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Prevalence of Class 1 Integrons and Antibiotic Resistance Patterns in Bacteria of Swine and Chicken in the US and Thailand

Sumalee Liamthong
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Sumalee Liamthong entitled "Prevalence of Class 1 Integrons and Antibiotic Resistance Patterns in Bacteria of Swine and Chicken in the US and Thailand." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Alan G. Mathew, Major Professor

We have read this dissertation and recommend its acceptance:

Arnold M. Saxton, David A. Bemis, Charles H. Goan, Michael P. Davidson

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Sumalee Liamthong
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ABSTRACT

Studies were conducted to investigate the prevalence and potential for transfer of class 1 integrons and antimicrobial resistance in bacteria of broiler chickens and swine from the US and Thailand. Antibiograms were characterized and integron sequences were detected using standard methods. To determine if transfer of integrons occurred between bacterial species the location of the integrons (plasmid versus chromosome) was determined, and when integron-positive *E. coli* and *Salmonella* isolates possessed identical amplicon patterns, PCR products were sequenced to determine homology. Class 1 integrons were detected in 1,732 of 3,824 isolates from broiler chickens and 1,782 of 4,253 isolates from swine. Simultaneous presence of three conserved class 1 integron genes was found in 1,044 and 215 of isolates from chickens and swine, respectively. A high proportion of bacterial isolates from chickens demonstrated resistance to tetracycline, sulfamethoxazole, cephalothin, and ampicillin. A high proportion of isolates from swine demonstrated resistance to tetracycline, sulfamethoxazole, streptomycin, and ampicillin. Nine integron amplicons, with sizes ranging from 0.5 to 2.5 kb, were found, and we discovered a single swine farm on which similar integrons were observed in both *E. coli* and *Salmonella*. Sequence analysis revealed that a 1.0 kb amplicon found in both bacterial species contained an *aadA1* gene cassette encoding aminoglycosides 3'-adenyltransferase, confering resistance to streptomycin and spectinomycin. A 2.0 kb amplicon was also found in both types of bacteria containing the *aadA5* gene encoding aminoglycosides 3'-adenyltransferase, an additional reading frame with unknown function, *orfD,* as well as a *dfrA17* gene encoding dihydrofolate reductase, conferring

resistance to trimethoprim. Our results indicate that class 1 integrons are common in commensal and foodborne bacteria in broiler chickens and swine, and that some, but not all antibiotic resistances are associated with the presence of class 1 integrons. Identical integrons found in *Salmonella* and *E. coli* from a single farm likely indicate transfer between these two organisms occurs via exchange of plasmids. This work provides additional knowledge regarding the complex nature of antibiotic resistance gene acquisition, reservoirs, and transfer that should aid in development of courses of action and strategies for control of these potential foodborne and zoonotic hazards.

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CHAPTER 1 : INTRODUCTION

Since their discovery early in the 20th century, antibiotics have remained in extensive use for human and animal therapies. Subsequently, in the 1950s, antimicrobial compounds were found to produce consistent benefits in livestock production and have thus been used extensively to enhance growth and efficiency of animals, and thus profitability of production systems. However, significant evidence exists indicating that continued and extensive use of antibiotics for human medicine and animal production has led to an increased prevalence of drug-resistant bacteria, possibly affecting the long term usefulness of these important compounds (1). Of primary concern is a loss of efficacy of antibiotics that may offer a last line of defense against multi-resistant bacteria (3, 7). Thus, the use of some antimicrobial compounds, such as vancomycin and fluoroquinolones, has come under great scrutiny. While a number of studies have been conducted to characterize factors affecting resistance to derive strategies for control, a common consensus remains that too little information is available to guide appropriate agencies in formulation of regulations for agricultural use of antimicrobials (4).

A number of factors appear to affect the dissemination of resistance genes and thus prevalence of antimicrobial resistance in bacteria associated with livestock. These factors may include animal age, stress, husbandry practices, and on-farm use of antimicrobials (4, 10-13). The majority of methods by which bacteria become resistant involve the acquisition of exogenous DNA that confers the ability to resist an antimicrobial drug. Such acquisition typically takes the form of transduction by phage, appropriation of transposable DNA, and/or the reception of plasmid DNA.

In recent years, many resistance genes isolated from bacteria have been mapped to specific genome sites known as integrons. Integrons have been found in plasmids,

transposons, and as independent units on bacterial chromosomes (2, 5, 8). By definition, integrons contain three elements that allow the site-specific recombination of antibiotic resistance genes. These elements include: 1) a recombination or attachment site; 2) an integrase, which recognizes specific sequences on the extra-integron gene cassette and the recombination site; and 3) a strong promoter that allows the integron to act as an expression vector in the event of the incorporation of promoterless cassettes (9, 14). As such, bacteria that harbor integrons may have an enhanced ability to rapidly develop resistance to multiple antibiotics, and to promote the transfer of highly stable and self-promoting resistance factors across their own and other bacterial species. Thus, bacteria containing integrons pose a particularly insidious threat to the efficacies of current as well as future antimicrobials. And while it is proposed that the primary vehicle for transfer of bacterial resistance genes from animal hosts to human hosts is through food borne bacteria, the large pool of naturally occurring nonpathogenic bacteria in the gut, including *E. coli,* has been proposed to act as a reservoir of and/or vector for transferable resistance genes. These commensal bacteria, by their residence in the GI tract, are subject to exposure from antibiotics included in feeds and water, and they have been shown to acquire resistance following such exposure (10-12) demonstrating a higher prevalence of resistance genes and genetic resistance elements, including integrons (6). It is widely accepted that these genes are transferable across species of bacteria, and thus the potential exists for transfer from resident *E. coli* and other naturally occurring bacteria to transient animal and human pathogens, including *Salmonella*. However, to date, little evidence has been presented to clearly show that such transfer is common in vivo.

Understandably, representatives from consumer groups, the livestock industry, and government agencies continue to call for more information in order that sensible and effective recommendations to reduce antimicrobial resistance can be formulated. Through this proposed work, we hope to further the understanding of factors that promote antibiotic resistance and the prevalence of integrons and other resistance elements in bacteria associated with livestock.

The U.S. and Thailand, while occupying very diverse geographic regions, share many commonalities in livestock production practices. Both swine and poultry production tends to be intensive, with closed confinement, high biosecurity and similar high production genetic lines. Feed-based antibiotics are commonly used in both countries, although some differences occur in drugs of choice and/or availability. A comparison of antibiotic resistance genes and resistance prevalence between these two countries may provide information regarding global implications of agricultural use of antibiotics with regard to antibiotic resistance.

The hypothesis of this study is that antimicrobial resistance genes and transferable genetic units are common in bacteria associated with livestock, and prevalence may be affected by antibiotic use practices, livestock species, and geographic region. This study further proposes that resistance genes may be shared among non-pathogenic bacteria and foodborne pathogens that may concurrently reside within production animals. Therefore, this study was designed to investigate resistance patterns and prevalence of specific antimicrobial resistance genes and class 1 integrons in *E. coli* and *Salmonella* among swine and poultry in the US and Thailand. As a possible indication of gene transfer, we determined the degree of homology of resistance genes and integron sequences between

those two groups of bacteria within animals and farms. Data generated from the US and Thailand was compared to determine if similar patterns exist across these diverse geographic regions.

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CHAPTER 2 : LITERATURE REVIEW

I. ANTIBIOTICS

Definition and Classification of Antibiotics

The term antibiotic, from the Greek *anti*, meaning "against" and *biotikos*, meaning "concerning life", first appeared in a scientific letter in 1942 by Waksman (224, 225) to describe a newly discovered compound that specifically inhibited the growth of other microorganisms. The term antibiotic originally described only those compounds derived from microbial origin; however, it now extends to include any low-molecular weight compound, whether from microbial or other living organisms or even of semi-synthetic or synthetic origin, that can inhibit the growth or kill other microorganisms. The majority of antibiotics used in human and veterinary medicine are natural products produced by three main groups of microorganisms: actinomycetes, eubacteria, and filamentous fungi. The actinomycetes produce the largest number and greatest variety of antibiotics, with more than six thousand substances isolated from that group of microbes (114).

Antibiotic agents can be classified according to various criteria. Based on target microorganism, antibiotics are classified as antibacterial, antiviral, antifungal and antiparasitic. Although some drugs can target both bacteria and protozoa (e.g. ionophores used for control coccidiosis can also kill some Gram-positive bacteria), all antibacterials are inactive against viruses and fungi at normal therapeutic concentrations (84). On the basis of the spectrum of drugs, antibacterial agents can be classified as broad- or narrow-spectrum drugs (84, 122) . Broad-spectrum drugs are generally active against a wide variety of bacterial species, including Gram-positive, Gram-negative,

aerobic, and anaerobic microorganisms. Tetracycline, phenical, fluoroquinolones, third- and fourth-generation cephalosporins, and carbapenem are examples of broad-spectrum antibiotics. Drugs with narrower-spectra are mainly active against specific bacterial groups such as Gram-negative bacteria (polymyxins), Gram-positive bacteria (glycopeptide, natural penicillin), aerobes (aminoglycosides and sulfonamide), or anaerobe (nitroimidazoles) (84). Based on antibacterial activity of the drug, antibacterials are classified as bacteriolytic, bactericidal, and bacteriostatic. Bacteriolytic and bactericidal antibiotics both kill bacterial cells; however, bacteriolytic antibiotics induce killing by cell lysis and include those antibiotics that inhibit cell wall synthesis and those that damage cell membranes. In contrast, bactericidal antibiotics kill the organism but do not always rupture or lyse the cell. Antibiotics that inhibit the growth but do not kill are known as bacteriostatic. Bacteriostatic antibiotics often inhibit protein synthesis and act by binding to ribosomes. The type of antibacterial activity of a drug depends on how the drug binds to its target. Usually, drugs that kill bacterial cells bind to their target irreversibly or with high affinity and are not removed by dilution, whereas bacteriostatic drugs form unstable bonds, therefore when concentration is lowered, it becomes free from the target and the bacterium resumes growth. Sometimes, bactericidal and bacteriolytic drugs can appear as bacteriostatic if effective killing concentrations in blood and tissue are not achieved (155).

Antibiotics Use in Livestock Production

Antibiotics are used in food animals for four main purposes: therapeutic use to treat diseased animals, metaphylaxis or short term medication to treat diseased animals

and prevent infection in other animals, prophylactic use to prevent infections at times of risk, such as transport or weaning, and growth promotion to improve feed utilization and production (Table 1) (140, 219).

Benefits of Antibiotic Use in Agriculture

Potential benefits associated with using antibiotics in food animals include the treatment of disease, improvement of carcass quality, and improvement of feed efficiency (10). The use of feed-based antibiotics has consistently been shown to benefit livestock production, increasing the ability of farms to maintain profitable margins (52, 155, 165), lowering manure output and thus reducing effects of animal wastes on the environment (180), and lowering animal pathogen loads and carriage of foodborne pathogens in livestock (68, 87, 123). Many foodborne pathogens are not easily controlled in livestock by vaccines, and as these organisms have a commensal association with their food animal hosts, making eradication difficult, if not impossible. However, limiting their numbers in the gut with feed based antibiotics or water additives may be a practical approach to limiting foodborne transfer of these organisms (165). Given the above, it appears prudent that such benefits be included in risk assessment models evaluating antibiotic use in food animals, so that more realistic evaluations result and a balance is achieved that provides the greatest protection from inherent risks while maximizing the overall benefits to society.

Table 1. Types of antimicrobials use in food animals. [1,2]

Type of antimicrobial use	Purpose	Route or vehicle of administration	Administration to individual or group[a]	Diseased animals
Therapeutic	Therapy	Injection, feed, water	Individual or group	Diseased individuals; in groups, may include some animals that are not diseased or are subclinical
Metaphylactic	Disease prophylaxis, therapy	Injection, feed, water	Group	Some
Prophylactic	Disease prevention	Feed	Group	None evident, although some animals may be subclinical
Subtherapeutic	Growth promotion	Feed	Group	None
	Feed efficiency	Feed	Group	None
	Disease prophylaxis	Feed	Group	None

[1] Adapted from McEwen and Cray. (140)
[2] Food animals are usually grouped by pen, flock, pond, barn, or other aggregate.

1. Antibiotic Use and Resistance Associated with Swine

USDA NAHMS data provide some indication of the prevalence of antibiotic use in US swine production (214). It was found that 92% percent of farms surveyed had used antibiotics in the six-month period prior to the survey, with most being delivered through the feed. More than 85% of sites used in-feed antibiotics in the grower/finish phase. Most commonly used were tylosin, chlortetracycline, and bacitracin, with 56%, 43%, and 35% of sites using each, respectively. However, recent changes in the swine industry, a growing awareness of issues surrounding nontherapeutic uses of antibiotic products, and changing trends in therapeutic and nontherapeutic regimens may have caused a change in the overall use of antibiotics in more recent times, particularly in grower/finisher units and in high health herds where advantages of extended antibiotic use are less easily demonstrated (51, 64)

Through applied studies, a link between antibiotic use in swine and increased prevalence of resistant bacteria can be clearly demonstrated (133, 134). While such studies have shown significant increases of antibiotic resistance in the gut flora following use of antibiotics, it has also been shown that rapid reversion to susceptibility in commensal microflora following drug withdrawal may also occur, depending upon drug type. Studies with the aminoglycoside drug apramycin have shown that the general population of fecal *E. coli* demonstrate an increase in apramycin resistance soon after initiating the use of that antibiotic; however, this increase was followed by a return to more normal susceptibility when the drug was withdrawn (134, 135). As such, and as this

antibiotic was used exclusively in young pigs, the impact of apramycin use would appear to be minimal with regard to resistance of *E. coli* in market animals.

A study conducted by Gellin et al. (81) examined antibiotic resistance in experimental swine herds and found that 36.4%, 74.3% and 99.6% of *E. coli* isolates obtained from a herd regularly exposed to antibiotics were resistant to ampicillin, streptomycin and tetracycline, respectively. The same study examined resistance levels in *E. coli* isolated from a different herd that had not been exposed to antibiotics in over 50 weeks and found that only 0.5%, 12.4% and 26.7% of isolates were resistant to those same antibiotics, respectively. Another study by Mathew et al. (136) showed that 98%, 64.4%, 86%, and 29% of *E. coli* isolated from commercial swine farms where antibiotics were used extensively were resistant to tetracycline, neomycin, gentamicin, and apramycin, respectively, by the time pigs reached 63 days of age.

2. Antibiotic Use and Resistance Associated with Poultry

Antibiotics are used in the poultry industry for therapeutic, nontherapeutic and growth promotion (55, 129). Growth promoting antibiotics used in US poultry production include chlortetracycline, bacitracin, bambermycin, tylosin, and virginiamycin (55). Bacterial diseases, including colibacillosis, enteritis caused by *Clostridium* spp., mycoplasmosis, and several forms of salmonellosis, cause significant economic loss to the poultry industry (20, 88) and are a primary reason for treatment with antibiotics (196). Common antibiotics used for control of these organisms include sulfonamides, amoxicillin, tetracyclines, tylosin, virginiamycin, neomycin, and penicillin. Until recently, enrofloxacin, a fluoroquinolone drug, was approved for control of colibacillosis;

however, concerns that fluoroquinolone use in poultry may be linked to antibiotic-resistant *Campylobacter* infections in humans (39, 148, 166) caused the FDA to ban the use of that drug in poultry in 2005 (75).

NARMS data indicate that *Salmonella* from chickens have demonstrated increased resistance to amoxicillin/clavulanic acid, ceftiofur, cefoxitin and tetracycline since the NARMS program was initiated (151). In 1997, 0.5% of slaughter isolates were resistant to amoxicillin/clavulanic acid, 0.5% were resistant to ceftiofur, 0% were resistant to cefoxitin, and 20.6% were resistant to tetracycline. Preliminary data from 2005 indicate that 12.1%, 12.2%, 12.0% and 28.3% of *Salmonella* isolates from poultry at slaughter were resistant to those same antibiotics, respectively. A slight increase in resistance to ampicillin was noted over that same time period, whereas little or no change was noted for resistance to chloramphenicol, ciprofloxacin, kanamycin, and streptomycin. Marked decreases have occurred for resistance to gentamicin and sulfa drugs over that same time period. In 1997, 17.8% and 24.8% of isolates were resistant to gentamicin and sulfamethoxazole; whereas in 2005, 4.3% and 8.5% were resistant to those same drugs, respectively.

Cui et al. (54) conducted a study comparing prevalence and resistance of bacteria from conventionally raised chickens to organically raised chickens in Maryland. In that study, all *Salmonella* isolates derived from conventionally raised birds were resistant to 5 or more antibiotics, whereas 79% of isolates from organically raised birds were sensitive to all 17 antibiotics tested. However, as isolates were derived from poultry products of retail markets, it is unknown what effects, if any, were the result of processing location or methods. Harwood et al. (97) observed vancomycin resistant *Enterococcus* (VRE) from

chicken feces, and from hospital waste water, but not from dogs, cattle, pigs, wild birds, raccoons feces, or surface water from 3 main waterways in Florida; however, the sources of chickens and other animals were not described. In that study, *Enterococcus* spp. resistant to "low" concentrations of vancomycin (3 μg/ml) and harboring the *vanC* gene were isolated from chickens. By comparison, VRE (*E. faecium* and *E. avium*) resistant to "high" levels of vancomycin (10 μg/ml) and harboring the *vanA* gene were readily isolated from hospital waste water. Two *Enterococcus* isolates from chicken feces that were resistant to high levels of vancomycin were identified as *E. gallinarum*.

Fairchild et al. (74) investigated effects of tetracycline administration on cecal commensal bacteria, *Enterococcus* spp., *E. coli* and *Campylobacter* spp. They observed that *Enterococcus* spp. and *E. coli*, resistant to tetracycline and harboring a number of different tetracycline resistance genes were readily isolated in chickens, regardless of exposure or non-exposure to that drug. Tetracycline treatment in test birds did not produce tetracycline resistance in *Campylobacter* spp. in their study; however, tetracycline-resistant *Campylobacter* spp. were readily isolated from flocks that received and did not receive that antibiotic. The investigators concluded that complex population dynamics and genetics in enteric bacterial populations contributed to the antibiotic resistance observed in commercial flocks.

Risks Posed to Humans by Antibiotic Use in Livestock

It has been well established that agricultural use of antibiotics results in increased prevalence of antibiotic-resistant bacteria in farm environments, thus contributing to the global pool of antibiotic resistant organisms. However, what risks this poses to human

health has not been clearly established. Foodborne transfer of bacteria carrying resistance genes is the most likely route through which agricultural use of antibiotics could affect human health. However, some evidence for direct animal to human transmission of antibiotic resistant bacteria has been reported (27, 102, 103, 111).

Beyond selection for antibiotic resistant bacteria, there have been some concerns that on-farm use of antibiotics may also increase pathogen load in animals by selecting for pathogens that are known to possess antibiotic resistant genes, integrons, or genetic islands containing resistance genes. These organisms may have an advantage under the selective pressure of antibiotic use, aiding in their colonization, which could then result in a greater pathogen load. A frequently cited study supporting this hypothesis is that of Williams et al. (231). Using swine infected with a chlortetracycline-resistant strain of *S.* Typhimurium, they showed that subsequent treatment of infected pigs with chlortetracycline increased both the quantity and duration of shedding of that challenge organism. However, several subsequent studies have failed to show that antibiotic use caused increased pathogen loads, and following an extensive review of the literature the US Food and Drug Administration determined that no evidence existed to support the need for pathogen load analysis as a part of their Guidance Document # 152 FDA-CVM (76). In fact, some studies have shown that antibiotic use in livestock reduced shedding of foodborne pathogens (68, 87, 123).

There are several confounding factors that make the assessment of the risks posed by agricultural use of antibiotics difficult. A primary difficulty is that a large number of the antibiotics used in livestock production are also used in human and pet medicines, thus presenting difficulties in determining the initial sources or reservoirs for the resistant

populations. For example, it would be difficult to assign blame for the increase in sulfonamide resistance to use of sulfa drugs in livestock when sulfonamides have been used extensively in humans for prevention of acne, urinary tract infections, and diarrhea, among other common uses (95, 215). Cross resistance within and across families of antibiotics is also a confounding factor, as some antibiotics used solely in human medicine can select for resistance to other drugs which may be used primarily in livestock, and vice versa. As indicated earlier, broad mechanisms of resistance, such as efflux pumps, may be increased by use of a single antibiotic, but may subsequently confer resistance to unrelated antibiotics, making it difficult to determine the initial agent of selection. The fact that antibiotic resistance develops from both therapeutic and nontherapeutic use (81, 119) presents some additional difficulty in establishing a point from which to consider risks. Risk assessments focusing on nontherapeutic uses, the primary concern of agricultural use, would likely be confounded by resistance selection caused by therapeutic uses commonly applied for chronic diseases. It follows then that elimination of veterinary use of some antibiotic products may not translate into reductions of some antibiotic resistance patterns in bacteria of concern.

Some efforts have been undertaken to model antibiotic resistance and determine quantitative or semi-quantitative risks associated with agricultural use of antibiotics (19, 222). Such efforts have been conducted using defined risk assessment approaches, as opposed to precautionary principle approaches, for assessment and development of control strategies (223). However, the current lack of numerical or empirical data in key areas has hampered those efforts and/or caused some to doubt the validity of such risk assessments. Still, these attempts have indicated that direct risks of on-farm antibiotic

use may not be as significant as originally projected, primarily due to low risk elements in the steps between the movement of resistant organisms off of farms and the projected failure of a human therapy as a result of the agricultural use (50, 105). As an example, Hurd et al. (105) conducted a semi-quantitative risk assessment of the potential impact of using the macrolide antibiotics tylosin and tilmicosin in the various livestock commodities. The analysis was conducted based on guidelines outlined in the Guidance for the Industry Document #152 (76). In that analysis, it was conservatively assumed that all occasions of tylosin and tilmicosin use in pigs or poultry would lead to macrolide-resistant bacteria, including *S. enterica* serovars, *Campylobacter* spp. and *E. faecium;* and that those bacteria would contaminate meat and poultry products, causing foodborne illness at rates cited by CDC FoodNet data. However, as human foodborne illnesses caused by those organisms are seldom, if ever, treated with macrolide antibiotics, the risk of failure of a macrolide-mediated antibiotic therapy was negligible. In the analysis, the overall risks for consumption of poultry, swine and beef were estimated to be 1 in 14 million, 1 in 53 million, and 1 in 236 million cases per year in the US, respectively, for *Campylobacter* (combined risk *C. coli* and *C. jejuni*), and 1 in 3 billion, 1 in 21 billion, and 1 in 29 billion cases per year in the US, respectively, for *E. faecium*. As a comparison, the FDA, in their risk assessment of fluoroquinolone-resistant *Campylobacter* attributed to the consumption of chicken, estimated the risk at 1 in 32,900 cases, and determined this level of risk to be "low" (77). Using similar techniques, Cox and Ricci (49) estimated that a ban on enrofloxacin use in poultry would prevent less than 1 severe incident per year in the US, while causing approximately 6,600 additional cases of campylobacteriosis and more than 40,000 excess illness days.

The Origin of Antibiotic Resistance

It well known that a number of bacterial and fungal species possess the ability to produce antibiotic compounds, typically to gain a competitive advantage in microorganism-rich environments, including soils and biofilms (8). It is likely then that naturally-occurring antibiotics were present in the environment long before the first antibiotic agents were introduced into clinical use (189). Many antibiotic products used in human and animal medicine today have their origins in antibacterial compounds produced by organisms such as *Streptomyces, Bacillus, Penicillium*, *Cephalosporium* and *Pleurotus*. As researchers have been able to identify those compounds and their active components, development of more potent analogs has been possible (190).

Antibiotic resistance likely also emerged in nature prior to human use of drugs as organisms producing antibiotic compounds required the means to survive in the presence of their own products, and competing species also found ways to counteract effects of those compounds (59). Thus, some antibiotic resistance genes likely originated long before the advent of man, modern medicine, and agricultural use of antibiotics. Trieu-Cuot et al. (212) have shown that resistance to kanamycin due to aminoglycoside-phosphotransferase-3' developed in antibiotic-producing bacteria *Bacillus circulans* and *Streptomyces fradiae* as a mechanism of self-protection. The ancestor gene of *erm* genes, responsible for macrolide antibiotic resistance, was implicated in erythromycin resistance in antibiotic–producing strains of *Streptomyces erythreus* and *Arthrobacter* as well as in Gram-positive cocci and bacilli bacteria (15). Some aminoglycoside–inactivating enzymes, responsible for aminoglycoside resistance, appear to have originated from aminoglycoside-producing bacteria, such as *Streptomyces*, *Micromonospora*, and

Bacillus (58, 117, 194). The genes, encoding ribosomal protection proteins mediating tetracycline resistance and found in pathogenic and saprophytic organisms, show high similarity to genes found in tetracycline-producing strains. (9). As indicated by many researchers, sensitive microorganisms can gain resistance to antibiotic agents by directly or indirectly acquiring these resistance genes from antibiotic-producing organisms. For example, D'Costa et al., (56) suggest that soil microbes provide a large reservoir of antibiotic resistance genes that can be quickly mobilized into other microbial communities, including enteric bacteria and pathogens, under the selection of antibiotic use. Benveniste and Davies (23) suggested that dissemination of antibiotic resistance genes has been accelerated due to the presence of bacterial DNA, carrying resistance genes in antibiotic preparations. Other research using PCR amplification of known resistance genes, demonstrated that contaminating DNA from *Streptomyces* spp. used in industrial production of antibiotics, resulted in a source of antibiotic resistance genes in commercial antibiotic preparations (226). When the resistance genes are transferred across species or genus of bacteria, they may undergo mutation in their new host resulting in a wide variety of differences in structural but similar functional resistance determinants (189). The evolution of efflux proteins associated with tetracycline resistance in both Gram-negative and Gram-positive bacteria is frequently cited as an example of such divergent mutation (177). The stepwise mutation of genes whose product plays a role in physiological cell metabolism is another way for the bacteria to develop resistance properties. The substrate spectrum of the gene products will change from metabolite of biosynthesis or biodegradative pathways to certain antibiotic agents only (189). The enzymes exhibiting acetyl-, adenyl- or phosphotransferase activities that

inactivate aminoglycosides or chloramphenicol are believe to have evolved through this mechanism (108). Modification of target structure is also a way that bacteria become resistant to antibiotic agents. This modification can occur either by single-step, e.g. in streptomycin resistance, or multiple-step, e.g. in fluoroquinolone resistance (7). As antibiotic use became commonplace in human medicine and food animal production, selection pressure increased the advantage of maintaining resistance genes in diverse groups of bacteria, and bacterial evolutionary progress eventually included mechanisms to retain, accumulate and disperse resistance genes among bacterial populations (1) (Figure 1).

Among the first reports to suggest that antibiotic use in livestock promoted resistance was that of Starr and Reynolds (200) who noted streptomycin resistance in coliform bacteria from turkeys that had been fed that antibiotic. Other researchers reported the association of resistance to antibiotics in fecal streptococci when growth-promoting levels of tetracycline were fed to chickens (70). Since that time, numerous studies have demonstrated a link between antibiotic use in livestock and increased prevalence of antibiotic resistant organisms associated with those animals, the farm environment, and in some cases agricultural products (140, 232, 233).

The Debate over Antibiotic Use in Livestock

With concern over antibiotic resistance growing, a number of organized deliberations on the issue began to occur, including that of the Netherthorpe Committee (47) and the Swann Committee (204). Both groups focused specifically on antibiotic use in food animals, and came to different conclusions regarding the risk of such to human

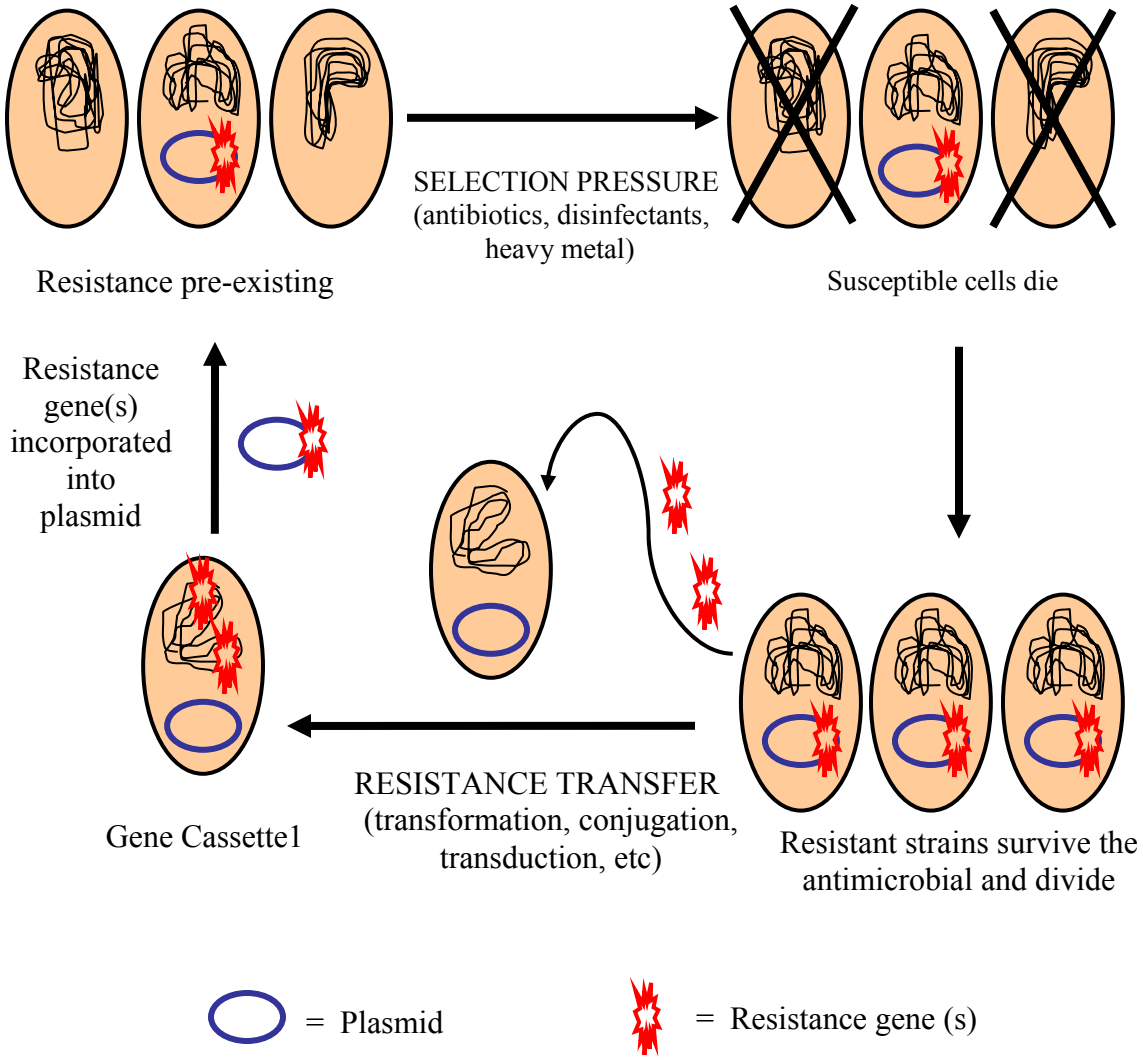

Figure 1. The emergence of antimicrobial resistance. Possible pathway depicting the emergence and transfer of antimicrobial resistance and/or antimicrobial resistant bacteria. Adapted from McDermott et al. (139).

Health. The Netherthorpe report concluded there was no evidence that agricultural use posed a risk to humans, whereas the Swann Committee concluded otherwise and indicated that the administration of antibiotics to livestock, particularly at nontherapeutic levels, posed a significant hazard to human and animal health. Among the recommendations of the Swann Committee were that antibiotics used for livestock production be available by prescription only and in-feed antibiotics should be limited to 100 ppm. Additionally, the Committee recommended that surveillance programs be established to monitor antibiotic resistance in bacteria of concern. The Swann report spawned much debate among the scientific community, as some noted the findings were partly based on anecdotal evidence and/or studies with little scientific rigor, and in some cases were more presumptive than substantive. This debate resulted in an increase in studies to investigate the issue of antibiotic use in livestock and risks associated with such use.

Over subsequent decades, other organizations became active in the debate. Among some of the most notable was the American Society of Microbiology (ASM) which formed the Task Force on Antibiotic Resistance, consisting of scientists from academia, the government and industry. Their initial report focused on critical issues and risks posed by the widespread and growing use of antibiotics in human medicine and agricultural production. That report provided a comprehensive set of recommendations for surveillance programs, as well as recommendations to address emerging resistant organisms and the development of new drugs and non-antibiotic therapies (16). In 1997, the World Health Organization (WHO) released a report that provided a strong statement against the use of antibiotics for growth enhancement, indicating that such use is

particularly conducive to selection for resistant bacteria (229). Later reports included the WHO Global Principals for the Containment of Antibiotic Resistance in Animals Intended for Food, which was formulated jointly with the Office of International des Epizooties (OIE) and the United Nations Food and Agricultural Organization (FAO) (230). That document, which was intended to provide a framework of recommendations to reduce the overuse and misuse of antibiotics for the protection of human health, outlined recommendations for pre-approval, manufacturing, distribution, sales, and prudent use of drugs, surveillance of resistance, and education of veterinarians and producers regarding use and hazards of food animal antibiotics. The report also recommended that in the absence of risks assessments, growth promoting antibiotics that are also used in human medicine should be rapidly phased out, preferably through voluntarily programs, but if necessary, by legislation. In 2002, the American Academy of Microbiology, representing the highest leadership within ASM, issued a report titled The Role of Antibiotics in Agriculture (106). Among the recommendations of that report were a call for better estimates of antibiotic use in livestock and aquaculture production, the need for research into the economics of growth promoting antibiotics, a call for wider dissemination and education of judicious use principles among veterinarians and producers, and more research into reservoirs of resistance, resistance transfer, and quantitative risks assessments.

During the same period, US agencies also addressed the issue of antibiotic resistance. In 1997, surveillance, educational and research initiatives to address antibiotic resistance in foodborne pathogens were expanded through funds provided by the President's Food Safety Initiative (210). In 1999, an interagency Task Force on

Antibiotic Resistance was formed, headed by the Centers for Disease Control (CDC), the National Institutes of Health (NIH), and the FDA. In 2001, the Task Force released the Public Health Action Plan to Combat Antibiotic Resistance, with the main aspects to include surveillance, prevention and control, research, and product development related to antibiotic resistance (37). Since its initiation, the Task Force continues to expand in scope, and partner with other national and international agencies, addressing high priority issues relevant to antibiotic resistance (36). Soon after establishment of the Task Force, the FDA directly addressed the issue of risks caused by use of antibiotics in food animals with the release of the Guidance for Industry #152, which outlined steps for risk assessments for the evaluation of new animal drugs in terms of microbial food safety (FDA, 2003). While not mandated, the steps suggested by Guidance #152 have provided clear direction for pharmaceutical companies to assess the potential for emergence and selection of antibiotic resistant foodborne pathogens as a result of the drug use. Table 2 shows reports addressing the association of antibiotic use in food animal and public health.

Table 2. Report on the use of antibiotics in animals and associated public health implication. [1]

Year	Report (Source)
1969	Swann Committee Report (Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine)
1969	The Use of Drug in Feed animals (National academy of Sciences)
1972	The Use of Antibiotic in Animal Feeds (FDA task force)
1977	Need to Establish Safety and Effectiveness of Antibiotic Used in Animal Feeds (U.S. General Accounting Office Report)
1979	Drugs in Livestock Feed (Office of Technology Assessment)
1980	The Effects on Human Health of Subtherapeutic Use of Antibiotic in Animal Feed (Institute of Medicine Report)
1981	Antibiotic in Animal Feeds (Council for Agricultural Science and Technology)
1989	Human Health Risks with the Subtherapeutic Use of Pennicillin or Tetracyclines in Animal Feed (Institute of Medicine Report)
1995	Impacts of Antibiotic-Resistant Bacteria (Office of Technology Assessment)
1997	The Medical Impact of the Use of Antibiotic in Food Animals (World Health Organization)
1997	Antibiotic Feed Additives (Ministry of Agriculture, Commission on Antibiotic Feed Additive)
1998	Fluoroquinolone Use in Food Animals (World Health Organization)
1998	A Review of Antibiotic Resistance in the Food Chain (Ministry of Agriculture, Fisheries, and Food)
1998	Use of Drug in Food Animals: Benefits and Risks (National Research Council)
1999	The Agricultural Use of Antibiotics and its Implications for Human Health (U.S. General Accounting Office Report)
2000	The Use of Antibiotics in Food-Producing Animals: Antibiotic-Resistant Bacteria in Animal and Humans (Joint Expert Advisory Committee on Antibiotic Resistance)
2001	Opinion of the Scientific Committee on Animal Nutrition on the Criteria for Assessing the Safety of Microorganism Resistant to Antibiotics of Human Clinical and Veterinary Importance (EU SCAN Report)
2001	Risk Assessment on The Human Health Impact of Fluoroquinolone Resistant Campylobacter Associated with the Consumption of Chicken (Center for Veterinary Medicine)
2002	The Need to Improve Antibiotic Use in Agriculture: Ecological and Human Health Consequences (The FAAIR Report)

Table 2. (continued) Report on the use of antibiotics in animals and associated public health implication.

Year	Report (Source)
2002	Uses of Antibiotic in Food Animal in Canada: Impact on Resistance and Human Health (Health Canada Advisory Committee on Animal Uses of Antibiotics and Impact on Resistance and Human Health)
2002	Food Safety and Pig Production in Denmark: controls on Antibiotics, Veterinary Medicines and Salmonella (Verner Wheelock Associates Limited; Danish Bacon and Meat Council)
2003	Joint FAO/OIE/WHO Expert Workshop on Non-Human Antibiotic Usage and Antibiotic Resistance: Scientific Assessment (World Health Organization)
2003	Impact of Antibiotic Growth Promotion Termination in Denmark (World Health Organization)

[1] Compiled from Prescott (171), and McDermott et al. (228).

II. ANTIBIOTIC ACTIVITIES AND MECHANISMS OF ANTIBIOTIC RESISTANCE DEVELOPMENT AND TRANSFER

Antibiotic Targets and Mechanisms of Action

Most antibiotics target bacterial structures and metabolic pathways that are essential for bacterial growth, survival, or both, while causing little or no effect to eukaryotic hosts harboring the bacteria. Antibiotics interfere or block the growth of bacteria by binding to specific target sites in or on bacteria forming a complex structure that no longer displays the original functions. The four principle targets for the main classes of antibiotic are: 1) bacterial cell-wall biosysnthesis; 2) bacterial protein synthesis; 3) bacterial DNA replication; and 4) folic acid synthesis.

1. Inhibition of Cell Wall Synthesis

The cell wall is an essential structure of bacteria because it confers mechanical protection and provides a solid surface for protein and appendages necessary for cell adhesion, motility, host infection, and horizontal gene transfer (84). The bacterial cell wall differs from that of all other organisms by the presence of peptidoglycan (poly-*N*-acetylglucosamine and *N*-acetylmuramic acid), which is located immediately outside of the cytoplasmic membrane. There are two main types of bacteria, Gram-positive and Gram-negative, according to their cell walls components. Gram-positive organisms are characterized by the presence of a thick peptidoglycan layer, while Gram-negative organisms contain a thin peptidoglycan but have an additional outer membrane composed by phospholipids and lipopolysaccharides. Multiple enzymes are required for

peptidoglycan synthesis and attachment to the cell wall. Enzymes involved in the final stage of cell wall synthesis are called transpeptidases. Beta-lactam antibiotics (penicillins, cephalosporins, and other classes used in human medicine, such as carbapenems and monobactams) bind to transpeptidases and inhibit peptidoglycan formation, thus interfering with cell wall synthesis. Glycopeptide antibiotics, including vancomycin, teicoplanin, and the growth promoter avoparcin, inhibit cell wall synthesis by forming a complex with residues of peptidoglycan precursors, making it inaccessible to transpeptidase. (24, 84, 153, 175).

2. Inhibition of DNA Replication

DNA replication is a vital function of the bacterial cell. DNA gyrase and DNA topoisomerase IV are enzymes that control the folding or supercoiling of the DNA during DNA replication. They are essential for preventing the DNA molecule from becoming entangled during replication of circular chromosomes in bacteria. Quinolone antibiotics irreversibly bind to the DNA molecule-gyrase complex, thereby preventing DNA replication and leading to bacterial cell death. The original quinolone was naladixic acid, which only acts on aerobic Gram-negative species. The newer fluoroquinolones, such as ciprofloxicin, norfloxacin, and ofloxacin, have a much broader spectrum of activity (84, 153).

3. Inhibition of Protein Synthesis

Proteins play an essential role in bacterial cells since enzymes and most cellular structures are composed of protein. Protein synthesis consists of two biological processes starting with transcription of DNA to mRNA and translation of mRNA into protein.

Several classes of antibiotics inhibit protein synthesis by acting on the ribosome during the translation process. Bacterial ribosomes contain two subunits, the 50S and 30S subunits. Antibiotics bind to one or both subunits, and cause misreading of the genetic code or formation of abnormal, nonfunctional protein complexes. Aminoglycosides (gentamicin, tobramycin, amikacin, streptomycin) and tetracyclines are antibiotic classes that act primarily by binding to the 30S subunit. Tetracyclines are bacteriostatic rather than bactericidal because their binding to the ribosome is transient. Several other classes of antibiotics inhibit the 50S ribosomal subunit. Macrolides (erythromycin, tylosin, tilmicosin), chloramphenicol and clindamycin are primarily bacteriostatic and attach reversibly to the 50S subunit and interfere with the linking of amino acids (84, 153).

4. Inhibition of Folic Acid Synthesis

Folic acid is an essential precursor of pyrimidine rings in nucleic acids; therefore inhibition of folic acid synthesis will result in indirect inhibition of nucleic acid formation and function. Unlike humans and animals, bacteria usually lack the ability to take up folic acid from the environment and must synthesize it internally. Sulfonamides and diaminopyrimidine (trimethoprim and similar compounds) interfere with folate metabolism by competitively and irreversibly binding to dihydrofolate synthase or dihydrofolate reductase, two enzymes necessary for production of tetrahydrofolate. Trimethoprim and sulfonamides are usually administered together because they display a synergic therapeutic effect (84, 153).

Mechanisms of Antibiotic Resistance Development and Transfer

The two most commonly used criteria to describe antibiotic resistance are based on microbiological (*in vitro* resistance) and clinical (*in vivo* resistance) factors (84). For the microbiological definition, a strain is defined as resistant if it is no longer inhibited by the minimal concentration of the antibiotic that inhibits growth of typical strains of that species (83). However, for clinical definitions, a strain can be defined as resistant when it survives under chemical therapy. Under these conditions, a strain may be either sensitive or resistant depending on its location, the dosage and the mode of drug administration, tissue distribution of the drug, and the immune status of the patient (84).

Antibiotic susceptibility can be tested under laboratory conditions by exposing a known concentration of bacterial culture to increasing concentrations of a select antibiotic drug. These testing methods can be performed based on one of the three approaches: broth microdilution, agar dilution, and agar diffusion (228). The endpoint measurement based on inhibition of bacterial growth, is reported either qualitatively (sensitive, intermediate, resistant) or quantitatively as minimum inhibitory concentration (MIC), which is the lowest drug concentration that completely inhibits growth of the bacterial isolate under test (114, 228).

Two aspects are typically discussed regarding antibiotic resistance. One is the mechanism(s) by which the bacteria become resistant. The other is the development and spread of antibacterial resistance genes. The following will discuss these two aspects in detail.

Mechanisms of Bacterial Antibiotic Resistance

Bacterial cells use several mechanisms to resist the effects of antibiotics. The most widespread mechanisms are modification or replacement of the drug targets, enzymatic drug inactivation, active drug efflux and reduced drug uptake. Other mechanisms, including drug protection and overproduction of the drug's targets, are less common (Figure 2) (84, 153, 206).

1. Modification or Replacement of Drug Targets

By modification or replacement of the target receptor, the antibiotic no longer binds and therefore does not have the intended effect. Target modification can be linked to resistance mechanisms for almost all classes of antibiotics. This mechanism has been known to be of importance for resistance to penicillin, glycopeptides, and macrolides lincosamides and streptogramins (MLS) in Gram-positive bacteria, and for resistance to quinolones in both Gram-positive and Gram-negative bacteria. Methylation of the drug's target, the ribosome (MLS and aminoglycoside resistance) and gene mutation of the drug's targeted bacterial enzyme in quinolone resistance are examples of target modification. Glycopeptide resistance in enterococci and methicillin resistance in *Staphylococcus aureus* are examples of replacement of drug target with a compound of lower affinity (84).

2. Enzymatic Drug Inactivation

Enzymatic inactivation is the main mechanism that bacteria use to escape the effect of β-lactams, aminoglycosides, and phenicols. Although not prevalent, these

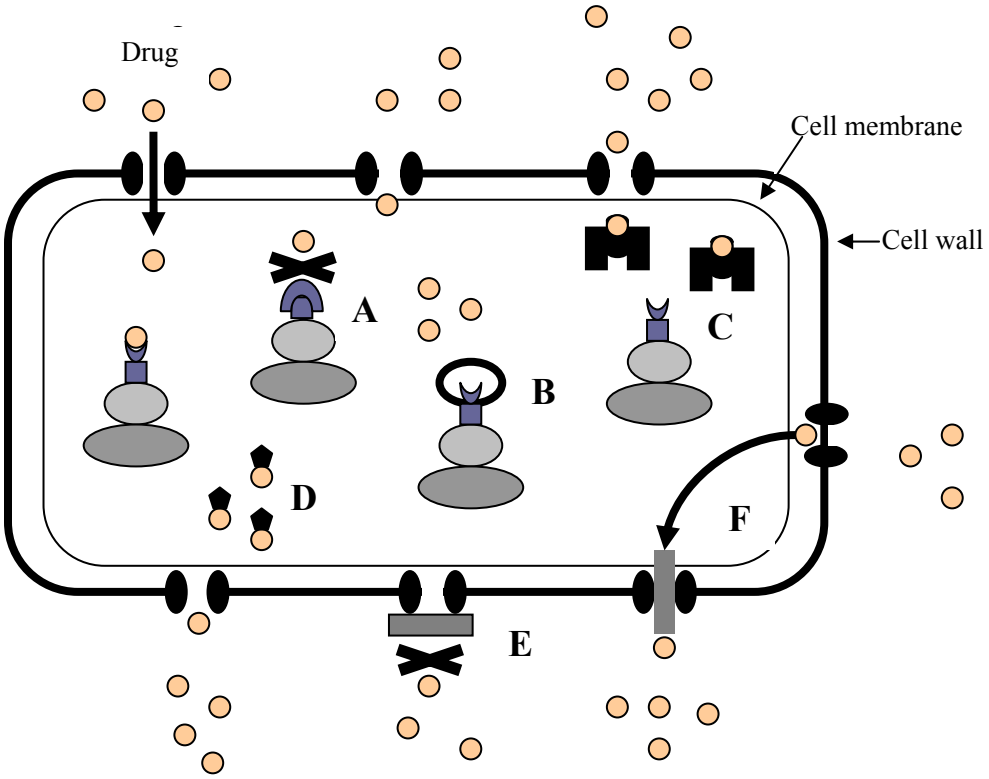

Figure 2. Mechanisms of antibiotic resistance development.

A) Target modification. B) Target protection. C) Drug trapping. D) Enzymatic drug inactivation. E) Reduced permeability. F) Active efflux. Adapted from Guardabassi and Courvalin (84).

mechanisms are also known to be involved in resistance to tetracycline, MLS, and fosfomycin. Enzymes can modify the active site of the drug by cleaving the molecule or adding chemical groups that prevent the drug from binding to its target site, resulting in the loss of antibacterial ability. The most important drug-inactivating enzymes are β-lactamase and aminoglycoside-modifying enzyme. β-lactamase destroys beta-lactam antibiotics before they reach the bacterial target, preventing it from binding to its target. Aminoglycside-modifying enzymes function by catalyzing transfer of the acetyl group to amino groups or phosphoryl groups or nucleotides to amino or hydroxyl groups in the aminoglycoside molecule, resulting in poor binding of this drug to ribosome. (17, 84, 186)

3. Drug Efflux Pumps

By actively pumping out antibiotic molecules, drug efflux systems prevent the intracellular accumulation necessary for antibiotics to exert their lethal activity. Drug efflux pumps are energy-dependent mechanisms. These pumps may be specific for one substrate (drug specific resistance pumps) or may transport a range of structurally dissimilar compounds, including antibiotics of multiple classes, imparting multiple drug resistance (multiple drug resistance pumps). Drug specific resistance pumps are the most important mechanism of resistance to tetracycline. These pumps generally confer a high level of resistance and are mostly associated with mobile genetic elements. Multiple drug resistance pumps may have several substrates, however these pumps generally confer low-level resistance and are usually encoded on the chromosome (84, 120, 227).

4. Reduced Drug Uptake

Reduced drug uptake is another mechanism the bacteria use to reduce concentrations of drugs accumulating in the cell. This may occur through several mechanisms such as reduced outer membrane permeability, as in *Pseudomonas aeruginosa* and *E. coli* O157:H7, porin mutation that leads to loss, reduced size, or decreased expression of porin proteins, as in reduction of OmpF porin expression, which has been shown to increase resistance of *E.coli* to quinolones, β-lactams, tetracyclines and chloramphenicol. Lack of an electrical potential gradient, which is required to drive the drug across the bacterial membrane, as in the case of aminoglycoside resistance, is also a way that reduced drug uptake by bacteria can result (69, 84, 132)

5. Target Protection

Target protection has been reported to be involved in resistance to tetracyclines and quinolones. Resistance to tetracyclines by this mechanism results from the presence of ribosomal protection proteins. At least eight ribosomal protection proteins have been found to be associated with tetracycline resistance. Among them, Tet(M) and Tet(O) are the most prevalent and well-studied. The presence of a DNA gyrase protection protein has been reported in quinolone resistant Enterobacteriaceae (48, 84, 85, 118, 211).

6. Drug Trapping

Bacteria can trap the drug by over production of the drug target or other molecule with high affinity for the drug. Over production of targets for sulfonamides and diaminopyrimidine have been reported in several bacterial species. A mutation resulting in the thicker cell wall with many binding sites for vancomycin has been shown to trap

antibiotic molecules, thereby reducing the number of vancomycin molecules that reach the cytoplasmic membrane where the transglycosylase targets are located (18, 53).

Families of Antibiotics

Antibiotics can be grouped based on mechanisms of action but more often by chemical structure. Antibiotics of the same chemical structure usually share many biological properties making this classification of antibiotics useful in practice. Below are descriptions of major antibiotic families, their modes of action, and mechanisms bacteria employ to resist them.

1. β–Lactams

β-lactams comprise a family of bactericidal antibiotics that contain a β-lactam ring in their structure. The principle antibiotics of this family include penicillins, cephalosporins, carbapenems and monobactams. The β-lactam ring inhibits bacterial cell wall synthesis by disruption of peptidoglycan cross-linkage; therefore these antibiotics tend to be more active against Gram-positive bacteria which have a high concentration of peptidoglycan in their cell wall. The spectrum of the penicillin family varies. Some penicillins, such as Benzyl penicillin (Penicillin G) and phenoxymethyl penicillin (Penicillin V), have a narrow spectrum, with activity against aerobic and most anaerobic Gram-positive bacteria. Others, such as ampicillin and amoxycillin, have a broader spectrum of activity against a range of Gram-positive and Gram-negative bacteria. Cephalosporins are usually classified as generations, according to their antibiotic properties. First-generation cephalosporins are predominantly active against Gram-

positive bacteria, and successive generations have increased activity against Gram-negative bacteria. Fourth generation cephalosporins, have true broad spectrum activity active against Gram-positive and Gram-negative bacteria. Carbapenems have the broadest antibacterial spectrum compared to penicillins and cephalosporins (25, 32, 88)

Bacteria can become resistant to β-lactam antibiotics by expressing beta-lactamase enzymes that degrade the β-lactam ring. Resistance to β-lactam drugs is not only found in food-associated pathogens but also in commensal bacteria and it occurs in bacteria of both animals and man. The widespread occurrence of bacteria carrying β-lactamases is well documented around the world (13, 40, 98, 121, 145, 176, 179).

2. Aminoglycosides

Aminoglycosides are bactericidal antibiotics that include amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, spectinomycin, tobramycin and apramycin. These antibiotics usually contain a cyclic nucleus with one or two amino acids attached by glycosidic linkages. The aminoglycosides interfere with protein synthesis by binding to the 30S ribosomal subunit. Aminoglycosides are primarily active against aerobic bacteria, especially Gram-negative organisms such as *Salmonella*, *E. coli*, and *Pasteurella*. They are also active against *Pseudomonas* and *Staphylococci*. In addition, some mycoplasma, and some spirochetes are susceptible to aminoglycosides (30, 88, 172).

Resistance to aminoglycosides usually occurs via enzymatic modification. The majority of the enzymes that promote such are acylases or acetylases which modify the conformation of the aminoglycosides thereby preventing them from binding to

ribosomes. Resistance to some aminoglycosides, such as streptomycin and spectinomycin, is widespread and the genes responsible for resistance to these antibiotics are often found as gene cassettes carried in integrons (38, 82, 115, 147, 157).

3. Tetracyclines

Tetracyclines are a large family of broad spectrum antibiotics including tetracycline, oxytetracycline, chlortetracycline and doxycycline. Tetracyclines are named for their four hydrocarbon rings. More specifically, they are defined as a subclass of polyketides having an octahydrotetracene-2-carboxamide skeleton. Tetracyclines are bacteriostatic. They work by binding to the 30S ribosome and inhibit the function of such by blocking the binding steps of molecules needed to initiate the process of protein synthesis (translation). They are active against wide variety of bacteria such as Gram-positive Gram-negative aerobes and anaerobes, and show activity against mycoplasmas, chlamydia, protozoa and ricketsia. The various tetracyclines have a similar spectrum of activity; however, they are different in degree of lipid solubility and therefore absorption and ability to cross membrane (14, 78, 88, 152).

Resistance to tetracyclines can occur by at least three mechanisms, including enzymatic inactivation, efflux pumps, and ribosomal protection. Resistance to these antibiotics is widespread in both animals and humans and is well documented (2, 5, 6, 31, 35, 99, 107, 128, 130, 191).

4. Sulfonamides

Sulfonamides are groups of antibiotics that contain sulfonamide in their structure. They include sulfaodiazine, sulfamethazine, sulfadimidine, sulfadimethoxine,

sulfadoxine, sulfachloropyridazine, and sulfamethoxazone. Sulfonamides are often used together with trimethoprim, or one of the other diaminopyrimidines, known as potentiated sulfonamides. While both compounds on their own are bacteriostatic, the combination of these two compounds provides a synergistic effect resulting in bactericidal action. Sulfonamides interfere with the folic acid synthesis in bacteria preventing multiplication of the bacterial cell. Sulfonamides are broad spectrum antibiotics inhibiting both Gram-positive and Gram-negative bacteria, as well as some protozoa such as coccidia; however, they are ineffective against obligate anaerobes (11, 88, 172).

As with tetracycline, resistance to sulfonamides is widespread in bacteria associated with humans and animals (26, 71, 86, 207). Several studies have shown the presence of sulfonamide resistance genes in class 1 integrons as part of the integron's conserved sequence, and these elements are known to be responsible for widespread dissemination of this sulfonamide resistance (86, 125, 197, 209).

5. Quinolones and Fluoroquinolone

Nalidixic acid was the first quinolone developed in 1962. Second generation 4-quinolones are a group of compounds that include as oxolinic acid, pipemidic acid and cinoxacin. The quinolones have narrow spectrum activity, affecting only Gram-positive bacteria. Third generation constructs of these compounds are classified as fluoroquinolones, which are broad spectrum bactericidal drugs. The newer fluoroquinolones, such as enrofloxacin and danofloxacin, are highly active against Gram-positive and Gram-negative bacteria, including *Salmonella*, *Pseudomonas* and

mycoplasmas. The quinolones and fluoroquinolones inhibit bacterial growth by binding to DNA gyrase and DNA topoisomerase IV, thereby inhibiting DNA replication and transcription (88, 162).

Bacterial resistance to this group of antibiotics is most commonly due to alteration of DNA-gyrase via mutation (gyr-A). Less common but perhaps more important for Gram-positive bacteria, mutation occurs to topoisomerase IV (parC). Other mechanisms of resistance occur via decreased drug entry or increased active transport out of cell (29, 101, 142).

6. Phenicols

Phenicols include chloramphenicol, florfernicol and thiamphenicol. They are bacteriostatic; however may be bactericidal in high concentrations or when used against highly susceptible organisms. Chloramphenicols inhibit protein synthesis by binding to 50S ribosomal subunit. They are broad spectrum antibiotics, active against a wide range of Gram-negative and Gram-positive bacteria and also some chlamydia spirochetes, and ricketsia (88).

Bacteria become resistant to chloramphenicol by becoming impermeable to the drug or by producing an inactivating enzyme, chloramphenicol acetyltransferase (195, 218).

Antimicrobial resistance mechanisms for each antibiotic drug group are shown in Table 3. Table 4 provides a list of drugs licensed by USDA for animal use. A listing of antibiotics licensed in Thailand is not available (149).

Table 3. Antimicrobial Resistance Mechanisms.[1]

Antimicrobial Group	Anitimicrobial Resistance Mechanism
Aminoglycosides	Modifying enzymes
Gentamicin	Decrease permeability
Streptomicin	Target resistance (ribosome)
Kanamycin	Efflux
B-lactams	Reduce permeability
Cephalothin	Altered penicillin-binding proteins
Cefoxitin	B-lactamase, cephalosporinases
Ceftiofur	Efflux
Cefquinome	
Folate pathway inhibitors	Decrease permeability
Sulfonamides	Production of drug-insensitive enzymes
Macrolide-lincosamide-streptgramin	Decrease ribosomal binding
Erythromycin	Decrease permeability
Lincomycin	Modifying enzymes
Virginiamycin	Efflux
Phenicols	Decrease ribosomal binding
Chloramphenicol	Decrease permeability
Florfenicol	Modifying enzymes
	Efflux
Quinolones and fluoroquinolones	Target resistance(DNA gyrase, Topoisomerase IV)
Nalidixic acid	Decreased permeability
Ciprofloxacin	Efflux
Enrofloxacin	
Tetracyclines	Target resistance (ribosome)
Chlortetracycline	Drug detoxification
Tetracycline	Efflux
Doxycycline	

[1]Adapted from McDermott et al. (139).

Table 4. Antibiotic drugs licensed for avian and porcine use in the US and of human health importance. [1]

Antibacterial class	Licensed drugs	Animal used	Human health importance
Aminoglycosides	Gentamicin	Avian and Porcine	High
	Streptomycin	Avian and Porcine	Low
	Kanamycin	Avian and Porcine	Medium
	Neomycin	Avian and Porcine	Rarely used
	Apramycin	Porcine	Not used
Aminocyclitols	Spectinomycin	Avian and Porcine	Low
Cephalosporins	Narrow spectrum		
	Cefalexin	Porcine	Medium
	Expanded spectrum		
	Ceftiofur	Avian and Porcine	Very high
Lincosamides	Lincomycin	Avian and Porcine	High
Macrolides	Erythromycin	Avian and Porcine	High
	Tylocin	Avian and Porcine	Not used
	Tilmicosin	Avian and Porcine	Not used
Penicillins	Penicillin G and V	Avian and Porcine	Low
	Ampicillin[2]	Avian and Porcine	Medium
	Amoxicillin[2]	Avian and Porcine	Medium
Phenicols	Florfenicol	Porcine	Not used
Pleuromutilins	Tiamulin	Avian and Porcine	Not used
	Valnemulin	Avian and Porcine	Not used
Polypeptides	Bacitracin	Avian and Porcine	Very low
	Colistin	Avian	Low
Quinoxalines	Carbadox	Avian and Porcine	Not used
	Second generation		
	Enrofloxacin	Avian and Porcine	Very high
Streptogramins	Virginiamycin	Avian and Porcine	High
Sulfonamides	Many compound	Avian and Porcine	Medium
Tetracyclines	Oxytetracycline, chlortetracyclines, tetracycline	Avian and Porcine	Medium
Miscellaneous	Avilanycin	Porcine	Not used
	Bambermycin	Porcine	Not used

[1] Adapted from Guardabassi and Courvalin (84).
[2] With or without the beta-lactamase inhibitor clavulanic acid.

Resistance to Antibiotic Agents

Resistance to antibiotic agents can be subdivided into two groups, intrinsic resistance and acquired resistance (190). Intrinsic resistance is a genus- or species-specific property of bacteria which describes the status of bacteria with regard to inherent insensitivity to a particular antibiotic agent or class of agents (84, 190). Such may be due to the lack of target for certain antibiotic agents (e.g. resistance to β-lactam by bacteria that lack a cell wall), their inaccessibility in specific bacteria (e.g. impermeability to glycopeptides by the outer membrane of Gram-negative bacteria), extrusion of the antibiotic by chromosomally encoded active exporters (e.g. resistance to tetracyclines, chloramphenicol, and quinolones in *Psuedomonas aeruginosa)*, or innate production of enzymes inactivating the drug (e.g. AmpC β-lactamase in some Enterobacteriaceae) (84).

Unlike intrinsic resistance, acquired resistance is a strain-specific property of a particular bacterial genus or species. Acquired resistance is a major threat to human and animal health because it results in the emergence and spread of resistance in normally susceptible bacteria populations, and can consequently result in therapeutic failure (84, 190). Acquired resistance is the result of genetic change in the bacterial genome which can occur by mutation of chromosomal target genes, or by horizontal acquisition of foreign resistance genes (34). Mutations can occur spontaneously in any gene of the bacterial genome during the replication of bacteria. The frequency of mutation and the expression of an antibiotic resistance phenotype will depend on environmental factors, cell physiology, bacterial genetics, and population dynamics (131). Although the rate of mutation is low (in vitro rates of 10^{-6} to 10^{-12}), it may be relatively common in the

ubiquitous and vast bacterial population. The resistance caused by mutations plays an essential role in bacteria that are not known to acquire foreign DNA under natural conditions (such as *Mycobacterium* spp.). This is the major mechanism of acquiring resistance when a high level resistance is not conferred by mobile genetic elements, as in the case with fluoroquinolone resistance. (84). In many cases, different species or genera of bacteria are found to contain closely related or even the same antibiotic resistance genes, indicating the exchange of resistance genes by horizontal transfer. Such resistance gene transfer events occur not only in pathogenic bacteria but also in harmless commensal normal flora (190). Many antibiotic resistance genes are located on mobile genetic elements, such as plasmids, transposons, and integrons, which can act as vectors which promote gene transfer between bacteria (158).

Horizontal Gene Transfer Mechanisms

Antibiotic resistance genes located on plasmids, transposons, gene cassettes in integrons, or genomic islands are spread vertically during division of host cells. However, they can also transfer horizontally between the same or different species or genera of bacteria through three main mechanisms: transformation, transduction, and conjugation (Figure 3). Transformation occurs via uptake of exogenous DNA from the immediate surrounding environments, which can be incorporated into naturally transformable bacteria, and by bacteria which become competent for transformation under special physical or chemical conditions (21, 178). Conjugation results from the transfer of plasmids, which are typically exchanged between donor and recipient cells through physical contact (234). Transduction is facilitated by bacteriophages, which inject their

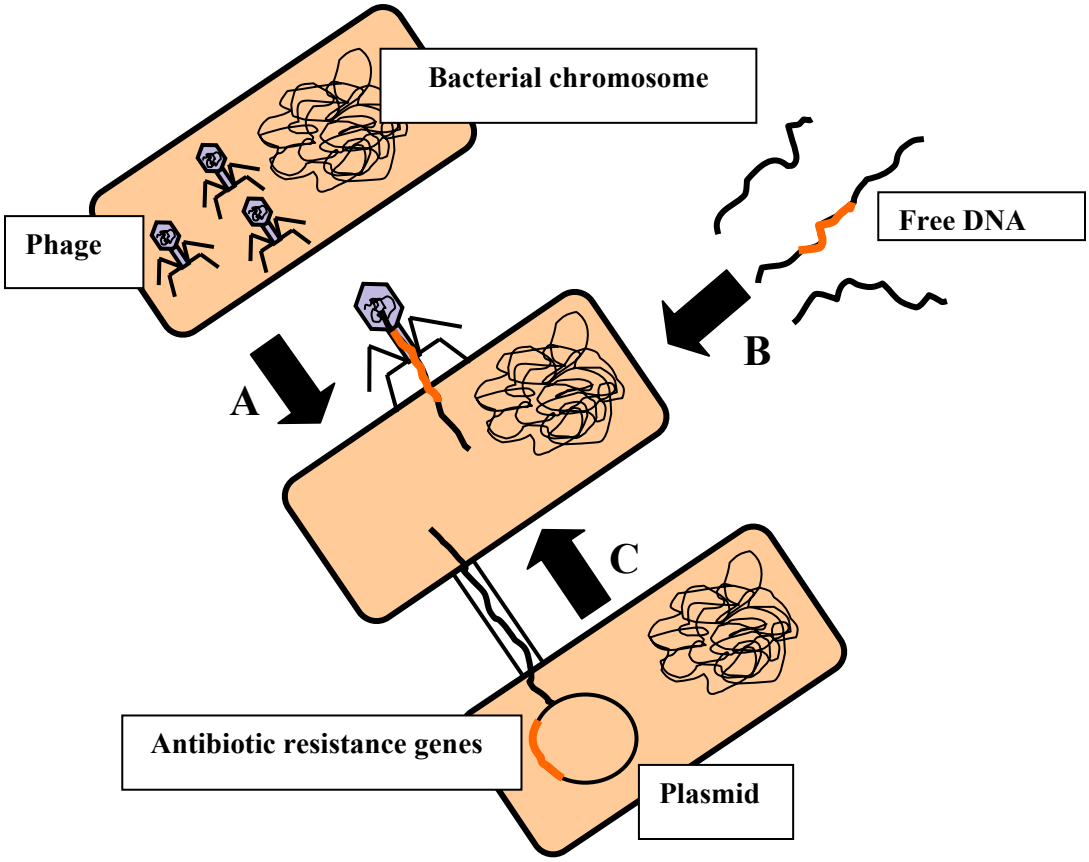

Figure 3. Three main mechanisms by which antibiotic resistance genes are transferred horizontally among bacteria.

A) Transduction; bacteriophage containing bacterial genes transduce bacteria though phage lytic cycle; B) Transformation: free DNA is taken up by bacterial cell and incorporated into the genome through recombination; C) Conjugation: plasmid containing antibiotic resistance genes is passed from donor to recipient bacteria through pilus.

DNA into the genome of a host bacterium, after which replication and re-packaging of the bacteriophage DNA occurs. In that process, bacterial DNA may be incorporated into the viral DNA and upon dispersion of new bacteriophages and injection of repackaged DNA into new hosts, resistance genes from the original host may be disseminated into a new population (66).

Transduction and transformation do not require viability of the donor cell nor the simultaneous presence of donor and the recipient cell in a given environment (158). However, since both mechanisms require homology between donor and recipient DNA for recombination to occur, and the host range of transduction is limited by the high host specificity of bacteriophage (60), the spread of antibiotic resistance via transduction and transformation is limited to closely related bacteria belonging to the same species or genus (184). As antibiotic resistance genes are often located on conjugative genetic elements such as plasmids or transposons and these elements can maintain themselves in the new hosts without requiring large regions of sequence similarity to integrate into the new host's genome, conjugation likely plays a more important role in dissemination of antibiotic resistance genes across species and genus barriers (184, 185).

Elements Involved in Horizontal Transfer of Resistance Genes

Plasmids, transposons, integrons/gene cassettes, and chromosomal genomic islands play major roles in horizontal transfer of antibiotic resistance genes (189). These four types of elements are composed of double-stranded DNA, but differ distinctly in their sizes, structures, biological properties as well as mechanisms of spreading (190).

1. Plasmids

Plasmids are autonomously replicating extra-chromosomal elements that vary in size from less than 2 kilobase pairs (kb) to more than 100 kb. Plasmids have been detected not only in nearly all bacterial genera of clinical importance but also in normal flora of the skin and various mucosal tissues in humans and animals. Plasmids code for resistance to antibiotic agents, disinfectants, heavy metal cations, anions, nucleic acid-binding substances, or bacteriocins, providing mechanisms for the bacteria to survive under environmental stress (190). Plasmids can also code for metabolic (22, 61) or virulence properties (150, 170, 188, 235), allowing recipient bacteria to exist in new environments or gain novel virulence properties. Resistance plasmids are known to carry one or more resistance genes, sometimes in addition to genes for other traits. Large plasmids can carry a *tra* gene complex that enables them to move from one host cell to another by conjugation. Such plasmids are called conjugative plasmids. In most instances, the genes necessary for transfer are located together with resistance determinants on the same plasmid, creating a highly efficient mechanism for antibiotic dissemination among bacteria (113). The broad host-range, conjugative plasmids are possibly the most active vehicle for a potential horizontal gene pool of antibiotic resistance genes that are available to many bacteria of many species and families (158, 208). It is important to note that not every plasmid can replicate in every bacterium. Therefore, when transferred into a new host cell, plasmids may stably replicate, co-integrate with other plasmids; or integrate, either in part or completely, into the chromosomal DNA (190). Plasmids usually act as vectors for transposons and

integrons/gene cassettes, thus promoting rapid spread of multiple antibiotic resistance genes (114).

2. Transposons

Since transposons do not possess replication systems, they must integrate into chromosomes or plasmids to maintain their stability. Transposons also vary in their structures and sizes ranging from less than 1 kb to 60 kb. The smallest type of transposon is insertion sequence (IS), which usually carries only the transposase genes whose products mediate transposition of the elements. The insertion of additional genes, such as toxin genes, and more often antibiotic resistance genes, creates larger transposons (114). Composite transposons, such as Tn9, Tn10, and Tn5706, usually have one or more central antibiotic resistance genes and insertion sequences at the termini. Complex transposons, such as Tn1721, are commonly characterized by terminal inverted repeats and occasionally also internal repeats that separate the part carrying resistance gene(s) from the part carrying transposase genes. Some transposons integrate site specifically, whereas others can insert at various positions in the chromosomal or plasmid DNA. Similar to the situation among plasmids, there are also non-conjugative and conjugative transposons. Conjugative transposons, for instance Tn916, transfer not only among species within the Gram-positive or Gram-negative bacteria, but also between both groups. The tetracycline resistance gene, (*tetM*), located on a conjugative transposon, can transfer across several genera of bacteria such as *Enterococcus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Actinomyces* spp., *Bifidobacterium* spp., *Clostridia* spp.,

Campylobacter spp., *Fusarium nucleatum*, *Gardenella vaginalis*, *Haemophilus* spp., *Neisseria* spp., and *Veillonella* spp (184).

3. Gene Cassettes/ Integrons

Gene cassettes represent small mobile elements of less than 2 kb and were previously detected only in Gram-negative bacteria (174), but recently have been found in both Gram-negative and Gram-positive bacteria. They commonly consist of only a specific recombination site and a single gene, which most often is an antibiotic resistance gene (45). Gene cassettes do not have replication systems or transposition systems, but move by site-specific recombination. Gene cassettes can occur as a free circular DNA molecule, but they are usually found integrated into an integron (45). In recent years many different and diverse genes responsible for antibiotic resistance have been found in integrons.

4. Mobile Genomic Islands

Since the 1990s, several mobile genomic islands carrying antibiotic resistance genes have been reported. These genomic islands integrate site-specifically into the chromosome. The most well-studied is the *Salmonella* genomic island 1 (SGI1). This 43-kb SGI1 was first identified in epidemic multidrug-resistant strains of *S. enterica* serovar Typhimurium DT104. Several studies have shown a high prevalence of SGI1 in *S.* Typhimurium DT104. SGI1 has also been identified in other *S. enterica* serovars, such as Agona, Albany, Derby, Newport, Meleagridis, and Paratyphi B (28, 67, 144, 220). Recently, SGI1 has been identified as an integrative mobilizable element. The 13-kb antibiotic resistance gene cluster within SGI1 consists of a complex integron related to

the In4 group of integrons. In most known cases, it mediates resistance to a pentadrug pattern (ACSSuT), including ampicillin ($bla_{\text{PSE-1}}$), chloramphenicol and florfenicol (*floR*), streptomycin and spectinomycin (*aadA2*), sulfonamide (*sul1*), and tetracycline (*tetG*). However, variant clusters have been identified containing additional or other resistance genes, such as *dfrA1* and d*frA10*, conferring resistance to trimethoprim; *aadA7*, conferring resistance to streptomycin, and *aac*(3)-*ID*, conferring resistance to gentamicin (63, 144, 221).

III. INTEGRONS AND GENE CASSETTES

Plasmids and transposons are genetic elements that have been found to carry antibiotic resistance genes. However, numerous studies have shown that many antibiotic resistance genes found on plasmids and transposons in Gram-negative bacteria are located at a unique site within the conserved DNA sequence, leading to the discovery of a new genetic element called an integron. Integrons are natural genetic engineering systems that capture and incorporate open reading frames and convert them into functional genes (1). Aside from antibiotic resistance genes, integrons have been implicated in the acquisition of virulence determinants by *V. cholerae* (138) and *E. coli* (198).

Classification of Integrons

Integrons are believed to play a major role in the rapid dissemination of multi-drug resistance (MDR) among bacteria (158). Two major groups of integrons exist. Super-integrons (SI) are chromosomally located and may contain hundreds of gene cassettes encoding for a variety of functions, and there is a high degree of identity (> 80%) observed between the *attC* sites of these cassettes (137, 181). Resistance integrons (RI) typically carry gene cassettes encoding resistance to antibiotics and disinfectants and are currently divided into three classes, based on variations in sequence of primary elements, gene cassettes, and associations with transposons (4, 12, 44, 79, 80, 173, 192). RI can occur on bacterial chromosomes, transposons, or plasmids. The most common and widely distributed of RI are class 1 integrons (33, 79, 164, 217). Table 5 provides examples of

Table 5. Bacteria found to carry integrons.[1]

Integrons		Bacteria
Resistance integrons	Class 1	*Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Burkholderia*, *Campylobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio, Corynebacterium glutamicum,* and *Mycobacterium fortuitum*
	Class 2	*Acinetobacter*, *Shigella*, and *Salmonella*
	Class 3	*Pseudomonas aeruginosa*, *Serratia marcescens*, *Alcaligenes xylosoxidans*, *Pseudomonas putida*, and *Krebsiella pneumoniae*
Super integrons		*Geobacter sulfurreducens, Listonella pelagia, Nitrosomonas eurapaea, Pseudomonas alcaligenes, Pseudomonas mendocina, Pseudomonas stutzeri, Shewanella oneidensis, Shewanella putrefaciens, Treponema denticola, Vibrio anguillarum, Vibrio cholerae, Vibrio fischerii, Vibrio metschnikovii, Vibrio mimicus, Vibrio parahaemolyticus,* and *Xanthomonas campestris*

[1] Adapted from Fluit and Schmitz (80).

bacteria that have been reported to carry integrons. Class 1 integrons are widespread and have been found on all continents (65, 109, 112, 124, 193). Class 1 integrons possess two conserved segments separated by a variable region that often includes antibiotic resistance genes. The construct of class 1 integrons includes a conserved *intI* (integrase) gene and a complimentary strand containing a common promoter region that is directed toward the site of integration (127, 159). Site-specific recombination events occur within integrons enabling promotorless cassettes encoding for a wide range of antibiotic resistance genes to be inserted downstream of the integron promoter region, thereby providing for simultaneous resistance to multiple antibiotics (80). Integrons can be exchanged indiscriminately between similar bacteria as well as among bacteria of different taxa (110, 236), thus causing concern for wide dissemination
of these genetic resistance elements.

The Structure of Integrons

Class 1 integrons consist of two conserved segments, 5' and 3' conserved sequences, separated by a variable region, which includes one or more genes (Figure 4). The most common genes inserted into variable regions as cassettes are antibiotic resistance genes. (43, 127). Gene cassettes are not necessarily part of integrons, but once incorporated, they become part of that integron (79). Integrons can carry several different gene cassettes and therefore play an important role in the dissemination of multiple antibiotic resistance genes.

1. 5'Conserved Sequences

The 5' conserved sequence of integrons consists of three elements necessary for the capture of gene cassettes; a gene encoding a site specific recombinase integrase enzyme (*intI*); an attachment site where horizontally acquired sequences are integrated (*attI*); and a promoter that drives expression of the incorporated sequence (P) (89, 137, 158, 182).

Integrase

The *IntI* gene encoding for the integrase enzyme belongs to the tyrosine-recombinase family, which is the same as a well known λ-integrase (72, 156). The integron integrase is similar in length; however, it shares only 40-60% homology of amino acid identity. The *IntI2* and *IntI3* are 46% and 59% identical to *IntI1,* respectively (46). It is important to note that despite the differences in the integrase, gene cassettes are thought to be acquired by all integron classes, as identical genes cassette have been found in different classes of integron (12, 174, 203).

Integron Attachment Sites

Integrase enzymes catalyze the excision and integration of antibiotic resistance genes in integrons by a site-specific recombination system. These reactions occur by the integrase interacting with the two different recombination sites, the integron attachment site (*attI*) in the 5' conserved segment of the integron and the cassette attachment site (*attC*) or 59 base element (59-be) of the gene cassettes (45, 143, 163). The integrase cleaves double stranded DNA in both *attI* and *attC* sites between G and the first T within a 7-bp sequence (GTTRRRY, where R is a purine and Y is a pyrimidine), known as a

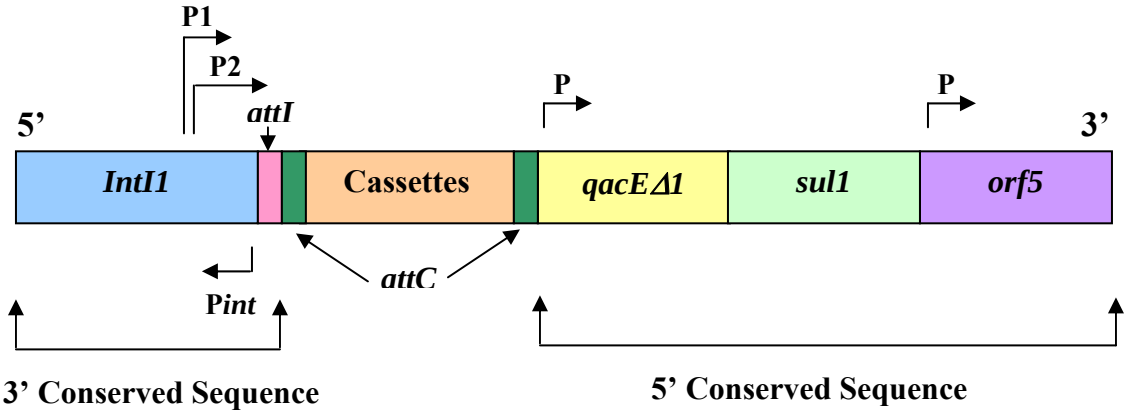

Figure 4. Typical genetic organization of class 1 integrons.

core site (201) creating a cross-over recombination point (91, 163). A diagram of the gene cassette capture and expression mechanism of class 1 integrons is show in Figure 5.

Promoters

Most antibiotic resistance gene cassettes are inserted into integrons as promoterless elements, therefore the expression of the genes located in the cassettes depend on the promoter located downstream of *attI* site. In class 1 integrons the 5'-CS contains 2 potential promoters, P_1 (also called P_{ANT}) and P_2. These promoters vary in strength to up to 20 times with P1 generally being a strong promoter and P_2 being a weaker Promoter, or frequently, inactive because only 14 nucleotides are present between the -35 and -10 boxes of this promoter, instead of the optimal 17 nucleotides. At least five different P_1 and two different P_2 sequences have been described, which may occur in varying combinations (42, 94, 126, 167, 201).

2. Gene Cassettes

Gene cassettes are discrete genetic elements that may exist as free, circular, non-replicating DNA molecules when moving from one genetic site to another (43); but they are normally found as linear sequences that constitute part of a larger DNA molecule, such as a plasmid or bacterial chromosome. Gene cassettes normally contain only a single gene and an additional short sequence, *attC*, that functions as a specific recombination site (90). Accordingly, the cassettes are small, normally of the order of 500–1000 bp.

Most gene cassettes lack the promoter in front of the coding sequence, therefore the expression of the genes located in the cassettes depends on the promoter of the integron (79). The number of identifie d resistance gene cassettes associated with RI

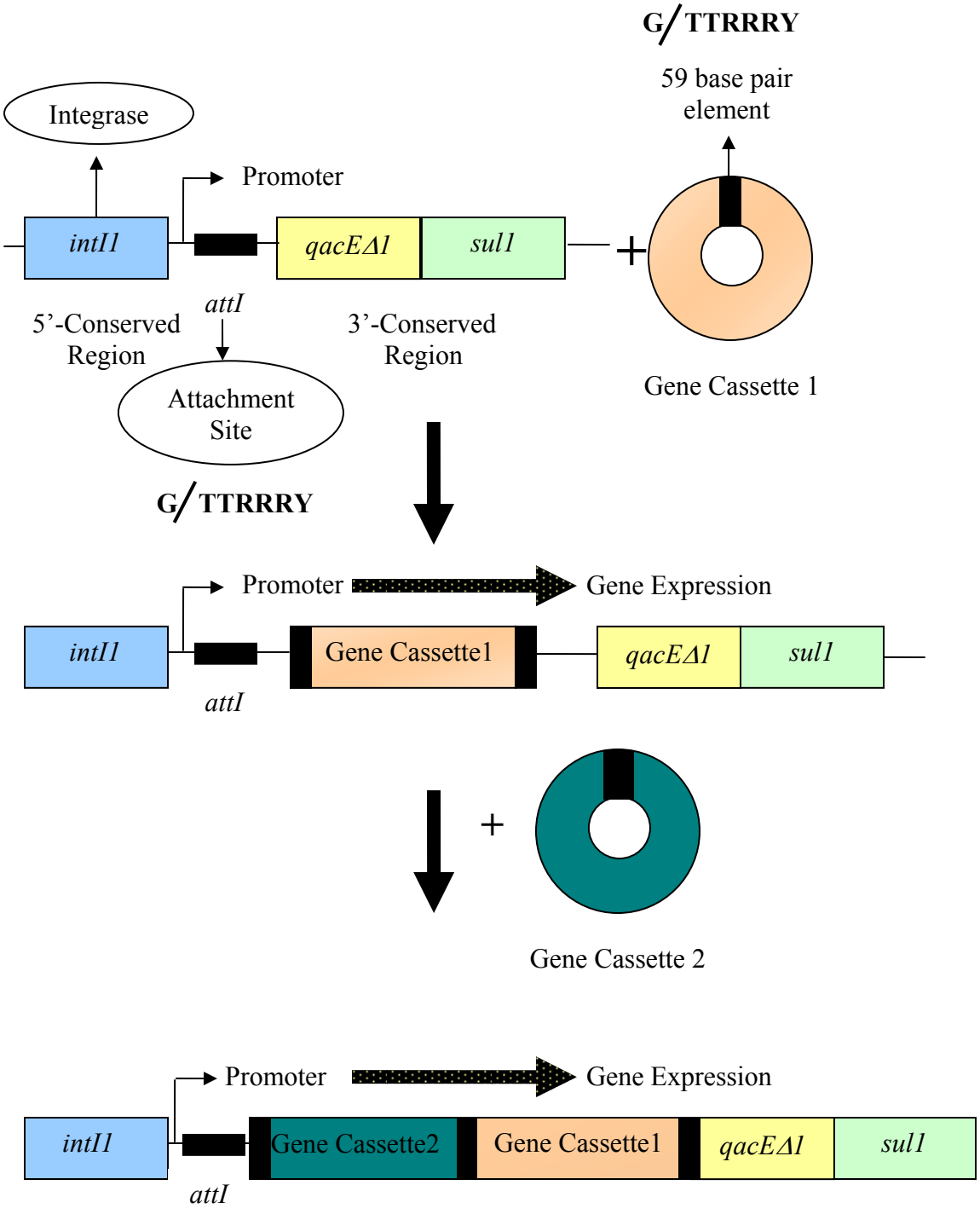

Figure 5. Schematic representation of gene cassette capture and expression mechanism by class 1 integrons. Adapted from Harbottle et al. (96), and Rowe-Magnus and Mazel (182).

increased dramatically from 40 different genes cassettes in 1998 (92) to 60 in 1999 (79), and over 80 have been reported to be associated with class 1 integrons in 2006 (137). Incorporation of gene cassettes into integrons occurs by *IntI*-mediated site-specific recombination between a 59-base element site in the cassette and an integron attachment site (*attI*) site in the integron (41). The following are details of *att*C and varying types of gene cassettes.

Cassette Attachment Site

The *attC* sites present at the 3'-end of a cassette are a diverse family of nucleotide sequences that function as recognition sites for the site-specific integrase. They vary in length from 57 bp to 141 bp; however, they share a region of about 25 bp at each end which conforms to consensus sequences (43, 90, 202). The consensus sequences are imperfect inverted repeats of one another, which begin with an inverted core site (RYYYAAC) separated by spacer of 7 or 8 bp and end with a core site (GTTRRRY) (202). Although they have similar DNA sequences at their extremities, each gene cassette has its own version of *attC* (90, 202). However *attC* can be recognized by the same integrase, as the same gene cassettes have been found located within different classes of integrons, indicating that the pool of cassettes is shared.

Type of Gene Cassettes

More than 70 gene cassettes with nucleotide sequences that differ by more than 5% have been found associated with class 1 integrons. Among them they confer resistance to all known β-lactams, all aminoglycosides, chloramphenicol, trimethoprim, streptothricin, rifampin, erythromycin, fosfomycin, and lincomycin and are also found to

carry genes coding for resistance to antiseptics of the quaternary-ammonium-compound family (80, 137, 183). Following are descriptions of four major resistance gene cassettes.

1. β-Lactams Resistance Gene Cassettes: Recently twenty-five gene cassettes conferring resistance to β-lactam antibiotics were characterized (80). Resistance to this group of antibiotics is not only caused by classical β-lactamase but also by extended spectrum β-lactamases. Carbapenamases, β-lactamases that have zinc at their active centers instead of serine, as in most β-lactamases, and have the ability to hydrolyze most beta-lactams including carbapenems (79), have also been described. Among four classes of carbapenamases, the metallo-beta-lactamases (mostly of the IMP and the VIM series) are the most clinically-significant and have been reported worldwide (154, 160, 168, 205).

2. Aminoglycoside Resistance Gene Cassettes: A large number of gene cassettes confer resistance to aminoglycosides. Twenty-six aminoglycoside resistance gene cassettes have been reported (79, 80). Aminoglycoside adenyltransferase (*aad*) conferring resistance to streptomycin and spectinomycin is the most common gene cassette found in this group. The second common aminoglycoside resistance gene cassette is aminoglycoside acetyltransferase (*aac*). Its products confer resistance to neomycin, gentamicin, tobramycin, and kanamycin (79).

3. Trimethoprim Resistance Gene Cassettes: Two main families of dihydrofolate reductase (*dfr*) comprise gene cassettes that confer resistance to the drug trimethoprim. They are grouped into two main families, A and B. At least 20 different *dfr*A sequences and 5 *dfr*b have been reported so far. Trimethoprim resistance in clinically significant Gram-negative bacteria is usually caused by horizontally

transferable resistance genes (*dfr* genes) coding for alternative resistant dihydrofolate reductases (104, 172).

4. Chloramphenicol Resistance Gene cassettes: This gene cassette's product confers resistance to chloramphenicol either via acetylation or efflux pump. Two main families of gene cassettes are responsible for chloramphenicol resistance, *cat* and *cml*. The former families are involved in drug modification, whereas the latter families produce products that promote activity of a chloramphenicol efflux pump (79, 183).

3. 3'Conserved Sequence

Most class 1 integrons have a 3' conserved sequence (3'CS). The 3'CS carries *qacEΔ1* gene, a semi functional derivative of the quaternary ammonium compounds resistance gene, *qacE*, the sulfonamide resistance gene (*sul1*), and an open reading frame of unknown function called ORF5. Similar 3'CS are not found in other classes of integrons. (91, 93, 183)

IV. THE BACTERIA

The population of resistant microorganisms in a host organ depends upon the ability of the system to harbour a large commensal flora (57). The digestive tract is the largest reservoir of commensal bacteria within the host and has been found to be the largest reservoir of resistance genes (216). This has been ascribed to the fact that the huge intestinal microflora forms an ideal environment for horizontal gene transfer (34). Three types of bacteria were included in this study, *Escherichia coli*, *Salmonella* spp. and *Proteus mirabilis,* and thus a discussion of those organisms follows.

Escherichia coli

Although normally commensal, some strains of *E. coli* are associated with infections in humans and animals. In swine, pathogenic *E. coli* can cause enteric colibacillosis, edema disease or mastitis metritis agalactia syndrome (MMA). Enteric colibacillosis, or piglet scours, causes diarrhea and dehydration in first few days of life and in piglets 1-2 weeks of age. This diarrhea is responsible for significant economic losses due to mortality, morbidity, decreased growth rate, and cost of medication (62, 73, 88). Edema disease, or *E. coli* enterotoxaemia, affects growing pigs several weeks after weaning. It is often rapidly fatal (73). MMA occurs in sows just after farrowing and results in a lack of milk which causes high piglet mortality. In chickens, *E. coli* may cause infections of the respiratory tract and soft tissues, resulting in colibacillosis, air sacculitis, and cellulitis. *E. coli* can also cause yolk sac infections, which is a common cause of chick mortality in the first week of life.

Salmonella spp.

Salmonella is a genus of rod-shaped Gram-negative enteric bacteria. There are currently 2,463 known *Salmonella* serotypes (169). This genus of bacteria can be divided into 3 groups according to clinical syndrome in humans: typhoid fever, bacteremia, and enterocolitis (187). Among those syndromes only bacteremia and enterocolitis, caused by non typhoidal *S. enterica* serotypes, are associated with animal reservoirs and human disease outbreaks caused by this bacterium, commonly resulting from animal to human transmission. In contrast, typhoid fever, caused by typhoidal *S. enterica* serotypes, do not have animal reservoirs and are maintained in human populations by person to person transmission (146). Salmonellosis is a major cause of bacterial enteric illness in both humans and animals. On the global scale, it has been estimated that each year about 1.3 billion cases of gastroenteritis due to nontyphoidal salmonellosis occur, resulting in 3 million deaths (161). Among humans in the United States, an estimated 1.4 million cases of salmonellosis occur annually (141). Furthermore, Salmonella-induced enterocolitis is the single most common cause of death from foodborne illness associated with viruses, parasites or bacteria in the United State (141). Transmission of *Salmonella* in food animals typically occurs through fecal-oral or aerogenous transmission. Rodents, insects, cats, birds, humans, contaminated feed, and transportation vehicles can act as vectors for dissemination of *Salmonella.* Although animal to human transmission may occur through direct contact, the most important source of human infection is contaminated food products of animal origin (100). In the United States, chicken, beef, turkey and eggs are the most common food vehicles for transfer of *Salmonella* to humans. *Salmonella*

contamination of food and food products may occur either because animals are infected or because fecal contamination occurs during food processing.

Proteus mirabilis

Proteus mirabilis is a rod-shaped Gram-negative, facultative anaerobic bacterium belonging to family *Enterobacteriaceae*. This bacterium is part of the normal gut flora of a healthy individual, but is also an opportunistic pathogen. *P. mirabilis* is an important opportunistic uropathogen that is frequently isolated from patients with complications from urinary tract infections (199, 237). Recently, several research groups have reported the presence of mobile genetic elements involved in antibiotic resistance dissemination in *P. mirabilis*. For example, clinical *P. mirabilis* isolates harboring *Salmonella* genomic island 1 containing the multiple antibiotic resistance regions have been reported in Japan (3). Using PCR targeting conserved sequences of integrons, Tsakris et al. (213) noted that the $bla_{\text{VIM-1}}$ allele in *P. mirabilis* strains that were phenotypically metallo-ß-lactamase (MBL)-positive, was located in a common integron structure. Class 1 integrons were also detected as common carriers of antibiotic resistance genes, such as *aadA1*, *aadB*, and *aadA2*, in multidrug-resistant isolates of *P. mirabilis* collected from retail meat in Oklahoma, Japan (116).

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CHAPTER 3: PREVALENCE OF CLASS 1 INTEGRONS AND ANTIBIOTIC RESISTANCE PATTERNS IN BACTERIA OF BROILER CHICKENS IN THE US AND THAILAND

ABSTRACT

The objective of this study was to investigate the prevalence of class1 integrons and antimicrobial resistance patterns in *E. coli, P. mirabilis,* and *Salmonella* isolates of commercial chickens from the US and Thailand. Class 1 integrons, as indicated by the presence of *intI1*, were detected in 1,732 isolates (45.4%) of the total 3,824 isolates. Simultaneous presence of all three conserved genes (*intI1, qacEΔ1*, and *sul1*) was found in 1,044 (27.3%) isolates. The prevalence of class 1 integrons in *E. coli* and *Salmonella* spp. isolates was found to differ with isolate origin (P<0.001). In both types of bacteria, class 1 integrons were more prevalent in isolates from Thailand compared with the US (70.3% and 44.6% of *E. coli* isolates and 48.1% and 0.5% of *Salmonella* spp. isolates from Thailand and the US, respectively). Among *P. mirabilis*, 29.9% of isolates from the US carried integrons; however, this prevalence was not different (P=0.81) from that of isolates from Thailand (38.2%). Most isolates were found to be multi-antibiotic resistant. A high proportion of isolates demonstrated resistance to tetracycline (80.2%), sulfonamides (67.7%), cephalothin (62.5%), and ampicillin (57.8%), whereas no isolates were resistant to amikacin. Integron-positive isolates were more likely to be resistant to sulfamethoxazole than integron-negative isolates. Thailand isolates had a greater occurence of resistance (P<0.01) to chloramphenicol, nalidixic acid, and trimethoprim/sulfamethoxazole than isolates from the US. Whereas, both *E. coli* and *Salmonella* isolates from the US demonstrated a greater prevalence of resistance (P<0.01) to amoxicillin/clavulanic acid, cefoxitin, and ceftiofur than those from Thailand. *Salmonella* and *P. mirabilis* isolates from the US were also more frequently resistant

($P<0.01$) to cephalothin than isolates from Thailand. Among 85 antibiotic resistance patterns found in antibiotic susceptibility tests, 11 patterns were found in both integron-positive and integron-negative isolates, and only one pattern, resistance to tetracycline, was found among isolates from both countries. These results indicate that class 1 integrons are common in commensal and foodborne bacteria in chickens, and that some, but not all, antibiotic resistances in those isolates were found in the presence of class 1 integrons.

I. INTRODUCTION

Food animals are often exposed to antimicrobials to treat and prevent infectious diseases or to promote growth (37). Antimicrobial usage in food animals provides selection pressure that favors an increase of antimicrobial resistance in bacteria (23, 29, 32, 34). Antimicrobial resistance has emerged not only in commensal bacteria and bacterial pathogens of animals but also in zoonotic enteropathogens (37). Resistant bacteria from animals can infect humans by direct contact and also via consumption of food products of animal origin (60, 63). These resistant bacteria can colonize humans and/or transfer their resistance genes to other bacteria of humans, which can result in treatment failure as a consequence of that antimicrobial resistance (61).

Integrons are genetic elements that mediate integration of antibiotic resistance genes through site specific recombination and convert them into functional genes (14, 20, 27, 35, 49). More than 70 different genes imparting resistance to most classes of antibiotics have been found as gene cassettes in the central region of different integrons from diverse bacteria (51). Moreover, these genetic elements can incorporate several resistance genes, allowing them to transfer as a single gene. As many as seven gene cassettes have been found within an integron (58). Integrons are often found to be carried on mobile elements such as plasmids and transposons; therefore integrons are believed to play a major role in the rapid dissemination of multi-drug resistance among bacteria (43). Four different classes of integrons have been identified based on variations in sequence of primary elements, gene cassettes, and associations with transposons (3, 5, 15, 19, 20,

47, 52). Class 1 integrons are the most common and widely distributed type (10, 19, 45, 55).

The U.S. and Thailand, while occupying very diverse geographic regions, share many commonalities in livestock production practices. Broiler chicken production tends to be intensive, with closed confinement, high biosecurity and similar high production genetic lines. Feed-based antibiotics are commonly used in both countries, although some differences occur in drugs of choice and/or availability. A comparison of class 1 integron prevalence and antibiotic resistance gene patterns of *Escherichia coli*, *Salmonella* spp., and *Proteus mirabilis* between these two countries may provide information regarding global implications of agricultural use of antibiotics with regard to antibiotic resistance.

II. MATERIALS AND METHODS

Bacterial Isolates

A total of 2,137 *E. coli*, *P. mirabilis* and *Salmonella* spp. isolates were collected via fecal swab from 1,270 broiler chickens from 7 farms at abattoirs in the US, and 1,050 isolates were collected from 427 broiler chickens from 2 farms in Thailand. All bacterial collections from Thailand took place in southern Thailand during the period of May 2003 to August 2004, whereas bacterial collections from the US took place in Tennessee during the period of January to May 2005. All isolates were recovered from fecal swabs by standard microbiological procedures. The primary isolation method for *E. coli* has been described previously (36). For *Salmonella* and *P. mirabilis* isolation, fecal swabs were first enriched in selective tetrathionate broth (Difco, Sparks, MD) and incubated at 41.5$\pm$1ºC for 18-24 h. Enriched broth cultures were then plated on Xylose Lysine Tergitol 4 agar (XLT4; Difco) for selective culture. Plates were incubated at 37$\pm$1ºC and examined after 18-24 h. Presumptive *Salmonella* and *P. mirabilis* colonies were subcultured and then plated again, and identity was confirmed with API 20E strips according to the manufacture's specification (bioMérieux Vitek, Inc., Hazelwood, MO). Up to 6 colonies of each bacterial type were selected for analysis. Bacterial cultures were maintained at -80°C in 10% glycerol until analysis.

Multiplex PCR (MP-PCR)

Integron harboring isolates were detected using a MP-PCR targeting three conserved sequences of class 1 integrons, *intI1, qacEΔ1*, and *sul1* (18). Primer pairs were

manufactured by Operon, Inc. (Alameda, CA) (Table 1). Total DNA was prepared by boiling overnight cultures in 2YT broth (Difco) in an equal volume of 0.2% (wt/vol) Triton X-100 (Mallinckrodt, Paris, KY) for 5 min (30). Boiled cultures were cooled on ice for 5 min and used immediately for PCR. PCR reagents, excluding template DNA, were combined in a master mix prior to aliquoting. The final reaction volumes for each aliquot included: 1) 1 μL of each primer pair (50 pmol (each primer) μL^{-1}); 2) 1 μL of *Taq* DNA polymerase (0.5U μL^{-1}; Promega, Madison, WI); 3) 10 μL reaction buffer (12.5mM $MgCl_2$, pH 8.5; Invitrogen, Carlsbad, CA); 4) 5 μL dNTPs solution (2.5mM of each dNTP, pH 8.0; Invitrogen); and 5) 32 μL sterile H_2O. Sample DNA (1 μL) was then added to each aliquot. Reactions were conducted in a Mastercyler Gradient thermocycler (Eppendorf, Westbury, NJ) with the following conditions: 1) 1 cycle of 94°C for 4 min; 2) 10 "touchdown" cycles of 94°C for 1 min, 65°C for 30s (decreasing 1°C/cycle), 70°C for 2 min; 3) 24 cycles of 94°C for 1 min, 55°C for 30s, 70°C for 2 min; and 4) 1 final cycle of 70°C for 5 min. *Salmonella* Typhimurium DT104 known to contain two class 1 integrons (23) was used as a positive control. A blank containing only PCR reagents and Triton X-100 was used as a negative control. Reaction products were separated by conventional electrophoresis in 1.5 % agarose and stained with ethidium bromide for visualization (Figure 1). Prevalence of class 1 integrons was based on the presence of the *IntI1* gene.

The integron prevalence in each bacterial species was compared between Thailand and the US using the freq procedure of SAS (SAS 8.2, SAS Institute Inc, Cary,

Table 1. Primer pairs used in MP-PCR.

Target	Sequence (5' to 3')	PCR Product (bp)
int1	(F) GGTTCGAATGTCGTAACCGC	248
	(R) ACGCCCTTGAGCGGAAGTATC	
sul1	(F) ATCAGACGTCGTGGATGTCG	346
	(R) CGAAGAACCGCACAATCTCG	
qacEΔ1	(F) GAGGGCTTTACTAAGCTTGC	200
	(R) ATACCTACAAAGCCCCACGC	

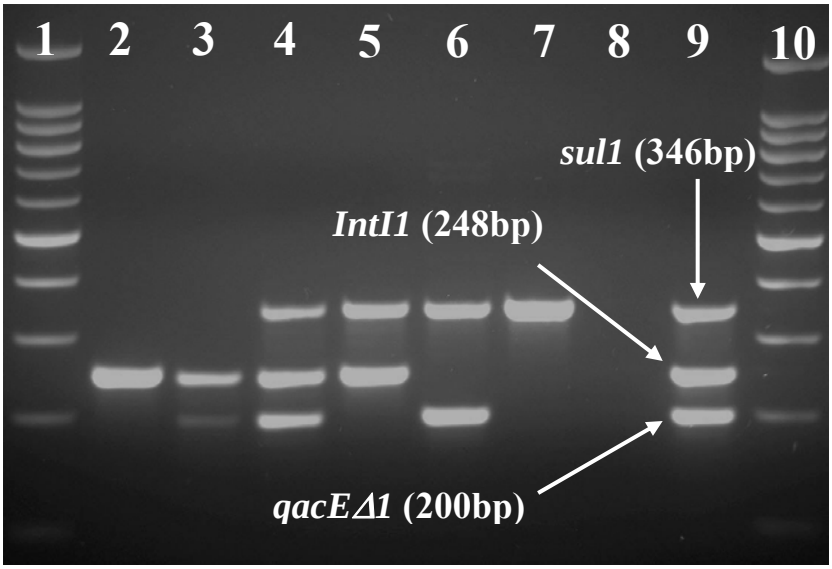

Figure 1. Multiplex PCR detecting class 1 integrons gene sequence.
Lane 1 and 10; 100 bp DNA ladders; Lane 2-7 wild type isolates; lane 8; negative control; and lane 9; *Salmonella enterica* Typhimurium DT104 (positive control)

NC) Comparisons were made using the Fisher's two-sided exact tests. Differences were considered significant at $P < 0.05$.

Antibiotic Susceptibility Testing

Antibiotic MICs were determined for subsets of each bacterial type using the National Antimicrobial Resistance Monitoring System (NARMS) microdilution sensititre plates, CMV7CNCD, (Sentititre, Trek Diagnostic System Inc., Cleveland, Ohio) according to Clinical and Laboratory Standards Institute (CLSI), (formerly National Committee on Clinical Laboratory Standards, NCCLS) broth microdilution guidelines. *E.coli* ATCC 25922 was used as a reference strain. The isolates were tested for resistance to 16 antibacterials or antibacterial combinations that included: amoxicillin/clavulanic acid (Aug), ampicillin (Amp), ciprofloxacin (Cip), cefoxitin (Fox), ceftiofur (Tio), ceftriaxone (Axo), cephalothin (Cpl), chloramphenicol (Chl), amikacin (Ami), gentamicin (Gen), kanamycin (Kan), nalidixic acid (Nal), streptomycin (Str), sulfamethoxazole (Sul), tetracycline (Tet), and trimethoprim/sulfamethoxazole (Sxt). The CMV7CNCD plate layout is shown in Figure 2. Results were interpreted using CLSI guidelines for broth microdilution methods for veterinary *E. coli* (Table 2). In the phenotypic analysis, isolates with intermediate MICs were not considered as resistant.

The frequency of antibiotic resistance in integron-positive isolates was compared to that of integron-negative isolates and frequency of antibiotic resistance in isolates from Thailand was compared to the US using the Analysis and Statcalc programs of the Epi Info™ version 3.4.1 software package from the Centers for Disease Control and

Prevention (11). Comparisons were made using Fisher's two-sided exact tests. Differences were considered significant at $P<0.05$.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Fox 16	Ami 1	Tet 16	Axo 2	Aug 2/1	Cip 0.06	Gen 0.5	Nal 0.5	Sul 512	Sxt 1/19	Cpl 2	Amp 4
B	Fox 8	Ami 0.5	Tet 8	Axo 1	Aug 1/0.5	Cip 0.03	Gen 0.25	Tio 8	Sul 256	Sxt 0.5/9.5	Kan 64	Amp 2
C	Fox 4	Chl 32	Tet 4	Axo 0.5	Cip 4	Cip 0.015	Nal 32	Tio 4	Sul 128	Sxt 0.25/4.75	Kan 32	Amp 1
D	Fox 2	Chl 16	Axo 64	Axo 0.25	Cip 2	Gen 16	Nal 16	Tio 2	Sul 64	Sxt 0.12/2.38	Kan 16	Str 64
E	Fox 1	Chl 8	Axo 32	Aug 32/16	Cip 1	Gen 8	Nal 8	Tio 1	Sul 32	Cpl 32	Kan 8	Str 32
F	Fox 0.5	Chl 4	Axo 16	Aug 16/8	Cip 0.5	Gen 4	Nal 4	Tio 0.5	Sul 16	Cpl 16	Amp 32	Pos
G	Ami 4	Chl 2	Axo 8	Aug 8/4	Cip 0.25	Gen 2	Nal 2	Tio 0.25	Sxt 4/76	Cpl 8	Amp 16	Pos
H	Ami 2	Tet 32	Axo 4	Aug 4/2	Cip 0.12	Gen 1	Nal 1	Tio 0.12	Sxt 2/38	Cpl 4	Amp 8	Pos

Figure 2. Sensititre non-fastidious Gram negative plate (CMV7CNCD) format.
Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline; Pos, growth control wells.

Table 2. Minimum Inhibitory Concentration (MIC) breakpoint. [1]

Drugs	Susceptible (μg/ml)	Intermediate (μg/ml)	Resistant (μg/ml)
Aminoglycoside			
Amikacin (Ami)	≤ 16	32	≥ 64
Gentamicin (Gen)	≤ 4	8	≥ 16
Kanamycin (Kan)	≤ 16	32	≥ 64
Streptomycin (Str)	≤ 32		≥ 64
β-Lactams			
Ampicillin (Amp)	≤ 8	16	≥ 32
Amoxicillin/Clavulanic acid (Aug)	≤ 8/4	16/8	≥ 32/16
Cephalothin (Cpl)	≤ 8	16	≥ 32
Cefoxitin (Fox)	≤ 8	16	≥ 32
Ceftiofur (Tio)	≤ 2	4	≥ 8
Ceftriaxone (Axo)	≤ 8	16,32	≥ 64
Chloramphenicol (Chl)	≤ 8	16	≥ 32
Quinolone			
Ciprofloxacin (Cip)	≤ 1	2	≥ 4
Nalidixic Acid (Nal)	≤ 16		≥ 32
Sulfonamides			
Sulfamethoxazole (Sul)	≤ 256		≥ 512
Trimethoprim/Sulfamethoxazole (Sxt)	≤ 2/38		≥ 4/76
Tetracycline (Tet)	≤ 4	8	≥ 16

[1] MICs determined via microdilution broth methods in accordance with CLSI standards (12, 13).

III. RESULTS

Prevalence of Bacterial Isolates

The frequency of *E. coli* isolates from chickens was high in both countries with 97.4% and 95.8 % of chickens from Thailand and the US, respectively, carrying *E. coli*. However, the prevalence of *Salmonella* spp. and *P. mirabilis* in chickens was considerably lower than *E. coli.* The prevalence of *Salmonella* was higher in the US, with that organism being detected in 21.2% of chickens, whereas only 3% of chickens in Thailand were found to carry *Salmonella*. A similar prevalence of *P. mirabilis* was found in Thailand and the US with 12.2 and 11.8% of chickens from Thailand and the US, respectively, were found to carry these bacteria. However, the prevalence of both bacteria varied between farms ranging from 0 to 66% and 0 to 5.2% for *Salmonella* isolates and 1.3 to 24.3% and 7.0 to 20.6% for *P. mirabilis* isolates from the US and Thailand, respectively. Table 3 shows the number of positive samples for each type of bacteria and their distribution by country and farm.

Table 3. Prevalence of *E. coli*, *Salmonella,* and *P. mirabilis* of chickens in the US and Thailand.

Countries	Farm	# of animals sampled	# of animals carrying bacteria (%)		
			E. coli	*Salmonella*	*P. mirabilis*
US	US 1	70	67 (95.7)	8 (11.4)	4 (5.7)
	US 2	150	145 (96.7)	99 (66.0)	2 (1.3)
	US 3	150	147 (98.0)	24 (16.0)	12 (8.0)
	US 4	150	142 (94.5)	27 (18.0)	14 (9.3)
	US 5	150	139 (92.7)	9 (6.0)	23 (15.3)
	US 6	300	287 (95.7)	102 (34.0)	22 (7.3)
	US 7	300	290 (96.7)	0 (0)	73 (24.3)
	Total	1,270	1,217(95.8)	269 (21.2)	150 (11.8)
Thailand	TH 1	267	267 (100)	14 (5.2)	19 (7.0)
	TH 2	160	149 (93.0)	0 (0)	33 (20.6)
	Total	427	416 (97.4)	14 (3.3)	52 (12.2)

Prevalence of Class 1 Integrons

Prevalence of class 1 integrons in *E. coli*, *Salmonella* spp., and *P. mirabilis* in chickens from the US and Thailand is shown in Table 4. Class 1 integrons, as indicated by the presence of *intI1*, were detected in 1,732 isolates (45.4%). Simultaneous presence of all three conserved genes (*intI1, qacEΔ1*, and *sul1*) was found in 1,044 (27.3%) of the total of 3,824 isolates. The prevalence of class 1 integrons in *E. coli* and *Salmonella* spp. was found to differ with isolate origin ($P<0.001$). In both types of bacteria, class 1 integrons were more prevalent in isolates from Thailand when compared with the US. (70.3 and 44.6% for *E.coli* isolates, 48.1 and 0.5% for *Salmonella* spp. isolates from Thailand and the US, respectively). Among *P. mirabilis*, 29.9% of isolates from US carried integrons; however this was not different from Thailand (38.2%) ($P=0.81$). There were no differences in prevalence of class 1 integrons across farms among *Salmonella* spp. ($P=0.99$). In contrast, the prevalence of integrons across farms in both *E. coli* and *P. mirabilis* isolates differed ($P<0.001$) (data not shown)

Table 4. Prevalence of integrons and integron component genes in bacteria of chickens from the US and Thailand. [1]

	E. coli		*P. mirabilis*		*Salmonella*	
	The US	Thailand	The US	Thailand	The US	Thailand
No. of isolates	1161	1341	279	334	576	133
No.(%) carrying						
- *IntI1* (*I*)	518 (44.6)[b]	942 (70.3)[a]	83 (29.9)[a]	127 (38.2)[a]	3 (0.5)[b]	64 (48.1)[a]
- All 3 conserved genes (*I, Q, S*)	502 (43.2)[a]	307 (22.9)[b]	83 (29.9)[a]	96 (28.8)[a]	3 (0.5)[b]	53 (39.9)[a]

[1] Results are reported as numbers and percentage of bacteria carrying integrons and genes associated with integrons, as determined by multiplex PCR. ***I*****,** *intI1*; *Q*, *qacEΔ1*; *S*, *sul1*.
[a,b] Values within the same bacteria and row not sharing like superscripts differ (P<0.05).

Antibiotic Susceptibility

E. coli isolates

A comparison of antibiotic resistance patterns between integron-positive and integron-negative *E. coli* isolates is shown in Figure 3. The highest percentage of resistance in integron-positive *E. coli* was found to tetracycline (100%), followed by sulfamethoxazone (97.8%), cephalothin (75.6%), streptomycin (71.1%) and gentamicin (62.2%); whereas 100, 82.2, 68.9, 55.6, and 51.1 % of integron-negative isolates showed resistance to tetracycline, cephalothin, ampicillin and sulfamethoxazone, respectively. All integron-negative isolates were susceptible to chloramphenicol and amikacin, whereas all integron-positive isolates were only susceptible to amikacin. Integron-negative isolates demonstrated a low percentage of resistance (2.2–20%) to ceftriaxone, gentamicin, kanamycin, ciprofloxacin, trimetroprim/sulfamethoxazole and nalidixic acid. Among integron-positive isolates, 2.2 to 28% of isolates were resistant to ceftriaxone, chloramphenicol, ciprofloxacin, kanamycin and nalidixic acid. Resistance to gentamicin, kanamycin, chloramphenicol, and sulfamethoxazole was more common in integron–positive isolates ($P<0.05$).

Compared to Thailand, US isolates showed a higher percentage of resistance to tetracycline, cephalothin, streptomycin, sulfamethoxazole, ampicillin, amoxicillin/clavulanic acid, ceftiofur and cefoxitin (100, 81.4, 77.1, 70, 61.4, 58.6, 57.1 and 54.3%, respectively). All US isolates were susceptible to amikacin, chloramphenicol, and ciprofloxacin and showed a high level of susceptibility (85.7-97.3%) to kanamycin, trimethoprim/sulfamethoxazole, nalidixic acid, and ceftriaxone.

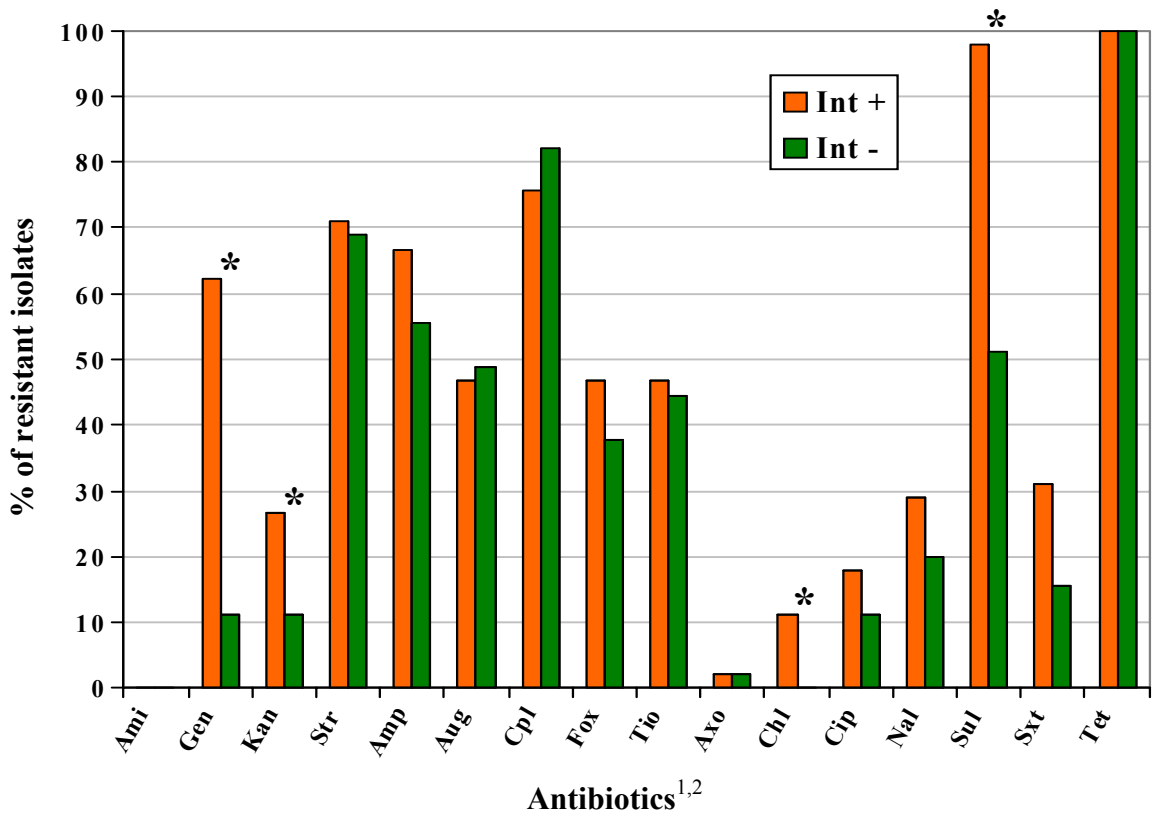

Figure 3. Frequency of resistance to 16 antimicrobials in integron-positive and integron-negative *E. coli* from chickens.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within the same antibiotic with * differ ($P<0.05$)

All Thailand isolates were resistant to tetracycline and showed a high resistance to nalidixic acid, sulfamethoxazole, cephalothin, ciprofloxacin nalidixic acid, sulfamethoxazole, cephalothin, ciprofoxacin, trimethoprim/sulfamethoxazone and ampicillin (95, 90, 70, 65, 65 and 60%, respectively). All *E. coli* isolates from Thailand were susceptible to amikacin, gentamicin, cefoxitin, and ceftriaxone. Only 5, 10, 25 and 35 % of *E. coli* isolates from Thailand were resistant to ceftiofur, amoxicillin/clavulanic acid, chloramphenicol, and kanamycin. Resistance to gentamicin, streptomycin, amoxicillin/clavulanic acid, cefoxitin and cetriofur was more common in US isolates ($P<0.05$); whereas isolates from Thailand showed a higher percentage of resistance to kanamycin, chloramphenicol, ciprofloxacin, trimethoprim/sulfamethoxazole, and nalidixic acid than isolates from the US ($P<0.0001$) (Figure 4).

Overall, all *E. coli* isolates from chickens were resistant to at least one antibiotic. Integron-positive and -negative isolates were resistant to 2 to 10 and 1 to 10 antimicrobials, respectively. The highest proportion of integron–positive isolates was resistant to 9 antimicrobials, whereas the highest proportion of integron-negative isolates was resistant to 6 antimicrobials. *E. coli* isolates from the US and Thailand were resistant to 2 to 10 and 1 to 9 antimicrobials, respectively. Whereas 90% of *E. coli* isolates from Thailand were resistant to more than 5 antimicrobials, only 62% of isolates from the US showed resistance to that number of antimicrobials (Figure 5).

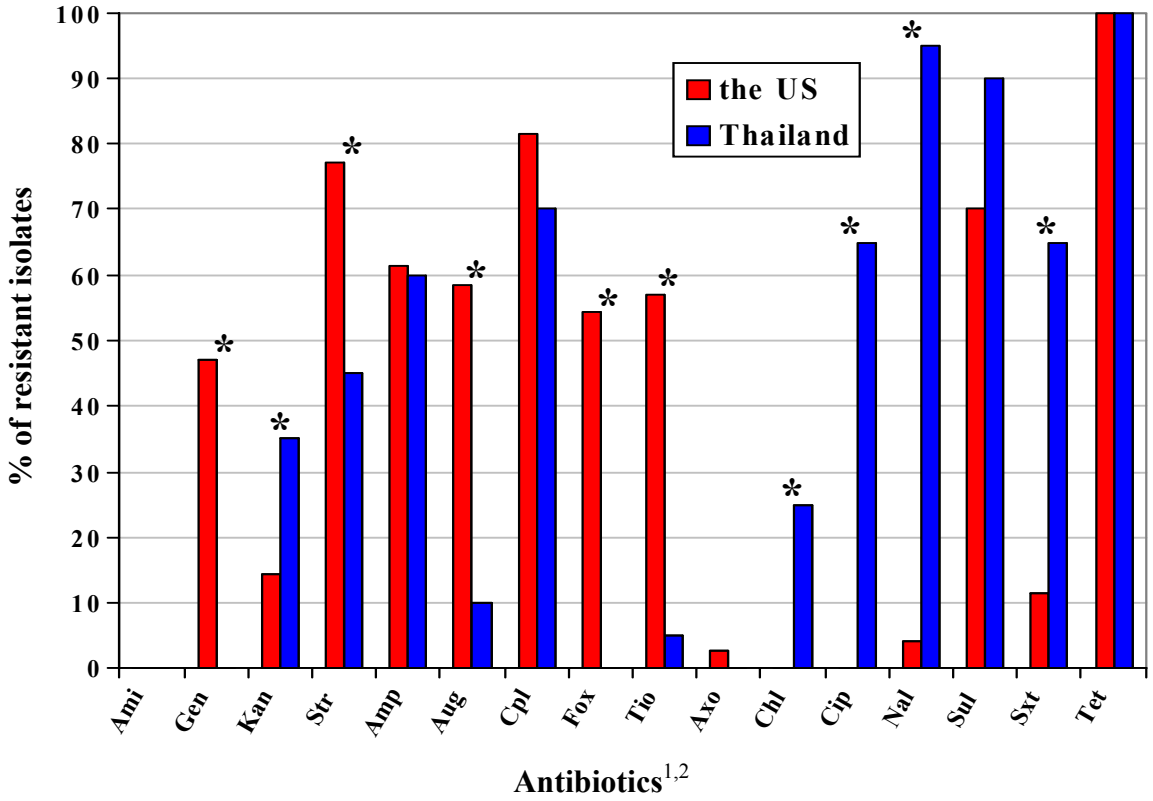

Figure 4. Frequency of resistance to 16 antimicrobials in *E. coli* of chickens from the US and Thailand.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within the same antibiotic with * differ ($P<0.05$)

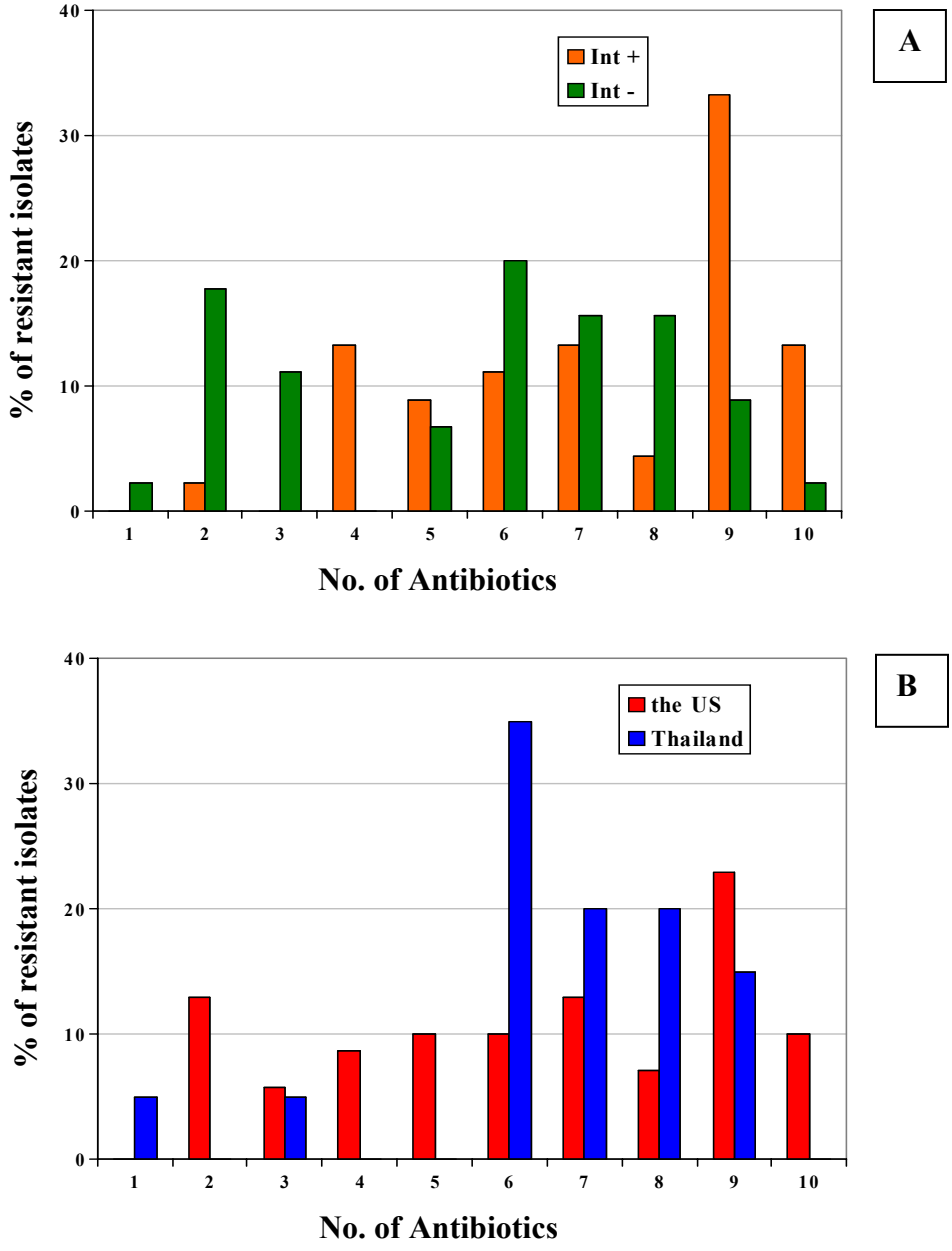

Figure 5. Multiple antimicrobial resistance of *E. coli* from chickens.

A. Integron-positive and integron-negative isolates.
B. Isolates from the US and Thailand.

A total of 47 patterns were observed among 90 *E. coli* isolates. The most frequent pattern was resistance to 9 antimicrobials, Aug-Amp-Fox-Tio-Cpl-Gen-Str-Sul-Tet, which was found in 11.1% of isolates. Seven isolates (7.8%) exhibited resistance to the highest number of antimicrobials (10) with those being Aug-Amp-Fox-Tio-Cpl-Kan-Gen-Str-Sul-Tet and Aug-Amp-Fox-Tio-Cpl-Gen-Str-Sul-Tet-Sxt. Among the 47 patterns, eight patterns were found in both integron-positive and integron-negative isolates. However, no common pattern was found among isolates from Thailand and the US (Table 5).

Table 5. Antibiograms of *E. coli* isolates of chickens from the US and Thailand.

Pattern No.	Antibiogram[1]	No. of isolate(s)				
		Integron		Location		Total
		+ (n=45)	- (n=45)	US (n=70)	Thailand (n=20)	(n=90)
1	Aug-Amp-Fox-Tio-Axo-Cpl-Str-Sul-Tet	0	1	1	0	1
2	Aug-Amp-Fox-Tio-Axo-Cpl-Sul-Tet-Sxt	1	0	1	0	1
3	Aug-Amp-Fox-Tio-Cpl-Kan-Gen-Str-Sul-Tet [2]	4	1	5	0	5
4	Aug-Amp-Fox-Tio-Cpl-Kan-Str-Sul-Tet	0	1	1	0	1
5	Aug-Amp-Fox-Tio-Cpl-Gen-Nal-Sul-Tet	1	0	1	0	1
6	Aug-Amp-Fox-Tio-Cpl-Gen-Str-Sul-Tet-Sxt	2	0	2	0	2
7	Aug-Amp-Fox-Tio-Cpl-Gen-Str-Sul-Tet [2]	9	1	10	0	10
8	Aug-Amp-Fox-Tio-Cpl-Str-Sul-Tet-Sxt [2]	1	1	2	0	2
9	Aug-Amp-Fox-Tio-Cpl-Str-Sul-Tet	0	3	3	0	3
10	Aug-Amp-Fox-Tio-Cpl-Str-Tet	0	2	2	0	2
11	Aug-Amp-Fox-Tio-Cpl-Sul-Tet-Sxt	0	1	1	0	1
12	Aug-Amp-Fox-Tio-Cpl-Sul-Tet[2]	3	1	4	0	4
13	Aug-Amp-Fox-Tio-Cpl-Tet	0	3	3	0	3
14	Aug-Amp-Fox-Cpl-Str-Sul-Tet	0	1	1	0	1
15	Aug-Amp-Tio-Cpl-Cip-Nal-Sul-Tet	0	1	0	1	1
16	Aug-Amp-Tio-Cpl-Gen-Str-Sul-Tet	0	1	1	0	1
17	Aug-Amp-Tio-Cpl-Str-Sul-Tet	0	1	1	0	1
18	Aug-Amp-Tio-Cpl-Str-Tet	0	1	1	0	1
19	Aug-Amp-Cpl-Cip-Nal-Sul-Tet-Sxt	0	1	0	1	1
20	Aug-Amp-Cpl-Str-Tet	0	1	1	0	1
21	Amp-Fox-Tio-Cpl-Tet	0	1	1	0	1
22	Amp-Cpl-Chl-Cip-Kan-Nal-Sul-Tet-Sxt	2	0	0	2	2

Table 5. (continued) Antibiograms of *E. coli* isolates of chickens from the US and Thailand.

Pattern No.	Antibiogram[1]	No. of isolate(s)				
		Integron		Location		Total
		+ (n=45)	- (n=45)	US (n=70)	Thailand (n=20)	(n=90)
23	Amp-Cpl-Cip-Nal-Sul-Tet-Sxt [2]	2	1	0	3	3
24	Amp-Cpl-Nal-Sul-Tet-Sxt	1	0	0	1	1
25	Amp-Chl-Cip-Kan-Nal-Str-Sul-Tet	1	0	0	1	1
26	Amp-Chl-Cip-Nal-Str-Sul-Tet-Sxt	1	0	0	1	1
27	Amp-Cip-Nal-Sul-Tet-Sxt	1	0	0	1	1
28	Amp-Gen-Str-Sul-Tet	1	0	1	0	1
29	Amp-Nal-Tet	0	1	0	1	1
30	Cpl-Chl-Cip-Kan-Nal-Str-Sul-Tet-Sxt	1	0	0	1	1
31	Cpl-Cip-Nal-Str-Sul-Tet	0	1	0	1	1
32	Cpl-Kan-Gen-Nal-Str-Sul-Tet	1	0	1	0	1
33	Cpl-Kan-Gen-Str-Sul-Tet [2]	1	1	2	0	2
34	Cpl-Kan-Nal-Str-Sul-Tet-Sxt	0	1	0	1	1
35	Cpl-Kan-Nal-Str-Sul-Tet	1	0	0	1	1
36	Cpl-Gen-Str-Sul-Tet-Sxt	1	0	1	0	1
37	Cpl-Gen-Str-Sul-Tet [2]	1	1	2	0	2
38	Cpl-Gen-Sul-Tet	1	0	1	0	1
39	Cpl-Na-Str-Sul-Tet-Sxt	0	2	0	2	2
40	Cpl-Str-Tet	0	4	4	0	4
41	Cpl-Tet [2]	1	3	4	0	4
42	Cip-Kan-Nal-Str-Sul-Tet	0	1	0	1	1
43	Kan-Str-Sul-Tet-Sxt	1	0	1	0	1
44	Gen-Nal-Str-Sul-Tet	1	0	1	0	1
45	Gen-Str-Sul-Tet	5	0	5	0	5
46	Str-Tet	0	5	5	0	5
47	Tet	0	1	0	1	1

[1] Aug, Amoxicillin/Clavulanic Acid; Amp, Ampicillin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Cpl, Cephalothin; Chl, Chloramphenicol; Cip, Ciprofloxacin; Gen, Gentamicin; Kan, Kanamycin; Nal, Nalidixic Acid; Str, Streptomycin; Sul, Sulfamethoxazole; Tet, Tetracycline; Sxt, Trimethoprim/Sulfamethoxazole.
[2] Resistance pattern found in both integron-positive and integron-negative isolates.

Salmonella isolates

A comparison of antibiotic resistance patterns between integron-positive and integron-negative *Salmonella* spp. isolates is provided in Figure 6. All *Salmonella* isolates were susceptible to amikacin, gentamicin, kanamycin, streptomycin, ceftriaxone and ciprofloxacin. In addition, all integron-negative isolates were also susceptible to chloramphenicol. All integron-positive isolates were resistant to sulfamethoxazole. Integron-positive isolates also showed a high percentage (55.6-66.7%) of resistance to ampicillin, chloramphenicol, nalidixic acid, trimethoprim/sulfamethoxazone and tetracycline, whereas only 11.5% of those isolates were resistant to amoxicillin/clavulanic acid, cephalothin, cefoxitin and ceftiofur. While a high percentage of resistance (51.5-60.6%) to ampicillin, sulfamethoxazole, amoxicillin/clavulanic acid, cephalothin, cefoxitin, and ceftiofur was found in integron-negative isolates, less than 10% of those isolates were resistant to nalidixic acid, trimetroprim/sulfamethoxazole and tetracycline. Resistance to sulfamethoxazone, chloramphenicol, nalidixic acid, tetracycline, and trimethoprim/sulfamethoxazole was more common in integron-positive isolates. In contrast, resistance to amoxicillin/clavulanic acid, cephalothin, cefoxitin, and ceftiofur was more frequent in integron-negative isolates ($P<0.05$).

A comparison between resistance frequencies of *Salmonella* isolates from Thailand and the US is shown in Figure 7. Resistance to ampicillin and sulfamethoxazole was found in *Salmonella* isolates from both countries, although no significant differences were noted ($P>0.05$). There were significant differences in resistances to amoxicillin/clavulanic acid, cephalothin, cefoxitin, ceftiofur, chloramphenicol, nalidixic

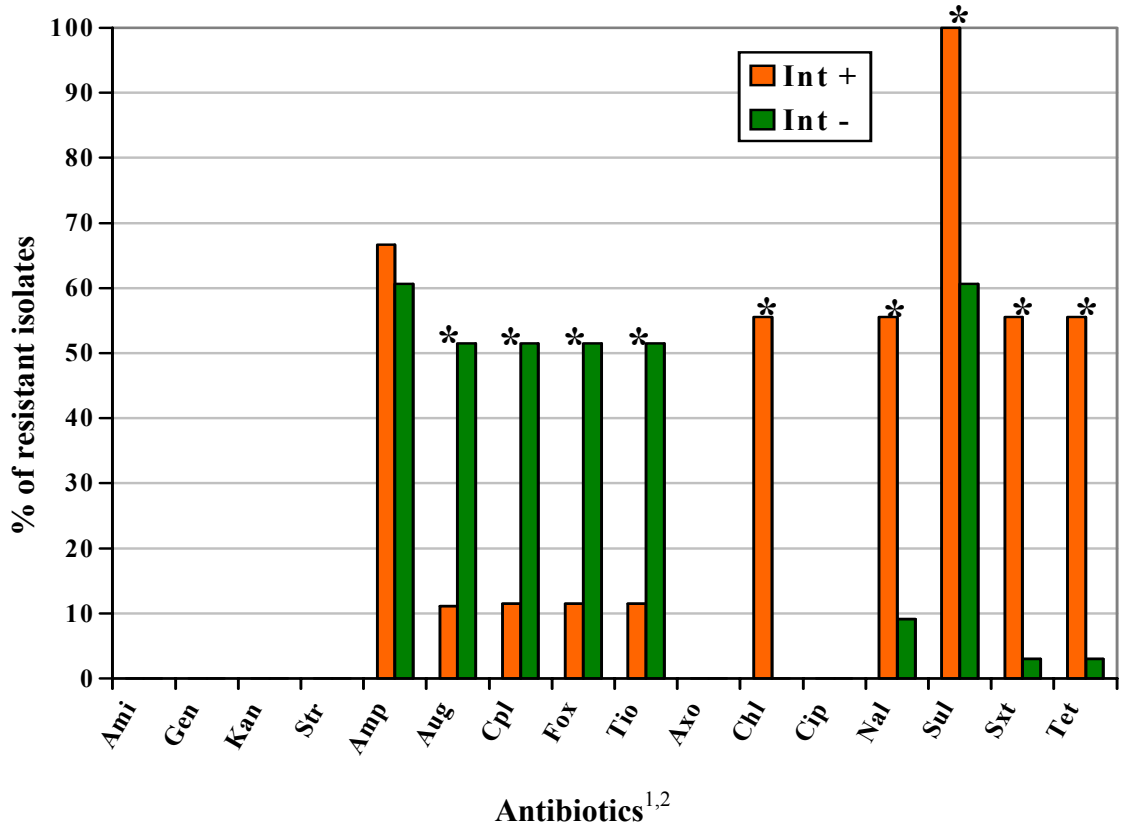

Figure 6. Frequency of resistance to 16 antimicrobials in integron-positive and integron-negative *Salmonella* spp. from chickens.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within the same antibiotic with * differ ($P<0.05$).

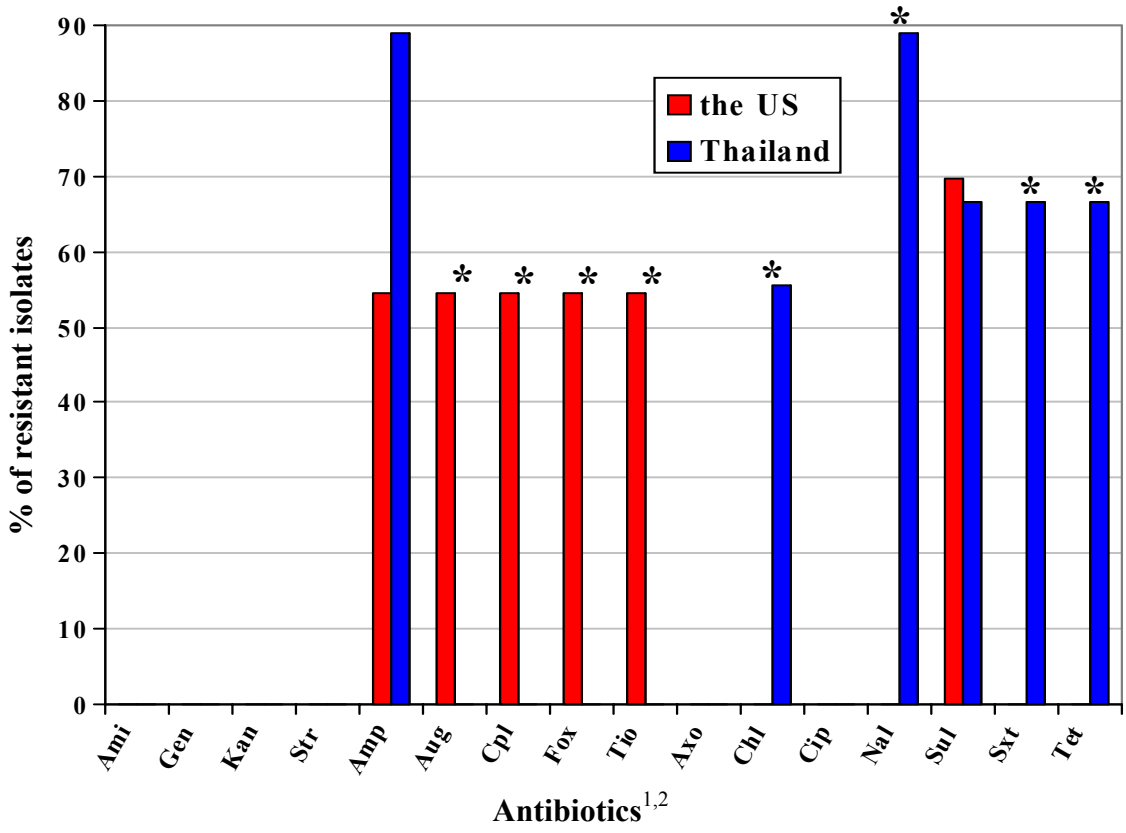

Figure 7. Frequency of resistance to 16 antimicrobials in *Salmonella* spp. of chicken isolates from the US and Thailand.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within the same antibiotic with * differ ($P<0.05$).

acid, trimethoprim/sulfamethoxazole and tetracycline between *Salmonella* isolates from Thailand and the US ($P<0.01$). Resistance to amoxicillin/clavulanic acid, cephalothin, cefoxitin, and ceftiofur was found only in isolates from the US; in contrast, resistance to chloramphenicol, nalidixic acid, trimethoprim/sulfamethoxazone and tetracycline was found only in Thailand.

All *Salmonella* isolates were resistant to 1 to 6 antibiotics (Figure 8). Among 41 *Salmonella* isolates investigated, 8 resistance patterns were observed. Fifteen isolates (36.6%) were found to have resistance to only sulfamethoxazole and appeared to be the most common resistance pattern in *Salmonella* isolates. Thirteen isolates (31.0%) demonstrated resistance to 6 antimicrobials with those being Aug-Amp-Fox-Tio-Cpl-Sul and Amp-Chl-Nal-Sul-Tet-Sxt. Two patterns were found in both integron–negative and integron-positive isolates with those being Aug-Amp-Fox-Tio-Cpl-Sul and Sul. Similar to *E. coli* isolates, no common patterns were found among *Salmonella* isolates from Thailand and the US (Table 6).

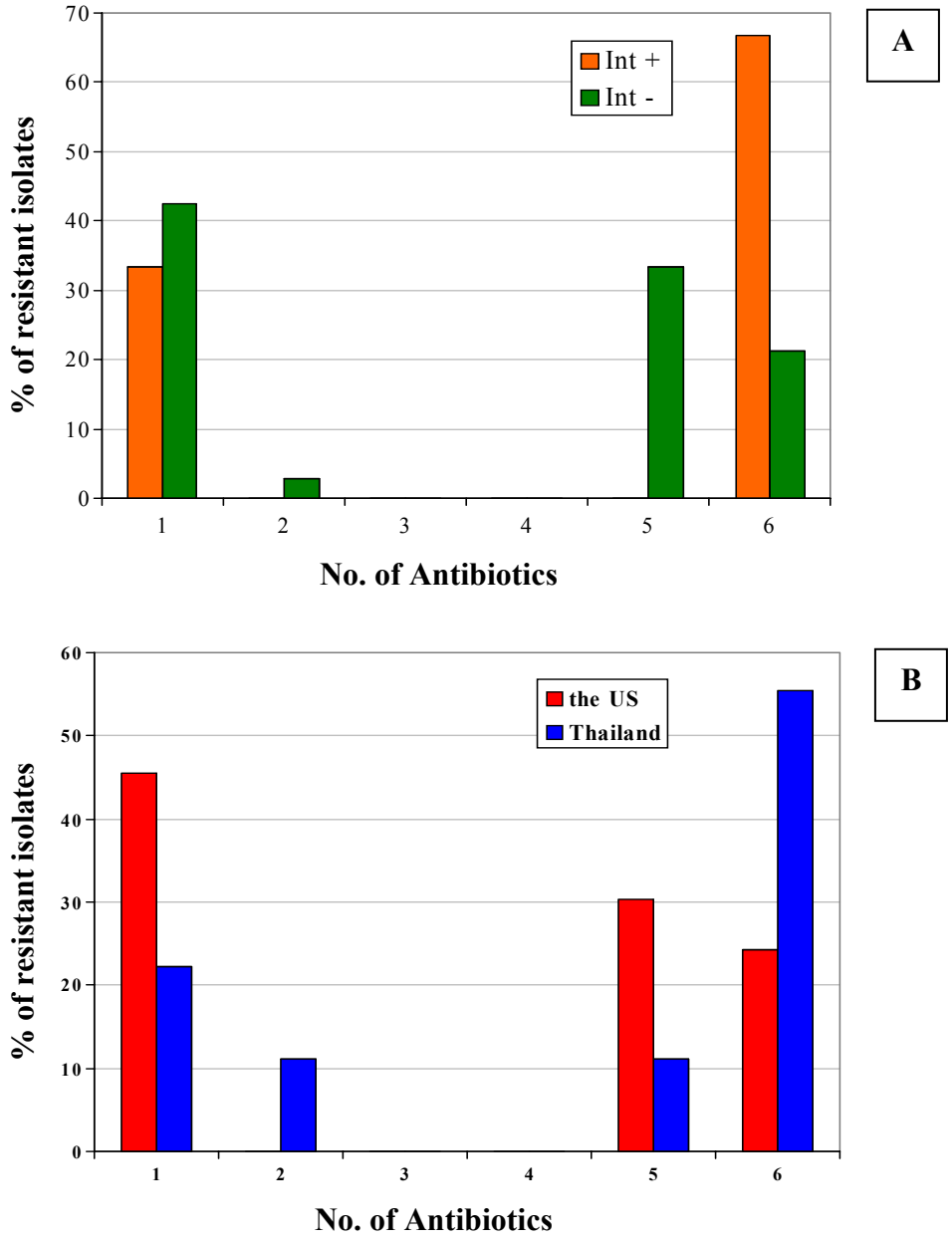

Figure 8. Multiple antimicrobial resistance of *Salmonella* spp. isolates from chickens

A. Integron-positive and integron-negative isolates.
B. Isolates from the US and Thailand.

Table 6. Antibiograms of *Salmonella* spp. isolates of chickens from the US and Thailand.

Pattern No.	Antibiogram[1]	No. of isolate(s)				
		Integron		Location		Total
		+ (n=9)	- (n=33)	US (n=33)	Thailand (n=9)	(n=42)
1	Aug-Amp-Fox-Tio-Cpl-Sul [2]	1	7	8	0	8
2	Aug-Amp-Fox-Tio-Cpl	0	10	10	0	10
3	Amp-Chl-Nal-Sul-Tet-Sxt	5	0	0	5	5
4	Amp-Nal-Sul-Tet-Sxt	0	1	0	1	1
5	Amp-Nal	0	1	0	1	1
6	Amp	0	1	0	1	1
7	Nal	0	1	0	1	1
8	Sul [2]	3	12	15	0	15

[1] Aug, Amoxicillin/Clavulanic Acid; Amp, Ampicillin; Fox, Cefoxitin; Tio, Ceftiofur; Cpl, Cephalothin; Chl, Chloramphenicol; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Tet, Tetracycline; Sxt, Trimethoprim/Sulfamethoxazole.
[2] Resistance pattern found in both integron-positive and integron-negative isolates.

P. mirabilis isolates

All 60 *P. mirabilis* isolates were susceptible to 4 of the test antibiotics, amikacin, cefoxitin, ceftiofur and ceftriazone. In addition, all integron-negative isolates were also susceptible to ciprofloxacin. All integron-positive isolates were resistant to sulfamethoxazole, which was higher than the 21.2% noted for integron-negative isolates ($P<0.001$). A high percentage (45.5-97%) of resistance to tetracycline, ampicillin and cephalothin was also found in both integron-negative and integron-positive isolates. In contrast, less than 35% of integron-negative and integron-positive isolates were resistant to gentamicin, kanamycin, streptomycin, amoxicillin/clavulanic acid, chloramphenicol, nalidixic acid and trimethoprim/sulfamethoxazole (Figure 9).

A comparison between Thailand and the US is shown in Figure 10. All Thailand and US isolates were susceptible to amikacin, cefoxitin, ceftiofur and ceftriazole, and all US isolates were also susceptible to chloramphenicol and ciprofloxacin. Resistance to nalidixic acid, trimethoprim/sulfamethoxazole, chloramphenicol, and streptomycin was more common in Thailand compared to the US ($P<0.05$); whereas isolates from the US showed a higher percentage of resistance to cephalothin ($P<0.05$).

The distribution of multiresistance among *P. mirabilis* isolates is shown in Figure 11. Overall, all *P. mirabilis* isolates from chickens were resistant to at least one antibiotic. Integron-positive and -negative isolates were resistant to 2 to 8 and 1 to 8 antimicrobials, respectively. Eighty-one percent of integron-positive isolates were resistant to at least four antimicrobials; whereas, a lower percentage was found in integron-negative isolates with 45% of those isolates demonstrated resistance to that number of antimicrobials.

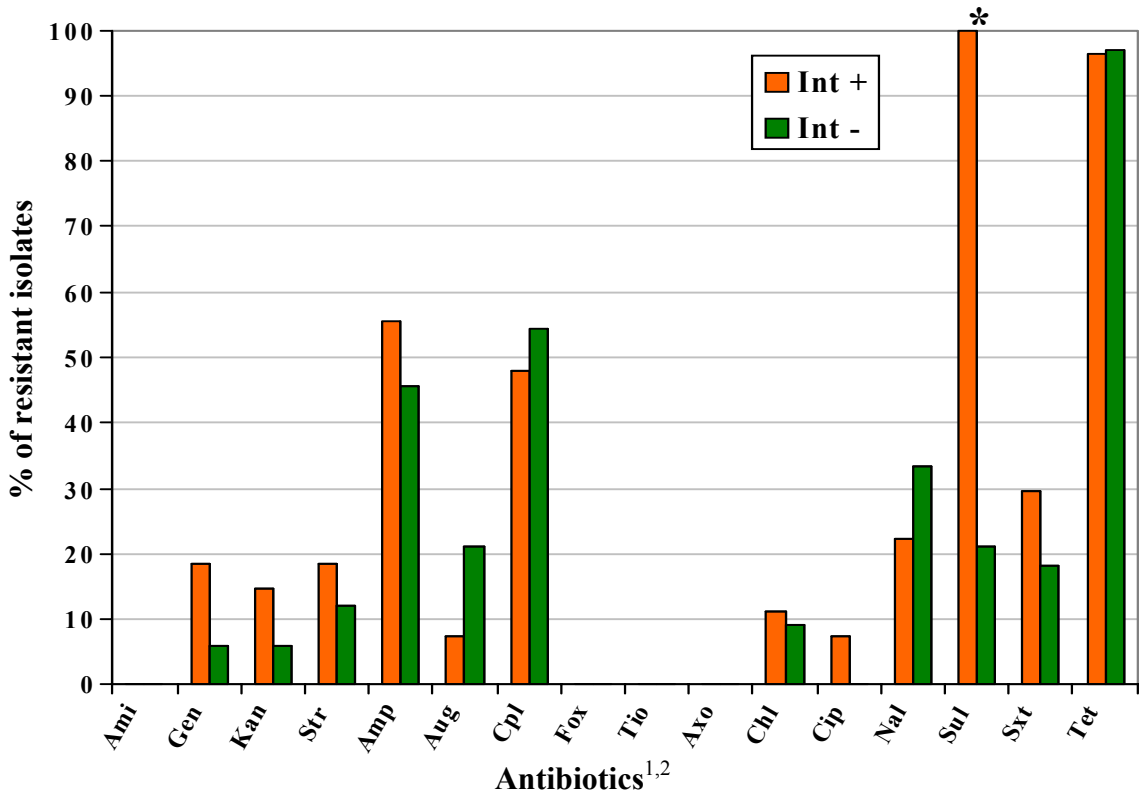

Figure 9. Frequency of resistance to 16 antimicrobials in integron-positive and integron-negative *P. mirabilis* from chickens.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within the same antibiotic with * differ ($P<0.05$).

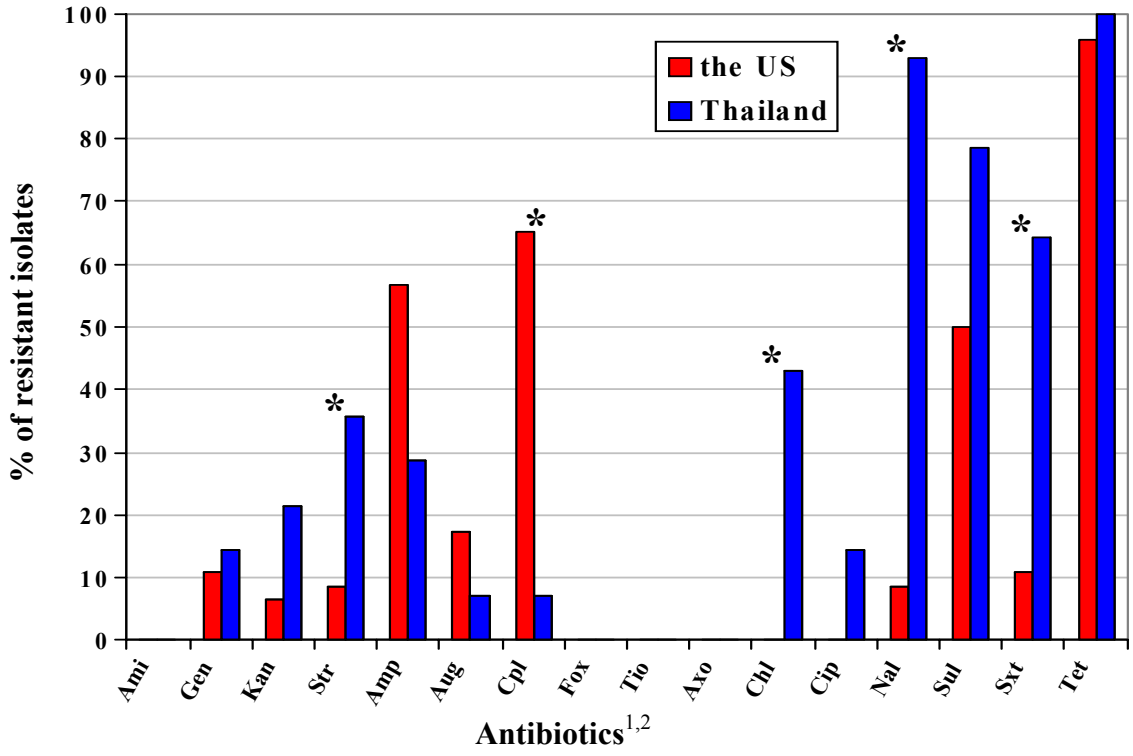

Figure 10. Frequency of resistance to 16 antimicrobials in *P. mirabilis* of chicken isolates from the US and Thailand.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within the same antibiotic with * differ ($P<0.05$).

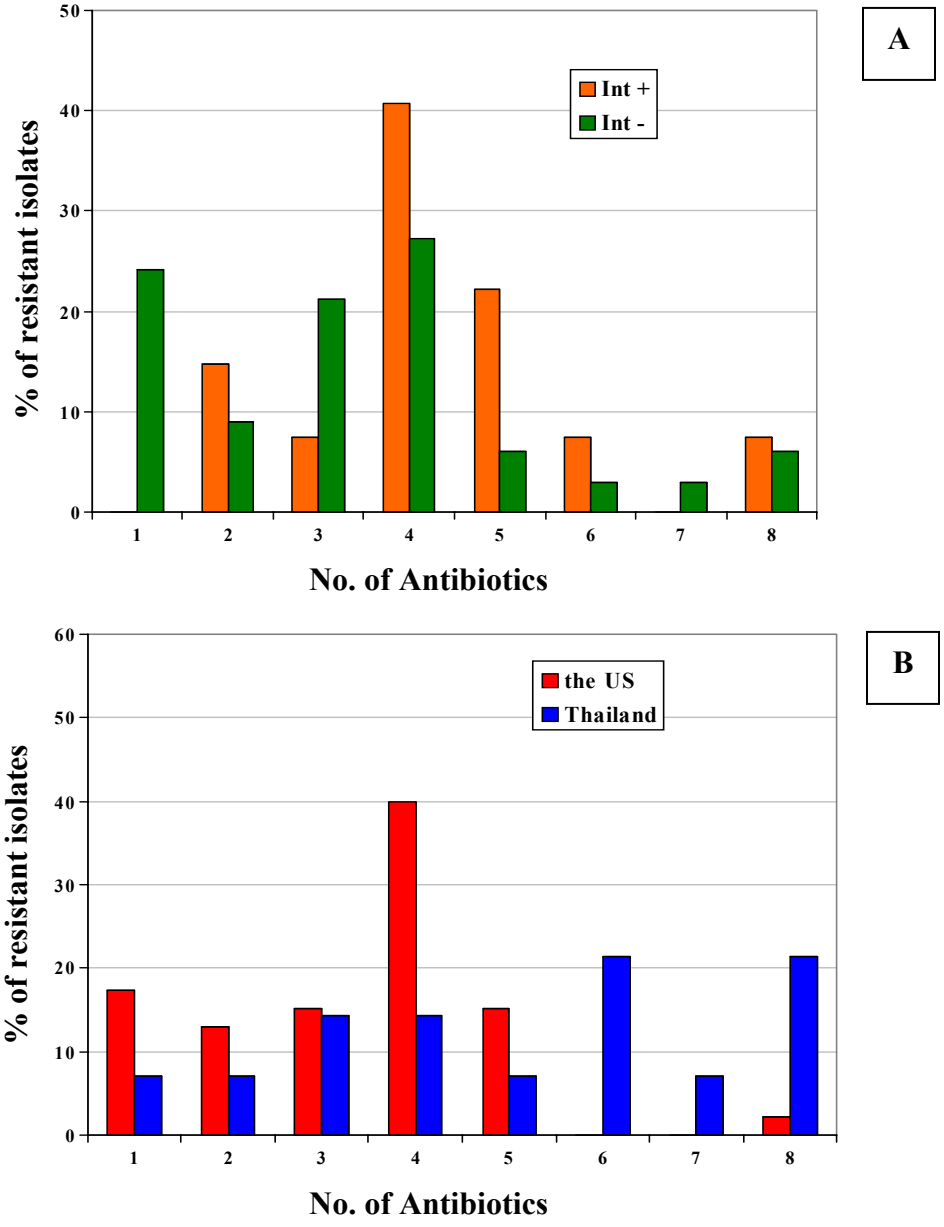

Figure 11. Multiple antimicrobial resistance of *P. mirabilis* isolates from chickens.

A. Integron-positive and integron-negative isolates.
B. Isolates from the US and Thailand.

Proteus mirabilis isolates from both the US and Thailand were resistant to 1 to 8 antimicrobials. A large portion of *P. mirabilis* from Thailand tended to be resistant to a higher number of antimicrobials than isolates from the US with over 50% of isolates from Thailand demonstrating resistance to more than 5 antimicrobials; whereas only 17% of isolates from the US were resistant to that number of antibiotics.

A total of 29 resistance patterns were noted among 60 *P. mirabilis* isolates. Thirteen patterns were found in integron-positive and 17 patterns were found in integron-negative isolates. Four isolates were resistant to 8 antimicrobials with those being Aug-Amp-Cpl-Kan Gen-Str-Sul-Tet, Aug-Amp-Cpl-Nal-Str-Sul-Tet-Sxt, Amp-Chl-Kan-Nal-Str-Sul-Tet-Sxt, and Amp-Chl-Gen-Nal-Str-Sul-Tet-Sxt. Among 29 patterns, only one antibiogram, Sul-Tet, was found in both integron-positive and integron-negative isolates. In contrast to *E. coli* and *Salmonella* isolates, one pattern, resistance to tetracycline, was found among isolates from Thailand and the US (Table 7).

Table 7. Antibiograms of *P. mirabilis* isolates of chickens from the US and Thailand.

Pattern No.	Antibiogram[1]	No. of isolate(s)				
		Integron		Location		Total
		+ (n=27)	- (n=33)	US (n=46)	Thailand (n=14)	(n=60)
1	Aug-Amp-Cpl-Kan Gen-Str-Sul-Tet	1	0	1	0	1
2	Aug-Amp-Cpl-Nal-Str-Sul-Tet-Sxt	0	1	0	1	1
3	Aug-Amp-Cpl-Nal-Tet	0	2	2	0	2
4	Aug-Amp-Cpl-Sul-Tet	1	0	1	0	1
5	Aug-Amp-Cpl-Tet	0	4	4	0	4
6	Amp-Cpl-Nal-Tet	0	1	1	0	1
7	Amp-Cpl-Sul-Tet-Sxt	3	0	3	0	3
8	Amp-Cpl-Sul-Tet	7	0	7	0	7
9	Amp-Cpl-Sul-Sxt	1	0	1	0	1
10	Amp-Cpl-Tet	0	6	6	0	6
11	Amp-Chl-Kan-Nal-Str-Sul-Tet-Sxt	1	0	0	1	1
12	Amp-Chl-Gen-Nal-Str-Sul-Tet-Sxt	0	1	0	1	1
13	Amp-Nal-Sul-Tet-Sxt	1	0	0	1	1
14	Cpl-Nal-Tet	0	1	1	0	1
15	Cpl-Sul-Tet-Sxt	0	1	1	0	1
16	Cpl-Tet	0	1	1	0	1
17	Cpl	0	1	1	0	1
18	Chl-Cip-Nal-Sul-Tet-Sxt	2	0	0	2	2
19	Chl-Kan-Nal_Str-Sul-Tet-Sxt	0	1	0	1	1
20	Chl-Kan-Nal-Tet	0	1	0	1	1
21	Kan-Gen-Str-Sul-Tet	1	0	1	0	1
22	Kan-Gen-Sul-Tet	1	0	1	0	1
23	Gen-Nal-Str-Sul-Tet-Sxt	0	1	0	1	1
24	Gen-Str-Sul-Tet	2	0	2	0	2
25	Nal-Sul-Tet-Sxt	0	1	0	1	1
26	Nal-Sul-Tet	2	0	0	2	2
27	Nal-Tet	0	1	0	1	1
28	Sul-Tet [2]	4	1	5	0	5
29	Tet [3]	0	8	7	1	8

[1] Aug, Amoxicillin/Clavulanic Acid; Amp, Ampicillin; Cpl, Cephalothin; Chl, Chloramphenicol; Cip, Ciprofloxacin; Gen, Gentamicin; Kan, Kanamycin; Nal, Nalidixic Acid; Str, Streptomycin; Sul, Sulfamethoxazole; Tet, Tetracycline; Sxt, Trimethoprim/Sulfamethoxazole.

[2] Resistance pattern found in both integron-positive and integron-negative isolates.

[3] Resistance pattern found in both Thailand and US isolates.

IV. DISCUSSION

Prevalence of Bacterial isolates

In this study, we determined the prevalence of three bacteria: *E. coli*, *Salmonella* spp., and *P. mirabilis,* in chickens from the US and Thailand. It was expected that the prevalence of *E. coli* in fecal samples would be high, and our results showed that more than 95% of chickens from both countries were found to carry *E. coli*. The prevalence of *E. coli* in our results compares with that reported by Bywater et al. (9) who noted the isolation rate for *E. coli* approached 100% in chickens from France, Netherlands, Sweden, and the UK. In contrast to *E. coli*, we found a much lower prevalence of *Salmonella* spp. in chickens, with only 3.3% of chickens from Thailand and 21.2% of chickens in the US being positive for *Salmonella*. The difference in *Salmonella* prevalence between countries may be due to differences in the methods of isolate collection. While *Salmonella* isolates in the US were collected from killed chickens at a slaughterhouse, *Salmonella* isolates from Thailand were isolated from live chickens on farms. The stress caused by transportation of animals from farm to slaughterhouse has been reported to result in increased *Salmonella* shedding (8, 44, 48). However, our results from the US show a higher prevalence compared to that found in studies conducted in Brazil and Europe. The study conducted in Brazil detected *Salmonella* in only 6.7% of carcasses prior to evisceration (50). Similarly the European study noted that 7.1% of chickens contained *Salmonella* (9). The prevalence of *Salmonella* in chickens from southern Thailand in our study was comparable to that found by another study conducted

in the northern part of Thailand, with that report indicating 4% of chickens carried *Salmonella* (44).

P. mirabilis is a bacteria often found in farm environments and intestinal tracts of both animals and humans. Although lacking the ability to cause disease in poultry, it can cause opportunistic urinary tract infections in humans (56, 66). Although less studied than *E. coli* and *Salmonella*, several recent studies have reported the presence of mobile genetic elements involved in antibiotic resistance dissemination in *P. mirabilis* (2, 31, 59). We therefore included *P. mirabilis* in our study.

Prevalence of Class 1 Integrons

It is well known that class 1 integrons play an important role in dissemination of antibiotic resistance in the *Enterobacteriaceae*. Our results show that class 1 integrons, as indicated by the presence of *intI1* gene, are widespread in enteric bacteria of broiler chickens. More than 70% and 40% of *E. coli* isolated from Thailand and the US, respectively, carried class 1 integrons. Bass et al. (7) noted that 63% of pathogenic *E. coli* isolates obtained from diseased poultry were positive for the class 1 integron markers *intI1* and *qacEΔ1*. The prevalence of class 1 integrons in *Salmonella* isolates was lower than that of *E. coli* in our study. Our results showed that only 48.1% and 0.5% of *Salmonella* isolates from Thailand and the US, respectively, carried class 1 integrons. Similar to our study, Diarrassouba et al. (16) noted a higher prevalence of class 1 integrons in *E. coli* isolates. That group studied the prevalence of class 1 integrons (*qacEΔ1-sul1*) in 74 sorbitol negative *E. coli* and 62 *Salmonella* isolates from nine broiler chicken farms in Canada and detected class 1 integron genes in 40% of sorbitol negative

E. coli, however they were not detected in any *Salmonella* isolates. A low prevalence of class 1 integrons was also noted in *Salmonella* isolates in other poultry varieties. Only 2 of 80 (2.5%) *Salmonella* isolates from turkeys at processing plants in the US carried class 1 integrons (42). Zhao et al. (65) noted a higher prevalence of class 1 integrons, with 43% of 380 *Salmonella* isolates recovered from animal diagnostic samples (swine, turkeys, cattle, chickens, and horses) obtained from four state veterinary diagnostic laboratories (AZ, NC, MO, and TN) between 2002 and 2003 carrying class 1 integrons. However, the prevalence of class 1 integrons was not reported for each animal group. The higher prevalence of class 1 integrons in these *Salmonella* may be due to their being isolated from diseased animals, which may have been exposed to antimicrobial treatments more frequently than healthy animals.

Our results show that 38.2% of *P. mirabilis* isolates from Thailand contained class 1 integrons and this was not different from the 29.9% of US isolates. Because of their ability to harbor class 1 integrons, *P. mirabilis* may provide a reservoir for these determinants and may account to some degree for rapid dissemination of antibiotic resistance genes.

It is important to note, however, that while only 32.5, 75.4, and 82.9% of *intI1*-positive *E. coli, P. mirabilis,* and *Salmonella* isolates from Thailand were found to carry *qacEΔ1* and *sul1,* almost all of the *E. coli* (96.8%), all *P. mirabilis,* and *Salmonella intI1*-positive isolates from the US carried *qacEΔ1* and *sul1*. In agreement with that found in Thailand isolates in our study, van Essen-Zandbergen noted that only a subset of *Salmonella* and *E. coli* isolates carrying *intI1* are also carried the 3' conserved sequence (62). Sunde (57) reported that a high proportion of class 1 integrons found in *E. coli*

lacked the *sul1* gene. Additionally, Guerra et al. (24) found defective integrons which lacked both *qacEΔ1* and *sul1,* or only *sul1,* in some *E. coli* isolates from Germany, suggesting that use of specific primers to detect conserved segments alone can produce false negatives when those specific gene components are not present in otherwise functional integrons**.**

Antibiotic Susceptibility

Antibiotic resistance was found in all bacterial isolates tested in this study. A high proportion (86-100%) of tetracycline resistance was found in *E. coli* and *P. mirabilis* from Thailand and the US and *Salmonella* spp. from Thailand. In agreement with our study, several research groups from different countries also found a high level of tetracycline resistance in broiler chickens. For example, Smith et al. (55) noted 97% of *E. coli* isolated from farms that used antibiotics in northeast Georgia were resistant to tetracycline. Mile et al. (41) found tetracycline resistance at a frequency of 82.4% in *E. coli* isolates from Jamaica, Georgnaras et al. (22) reported 90% of *E. coli* isolates from South Africa were resistant to tetracycline, and Yang et al. (64) noted all *E. coli* isolated from diseased chickens in China were also resistant to tetracycline. Asai et al. (6) reported that resistance to both oxytetracycline and dihydrostreptomycin accounted for 94.0% of the resistance patterns in *Salmonella enterica* serotype Infantis isolates from meat and broilers in Japan, and Diarrasouba et al. reported 75.8% of combined *E. coli* and *Salmonella* isolates from Canada were resistant to tetracycline (16). The high percentage of resistance to tetracycline may reflect the selection pressure caused by wide use of these drugs in farm animals (26, 53). A high percentage of tetracycline resistance

may also result from an ability of animals to serve as a reservoirs of tetracycline resistant bacteria for a long periods of time, as indicated by the survey conducted in United Kingdom in 1980, which found tetracycline-resistant *E. coli* in chickens and pigs 9 years following a ban on tetracycline use as a feed additive (54).

When comparing the frequency of antibiotic resistance between integron-positive and integron negative isolates, we found that integron-positive isolates were more likely to be resistant to sulfamethoxazole than integron-negative isolates in all three types of bacteria tested. This likely occurred because all integron-positive isolates contained *sul1* as an integral part of the integron. Similar to our study, Shahada et al. (53) reported all of the *intI1*-positive *S. enterica* serovar Infantis isolates collected were resistant to sulfamethoxazole. In addition, integron-positive *E. coli* and *Salmonella* isolates were also statistically more likely to be resistant to chloramphenicol compared to integron-negative isolates. While this study did not aim to characterize specific gene cassettes incorporated in the integrons, several research groups have reported *cat* and *cml* genes, responsible for chloramphenicol resistance, as a gene cassette within the integron (17, 25, 28, 33, 39, 40, 46). This might explain the high prevalence of chloramphenicol resistance in integron-positive bacteria.

It should, however, be noted that the resistance phenotypes in our study were only partially explained by the presence of integrons. In agreement with our study, a study of the occurrence of integrons and antimicrobial resistance genes among *Salmonella enterica* in Brazil revealed that integron-mediated resistance genes contributed to the multiresistance phenotypes observed in the isolates; however, most resistance genes were

located outside the integron structure as independent genes. Another researcher also suggested that the genes might also be located on the same conjugative plasmid (46).

When comparing isolates from Thailand and the US, we found that resistance patterns differed, with no common patterns occurring between each type of bacteria from those two countries. All three types of bacteria from Thailand had a higher prevalence of resistance to chloramphenicol, nalidixic acid, and trimethoprim/sulfamethoxazole than isolates from the US. *E. coli* and *Salmonella* isolates from the US showed a statistically greater prevalence of resistance to amoxicillin/clavulanic acid, cefoxitin, and ceftiofur than those from Thailand. *Salmonella* and *P. mirabilis* isolates from the US demonstrated a more frequent occurrence of resistance to cephalothin than isolates from Thailand. The distribution of antimicrobial resistance phenotypes can be expected to reflect different use patterns of antimicrobial agents (1, 21, 38).

Chloramphenicol was a popular veterinary product and was used particularly in difficult disease cases. However, due to concerns over transfer of antibacterial resistance in zoonotic pathogens, particularly in *Salmonella*, its use in animals has been restricted or banned in many countries. Chloramphenicol cannot be used in food animals in the US and Canada and can only be used as an eye ointment for small animals in the UK (26). In Thailand, chloramphenicol has been banned from animal feeds since 1999 (4). Thus, the prevalence of resistance to this antibiotic was noteworthy.

V. CONCLUSIONS

Antimicrobial resistance genes and class 1 integrons are common in bacteria associated with chickens in both Thailand and the US. While multi-antibiotic resistance is associated with integrons, and specific multi-resistance patterns were often characteristic of integron carriage, many multi-resistant isolates with a variety of antibiograms also lacked class 1 integrons. Our work indicates that integrons and multi-antibiotic resistant bacteria appear to be a significant aspect of microbial communities associated with chickens.

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CHAPTER 4 : PREVALENCE OF CLASS 1 INTEGRONS AND ANTIBIOTIC RESISTANCE PATTERNS IN BACTERIA OF SWINE IN THE US AND THAILAND

ABSTRACT

The objective of this study was to investigate the prevalence of class1 integrons and antimicrobial resistance patterns in *E. coli* and *Salmonella* isolates of commercial swine from the US and Thailand. In market pigs, class 1 integrons, as indicated by the presence of *intI1*, were detected in 1,130 isolates (39.9%). Simultaneous presence of all three conserved genes (*intI1, qacEΔ1*, and *sul1*) was found in 184 (6.5%) of the total of 2,833 isolates. The prevalence of class 1 integrons in *E. coli* and *Salmonella* spp. was found to differ with isolate origin (P<0.001). In both types of bacteria, class 1 integrons were more prevalent in isolates from Thailand compared with the US (68.8 and 25.9% for *E. coli* isolates, 37.8 and 10.3% for *Salmonella* spp. isolates from Thailand and the US, respectively). In sows, 46% of 1,420 bacterial isolates were found to carry the *intI1* gene; however only 4.8% of these bacteria carried all three conserved genes. Similarly, for market pigs the prevalence of class 1 integrons in *Salmonella* isolates was found to differ with isolate origin (P<0.001). Approximately 72% of *Salmonella* isolates from sows in Thailand contained class 1 integrons, which was higher than the 29.8% of the isolates from US. Among *E. coli* isolates, 64.8% of isolates from Thailand carried integrons; however this was not different from the US (56.6%) (P=0.08) Most isolates were multi-antibiotic resistant. A high proportion of isolates demonstrated resistance to tetracycline (95.8%), sulfamethoxazole (70.8%), streptomycin (55.7%), and ampicillin (52.6%). Integron-positive-isolates tended to be resistant to a higher number of antibiotics than integron-negative isolates. In addition, resistance to streptomycin, sulfamethoxazole, and trimethoprim/sulfamethoxazole is more common in integron-positive isolates than

integron-negative isolates. Among 113 antibiotic resistance patterns found in antibiotic susceptibility tests in integron-positive and integron-negative, 11 patterns (9.7%) were found in both integron positive and integron negative isolates. Among 39 antibiotic resistance patterns found in Thailand and the US, only 2 patterns (5.1%) were found in both countries. These results indicate that class 1 integrons are common in commensal and foodborne bacteria in swine, and that some, but not all, antibiotic resistances in those isolates are associated with the presence of class 1 integrons.

I. INTRODUCTION

Food animals are often exposed to antimicrobials to treat and prevent infectious disease or to promote growth (33). Antimicrobial usage in food animals provides selection pressure that favors an increase of antimicrobial resistant bacteria (17, 23, 28, 29). Antimicrobial resistance has emerged not only in commensal bacteria and bacterial pathogens of animals but also in zoonotic enteropathogens (33). Resistant bacteria from animals can infect humans by direct contact and also via consumption of food products of animal origin (53, 56). These resistant bacteria can colonize humans and/or transfer their resistance genes to other bacteria of humans, which can result in treatment failure as a consequence of antimicrobial resistance (54).

Integrons are genetic elements that mediate integration of antibiotic resistance genes through site specific recombination and convert them into functional genes (9, 15, 19, 30, 43). More than 70 different genes imparting resistance to most classes of antimicrobials have been found as gene cassettes in the central region of different integrons from diverse bacteria (44). Moreover, these genetic elements can incorporate several resistance genes, allowing them to transfer as a single gene. As many as seven gene cassettes have been found within an integron (51). Integrons are often found to be carried on mobile elements such as plasmids and transposons; therefore, integrons are believed to play a major role in the rapid dissemination of multi-drug resistance among bacteria (36). Four different classes of integrons have been identified based on variations in sequence of primary elements, gene cassettes, and associations with transposons (2, 3, 10, 14, 15, 41, 46). Class 1 integrons are the most common and widely distributed type (5, 14, 38).

The U.S. and Thailand, while occupying very diverse geographic regions, share many commonalities in livestock production practices. Swine production tends to be intensive, with closed confinement, high biosecurity and similar high production genetic lines. Feed-based antibiotics are commonly used in both countries, although some differences occur in drugs of choice and/or availability. A comparison of class 1 integron prevalence and antibiotic resistance gene patterns of *E. coli* and *Salmonella* spp. between these two countries may provide information regarding global implications of agricultural use of antibiotics with relevance to antibiotic resistance.

II. MATERIALS AND METHODS

Bacterial Isolates

A total of 2,820 *E. coli* and *Salmonella* spp. isolates were collected via fecal swab from 524 market pigs from 3 farms; and 120 sows from 3 farms at abattoirs in the US. In addition, 1,433 *E. coli* and *Salmonella* spp. isolates were collected from 436 market pigs from 6 farms and 130 sows from 2 farms in southern Thailand. All bacterial collections in Thailand took place in the southern Thailand during the period of May 2003 to August 2004, whereas bacterial collections from the US took place in Tennessee during the period of November 2004 to October 2005. All isolates were recovered from fecal swabs by standard microbiological procedures. The primary isolation method for *E. coli* has been described previously (31). For *Salmonella* isolation, fecal swabs were first enriched in selective tetrathionate broth (Difco, Sparks, MD) and incubated at 41.5$\pm$1ºC for 18-24 h. Enriched broth cultures were then plated on Xylose Lysine Tergitol 4 agar (XLT4; Difco) for selective culture. Plates were incubated at 37$\pm$1ºC and examined after 18-24 h. Presumptive *Salmonella* colonies were subcultured and then plated again, and identity was confirmed with API 20E strips according to the manufacturer's specification (bioMérieux Vitek, Inc., Hazelwood, MO). Up to 6 colonies of each bacterial type were selected for analysis. Bacterial cultures were maintained at -80°C in 10% glycerol until analysis.

Multiplex PCR (MP-PCR)

Integron harboring isolates were detected using a MP-PCR targeting three conserved sequences of class 1 integrons, *intI1, qacEΔ1*, and *sul1* (13). Primer pairs were manufactured by Operon, Inc. (Alameda, CA) (Table 1). Total DNA was prepared by boiling overnight cultures in 2YT broth (Difco) in an equal volume of 0.2% (wt/vol) Triton X-100 (Mallinckrodt, Paris, KY) for 5 min (24). Boiled cultures were cooled on ice for 5 min and used immediately for PCR. PCR reagents, excluding template DNA, were combined in a master mix prior to aliquoting. The final reaction volumes for each aliquot included: 1) 1 μL of each primer pair (50 pmol (each primer) μL^{-1}); 2) 1 μL of *Taq* DNA polymerase (0.5U μL^{-1}; Promega, Madison, WI); 3) 10 μL reaction buffer (12.5mM $MgCl_2$, pH 8.5; Invitrogen, Carlsbad, CA); 4) 5 μL dNTPs solution (2.5mM of each dNTP, pH 8.0; Invitrogen); and 5) 32 μL sterile H_2O. Sample DNA (1 μL) was then added to each aliquot. Reactions were conducted in a Mastercyler Gradient thermocycler (Eppendorf, Westbury, NJ) with the following conditions: 1) 1 cycle of 94°C for 4 min; 2) 10 "touchdown" cycles of 94°C for 1 min, 65°C for 30s (decreasing 1°C/cycle), 70°C for 2 min; 3) 24 cycles of 94°C for 1 min, 55°C for 30s, 70°C for 2 min; and 4) 1 final cycle of 70°C for 5 min. *Salmonella* Typhimurium DT104 known to contain two class 1 integrons (23) was used as a positive control. A blank containing only PCR reagents and Triton X-100 was used as a negative control. Reaction products were separated by conventional electrophoresis in 1.5% agarose and stained with ethidium bromide for visualization (Figure 1). Prevalence of class 1 integrons was based on the presence of the *IntI1* gene.

The integron prevalence in each bacterial species was compared between Thailand and the US using the freq procedure of SAS (SAS 8.2, SAS Institute Inc, Cary, NC) Comparisons were made using the Fisher's two-sided exact tests. Differences were considered significant at $P < 0.05$.

Table 1. Primer pairs used in MP-PCR.

Target	Sequence (5' to 3')	PCR product (bp)
intI1	(F) GGTTCGAATGTCGTAACCGC	248
	(R) ACGCCCTTGAGCGGAAGTATC	
sul1	(F) ATCAGACGTCGTGGATGTCG	346
	(R) CGAAGAACCGCACAATCTCG	
qacEΔ1	(F) GAGGGCTTTACTAAGCTTGC	200
	(R) ATACCTACAAAGCCCCACGC	

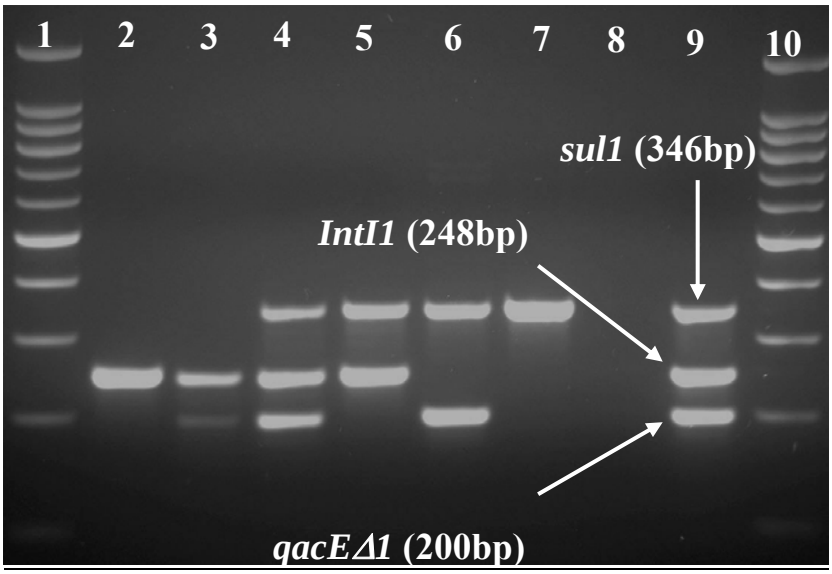

Figure 1. Multiplex PCR detecting class 1 integrons gene sequence.
Lane 1; 100 bp DNA ladders; Lane 2 -7 wild type isolates; lane 8; negative control; and lane 9; *Salmonella enterica* Typhimurium DT104 (positive control).

Antibiotic Susceptibility Testing

Antibiotic MICs were determined for subsets of each bacterial type using the National Antimicrobial Resistance Monitoring System (NARMS) microdilution sensititre plates, CMV7CNCD, (Sentititre, Trek Diagnostic System Inc., Cleveland, Ohio) according to Clinical and Laboratory Standards Institute (CLSI), (formerly National Committee on Clinical Laboratory Standards, NCCLS) broth microdilution guidelines. *Escherichia coli* ATCC 25922 was used as a reference strain. The isolates were tested for resistance to 16 antibacterials or antibacterial combinations that included amoxicillin/clavulanic acid (Aug), ampicillin (Amp), ciprofloxacin (Cip), cefoxitin (Fox), ceftiofur (Tio), ceftriaxone (Axo), cephalothin (Cpl), chloramphenicol (Chl), amikacin (Ami), gentamicin (Gen), kanamycin (Kan), nalidixic acid (Nal), streptomycin (Str), sulfamethoxazole (Sul), tetracycline (Tet), and trimethoprim/sulfamethoxazole (Sxt). The CMV7CNCD plate layout is shown in Figure 2. Results were interpreted using CLSI guidelines for broth microdilution methods for veterinary *E. coli* (Table 2). In the phenotypic analysis, isolates with intermediate MICs were not considered as resistant.

The frequency of antibiotic resistance in integron-positive isolates was compared to that of integron-negative isolates and frequency of antibiotic resistance in isolates from Thailand was compared to the US using the Analysis and Statcalc programs of the Epi Info™ version 3.4.1 software package from the Center for Disease Control and Prevention (6). Comparisons were made using the Fisher's two-sided exact tests. Differences were considered significant at $P<0.05$.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Fox 16	Ami 1	Tet 16	Axo 2	Aug 2/1	Cip 0.06	Gen 0.5	Nal 0.5	Sul 512	Sxt 1/19	Cpl 2	Amp 4
B	Fox 8	Ami 0.5	Tet 8	Axo 1	Aug 1/0.5	Cip 0.03	Gen 0.25	Tio 8	Sul 256	Sxt 0.5/9.5	Kan 64	Amp 2
C	Fox 4	Chl 32	Tet 4	Axo 0.5	Cip 4	Cip 0.015	Nal 32	Tio 4	Sul 128	Sxt 0.25/4.75	Kan 32	Amp 1
D	Fox 2	Chl 16	Axo 64	Axo 0.25	Cip 2	Gen 16	Nal 16	Tio 2	Sul 64	Sxt 0.12/2.38	Kan 16	Str 64
E	Fox 1	Chl 8	Axo 32	Aug 32/16	Cip 1	Gen 8	Nal 8	Tio 1	Sul 32	Cpl 32	Kan 8	Str 32
F	Fox 0.5	Chl 4	Axo 16	Aug 16/8	Cip 0.5	Gen 4	Nal 4	Tio 0.5	Sul 16	Cpl 16	Amp 32	Pos
G	Ami 4	Chl 2	Axo 8	Aug 8/4	Cip 0.25	Gen 2	Nal 2	Tio 0.25	Sxt 4/76	Cpl 8	Amp 16	Pos
H	Ami 2	Tet 32	Axo 4	Aug 4/2	Cip 0.12	Gen 1	Nal 1	Tio 0.12	Sxt 2/38	Cpl 4	Amp 8	Pos

Figure 2. Sensititre non-fastidious Gram negative plate (CMV7CNCD) format.
Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline; Pos, growth control wells.

Table 2. Minimum Inhibitory Concentration (MIC) breakpoint. [1]

Drugs	Susceptible (μg/ml)	Intermediate (μg/ml)	Resistant (μg/ml)
Aminoglycoside			
Amikacin (Ami)	≤ 16	32	≥ 64
Gentamicin (Gen)	≤ 4	8	≥ 16
Kanamycin (Kan)	≤ 16	32	≥ 64
Streptomycin (Str) [2]	≤ 32		≥ 64
β-Lactams			
Ampicillin (Amp)	≤ 8	16	≥ 32
Amoxicillin/Clavulanic acid (Aug)	≤ 8/4	16/8	≥ 32/16
Cephalothin (Cpl)	≤ 8	16	≥ 32
Cefoxitin (Fox)	≤ 8	16	≥ 32
Ceftiofur (Tio)	≤ 2	4	≥ 8
Ceftriaxone (Axo)	≤ 8	16,32	≥ 64
Chloramphenicol (Chl)	≤ 8	16	≥ 32
Quinolone			
Ciprofloxacin (Cip)	≤ 1	2	≥ 4
Nalidixic Acid (Nal)	≤ 16		≥ 32
Sulfonamides			
Sulfamethoxazole (Sul)	≤ 256		≥ 512
Trimethoprim/Sulfamethoxazole (Sxt)	≤ 2/38		≥ 4/76
Tetracycline (Tet)	≤ 4	8	≥ 16

[1] MICs determined via microdilution broth methods in accordance with CLSI standards (7, 8).

III. RESULTS

Prevalence of Bacterial Isolates

The prevalence of *E. coli* isolates from swine was high in both countries and both types of swine with all of market pigs and sows from the US and 82.3% and 97.4% of market pigs and sows, respectively from Thailand were found to carry *E. coli* (Table 3). However, the prevalence of *Salmonella* spp. in swine was considerably lower than that of *E. coli.* The prevalence of *Salmonella* was higher in sows from the US, with 60.9% of sows found to carry *Salmonella*, whereas that organism was detected in only 8.5% of sows in Thailand. A similar prevalence of *Salmonella* was found in market pigs from Thailand and the US with 3.4% and 5.7% of market pigs from Thailand and the US, respectively, carrying these bacteria.

Table 3. Prevalence of *E. coli* and *Salmonella* from market pigs in the US and Thailand.

Type of swine	Countries	Farm	# of animals sampled	# of animals carrying bacteria (%)	
				E. coli	*Salmonella*
Market pig	US	US-A	124	124 (100)	7 (5.6)
		US-B	200	200 (100)	4 (2.0)
		US-C	200	200 (100)	19 (9.5)
		Total	524	524 (100)	30 (5.7)
	Thailand	TH-A	110	94 (85.5)	8 (7.3)
		TH-B	176	151 (85.8)	4 (2.6)
		TH-C	10	10 (100)	0 (0)
		TH-D	10	10 (100)	2 (20.0)
		TH-E	10	10 (100)	0 (0)
		TH-F	120	84 (100)	1 (1.2)
		Total	436	359 (82.3)	15 (3.4)
Sow	US	US-D	60	60 (100)	35 (58.3)
		US-E	48	48 (100)	32 (66.7)
		US-F	12	12 (100)	6 (50.0)
		Total	120	120 (100)	73 (60.9)
	Thailand	TH-C	100	100 (100)	10 (10.0)
		TH-F	30	25 (83.3)	1 (3.3)
		Total	130	125 (97.4)	11 (8.5)

Prevalence of Class 1 Integrons

Prevalence of class 1 integrons in *E. coli* and *Salmonella* spp. in market pigs and sows from Thailand and the US is shown in Table 4. In market pigs, class 1 integrons, as indicated by the presence of *intI1*, were detected in 1,130 isolates (39.9%). Simultaneous presence of all three conserved genes (*intI1, qacEΔ1*, and *sulI1*) was found in 184 (6.5%) of the total of 2,833 isolates. The prevalence of class 1 integrons in *E. coli* and *Salmonella* spp. was found to be associated with isolate origin ($P<0.001$). In both types of bacteria, class 1 integrons were more prevalent in isolates from Thailand compared with the US (68.8% and 25.9% for *E. coli* isolates, 37.8% and 10.3% for *Salmonella* spp. isolates from Thailand and the US, respectively). The prevalence of integrons across farms in both *E. coli* and *Salmonella* spp. isolates differed ($P<0.001$) (data not shown)

In sows, forty-six percent of 1,420 bacterial isolates were found to carry *intI1* gene; however only 4.8% of these bacteria carried all three conserved genes. Similarly, for market pigs the prevalence of class 1 integrons in *Salmonella* isolates was found to be associated with isolate origin ($P<0.001$). Approximately 72% of *Salmonella* isolates from Thailand contained class 1 integrons, which was higher than the 29.8% of the isolates from US. Among *E. coli* isolates, 64.8% of isolates from Thailand carried integrons; however, this was not different from the US (56.6%) ($P=0.08$). The prevalence of integrons across farms in both *E. coli* and *Salmonella* isolates also differed ($P<0.01$) (data not shown)

Table 4. Prevalence of integrons and integron component genes in bacteria of pigs from the US and Thailand. [1]

Type of swine	No. of isolates	*E. coli*		*Salmonella* spp.	
		The US	Thailand	The US	Thailand
Market Pigs	No. of isolates	1,569	963	193	108
	No.(%) carrying				
	- *IntI1* (*I*)	406 (25.9)[b]	663 (68.8)[a]	20 (10.3)[b]	41 (37.8)[a]
	- All 3 conserved genes (*I,Q,S*)	102 (6.5)[a]	49 (5.1)[a]	0 (0.0)[b]	33 (30.6)[a]
Sows	No. of isolates	360	279	698	83
	No.(%) carrying				
	- *IntI1* (*I*)	204 (56.6)[a]	181 (64.8)[a]	208 (29.8)[b]	59 (71.7)[a]
	- All 3 conserved genes (*I,Q,S*)	11 (3.2)[a]	0 (0.0)[b]	15 (2.2)[a]	5 (6.4)[a]

[1] Results are reported as numbers and percentage of bacteria carrying integrons and genes associated with integrons, as determined by multiplex PCR. ***I*****,** *intI1*; *Q*, *qacEΔ1*; *S*, *sul1*.
[a,b] Values within the same bacteria and row not sharing like superscripts differ (P<0.05.)

Antibiotic Susceptibility

E. coli isolates

A comparison of antibiotic resistance prevalence between integron-positive and integron-negative *E. coli* isolates from market pigs is provided in Figure 3. All integron-negative *E. coli* were susceptible to amikacin, ceftiofur, amoxicillin/clavulanic acid, cefoxitin, ceftriaxone, and ciprofloxacin. In contrast, the only drug to which all integron-positive isolates were susceptible was cefoxitin. All integron-positive isolates were resistant to tetracycline. Integron-positive isolates also showed a high percentage (45-90%) of resistance to kanamycin, ampicillin, streptomycin, and sulfamethoxazole; whereas less than 30% of these isolates were resistant to the rest of antibiotics. Similar to integron-positive isolates, a high proportion (97.5%) of integron-negative isolates were resistant to tetracycline. Resistance to gentamicin, kanamycin, ampicillin ciprofloxacin, streptomycin, sulfamethoxazone, trimethoprim/sulfamethoxazone, and tetracycline was more common in integron-positive isolates ($P<0.05$).

A comparison between resistance frequencies of *E. coli* isolates from market pigs of Thailand and the US is shown in Figure 4. While resistance to amikacin, ceftiofur, ceftriaxone, and ciprofloxacin was found only in isolates from Thailand, resistance to amoxicillin/clavulanic acid was found only in the US. Resistance to kanamycin, streptomycin, sulfamethoxazole, and tetracycline was found in *E. coli* isolates in both countries, however no significant differences were noted ($P>0.05$). There were significant differences in resistance to gentamicin, ampicillin, cephalothin, chloramphenicol,

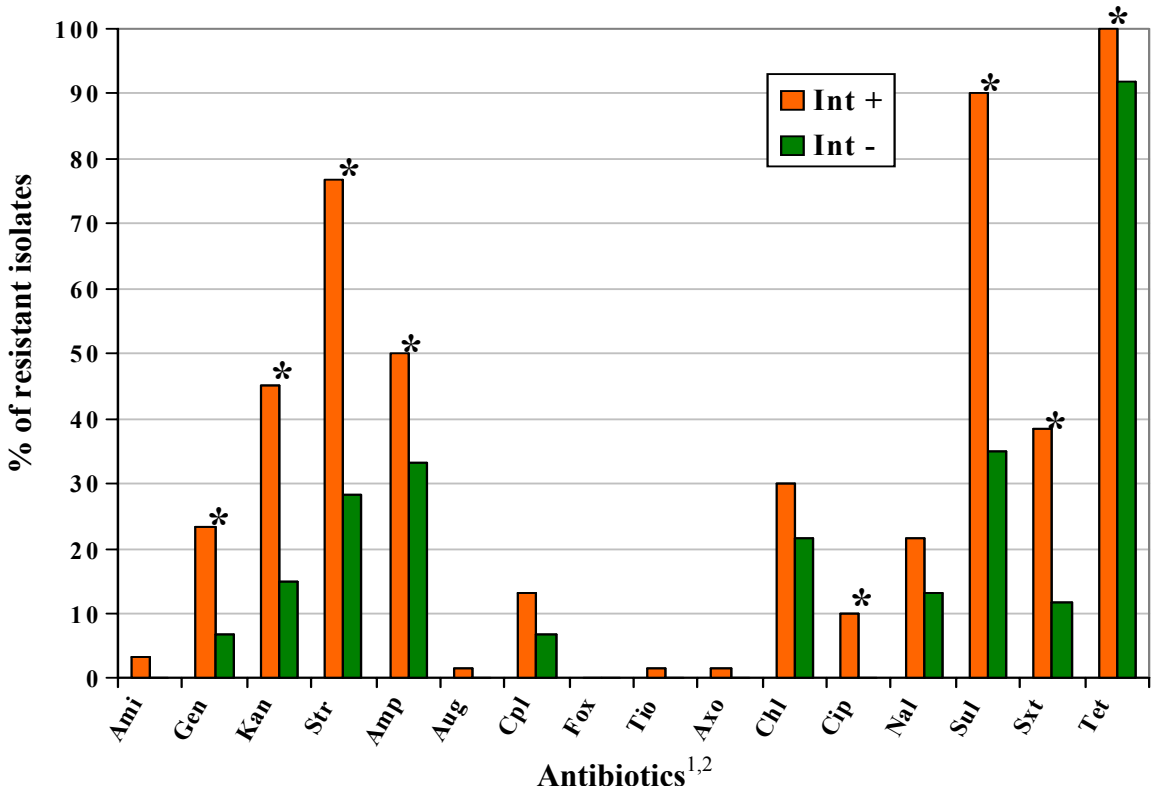

Figure 3. Frequency of resistance to 16 antimicrobials in integron-positive and integron-negative *E. coli* from market pigs.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within antibiotic with * differ ($P<0.05$).

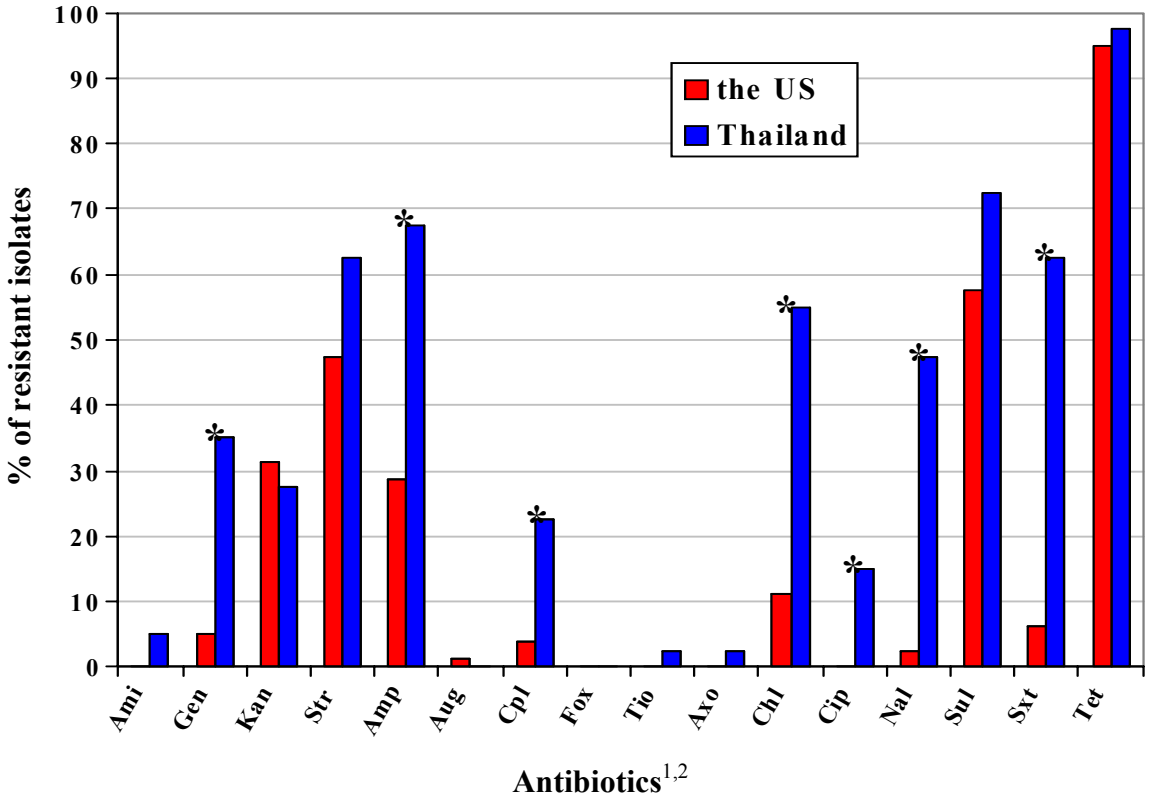

Figure 4. Frequency of resistance to 16 antimicrobials in *E. coli* of market pigs from the US and Thailand.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within antibiotic with * differ ($P<0.05$).

ciprofloxacin, nalidixic acid, and sulfamethoxazole with resistance being generally more common in isolates from Thailand ($P<0.01$).

The distribution of multiresistance among *E. coli* isolates from market pigs is shown in Figure 5. While integron-positive isolates were resistant to 1 to 10 antibiotics, four out of sixty integron-negative isolates (6.7%) were susceptible to all 16 antibiotics tested and the rest were resistant to 1 to 7 antibiotics. Ninety percent of integron-positive isolates were resistant to at least three antibiotics; whereas, a lower percentage was found in integron-negative isolates, with 51.7% of those isolates demonstrating resistance to that number of antimicrobials. A large proportion of *E. coli* isolates from Thailand tended to be resistant to a higher number of antimicrobials than isolates from the US, with over 75% of isolates from Thailand demonstrating resistance to 4 to 10 antibiotics; whereas only 27.5% of isolates from the US were resistant to 4 to 7 antibiotics.

A total of 58 patterns were observed among 120 *E. coli* isolates from market pigs. The most frequent pattern was resistance to 3 antimicrobials, Str-Sul-Tet, which was found in 35% of isolates. Three isolates (5.0%) exhibited resistance to the highest number of antimicrobials (10) with those being Ami-Amp-Chl-Kan-Gen-Nal-Str-Sul-Tet-Sxt and Amp-Cpl-Chl-Kan-Gen-Nal-Str-Sul-Tet-Sxt. Among the 58 patterns, seven were found in both integron-positive and integron-negative isolates. Furthermore, two resistance patterns (Amp-Str-Tet and Amp-Tet) were found in both countries (Table 5).

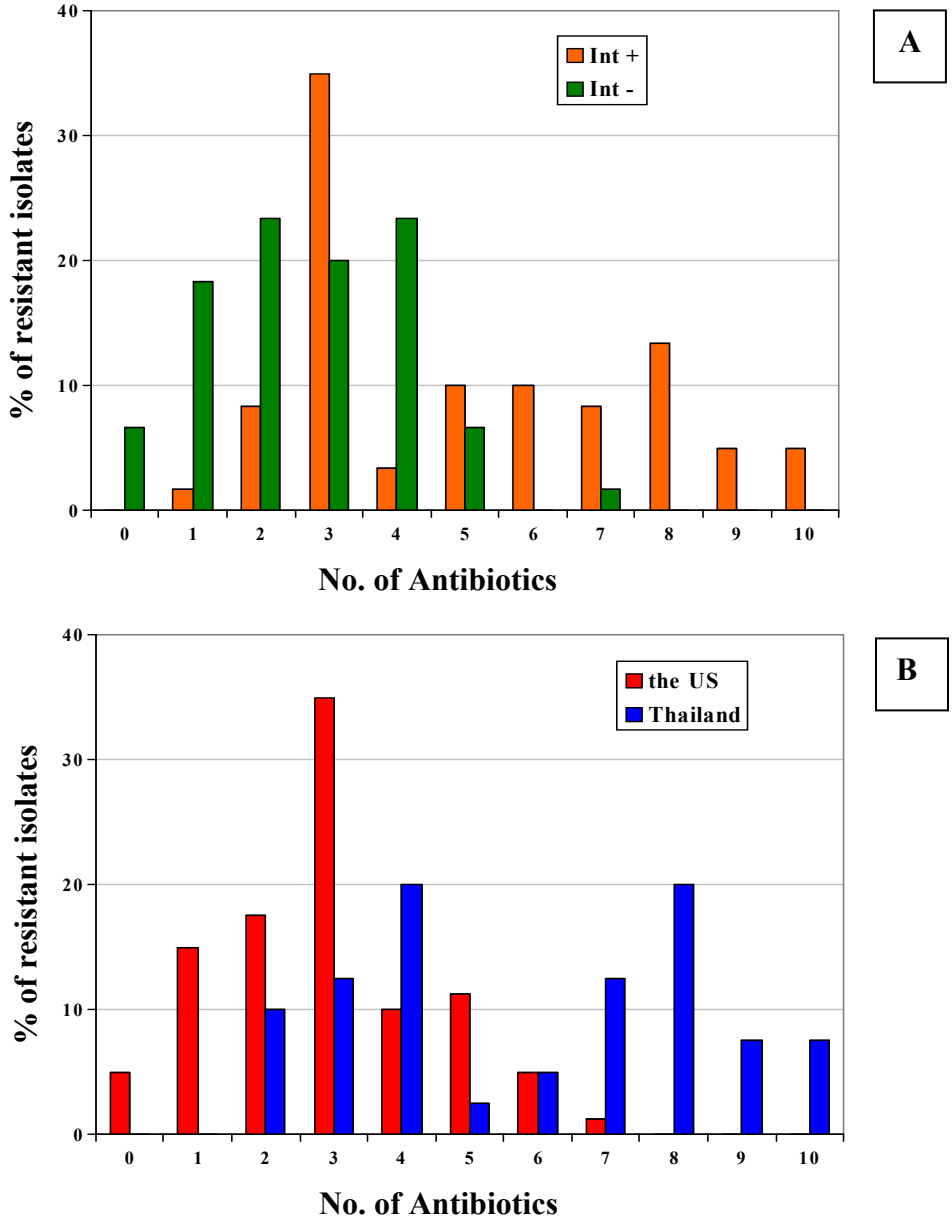

Figure 5. Multiple antimicrobial resistance of *E. coli* from market pigs.

A. Integron-positive and integron-negative isolates.
B. Isolates from the US and Thailand.

Table 5. Antibiograms of *E. coli* isolates of market pigs from the US and Thailand.

Pattern No.	Antibiogram[1]	No. of isolate(s)				
		Integron		Location		Total
		+ (n=60)	- (n=60)	US (n=80)	Thailand (n=40)	(n=120)
1	Ami-Amp-Cpl-Chl-Kan-Str-Sul-Tet-Sxt	1	0	0	1	1
2	Ami-Amp-Chl-Kan-Gen-Nal-Str-Sul-Tet-Sxt	1	0	0	1	1
3	Aug-Amp-Cpl-Kan-Str-Sul-Tet	1	0	1	0	1
4	Amp-Tio-Cip-Str-Sul-Tet-Sxt	1	0	0	1	1
5	Amp-Axo-Cip-Nal-Sul-Tet-Sxt	1	0	0	1	1
6	Amp-Cpl-Chl-Kan-Gen-Nal-Str-Sul-Tet-Sxt	2	0	0	2	2
7	Amp-Cpl-Chl-Kan-Gen-Str-Sul-Tet-Sxt	1	0	0	1	1
8	Amp-Cpl-Chl-Kan-Str-Sul-Tet-Sxt	1	0	0	1	1
9	Amp-Cpl-Chl-Sul-Tet	0	1	1	0	1
10	Amp-Cpl-Nal-Str-Sul-Tet	1	0	0	1	1
11	Amp-Cpl-Tet	1	0	1	0	1
12	Amp-Chl-Cip-Nal-Str-Sul-Tet-Sxt	3	0	0	3	3
13	Amp-Chl-Kan-Gen-Nal-Str-Sul-Tet-Sxt	1	0	0	1	1
14	Amp-Chl-Kan-Gen-Str-Sul-Tet-Sxt	2	0	0	2	2
15	Amp-Chl-Kan-Sul-Tet [2]	1	1	2	0	2
16	Amp-Chl-Gen-Nal-Sul-Tet-Sxt	0	1	0	1	1
17	Amp-Chl-Gen-Str-Sul-Tet-Sxt	1	0	0	1	1
18	Amp-Chl-Str-Sul-Tet-Sxt	1	0	0	1	1
19	Amp-Chl-Str-Sul-Tet	1	0	1	0	1
20	Amp-Chl-Str-Tet-Sxt	0	1	0	1	1
21	Amp-Chl-Sul-Tet	0	3	3	0	3
22	Amp-Chl-Tet	0	1	0	1	1
23	Amp-Cip-Nal-Str-Sul-Tet-Sxt	1	0	0	1	1
24	Amp-Kan-Gen-Nal-Str-Sul-Tet-Sxt	2	0	0	2	2
25	Amp-Kan-Gen-Str-Sul-Tet	3	0	3	0	3
26	Amp-Kan-Str-Sul-Tet	1	0	1	0	1
27	Amp-Kan-Sul-Tet	0	1	1	0	1
28	Amp-Nal-Sul-Tet	0	1	0	1	1
29	Amp-Str-Sul-Tet-Sxt	0	1	1	0	1

Table 5. (continued) Antibiograms of *E. coli* isolates of market pigs from the US and Thailand.

Pattern No.	Antibiogram[1]	No. of isolate(s)				
		Integron		Location		Total
		+ (n=45)	- (n=45)	US (n=70)	Thailand (n=20)	(n=90)
30	Amp-Str-Tet [3]	0	6	5	1	6
31	Amp-Sul-Tet	2	0	2	0	2
32	Amp-Tet [3]	0	3	1	2	3
33	Cph-Chl-Tet	0	1	0	1	1
34	Cpl-Gen-Nal-Tet	0	1	0	1	1
35	Cpl-Tet	0	1	0	1	1
36	Chl-Kan-Sul-Tet-Sxt	2	0	2	0	2
37	Chl-Nal	0	1	0	1	1
38	Chl-Str-Sul-Tet	0	1	0	1	1
39	Chl-Sul-Tet-Sxt	0	1	0	1	1
40	Chl-Tet	0	1	0	1	1
41	Kan-Nal-Str-Sul-Tet-Sxt	1	0	1	0	1
42	Kan-Nal-Sul-Tet	0	1	1	0	1
43	Kan-Str-Sul-Tet-Sxt	1	0	1	0	1
44	Kan-Str-Sul-Tet [2]	1	1	2	0	2
45	Kan-Str-Tet [2]	2	2	4	0	4
46	Kan-Sul-Tet	2	0	2	0	2
47	Kan-Tet [2]	1	3	4	0	4
48	Gen-Nal-Sul-Tet	0	1	0	1	1
49	Gen-Str-Sul-Tet	1	0	1	0	1
50	Gen-Str-Tet	0	1	0	1	1
51	Nal-Sul-Tet-Sxt	0	1	0	1	1
52	Nal-Sul-Tet	0	1	0	1	1
53	Str-Sul-Tet-Sxt	0	2	0	2	2
54	Str-Sul-Tet	14	0	14	0	14
55	Str-Tet [2]	1	2	3	0	3
56	Sul-Tet [2]	3	3	6	0	6
57	Tet [2]	1	11	12	0	12
58	None	0	4	4	0	4

[1] Ami, Amikacin; Aug, Amoxicillin/Clavulanic Acid; Amp, Ampicillin; Tio, Ceftiofur; Axo, Ceftriaxone; Cpl, Cephalothin; Chl, Chloramphenicol; Cip, Ciprofloxacin; Gen, Gentamicin; Kan, Kanamycin; Nal, Nalidixic Acid; Str, Streptomycin; Sul, Sulfamethoxazole; Tet, Tetracycline; Sxt, Trimethoprim/Sulfamethoxazole.

[2] Resistance pattern found in both integron-positive and integron-negative isolates.

[3] Resistance pattern found in both Thailand and the US.

No *E. coli* isolates from sows in Thailand carried all three genes, which prohibited a comparison between Thailand and the US. As a result, only integron-positive and –negative isolates from the US were used for the comparison.

All *E. coli* isolates from sows in the US were susceptible to at least half of the antibiotics tested in this study, with those being amikacin, amoxicillin/clavulanic acid, cephalothin, cefoxitin, ceftiofur, ceftriaxone, ciprofloxacin, and nalidixic acid. In contrast, all *E. coli* isolates were resistant to tetracycline. All integron-positive isolates were resistant to streptomycin, ampicillin, sulfamethoxazole, and tetracycline. Integron-positive isolates also demonstrated a high percentage of resistance to gentamicin (90.9%), kanamycin (90.9%), trimethoprim/sulfamethoxazole (72.7%), and chloramphenicol (54.5%). Integron-negative isolates demonstrated a high percentage of resistance to sulfamethoxazole (72.7%), ampicillin (63.6%), and streptomycin (54.5%). Resistance to gentamicin, kanamycin, streptomycin, ampicillin, trimethoprim/sulfamethoxazole and chloramphenicol was more common in integron–positive isolates ($P<0.05$) (Figure 6).

There was similarity between isolates from market pigs and isolates from sows, with integron-positive *E. coli* isolates from sows tending to be more resistant to a higher number of antimicrobials than integron-negative isolates. Integron-positive isolates demonstrated resistance to 5 to 8 antibiotics; whereas integron-negative isolates demonstrated resistance to 1 to 7 antibiotics (Figure 7).

Among 22 *E. coli* isolates investigated from sows in the US, 13 resistance patterns were observed. The pattern, Amp-Chl-Kan-Gen-Str-Sul-Tet-Sxt, was found in five isolates (4.5%). This pattern not only represented resistance to the highest number of antibiotics but it was also the most common resistance pattern in *E. coli* isolates.

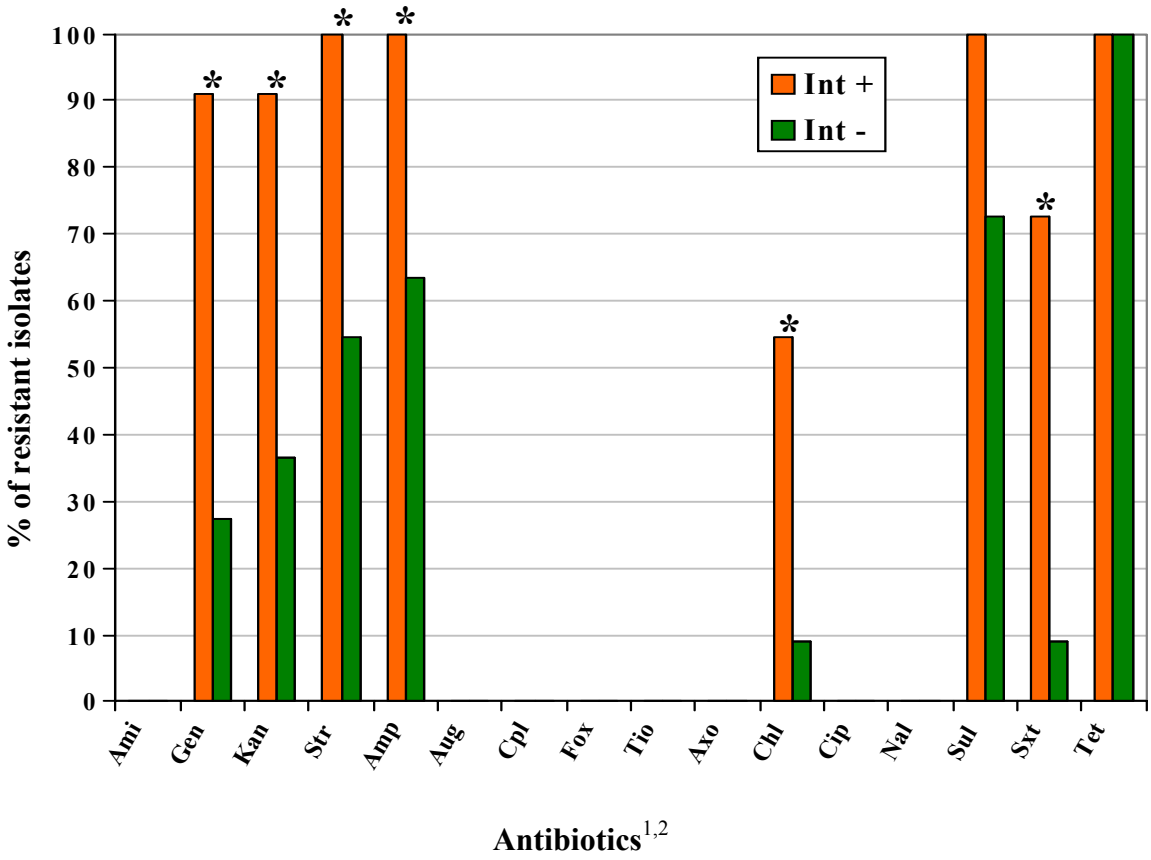

Figure 6. Frequency of resistance to 16 antimicrobials in integron-positive and integron-negative *E. coli* of sows from the US.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within antibiotic with * differ ($P<0.05$).

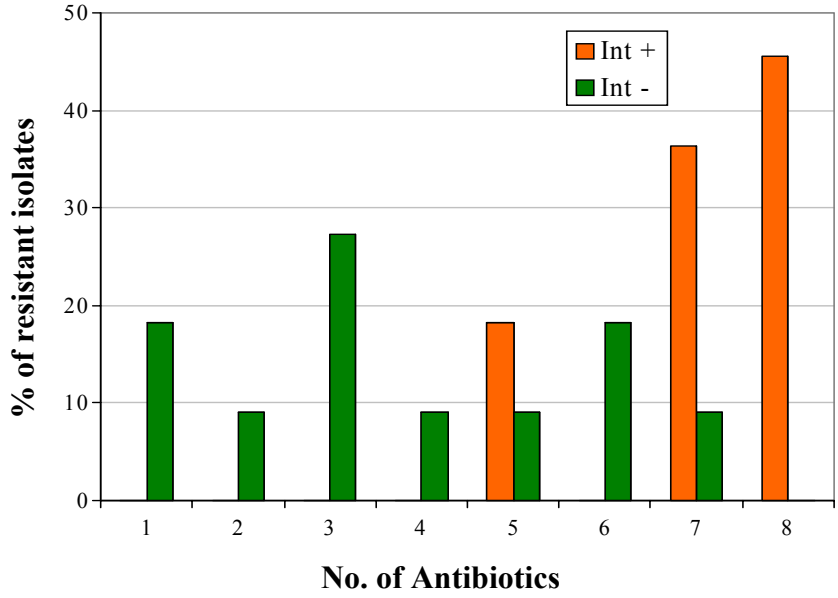

Figure 7. Percentage of integron-positive and integron-negative *E. coli* isolates from sows in the US demonstrating resistance to various numbers of antibiotics.

Only one pattern, Amp-Chl-Kan-Gen-Str-Sul-Tet, was found in both integron-positive and integron-negative isolates (Table 6).

Salmonella isolates

In contrast to *E. coli* isolates from sows, *Salmonella* isolates which carried all 3 class 1 integron conserved genes were only found in market pigs from Thailand. Therefore, only Thailand isolates were used for the comparison of antibiotic susceptibility tests between integron-positive and –negative isolates.

A comparison of the prevalence of antibiotic resistance between integron-positive and integron-negative *Salmonella* spp. isolates of market pigs from Thailand is given in Figure 8. All integron-positive and -negative isolates were susceptible to amikacin, amoxicillin/clavulanic acid, cephalothin, cefoxitin, ceftiofur, ceftriaxone and ciprofloxacin. The highest percentage of resistance in integron-positive *Salmonella* isolates was found to sulfamethoxazole (100%), followed by tetracycline (93.3%), streptomycin (80.0%), nalidixic acid (73.3%), trimethoprim/sulfamethoxazole (73.3%) ampicillin (66.7%), kanamycin (53.3%) and gentamicin (40.0%); whereas 86.7, 66.7, 53.3, 40.0, 33.3, 26.7, 26.7, and 20.0% of integron-negative isolates showed resistance to tetracycline, sulfamethoxazole, nalidixic acid, ampicillin, kanamycin, gentamicin, chloramphenicol, trimethoprim/sulfamethoxazole and streptomycin, respectively. Resistance to streptomycin, chloramphenicol, sulfamethoxazole and combination of trimethoprim and sulfamethoxazole was more common in integron–positive isolates ($P<0.05$).

Table 6. Antibiograms of *E. coli* isolates of sows from the US.

Pattern No.	Antibiogram[1]	No. of isolate(s)		
		Integron		Total
		+ (n=11)	- (n=11)	(n=22)
1	Amp-Chl-Kan-Gen-Str-Sul-Tet-Sxt	5	0	5
2	Amp-Chl-Kan-Gen-Str-Sul-Tet [2]	1	1	2
3	Amp-Kan-Gen-Str-Sul-Tet-Sxt	3	0	3
4	Amp-Kan-Gen-Str-Sul-Tet	0	2	2
5	Amp-Kan-Str-Sul-Tet	1	0	1
6	Amp-Kan-Tet	0	1	1
7	Amp-Gen-Str-Sul-Tet	1	0	1
8	Amp-Str-Sul-Tet-Sxt	0	1	1
9	Amp-Str-Sul-Tet	0	1	1
10	Amp-Sul-Tet	0	1	1
11	Str-Sul-Tet	0	1	1
12	Sul-Tet	0	1	1
13	Tet	0	2	2

[1] Amp, Ampicillin; Chl, Chloramphenicol; Kan, Kanamycin; Gen, Gentamycin; Str, Streptomycin; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.
[2] Resistance pattern found in both integron-positive and integron-negative isolates.

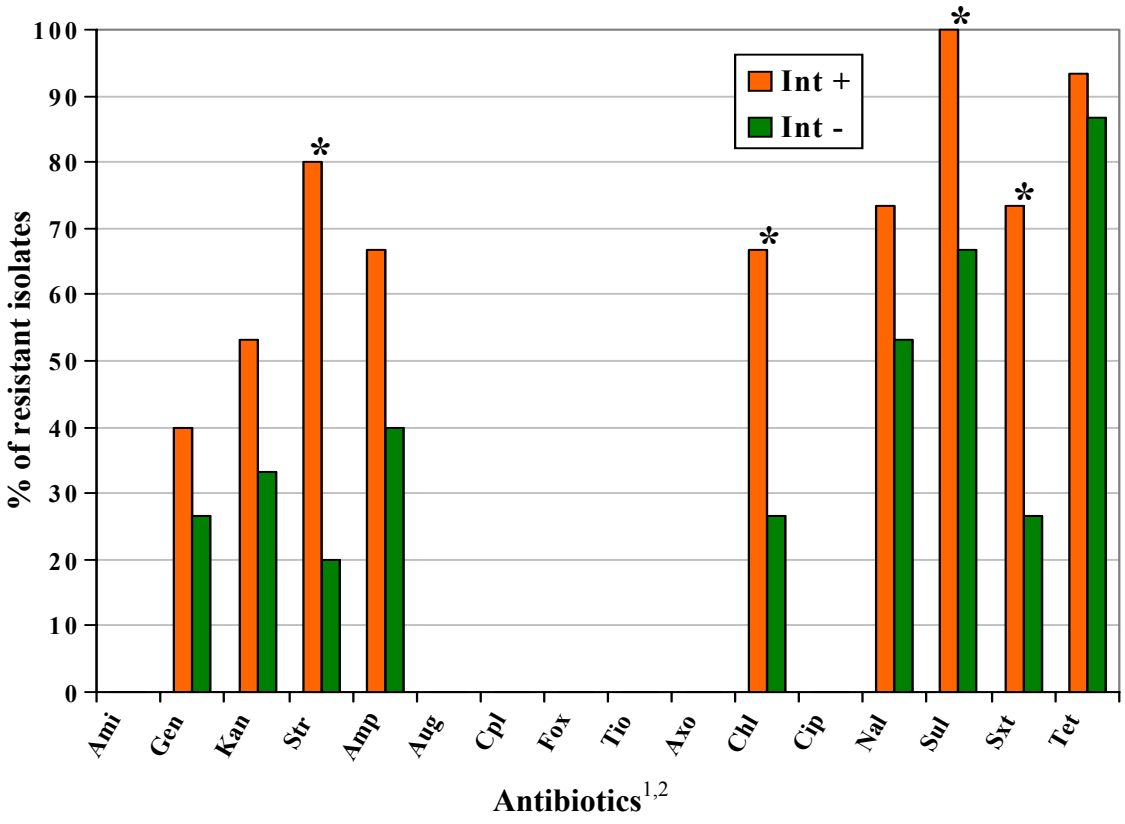

Figure 8. Frequency of resistance to 16 antimicrobials in integron-positive and integron-negative *Salmonella* spp. of market pigs from Thailand.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within antibiotic with * differ ($P<0.05$).

In agreement with *E. coli* isolates, integron-positive *Salmonella* isolates from market pigs from Thailand tended to be more resistant to a higher number of antibiotics than integron-negative isolates, with integron-positive isolates showing resistance to 3 to 9 antibiotics; whereas integron-negative isolates demonstrated resistance to 1 to 8 antibiotics (Figure 9).

There was a wide variety of resistance patterns found in *Salmonella* isolates from market pigs, with a total of 26 patterns being observed among 30 *Salmonell*a isolates. Only 4 patterns were found in more than one isolate, with those being Amp-Chl-Kan-Gen-Nal-Str-Sul-Tet-Sxt, Amp-Kan-Gen-Nal-Sul-Tet, Chl-Kan-Nal-Str-Sul-Tet-Sxt, and Tet. There was no common pattern found in both integron-positive and –negative *Salmonella* isolates from market pigs in Thailand (Table 7).

All 20 integron-positive and integron-negative *Salmonella* isolates from sows were susceptible to the same 7 antibiotics, with those being amikacin, amoxicillin/clavulanic acid, cephalothin, cefoxitin, ceftiofur, ceftriazole and ciprofloxacin. All integron-positive isolates were resistant to 6 antibiotics, kanamycin, streptomycin, ampicillin, trimethoprim/sulfamethoxazole, tetracycline, and sulfamethoxazole, whereas the only drug to which all integron-negative isolates were only resistant was tetracycline. A high percentage of resistance to gentamicin (80.0%), nalidixic acid (70.0%), and chloramphenicol (60.0%) was also noted in integron-positive isolates. Seventy percent of integron-negative isolates were resistant to ampicillin and sulfamethoxazole. Up to 30% of integron-negative isolates were resistant to gentamicin, kanamycin, streptomycin, chloramphenicol, trimethoprim/sulfamethoxazole and nalidixic acid. Resistance to gentamicin, kanamycin, streptomycin, nalidixic acid, and

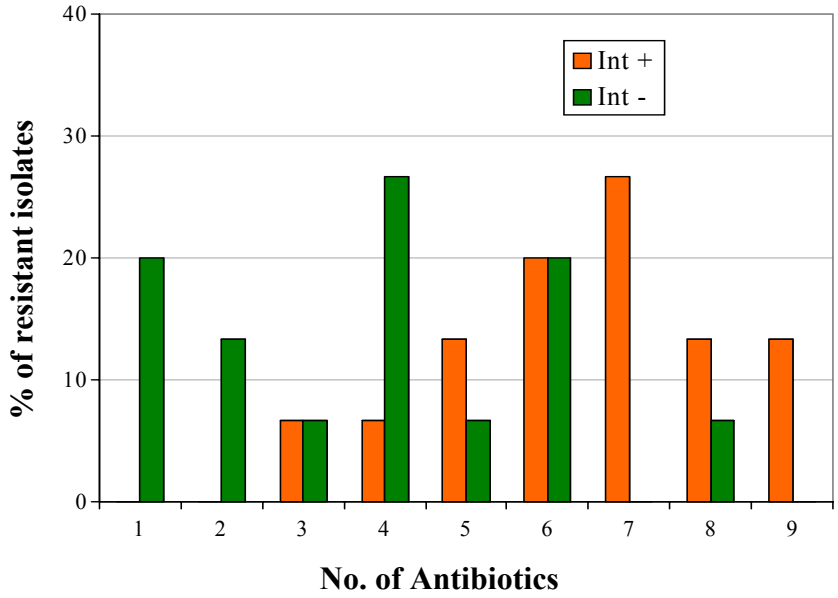

Figure 9. Multiple antimicrobial resistance of integron-positive and integron-negative *Salmonella* isolates of market pigs from Thailand.

Table 7. Antibiograms of *Salmonella* spp. isolates of market pigs from Thailand.

Pattern No.	Antibiogram[1]	No. of isolate(s)		
		Integron		Total
		+ (n=15)	- (n=15)	(n=30)
1	Amp-Chl-Kan-Gen-Nal-Str-Sul-Tet-Sxt	2	0	2
2	Amp-Chl-Kan-Gen-Nal-Str-Sul-Tet	0	1	1
3	Amp-Chl-Kan-Gen-Str-Sul-Tet-Sxt	1	0	1
4	Amp-Chl-Kan-Nal-Str-Sul	1	0	1
5	Amp-Chl-Gen-Nal-Sul-Tet-Sxt	1	0	1
6	Amp-Chl-Nal-Str-Sul-Tet-Sxt	1	0	1
7	Amp-Chl-Nal-Sul-Sxt	1	0	1
8	Amp-Kan-Gen-Nal-Str-Sul-Tet-Sxt	1	0	1
9	Amp-Kan-Gen-Nal-Sul-Tet	0	2	2
10	Amp-Kan-Sul-Tet	1	0	1
11	Amp-Gen-Str-Sul-Tet-Sxt	1	0	1
12	Amp-Nal-Tet	0	1	1
13	Amp-Str-Sul-Tet	0	1	1
14	Amp-Sul	0	1	1
15	Chl-Kan-Gen-Nal-Tet-Sxt	0	1	1
16	Chl-Kan-Nal-Str-Sul-Tet-Sxt	2	0	2
17	Chl-Kan-Nal-Sul-Tet	0	1	1
18	Chl-Nal-Str-Sul-Tet-Sxt	1	0	1
19	Chl-Sul-Tet-Sxt	0	1	1
20	Nal-Str-Sul-Tet-Sxt	1	0	1
21	Nal-Str-Sul-Tet	0	1	1
22	Nal-Sul-Tet-Sxt	0	1	1
23	Str-Sul-Tet	1	0	1
24	Sul-Tet	0	1	1
25	Tet	0	2	2
26	Sxt	0	1	1

[1] Amp, Ampicillin; Chl, Chloramphenicol; Kan, Kanamycin; Gen, Gentamycin; Nal, Nalidixic Acid; Str, Streptomycin; Sul, Sulfamethoxazole; Tet, Tetracycline; Sxt, Trimethoprim/Sulfamethoxazole.

trimethoprim/sulfamethoxazole was more common in integron-positive isolates compared with integron-negative isolates ($P<0.05$) (Figure 10).

A comparison between *Salmonella* isolates of sows from Thailand and the US is shown in Figure 11. Resistance to gentamicin, kanamycin, streptomycin, ampiciilin, chloramphenicol, nalidixic acid, sulfamethoxazole, trimetroprim/sulfamethoxazole and tetracycline was found in *E. coli* isolates in both countries. In addition, only resistance to nalidixic acid was found to be significantly higher in Thailand isolates ($P>0.05$).

In agreement with *E. coli* isolates and *Salmonella* isolates from market pigs, integron-positive isolates from sows tended to be resistant to a higher number of antibiotics than integron-negative isolates, with integron-positive isolates from sows demonstrating resistance to 7 to 9 antibiotics; whereas integron-negative isolates were resistant to 1 to 6 antibiotics. A comparison of isolates from the US and Thailand showed that US isolates were resistant to 1 to 8 antibiotics, whereas Thailand isolates were resistant to 2 to 9 antibiotics (Figure 12).

A total of 16 patterns were observed among 20 *Salmonella* isolates from sows. The most frequent pattern was resistance to 9 antimicrobials, Amp-Chl-Kan-Gen-Nal-Str-Sul-Tet-Sxt, which was found in 15% of isolates. No common pattern was found between both integron-positive and integron-negative isolates in both countries (Table 8).

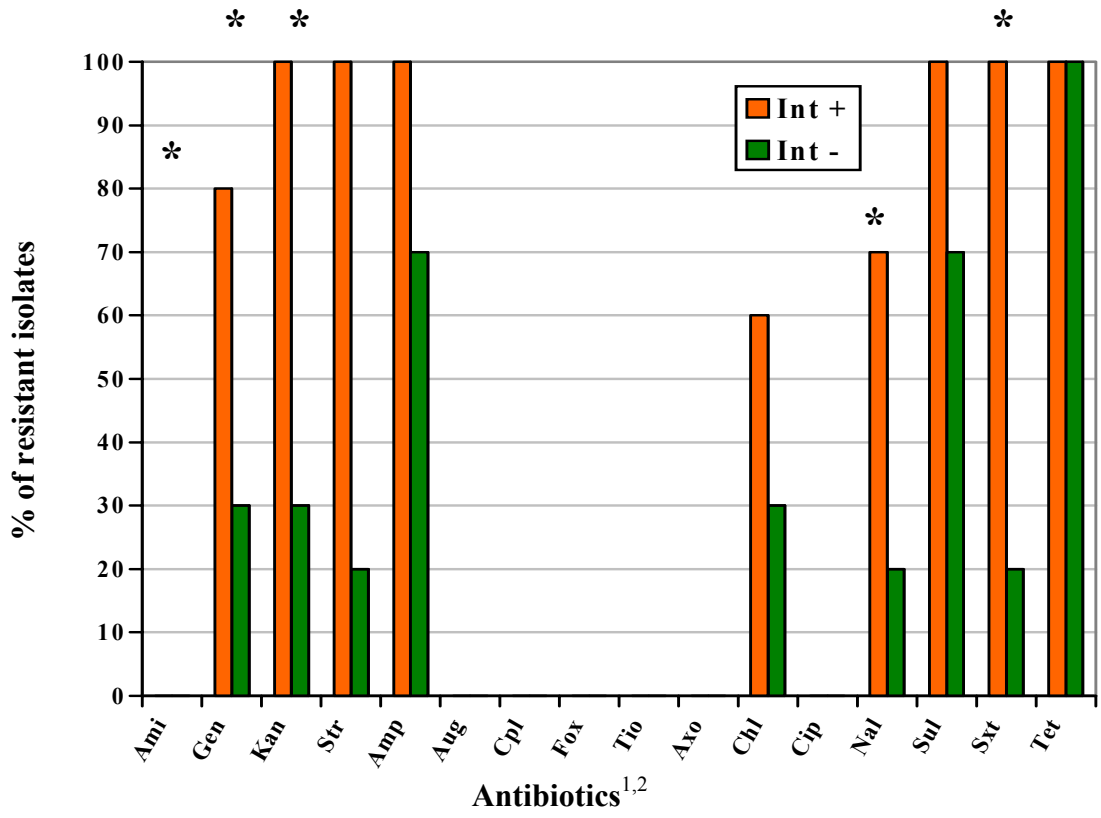

Figure 10. Frequency of resistance to 16 antimicrobials in integron-positive and integron-negative *Salmonella* spp. from sows.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline.

[2] Bars within antibiotic with * differ ($P<0.05$).

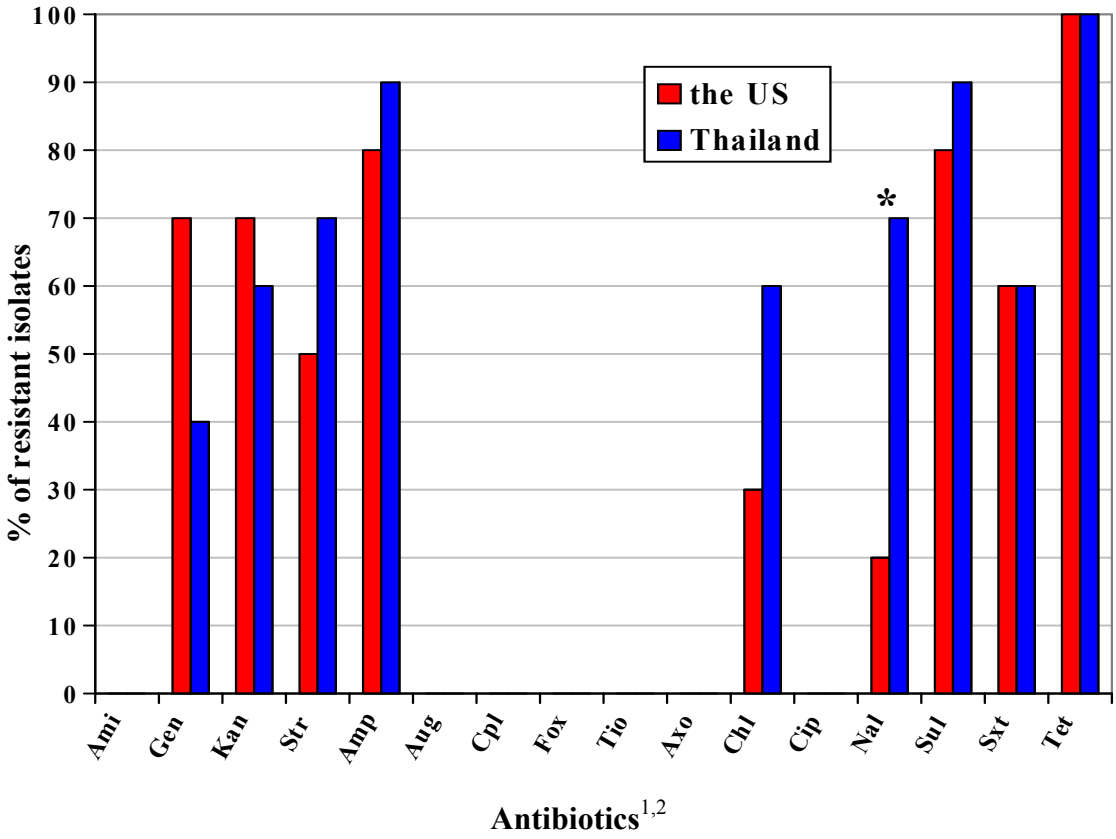

Figure 11. Frequency of resistance to 16 antimicrobials in *Salmonella* spp. of sow isolates from the US and Thailand.

[1]Ami, Amikacin; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Amp, Ampicillin; Aug, Amoxicillin/Clavulanic Acid; Cpl, Cephalothin; Fox, Cefoxitin; Tio, Ceftiofur; Axo, Ceftriaxone; Chl, Chloramphenicol; Cip, Ciprofloxacin; Nal, Nalidixic Acid; Sul, Sulfamethoxazole; Sxt, Trimethoprim/Sulfamethoxazole; Tet, Tetracycline

[2] Bars within antibiotic with * differ ($P<0.05$).

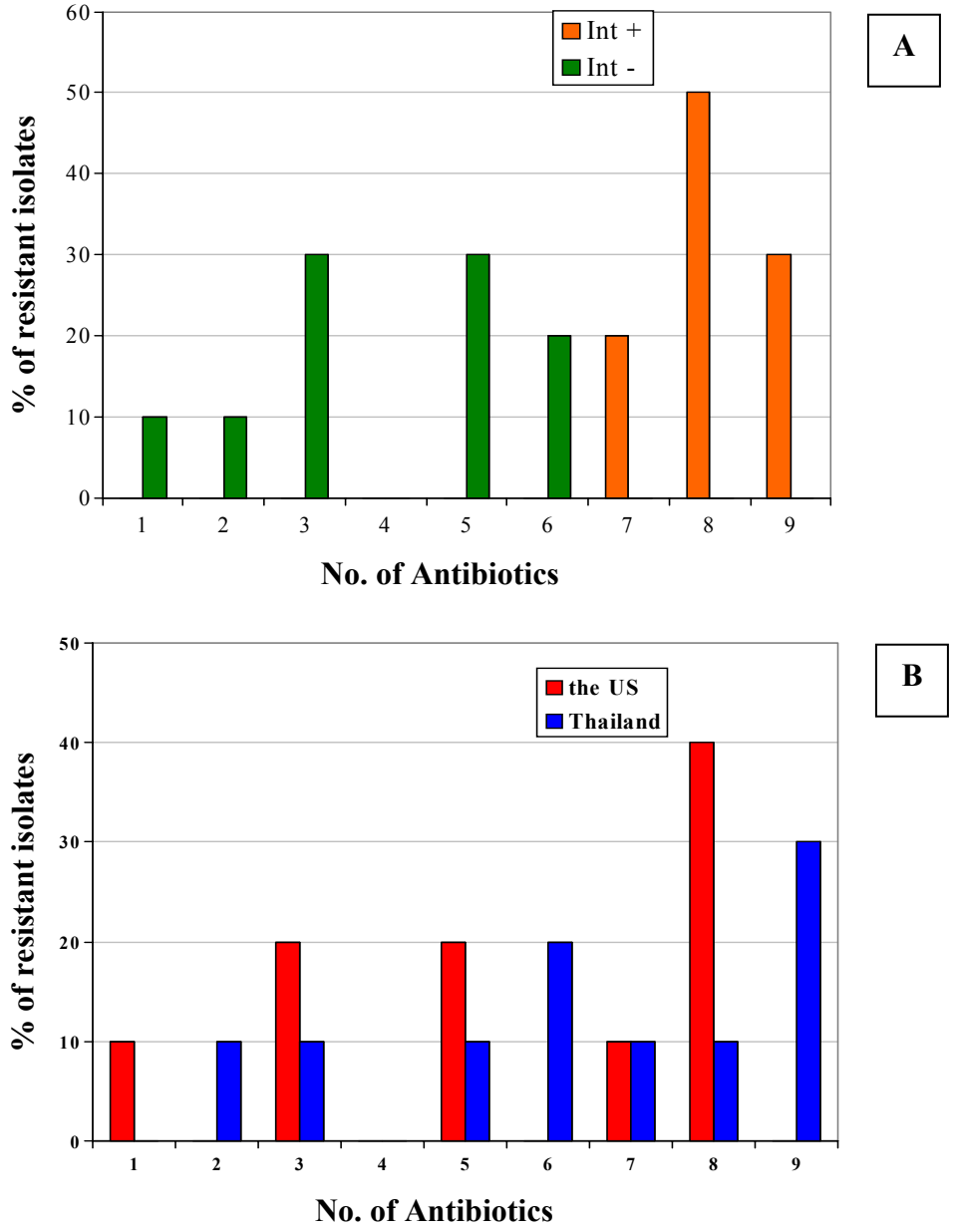

Figure 12. Multiple antimicrobial resistance of *Salmonella* spp. isolates from sows.

A. Integron-positive and integron-negative isolates.
B. Isolates from the US and Thailand.

Table 8. Antibiograms of *Salmonella* spp. isolates of sows from the US and Thailand.

Pattern No.	Antibiogram[1]	No. of isolate(s)				
		Integron		Location		Total
		+ (n=10)	- (n=10)	US (n=10)	Thailand (n=10)	(n=20)
1	Amp-Chl-Kan-Gen-Nal-Str-Sul-Tet-Sxt	3	0	0	3	3
2	Amp-Chl-Kan-Gen-Str-Sul-Tet-Sxt	2	0	2	0	2
3	Amp-Chl-Kan-Nal-Str-Sul-Tet-Sxt	1	0	0	1	1
4	Amp-Chl-Str-Sul-Tet-Sxt	0	1	0	1	1
5	Amp-Chl-Str-Sul-Tet	0	1	0	1	1
6	Amp-Kan-Gen-Nal-Str-Sul-Tet-Sxt	2	0	2	0	2
7	Amp-Kan-Gen-Nal-Sul-Tet	0	1	0	1	1
8	Amp-Kan-Gen-Str-Sul-Tet-Sxt	1	0	1	0	1
9	Amp-Kan-Gen-Sul-Tet	0	1	1	0	1
10	Amp-Kan-Nal-Str-Sul-Tet-Sxt	1	0	0	1	1
11	Amp-Kan-Sul-Tet-Sxt	0	1	1	0	1
12	Amp-Nal-Tet	0	1	0	1	1
13	Amp-Sul-Tet	0	1	1	0	1
14	Chl-Gen-Tet	0	1	1	0	1
15	Sul-Tet	0	1	0	1	1
16	Tet	0	1	1	0	1

[1]Amp, Ampicillin; Chl, Chloramphenicol; Kan, Kanamycin; Gen, Gentamycin; Nal, Nalidixic Acid; Str, Streptomycin; Sul, Sulfamethoxazole; Tet, Tetracycline; Sxt, Trimethoprim/Sulfamethoxazole.

IV. DISCUSSION

Prevalence of Bacterial Isolates

The prevalences of *E. coli* and *Salmonella* spp. in market pigs and sows from the US and Thailand were determined in this study. Our results show that the prevalence of *E. coli* is high in both types of swine and both countries with over 80% of animals were found to carry this bacterium, as was our expectation. The prevalence of *Salmonella* in market pigs was much lower than that of *E. coli* but was not different between the 2 countries with only 3.4% and 5.7% of market pigs from Thailand and the US respectively found to carry *Salmonella*. In sows, however, the prevalence of *Salmonella* was higher in the US when compared with Thailand, with 60.9% of sows from the US found to carry *Salmonella* isolates whereas only 8.5% of sows from Thailand were found to be positive for that organism. The differences in *Salmonella* prevalence in sows may be due to the differences of culture collection. While *Salmonella* isolates from sows in Thailand were collected from live animals on farm, *Salmonella* isolates from the US were collected from killed sows at the slaughterhouse. The stress caused by transportation of animals from farm to slaughterhouse has been reported to result in increased *Salmonella* shedding (4, 37, 42). Hurd et al. (21, 22) noted that a serotype of *Salmonella* not found in fecal samples from pigs on farms was isolated from intestinal contents at slaughter, suggesting infection occurred during transport or at the slaughterhouse. Davies et al. (11, 12) reported that although breeding herds may be a minor source of infection for finisher pigs, they can play an important role in the maintenance of *Salmonella* on farms and in its transmission to other farms.

Prevalence of Class 1 Integrons

It is well known that class 1 integrons play an important role in dissemination of antibiotic resistance in the enteric bacteria. Our results show that class 1 integrons, as indicated by the presence of *IntI1* gene, are widespread among *E. coli* and *Salmonella* isolates of swine. More than 60 and 30% of *E. coli* isolates, and more than 50 and 25% of *Salmonella* isolates from swine in Thailand and the US, respectively, carried class 1 integron.

The prevalence of class 1 integrons in both types of bacteria and both types of swine were associated with the isolate origin. Class 1 integrons were more prevalent in Thailand compared to the US.

With the exception of *E. coli* isolates from Thailand, we also noted a higher prevalence of integrons in isolates collected from sows. It may be that as sows spend a longer time on farm (3 to 5 years, compared with approximately 6 months for market pigs), they have more opportunity for exposure to antibiotics and thus present a greater opportunity for a resistant microflora to develop.

It is important to note that only 11.1% and 16.2% of *intI1*-positive *E. coli* and *Salmonella* isolates from swine were found to carry *qacEΔ1* and *sul1;* whereas almost all of the *E. coli* (96.8%) carried *qacEΔ1* and *sul1*. In agreement with this study, our earlier study in chickens showed that 32.5%, 75.4%, and 82.9% of *intI1*-positive *E. coli, P. mirabilis,* and *Salmonella* isolates from Thailand were carried *qacEΔ1* and *sul1*. van Essen-Zandbergen noted that only a subset of *Salmonella* and *E. coli* isolates carrying *intI1* also carry the 3' conserved sequence (55). Sunde (50) reported that a high

proportion of class 1 integrons found in *E. coli* lacked the *sul1* gene. Additionally, Guerra et al. (18) found defective integrons which lacked both *qacEΔ1* and *sul1,* or only *sul1,* in some *E. coli* isolates from Germany, suggesting that use of specific primers to detect conserved segments alone can produce false negatives when those specific gene components are not present in otherwise functional integrons**.**

Antibioticl susceptibility

Antibiotic resistance to at least 1 antibiotic was found in 97% of combined *E. coli* and *Salmonella* isolates from swine in this study. Resistance to tetracycline, sulfamethoxazole, streptomycin, and ampicillin was most common in swine, in agreement with other studies (18, 25, 27, 32, 45, 48, 52, 54)

When comparing the frequency of antibiotic resistance between integron-positive and integron-negative isolates, we found that integron-positive isolates were more likely to be resistant to streptomycin than integron-negative isolates in both bacteria tested and in both types of swine. A higher prevalence of resistance to sulfamethoxazole and trimetroprim/sulfamethoxazone was also noted in integron-positive *E. coli* isolates. This likely occurred because all integron-positive isolates contained *sul1* as an integral part of the integron. Similar to our study, Shahada et al. (47) reported all of the *intI1*-positive *S. enterica* serovar Infantis isolates were resistant to sulfamethoxazole. Streptomycin and trimethoprim are not only frequently found as a single gene cassette in integrons but also these two genes were often found in combination within the same integron, as reported by several research groups (20, 23, 26, 35, 49, 55). This co-selection may explain the high prevalence of resistance to these antibiotics in integron-positive bacteria.

It should, however, be noted that the resistance phenotypes were only partially explained by the presence of integrons. In agreement with our study, a study of occurrence of integrons and antimicrobial resistance genes among *Salmonella enterica* in Brazil revealed that integron-mediated resistance genes contributed to the multiresistance phenotypes observed in the isolates; however, most resistance genes were located outside the integron structure as independent genes. It was suggested that multiple resistance genes might also be located on a single conjugative plasmid (39). In addition, a study of antimicrobial resistance patterns and class 1 integrons among *E. coli* and *S. enterica* serovar Choleraesuis strains isolated from humans and swine in Taiwan discovered that class 1 integrons examined did not support the total resistance phenotypes observed among both bacterial isolates. The investigators suggested that this may be due to chromosomal mutation or the presence of other undetected integron classes (20).

There were no *E. coli* isolates from sows in Thailand or *Salmonella* isolates from market pig in the US which carried all three class 1 integron conserved genes. This prohibited a comparison of antibiotic resistance prevalence of those bacteria in Thailand and the US. Nevertheless, our results from *E. coli* from market pigs showed that resistance to gentamicin, ampicillin, cephalothin, chloramphenicol, ciprofloxacin, nalidixic acid, trimethoprim/sulfamethoxazole of *E. coli* isolates from market pigs and nalidixic acid of *Salmonella* isolates from sows were higher in Thailand isolates. Our earlier study of chickens indicated that *E. coli, P. mirabilis,* and *Salmonella* isolates from Thailand demonstrated a higher prevalence of chloramphenicol, nalidixic acid, and trimethoprim/sulfamethoxazole than isolates from the US. Whereas, both *E. coli* and *Salmonella* isolates from the US were statistically more likely to be resistant to

amoxicillin/clavulanic acid, cefoxitin, and ceftiofur than those from Thailand. *Salmonella* and *P. mirabilis* isolates from the US also demonstrated a higher prevalence of resistance to cephalothin than isolates from Thailand. It is interesting to note that all *E. coli* and *Salmonella* isolates from swine in the US and Thailand were susceptible to amoxicillin/clavulanic acid, cefoxitin, ceftiofur, ceftriazole, and less than 2% of bacterial isolates from swine were resistant to amikacin, which may be due to the limited use of this antibiotic in swine production. Low levels of ceftriaxone and ceftiofur resistance were also observed from previous studies conducted in Thailand (37, 40). The distribution of antimicrobial resistance phenotypes are expected to reflect different use patterns of antimicrobial agents in each type of animal and each countries (1, 16, 34)

V. CONCLUSIONS

Antimicrobial resistance genes and class 1 integrons are common in bacteria associated with swine in both Thailand and the US. While multi-antibiotic resistance is associated with integrons, and specific multi-resistance patterns were often characteristic of integron carriage, many multi-resistant isolates with a variety of antibiograms also lacked class 1 integrons. Our work indicates that integrons and multi-antibiotic resistant bacteria appear to be a significant aspect of microbial communities associated with swine.

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CHAPTER 5 : POTENTIAL FOR CLASS 1 INTEGRONS TRANSFER BETWEEN *E. COLI* AND *SALMONELLA* SPP.

ABSTRACT

A study was conducted to determine if homologous integrons could be detected in *E. coli* and *Salmonella* derived from the same animal or farm, from among swine or poultry farms in the US and Thailand. Class 1 integron variable regions were detected using a PCR targeting conserved integron sequences. When integron-positive *E. coli* and *Salmonella* isolates had identical amplicon patterns, the PCR product was sequenced to determine homology. Nine different amplicons with sizes ranging from 0.5 to 2.5 kb were observed in bacterial isolates, and we found a single farm on which similar integrons were found in both *E. coli* and *Salmonella*. Sequence analysis revealed that a 1.0 kb amplicon found in both *E. coli* and *Salmonella* isolated from the farm contained an *aadA1* gene cassette encoding aminoglycosides 3'-adenyltransferase, which confers resistance to streptomycin and spectinomycin. A 2.0 kb amplicon also found in both types of bacteria containing the *aadA5* gene encoding aminoglycosides 3'-adenyltransferase, an additional reading frame with unknown function, *orfD,* as well as a *dfrA17* gene encoding dihydrofolate reductase, conferring resistance to trimethoprim. Our results indicate that identical integrons were found in *Salmonella* and *E. coli* from a single farm, likely indicating transfer between these two organisms occurs *in vivo* via exchange of plasmids. Ours may be among the first studies to detect such transfer of integrons and resistance genes between commensal bacteria and a foodborne pathogen within a single farm.

I. INTRODUCTION

When antibiotics are used in animals, selection for resistance to those drugs may occur, not only in transient pathogenic bacteria, but also in commensal bacteria which continually live in intestinal tract. The result may be increased reservoirs of resistant organisms (32). In recent years, many resistance genes isolated from bacteria have been mapped to specific genome sites known as integrons. Integrons have been found in plasmids, transposons, and as independent units on bacterial chromosomes (4, 5, 17). Integrons contain three elements that allow site-specific recombination of antibiotic resistance genes. These elements include: 1) a recombination or attachment site; 2) an integrase, that recognizes specific sequences on the extra-integron gene cassette and the recombination site; and 3) a strong promoter that allows the integron to act as an expression vector in the event of the incorporation of promoterless cassettes (9, 14). As such, bacteria that harbor integrons may have an enhanced ability to rapidly acquire resistance to multiple antibiotics and to promote the transfer of highly stable and self-promoting resistance factors across their own and other bacterial species. Thus, bacteria containing integrons pose a particularly insidious threat to the efficacies of current as well as future antimicrobials. And while it is proposed that the primary vehicle for transfer of bacterial resistance genes from animal hosts to human hosts is through food borne bacteria, the large pool of naturally occurring nonpathogenic bacteria in the gut, including *E. coli,* have been proposed to act as a reservoir of and/or vector for transferable resistance genes. These commensal bacteria, by their residence in the GI tract, are subject to exposure from antibiotics included in feeds and water, and they have been shown to acquire resistance following such exposure (25, 26, 28) demonstrating a

higher prevalence of resistance genes and genetic resistance elements, including integrons (13).

It is widely accepted that resistance genes are transferable across species of bacteria, and thus the potential exists for transfer from resident *E. coli* and other naturally occurring bacteria to transient animal and human pathogens, including *Salmonella*. However, to date, little evidence has been presented to clearly show that such transfer is common *in vivo*. As a possible indication of gene transfer, we therefore determined the degree of homology of resistance genes and integron sequences between those two groups of bacteria within animals and farms.

II. MATERIALS AND METHODS

Bacterial Isolates

In this study *E. coli* and *Salmonella* spp. derived from a previous study were analyzed. We selected isolates for which both bacterial species within a farm were found to contain all three conserved sequences of class 1 integrons, *intI1, qacEΔ1*, and *sul1*. This criteria resulted in inclusion of 571 *E. coli* and 98 *Salmonella* spp. isolates from chickens from Thailand and the US, sows from the US, and market pigs from Thailand (Table 1).

Detection of Class 1 Integrons Variable Region by PCR

Class 1 integron variable regions were detected using a PCR targeting 5' and 3 conserved sequences of class 1 integrons (21). Primer pairs were manufactured by Integrated DNA Technologies, Inc. (San Diego, CA) (Table 2). Total DNA was prepared by boiling overnight cultures in 2YT broth (Difco, Spark, MD) in an equal volume of 0.2% (wt/vol) Triton X-100 (Mallinckrodt, Paris, KY) for 5 min (20). Boiled cultures were cooled on ice for 5 min and used immediately for PCR. The PCR solution was composed of 12.5 µl 2X PCR Master Mix (Promega, Medison, WI), 1.5 µl each primer (10 pmol/µl), 8.5 µl nuclease free water, and 1 µl DNA template. Reactions were conducted in a Mastercyler Gradient thermocycler (Eppendorf, Westbury, NJ) with the following conditions: 1) initial denaturation at 94°C for 3 min; 2) 35 cycles of denaturation at 94°C for 3 min, annealing at 60°C for 30 s; extension at 72°C for 2 min 30 s; and 3) final extension at 72°C for 5 min. Amplicons were analyzed via

Table 1. Source and number of bacteria possessing all three conserved sequence genes.

Animal	Countries	Farms	Bacteria	
			E. coli	*Salmonella*
Chickens	Thailand	TH1	353	53
	US	US3	58	1
		US6	146	2
Sows	US	USE	1	11
Market Pigs	Thailand	THA	12	25
		THD	1	6

Table 2. Primer pairs used for PCR.

Primer	Sequence (5' to 3')	Amplicon size (bp)
Class 1 integrons		
5'CS-F	GGCATCCAAGCACAAGC	Variable
3'CS-R	AAGCAGACTTGACTGAT	
E. coli housekeeping genes		
aspc-F	GTTTCGTGCCGATGAACGTC	594
aspc-R	AAACCCTGGTAAGCGAAGTC	
icdA-F	CTGCGCCAGGAACTGGATCT	669
icdA-R	ACCGTGGGTGGCTTCAAACA	
Salmonella housekeeping genes		
fhuA-F	AGAAGAAACCATTACCGTAACCG	402
fhuA-R	TGCTAACCATCGAAATGATACCG	
glnA-F	GTTATCGACCCGTTCTTCGC	579
glnA-R	GTTGGTGCCGTTCTTCGCC	

electrophoresis on a 1.0% agarose gel, and a 1 kb ladder (Promega, Medison, WI), was used as a molecular size marker. Gels were stained with ethidium bromide for visualization.

Location of Class 1 Integrons

When integron-positive *E. coli* and *Salmonella* isolates had identical amplicon patterns, further analysis was conducted to detect the location of the integron. To determine if class 1 integrons were carried on plasmids, plasmid DNA was extracted from bacteria cells using QIAprep Spin Miniprep kit (Qiagen, Maryland). Resulting DNA was subjected to a PCR analysis for detection of *aspc* and *icdA* chromosomal housekeeping genes for *E. coli* (27), and *fhuA* and *glnA* chromosomal housekeeping genes for *Salmonella* (35), using primers presented in Table 1, with PCR conditions used to detect class 1 integron variable regions. A positive control consisting of total DNA from each isolate was included to confirm efficiency of the PCR procedure. Absence of amplified products in the plasmid DNA prep and presence of appropriate amplified products (*aspc* and *icdA* for *E. coli* isolates, and *fhuA* and *glnA* for *Salmonella* isolates) in the control sample provided assurance that the plasmid solution was free of chromosomal contaminants. Plasmid DNA was then subjected to a PCR analysis for detection of class 1 integron variable regions, using the same primers and conditions as above. Successful amplification of the variable region gene products was considered proof that the gene was associated with the plasmid.

Cloning and Sequencing

When integron-positive *E. coli* and *Salmonella* isolates were found to possess identical amplicon patterns, randomly chosen CS-PCR products were sequenced. The CS-PCR amplicon product was purified from 1% agarose gel using QIAquick Gel Extraction Kit (Qiagen, Maryland) and cloned in the pGEM-T Vector (Promega, USA). Colonies carrying the target fragment were picked by blue-white screening from Luria-Bertani plates containing ampicillin 100 μg/ml, 40 μl 100 mM IPTG per plate and 40 μl 2% X-Gal per plate. Plasmid DNA from white colonies was checked for the presence of the target fragment by purification with a QIAprep Spin Miniprep kit (Qiagen, Germany).

The CS-fragment was sequenced using SP6 and T7 primers (Promega, USA) at the University of Tennessee Molecular Biology Resource Facility (Knoxville, TN). Sequences obtained were compared to those in the GenBank database using the BLAST algorithm available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov)

III. RESULTS

Six different variable region amplicon sizes ranging from 0.7 to 2.0 kb were found among 353 *E. coli* isolates from chicken farm in Thailand. Thus, class 1 integrons carrying gene cassettes were classified into 6 groups according to amplicon pattern. The most frequent pattern was a 0.7 kb amplicon which was found in 123 isolates (35%). *Salmonella* spp. isolates from chicken farms in Thailand possessed 2 sizes of amplicons, 1.0 and 1.25 kb, with the 1.0 kb amplicon being the most common (89% of isolates). *E. coli* isolates from one farm in the US produced three different sizes of amplicons, 0.9, 1.6, and 2.5 kb, and 1.0, 1.6, and 2.5 kb whereas the other produced 4 different sizes, 1.0, 1.6, 2.0, and 2.5 kb. The combination of 1.6 and 2.5 kb was the most common pattern in both farms. Only one amplicon, the 1.0 kb, was found in *Salmonella* from both chickens farms in the US. Only one *E. coli* isolate from US sows was analyzed for variable regions and those were found to carry integron patterns with combinations of 1.0 and 1.6 kb amplicons. All 11 *Salmonella* isolates from sows carried the 1.0 kb amplicon. Among two market pig farms in Thailand, all *E. coli* isolates from one farm carried the 1.6 kb amplicon, whereas *Salmonella* isolated from the same farm carried the 1.0 kb amplicon. Four different sizes of amplicons were found in *E. coli* isolates from another farm, with those being 0.5, 1.0, 1.6, and 2.0 kb in size. The presence of class 1 integrons carrying gene cassettes resulted in classification into 4 groups with the 2.0 kb, and co-existence of 1.0 and 2.0 kb, being the most common patterns found in this group. The same 2 patterns of gene cassettes were found in *Salmonella* isolates from this farm (Table 3).

Table 3. Gene cassette arrays of class 1 integrons among *E. coli* and *Salmonella* from chickens, sows, and market pigs.

Animal	Countries	Farms	Integron group	Approximate size of amplicon (kb)	No. (%) of strain carrying gene cassettes	
					E. coli	*Salmonella*
Chickens	Thailand	TH1	TH1-1	0.7	123 (35)	0 (0)
			TH1-2	0.7, 0.9	47 (13)	0 (0)
			TH1-3	0.7, 1.0	112 (32)	0 (0)
			TH1-4	0.9,1.6	13 (4)	0 (0)
			TH1-5	1.0, 1.75	47 (13)	0 (0)
			TH1-6	0.9, 1.25, 2.0	11 (3)	0 (0)
			TH1-7	1.0	0 (0)	47 (89)
			TH1-8	1.25	0 (0)	6 (11)
	US	US3	US3-1	1.6, 2.5	45 (78)	0 (0)
			US3-2	0.9, 1.6, 2.5	13 (22)	0 (0)
			US3-2	1.0	0 (0)	1 (100)
		US6	US6-1	1.6, 2.5	86 (59)	0 (0)
			US6-2	1.0, 1.6, 2.0	37 (25)	0 (0)
			US6-3	1.0, 1.6, 2.5	23 (16)	0 (0)
			US6-4	1.0	0 (0)	2 (100)
Sows	US	USE	USE-1	1.0,1.6	1 (100)	0 (100)
			USE-2	1.0	0 (0)	11 (100)
Market pigs	Thailand	THA	THA-1	1.6	1 (8)	0 (0)
			THA-2[1]	2.0	4 (33)	8 (32)
			THA-3[1]	1.0, 2.0	4 (33)	17 (68)
			THA-4	0.5, 1.0, 2.0	3 (25)	0 (0)
		THD	THD-1	1.6	1 (100)	0 (0)
			THD-2	1.0	0 (0)	6 (100)

[1] Integron group found in both *E. coli* and *Salmonella* spp.

Patterns of class 1 integrons carrying gene cassette in market pigs from Thailand are shown in Figure 1. Overall, among *E. coli* and *Salmonella* from 6 farms in this study only one market pig farm from Thailand possessed both integron-positive *E. coli* and *Salmonella* isolates with identical amplicon patterns; we therefore used isolates from that farm, and demonstrating that pattern, for further study.

E. coli and *Salmonella* housekeeping genes were detected in total DNA of each isolate; and were not detected in plasmid solutions. This provides assurance that the plasmid solution was free of chromosomal DNA, and suggests that integron sequences were associated with plasmids.

Sequence analysis revealed that the 1.0 kb amplicon from both *E. coli* and *Salmonella* contained the *aadA1* gene cassette encoding aminoglycoside 3'-adenyltransferase, which confers resistance to streptomycin and spectinomycin. In contrast to the 1.0 kb amplicon, the 2.0 kb amplicon found in both types of bacteria had an additional unknown reading frame, *orfD,* as well as a *dfrA17* gene cassette which encodes dihydrofolate reductase, conferring resistance to trimethoprim.

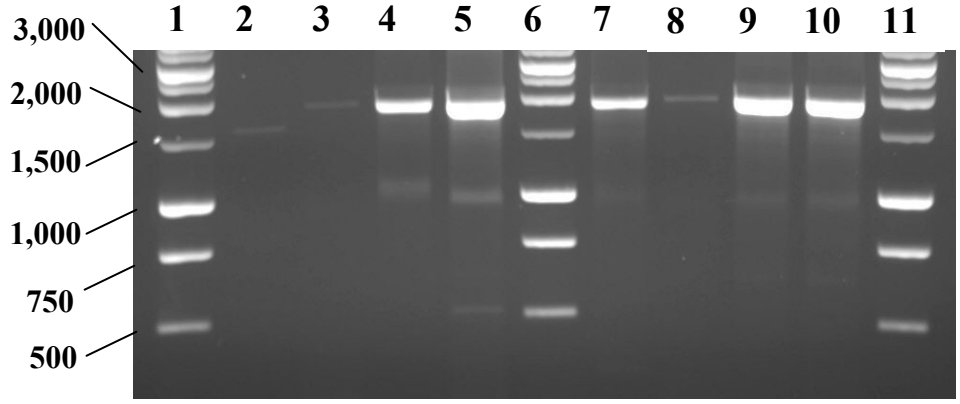

Figure 1. PCR detecting class 1 integron variable regions in bacterial isolates from market pigs.

Lane 1, 6, and 11; 1 kb DNA ladders; Lane 2-5; *E. coli* isolates; and Lane 7-10; *Salmonella* isolates.

IV. DISCUSSION

Antibiotic use in food animals targets pathogenic bacteria; however, when used, such compounds not only affect pathogenic bacteria, but also the commensal organisms that make up the major part of the gastrointestinal flora. These bacteria may function as a reservoir of resistance genes that can be transferred to pathogenic bacteria (25). It is now well established that many antibiotic resistance genes found in the *Enterobacteriaceae* are located on integrons (6). Integrons provide an efficient mechanism for capturing and exchanging a wide range of resistance genes. Four classes of integrons have been identified, and among them, class 1 integrons are the most prevalent (32).

Characterization of gene transfer DNA elements has mainly been conducted *in vitro*. However, the situation *in vivo* might be quite different, where a multitude of heterogeneous bacterial communities might facilitate, or dilute, the spread of resistance genes (1). We therefore investigated the potential for class 1 integrons transfer between commensal *E. coli* and pathogenic *Salmonella* in the farm environment.

The location of integrons within bacterial genetic structures has an effect on the stability and rate of dissemination of resistance genes. Integrons located on the chromosome can persist in bacteria for long periods of time; however transfer of such would typically be vertical. On the other hand, integrons located on plasmids are more easily transferred horizontally, as well as vertically. Our results show that class 1 integrons in this study were associated with plasmids. In agreement with our study, several researchers reported the presence of class 1 integrons on plasmids. For instance, a recent study of variable gene cassette patterns of class 1 integron-associated drug resistant *E. coli* in Taiwan revealed that class 1 integrons and *dfrA17-aadA5* gene

cassettes were located on the same transferable plasmids and were capable of transmission (5, 30). Vo et al. (29) investigated antimicrobial resistance, class 1 integrons and a novel variant of genomic island 1 in *Salmonella* isolates from Vietnam and found most integrons were associated with conjugative plasmids. They further suggested that those genes could transfer their antimicrobial resistance determinants to *E. coli* or *S.* Enteritidis; or to *Salmonella* Genomic Island 1 or variants of that genetic element.

Our sequencing results revealed that the 1.0 kb amplicon from both *E. coli* and *Salmonella* contained an *aadA1* gene cassette encoding for aminoglycoside 3'-adenyltransferase, conferring resistance to streptomycin and spectinomycin. A 2.0 kb amplicon also found in both types of bacteria containing the *aadA5* gene encoding aminoglycosides 3'-adenyltransferase*,* an additional reading frame with unknown function, *orfD,* as well as a *dfrA17* gene encoding dihydrofolate reductase, conferring resistance to trimethoprim. Those 2 types of amplicons were reported to be widespread in different hosts at several locations around the world (5, 12, 13, 18-20, 24, 26-31).

Because *E. coli* and *Salmonella* isolates isolated from the same farms had similar amplicon patterns and identical gene cassettes in the amplicon, and the integron was found to be located on the plasmid in both species, this may indicate that transfer of class 1 integrons between these two bacteria occurred in the farm environment. It is important to note; however, that the direction of transfer, whether from *Salmonella* to *E. coli* or *E. coli* to *Salmonella,* is unknown, as our current molecular techniques do not distinguish the direction of transmission (4).

While this study did not focus on *E. coli* and *Salmonella* which did not share similarity of class 1 integron patterns, a large proportion of bacterial isolates carried up to 3 amplicons in their integrons. This should be worrisome due to the fact that most amplicons in class 1 integrons carry antibiotic resistant gene cassettes. Thus, it could be suggested that a greater number of amplicons present in integrons would increase the number of resistance genes that could be disseminated.

V. CONCLUSIONS

Our study suggests that transfer of integrons between *E. coli* and *Salmonella* spp. can occur *in vivo* within hosts or in farm environments. While such has been theorized by experts, we may be among the first group to find evidence in that regard. Given the numerous samples, animals, and farms sampled in the companion study that produced the testable isolates for this study, as well as the number of integrons isolated, it appears that transfer of integrons between these species may not occur at a high rate. Additionally, it will be critical to determine the direction of transfer, as a primary concern is that commensal bacteria, such as *E. coli,* that may be continuously exposed to feed based or other antibiotic use in animal production, could acquire resistance and pass such to transient pathogens, such as *Salmonella*. That would appear to present a greater immediate risk than the reverse transfer.

In all, our work provides additional knowledge regarding the complex nature of antibiotic resistance gene acquisition, reservoirs and transfer, thus hopefully providing additional information from which to determine courses of action and strategies for control of this potential foodborne and/or zoonotic hazard.

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VITA

Sumalee Liamthong was born in Nakhon Si Thammarat, Thailand. She attended primary school in Chumphon and secondary school in Nakhon Si Thammarat, Thailand. Following graduation, she entered Prince of Songkhla University in Had Yai, Thailand to work towards an undergraduate degree. In 1993, she was awarded a Bachelor of Science degree in biology-chemistry from the faculty of Science. She then initiated her graduate study at that same university. In 1996, she received the Masters of Science degree in microbiology under the direction of Dr. Souwalak Phongpaichit. Sumalee then began a career as a lecturer in the Faculty of Science at Nakhon Si Thammarat Rajabhat University, in Nakhon Si Thammarat, Thailand. In 2003, she was accepted into the PhD program at the University of Tennessee in the Department of Animal Science under the direction of Dr. Alan Mathew. Upon completing the PhD, she will return to Thailand to continue her work at Nakhon Si Thammarat Rajabhat University.