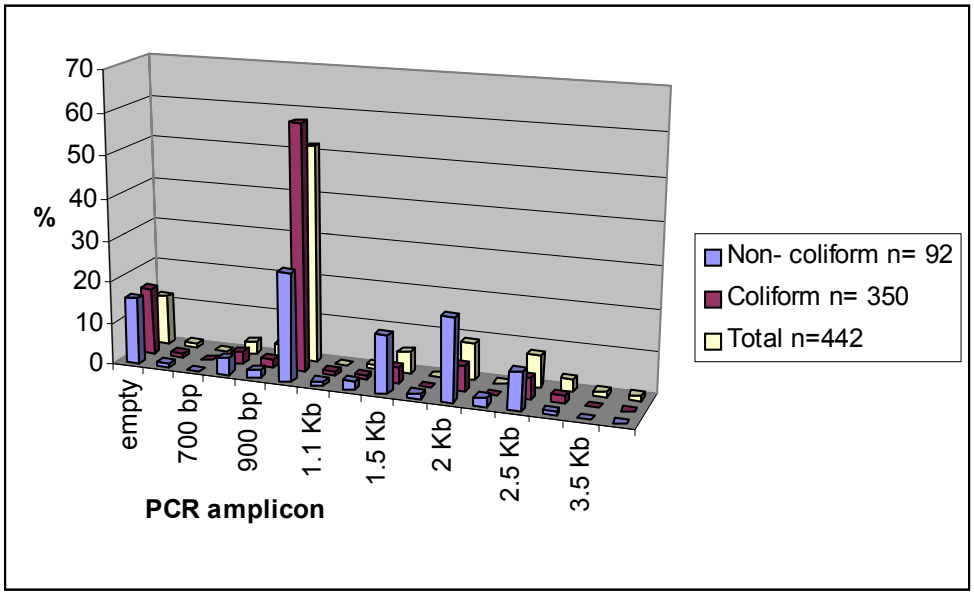


Figure 4. Class 1 integron, PCR amplicon in gram- negative enterics isolated from poultry litter. Integrons were amplified by PCR. PCR amplicons of less than 1.5 Kb contain single, antibiotic resistance genes while larger amplicions contain multiple antibiotic resistance genes. (A) Non- coliform gram- negative enterics (B) Coliform gram- negative enterics (C) Non- colifrom and Coliform gram- negative enterics.

Chapter 3

Conclusion

Some people say that one of the immediate causes of the collapse of the Roman Empire was a series of epidemics and plagues. The doctors of the day were powerless in the face of these disasters, which led to an almost universal reaction against the scientific and rational approach to disease, which was still slowly evolving. In fact, epidemics have played major roles in the world's history. Besides the Black Death, there was dancing mania, St. Vitus' dance, St. Anthony's fire, the English sweating sickness, and king's evil. Each of these played a role in the devastation of Europe at some point. Could we be bound to repeat history yet again? Antibiotic resistance may seem miniscule in the mind of the average person. But if pathogens are defiant to what treatments we have, won't we be in the same situation as the Roman Empire where the doctors will be helpless and completely unable to cure those that become infected. Maybe the mad cycle is simply starting with the less harmful organisms such as *E. coli, Salmonella, Proteus,* and *Klebsiella*. Who is to say that it won't spread further over time to pathogens such as *Bacillus, Clostridium, Mycobacterium, Borrelia,* etc. The list could go on forever and the potential of how large this chaos could become.

Maybe it's paranoia and far fetched, but when there is preventive maintenance available, why would someone even think of taking that risk? Doctors should prescribe

more specific antibiotics and only when its necessary. They don't need to give a child ampicillin for a viral infection to make the mother feel better. He is the doctor, not her. When someone is prescribed a medication, take it all for the full period of time, and don't share your medication with someone who comes over with a little cough. Maybe our farmers don't need to be using antibiotics in their feed to raise larger animals. Maybe the citrus farmers shouldn't use antibiotics on their trees, which end up blowing all around the trees and land. Darwin's theory of "survival of the fittest" still applies to bacteria. If we don't expose ourselves to a little dirt and grime, we don't build up our immune system to fight off foreign invaders.