ABSTRACT

Title of Dissertation: CHARACTERIZATION AND MOLECULAR MECHANISMS OF ANTIMICROBIAL RESISTANCE IN FOODBORNE PATHOGENS

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The emergence of antimicrobial resistance bacteria in both the medical and agricultural fields has become a serious problem worldwide. The use of antimicrobial agents in animals for prophylaxis and growth promotion favors the selection of antimicrobial resistance in bacteria. These resistant bacteria may subsequently transmit to humans through food chain or human-animal interactions. The objectives of this study were to characterize and determine the molecular mechanisms of antimicrobial resistance in *Salmonella* and *Escherichia coli E. coli* isolates recovered from diseased piglets and chicken in China were characterized for the antimicrobial resistance phenotype and the presence of mutation in their *gyrA, gyrB, parC* and *parE* genes. Results indicated that multiple- antimicrobial-resistant *E. coli*, including fluoroquinolone-resistant variants, are commonly present among diseased swine and chickens in China. DNA sequencing revealed that double *gyrA* mutations coupled with *parC* mutation conferred high-level resistance to fluoroquinolones. In addition, *Salmonella* isolates recovered from retail meats in the United States and China were

characterized for antimicrobial resistance genotypes and the horizontal transfer of resistance determinants. The antimicrobial resistance genotypes of *Salmonella* were consistent to their phenotypes. Genes conferring antimicrobial resistance in *Salmonella* are often carried on integrons and plasmids, and could be transmitted through conjugation. To rapidly screen for the antimicrobial-resistant and virulence genes from bacteria, a DNA microarray was developed to analyze the antimicrobial resistance and virulence genes from *Salmonella* and *E. coli*. Results indicated that microarrray was an effective method to rapidly screen antimicrobial resistance and virulence genes in *Salmonella* and *E coli*. Finally, molecular mechanisms of fluoroquinolone resistance, including over-expression of efflux pumps and target gene mutations, were characterized among laboratory-induced and field-acquired fluoroquinolone-resistant *Salmonella*. The efflux pumps which were overexpressed in resistant strains were deleted and mutated *gyrA* and *parC* genes were replaced to determine the contribution of efflux pump and target gene mutations in fluoroquinolone-resistant *Salmonella*. When *Salmonella* are exposed to fluoroquinolone, certain efflux pumps are overexpressed in tandem with particular mutations in topoisomerase genes (*gyrA* and *parC*). Based on deletion mutagenesis studies, it appears that the most relevant genes with regards to the selection of fluoroquinolone resistance phenotypes among *Salmonella* are the *AcrAB-tolC* efflux pump and the *gyrA* portion of DNA gyrase gene.

CHARACTERIZATION AND MOLECULAR MECHANISMS

OF

ANTIMICROBIAL RESISTANCE IN FOODBORNE PATHOGENS

By

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To my father who left me before my finishing the Ph.D. degree, to my mother and to my selfless wife and my lovely son

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Alanine
AAD	Aminoglycoside adenyltransferase
ACSSuT	Ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline
Amo	Amoxicillin / clavulanic acid
Amp	Ampicillin
ATCC	American Type Culture Collection
BAP	Blood agar plates
BHI	Brain heart infusion
Cef	Ceftiofur
Cep	Cephalothin
Cet	Ceftriaxone
Cml	Chloramphenicol
CNF1,2	Cytotoxic necrotizing factors
D	Aspartic acid
DHFR	Dihydrofolate reductase
DNA ABC	ATP-binding cassette
DT104	Phage definitive type 104
F4,5,6	Fimbrial types
Fox	Cefoxitin
G	Glycine
Gen	Gentamicin
gyrA,B	DNA gyrase units A & B

HACCP	Hazard Analysis and Critical Control Point
HlyE	Hemolysin E
I	Isoleucine
ISS	Increased serum survival factor MDR
Kan	Kanamycin
L	Leucine
LPS	Lipopolysaccharide
LT	Heat-lable toxin
MAR	Multi antimicrobial resistance
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug resistance
MFS	Major facilitator superfamily
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentrations
N	Asparagine
Nal	Nalidixic acid
NARMS	National Antimicrobial Resistance Monitoring System
NCCLS	National Committee for Clinical Laboratory Standards
ORF	Open reading frame
parC, E	Topoisomerase IV unit C & D
PCR	Polymerase chain reaction
QRDR	Quinolone resistance determine region
R	Arginine

RNAi	RNA interference
RND	Resistance nodulation division
S	Serine
SGI	*Salmonella* genomic island
SMR	Small multidrug resistance
STa	Heat-stable toxin a
STb	Heat-stable toxin b
Stx	Shiga toxin
Str	Streptomycin
Sul	Sulfamethoxazol
Tet	Tetracycline
Tri	Trimethoprim / sulfamethoxazole
Tsh	Temperature sensitive hemagglutination
Y	Tyrosine

Chapter 1 Introduction

Origins and consequence of antimicrobial resistance in food borne pathogens

The use of antimicrobials in human and animal since the discovery of penicillin in the late 1920s dramatically reducing the morbidity and mortality associated with numerous infectious diseases. This has also resulted in an unprecedented global increase in the incidence of clinical bacterial strains that are multiply resistant to antibiotics. Bacterial antimicrobial resistance in both the medical and agricultural fields has become a serious problem worldwide (125). Antibiotic resistant strains of bacteria are an increasing threat to animal and human health, with resistance mechanisms having been identified and described for all known antimicrobials currently available for clinical use. The administration of therapeutic and sub-therapeutic antimicrobials to animals caused the emergence and dissemination of multiple-antibiotic-resistant zoonotic bacterial pathogens, which currently has become a increased public and scientific interest (155, 191). Although there is still lack of evidences of the use of antimicrobials in animals on the development and dissemination of antibiotic resistance among human bacterial pathogens, research has linked the use of antibiotics in agriculture to the emergence of antibiotic-resistant foodborne pathogens (155, 191, 230). To date, bacterial pathogens of animal and human origin are becoming increasingly resistant to most frontline antimicrobials, including expanded-spectrum cephalosporins, aminoglycosides, and fluoroquinolones. The increasing incidence of antimicrobial-resistant bacterial pathogens has severe implications for the future treatment and prevention of infectious diseases in animals and humans.

Antimicrobial use in animal husbandry

There are three ways in which substances exhibiting antimicrobial activity are used in animals: for therapy, prophylaxis, and growth promotion (187). Therapeutic use of antimicrobial agents is intended to cure an existing bacterial infection. In food-producing animals, antimicrobials are given for group treatment because individual animal treatment is often impractical. Metaphylaxis is the application of antimicrobials to a group of animals when signs of disease are present in some animals to prevent spread of the infections. Early medication to the entire animal group may reduce the numbers of sick and/or dead animals and also may decrease the amount of antimicrobial agents needed to treat large numbers of the symptomatically ill population, consequently, reducing treatment costs.

In contrast to therapeutic use of antimicrobials, prophylaxis is a solely preventive measure which should be used with discretion. In the pork- and beef-producing industry, the antimicrobials were prophylactic used at the key time, such as weaning, or mixing of animals from different herds. Although animals usually do not exhibit symptoms of a disease at such time, it can be expected that the animals will become infected shortly thereafter based on experience. Antimicrobial prophylaxis at these times is essential in many piggeries and byres because frequently-occurring respiratory and enteric disease in cattle and pigs cannot be effectively controlled without prophylactic use of antimicrobials (46, 99). Although prophylactic herd treatment is criticized for its role on the selection of resistance among pathogenic bacteria (211), antimicrobial prophylaxis at these key periods for disease incidence is an unavoidable measure in the current pork and beef producing systems.

Even more critically assessed is the use of antimicrobial agents for growth promotion in food animals (211). The mode of action of antimicrobial growth promoters is not fully understood. However, the main effects are believed to be a reduction of the growth of bacteria in the intestinal tract and thereby less microbial degradation of useful nutrients, and the prevention of infections with pathogenic bacteria (3). Numerous studies on the economic benefit of the use of antimicrobial growth promoters have been performed. In general, an improvement in growth rate and feed utilization has been observed.

The association between the use of antimicrobials in food animals and the occurrence of antimicrobial-resistant bacteria

More than half of all antimicrobials used are associated with the production of food animals and a substantial portion of this is used for growth promotion and prophylactic purposes (131, 142). Thus, bacterial species related to food animals are exposed to a substantial and in many cases constant, selective pressure. This selective pressure favors the emergence of antimicrobial resistance in zoonotic pathogens such as *Salmonella*, *Campylobacter*, *Yersinia*, *Listeria* and diarrheagenic *E. coli*, which are frequently harbored in the animal intestinal tract. It also favors the selection of antimicrobial resistance genes in non-pathogenic bacteria (2, 3), which later may transfer the acquired resistance to different pathogenic bacterial species. Knowledge regarding associations between use of antimicrobial agents and occurrence of resistance is in many cases based on experience of changes in occurrence of resistance in relation to introduction of new agents.

There are some good examples of the introduction of antimicrobials for animal use and the emergence of resistant bacteria. In the United States, the increase in the occurrence of fluoroquinolone-resistant *Campylobacter* and *E. coli* has been observed after the introduction of fluoroquinolones into veterinary medicine in 1995 and 1996 (http://www.hhs.gov/news/press/1995pres/950818b.html). Because of the emergence of fluoroquinolone resistance in *Campylobacte*, the FDA proposed withdrawing the approval of fluoroquinolones for all poultry 2002. In Germany, an increase in the occurrence of fluoroquinolone resistance among *S*. Typhimurium DT204 occurred after the introduction of enrofloxacin for veterinary use in 1989 (87). Most recently, in the UK substantial increases in resistance to fluoroquinolones in *S*. Hadar and *S*. Virchow and also in multiple-resistant *S*. Typhimurium DT104 have followed the licensing for veterinary use of enrofloxacin in 1993 and danofloxacin in 1996 (204).

Another example is the use of aminoglycosides in Europe. After the introduction of the aminoglycoside antibiotic apramycin for veterinary use at the begining of the 1980s, resistance to apramycin emerged among *E. coli* isolates found in cattle and pigs in France and the UK. Although apramycin has never been used for treatment of infections in humans, the gene *aac*(3)-IV encoding resistance to apramycin also conferred resistance to gentamicin, which was widely used in humans (123). The same resistance gene and plasmid have since been found in *Salmonella* from animals and in human clinical *E. coli* isolates (123). These observations strongly suggest that this resistance gene primarily emerged among food animals because of the selection by use of apramycin in food animals, and was then transmitted to humans.

We characterized the phenotypes and genotypes of *Salmonella* isolated from retail meats in the US and China (33). Nineteen percent of the *Salmonella* isolates from the US were resistant or intermediately susceptible to ceftriaxone and harbored the bla_{CMY-2} gene. Conversely, each of the *Salmonella* isolates from China was susceptible to ceftriaxone (and other cephalosporins), and none harbored bla_{CMY-2}. A possible explanation for this observation is that ceftriaxone-resistant *Salmonella* in meats have arisen due to cross-resistance between ceftriaxone and ceftiofur, a cephalosporin used in food animals (205, 234). Ceftiofur, the only cephalosporin approved for therapeutic use in cattle, has been approved for use in the US since 1988 and for use in China in 2002 (www.agri.gov.cn/blgg/t20021219_36976.htm). Quinolones and fluoroquinolones have been used in veterinary medicine in China since the 1980s. In contrast, they were not approved for therapeutic use in animals in the US until 1995. The differences in fluoroquinolone susceptibility between the US and China isolates likely reflect the different approval dates in the two countries. Thirty-two of the *Salmonella* isolates from China were resistant to nalidixic acid and displayed increased minimum inhibitory concentration (MIC) for ciprofloxacin, while all of the isolates from the US were susceptible to these drugs.

Potential mechanisms of increased disease in humans from antimicrobial resistance in food animals

Simply showing that a growing proportion of pathogens and commensal organisms isolated from food animals are resistant to antimicrobial agents is not enough to prove a human health hazard. Rather, it must be demonstrated that as a result of such antimicrobial resistance infections are more numerous or are more severe or are less

easily treated than would be the case otherwise. There are some potential mechanisms of increased disease in humans from antimicrobial resistance in food animals, which are discussed in the following paragraphs (14).

First, exposure of food animals to antimicrobial agents can not only select antimicrobial-resistant pathogens, but also might lead to increased colonization of the animals by antimicrobial-resistant pathogens. This is because treatment with antimicrobial agents often results in the reduction of various components of the commensal flora, which normally exerts a protective effect against colonization and infection of exogenous organisms (207). This increase in the number of pathogens in food animals could lead to an increase in the burden of pathogens in the environment and in the food chain up to human consumers. Furthermore, as in humans, most of these pathogens would presumably be resistant to antimicrobial agents.

Second, antimicrobial resistance arising in food animals could involve not just obvious pathogens but relatively nonpathogenic bacteria such as generic *E. coli* or entercocci. These organisms could become reservoirs of mobile antimicrobial resistance elements that could colonize humans via food or environmental exposure. These resistance elements could then be transferred to pathogens in the gut (212).

Third, infection by antimicrobial-resistant microorganisms might have a worse outcome due to ineffective initial treatment, the need to use less desirable treatment options, or both. The initial empiric treatment choice might be an antimicrobial agent to which the pathogen is resistant, leading to a delay in effective therapy (154). Effective agents might be more toxic, more expensive, or more difficult to administer than traditional choices.

Last, there might be genetic linkage of resistance traits and virulence factors, resulting in increased virulence of resistant strains (48, 60, 194, 219). An increase in virulence would arise not directly from an antimicrobial resistance mechanism itself but from linkage of resistance genes to other virulence genes which are maintained by drug use. Co-transfer of resistant traits and virulence genes could make drug-resistant pathogenic strains intrinsically more virulent than drug-susceptible strains. In addition, it is possible that bacterial adaptation to high concentrations of antimicrobials can somehow enable them to better survive in the host system. For instance, antimicrobial-resistant bacteria may have increased resistance to antibacterial peptides or bile salts secreted by the host system.

Antimicrobial resistance in Salmonella and Commensal bacteria

The antimicrobial resistance in bacteria is increasing in recent years. At least 17 classes of antimicrobial agents are approved for growth promotion and feed efficiency in the US, including: tetracyclines, penicillins, macrolides, lincomycin (an analog of clindamycin), and virginiamycin (an analog of quinupristin/dalfopristin). The use of antimicrobial agents in food animals creates a selective pressure for the emergence and dissemination of antimicrobial-resistant bacteria including animal pathogens, humans pathogens which have food animal reservoirs, and other bacteria which are present in food animals (43, 110, 211). The National Antimicrobial Resistance Monitoring System (NARMS) was launched in 1996 to monitor antimicrobial resistance in foodborne enteric pathogens. 50 state and 4 local public health laboratories currently participate in NARMS

and the antimicrobial resistance of human enteric bacteria, including *Campylobacter*, *Salmonella*, *Escherichia coli* O157, and *Shigella* were monitored.

Antimicrobial resistance in *Salmonella*

Salmonella was the first organism that was monitored for antimicrobial resistance by NARMS. From the past five NARMS annual reports, the highest prevalence of resistance in *Salmonella* were tetracycline, sulfamethoxazole, streptomycin, and ampicillin, ranging from 15~25%. The emergence of multidrug-resistant *S.* Typhimurium DT104 in the US and UK, which is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT), constitutes a considerable portion of the *Salmonella* isolates analyzed in NARMS. The prevalence of *S.* Typhimurium isolates with the five-drug pattern of resistance increased from 0.6% in 1979-1980 to 34% in 1996 (163). This strain remains common; among *S.* Typhimurium isolates submitted to NARMS, the prevalence of the ACSSuT resistance pattern was 29% in 2001 (164).

In recent years, the portion of *Salmonella* which is resistant to clinically important drugs such as fluoroquinolones (e.g., ciprofloxacin) and third-generation cephalosporins (e.g., ceftriaxone) is increasing. Antimicrobial agents are commonly used empirically for treatment of patients with *Salmonella* infections and may be life-saving for persons with invasive infections. Fluoroquinolones are the most commonly used antimicrobial agent for the treatment of invasive *Salmonella* infections in adults (9). Although few non-Typhi *Salmonella* isolates in NARMS from 1996-2001 were resistant to fluoroquinolones (MIC > 4 µg/ml), 1% of isolates in 2001 had a decreased susceptibility to fluoroquinolones

(MIC >0.25 µg/ml), an increase from 0.4% in 1996(163) . Patients infected with *Salmonella* strains with a decreased susceptibility to fluoroquinolones may respond poorly to treatment with fluoroquinolones and have been associated with apparent treatment failures (47, 133). Because of this, it has been proposed that the resistance breakpoint for ciprofloxacin be lowered.

Third-generation cephalosporins, such as ceftriaxone, are commonly used for treatment of invasive *Salmonella* infections in children because of their pharmacodynamic properties and low prevalence of resistance to these agents. There is therefore concern about the potential emergence of ceftriaxone-resistant *Salmonella*. The first reported case of domestically acquired ceftriaxone-resistant *Salmonella* was in a 12-year-old child in Nebraska (59). Investigation by public health officials revealed that the *Salmonella* strain most likely came from cattle herds. The use of ceftiofur or other antibiotics likely selected for and maintained the ceftriaxone-resistant determinant within the intestinal flora of the involved herds, after which the ceftriaxone-resistant strains was transmitted to the child through his father, a veterinarian.

The Nebraska child's ceftriaxone-resistant infection was not an isolated event. The percentage of non-Typhi *Salmonella* isolates in NARMS resistant to ceftriaxone increased 20 fold from 0.1% in 1996 to 2% in 2001(164). When patients from whom isolates were received in 1996-1998 were interviewed, few reported international travel, suggesting that most infections were domestically acquired (54). Furthermore, ceftriaxone resistance in most domestically acquired infections, including the infection in the child in Nebraska, is due to a unique AmpC-type resistance gene (bla_{CMY-2}), which resides on a plasmid (54, 59). A 1999 study at the University of Iowa found multidrug-

resistant, cephalosporin-resistant bovine, porcine and humans *Salmonella* isolates from the same geographic region. All humans and animal resistant isolates encoded a bla_{CMY-2} AmpC-like gene (233). Interestingly, a study conducted in our lab indicated that ceftriaxone-resistant *Salmonella* carrying a bla_{CMY-2} coded on a conjugative plasmid was prevalent in ground meats in the greater Washington DC area (33, 230). The finding of a similar molecular mechanism of resistance among different *Salmonella* strains supports the hypothesis of dissemination of a resistance determinant and the transmission of antimicrobial-resistant *Salmonella* from animals to humans through the food supply (54).

Another multidrug-resistant *Salmonella* that has emerged recently in the United States is MDR-AmpC *Salmonella* Newport. These multidrug-resistant strains are resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazle, and tetracycline (ACSSuT), and have apparently acquired the bla_{CMY-2}AmpC-like gene conferring additional resistance to cephalothin, amoxicillin-clavulanic acid, cefotoxin, and ceftiofur (243). Of *S.* Newport isolates submitted to NARMS in 2001, a remarkable 25% were MDR-AmpC S. Newport (164). The MDR-AmpC *S.* Newport has been detected in very high frequency in meat products and food animals. Recently, we also isolated MDR-AmpC *S.* Newport from organic chicken samples.

Antimicrobial resistance in commensal bacteria

Pathogenic bacteria, such as *Campylobacter* and *Salmonella*, are not the only concern when considering antimicrobial resistance in bacteria with food animal reservoirs. Commensal bacteria, which are naturally occurring host flora, constitute an enormous potential reservoir of resistance genes for pathogenic bacteria. The prevalence

of antibiotic resistance in the commensal bacteria of humans and animals is considered to be a good indicator of the selective pressure of antibiotic usage and reflects the potential for resistance in future infections (92, 108). Most resistant bacteria have mobile genetic elements such R-plasmids and transposons. As the reservoir of resistant commensal bacteria increases, the plasmid reservoir becomes larger and enables more frequent transfer of resistance to pathogenic bacteria including *Salmonella* and *Shigella*. *E coli*, which is the predominant isolate of aerobic fecal flora in humans and most animals, has demonstrated its ability to transfer resistance genes to other species, including pathogenic bacteria (92, 146, 190, 202, 235).

Quinupristin/dalfopristin was approved for use in humans in 1999 for treatment of vancomycin-resistant *Enterococcus faecium* infections. Virginiamycin, an analog of quinupristin/dalfopristin, has been used as a growth promoter in food animals in the United States since 1974. A study conducted by the CDC in 1998-1999, before the approval of Quinupristin/dalfopristiṇ use in humans, found quinupristin/dalfopristin-resistant *E. faecium* on 58% of chickens purchased in grocery stores from four different states. Additionally, quinupristin/dalfopristin-resistant *E. faecium* was found in 1% of the stools from non-hospitalized people who submitted a stool specimen to clinical laboratories (126). Similar data in Europe led the European Union to ban the subtherapeutic use of virginiamycin in food animals in 1998 (225). These findings suggest virginiamycin use in food animals has created a large reservoir of quinupristin/dalfopristin-resistant *E. faecium*. The high carriage of quinupristin/dalfopristin-resistant E. faecium on chickens in grocery stores, and the frequent handling of chicken from grocery stores by consumers, suggests that humans are

commonly exposed to these resistant bacteria. The use of quinupristin/dalfopristin in humans for the treatment of vancomycin-resistant E. faecium and other serious infections may contribute additional selective pressure leading to an increased prevalence of quinupristin/dalfopristin resistance in humans.

Recent studies have also shown an emerging resistance in *E. coli* to fluoroquinolones and third-generation cephalosporins. A study by Garau et al., demonstrated an increase in quinolone resistance among *E. coli* isolates in Spain from 9% to 17% over the course of five years. This study also showed a high prevalence of quinolone-resistant *E. coli* in healthy children and adults (26% and 24% respectively) which could not be explained by previous use of quinolones. Animal testing from slaughterhouses in the area found a high rate of quinolone-resistant *E. coli* in swine and chickens (45% and 90%, respectively) (64). Winokur et al., found 16% of clinical *E. coli* isolates from cattle and swine and 1% of clinical human *E. coli* isolates collected in Iowa to be resistant to extended spectrum cephalosporins. This study also identified identical bla_{CMY-2} genes in resistant isolates from both humans and animals suggesting transfer of the resistance gene between food animals and humans (235). The frequency of fluoroquinolone resistance in animal *E. coli* isolates from developing countries is much higher than the clinical human isolates. Around 90% of *E. coli* isolates from chicken and 50% of *E. coli* from swine have been reported to be resistant to fluoroquinolones in Spain and China (178, 221).

Mechanisms of antimicrobial action and resistance

An ideal antimicrobial drug exhibits selective toxicity. This term implies that the drug is harmful to a parasite without being harmful to the host. The most successful antimicrobial agents are those whose targets are anatomic structures or biosynthetic functions unique to microorganisms. Resistance to antimicrobial agents in bacteria is mediated by several mechanisms, including 1) changes of bacterial cell wall permeability, 2) energy-dependent removal of antimicrobial agents via membrane bound efflux pumps, 3) modification of the site of drug action, and 4) destruction or inactivation of antimicrobial agents (13, 186). The antimicrobial-resistant phenotypes is gained from extra-chromosomal genes that may confer resistance to an entire antimicrobial class. Most of these resistance genes are associated with transferable plasmids, on which may be other DNA mobile elements, such as transposons and integrons (94, 182, 183). These DNA mobile elements played an important role on dissemination of resistance genes among different bacteria. The mechanisms of action and resistance of six major categories of antimicrobial agents are described below.

β-Lactams

β-Lactams represent groups of antibiotics that include penicillins, cephalosporins, monobactams and carbapenems. These groups of drugs inhibit a number of bacterial enzymes, namely, penicillin-binding proteins (PBPs), that are essential for peptidoglycan synthesis, thereby interfering with synthesis of petidoglycan of the cell wall (223). Of the various mechanisms of acquired resistance to β-lactam antibiotics, resistance due to production of β-lactamase by the cell is the most prevalent (25, 114). β-Lactamase

included a family of enzymes with tremendous diversity. The *bla*$_{TEM-1}$ and *bla*$_{SHV-1}$, which efficiently hydrolyze penicillins and narrow-spectrum cephalosporins but poorly hydrolyzes extended-spectrum cephalosporins, are the most prevalent and most common in Gram negative bacteria (193). Recently, a plasmid-mediated AmpC β-lactamase *bla*$_{CMY-2}$, which causes resistance to extended spectrum β-lactams (ESBLs) including ceftriaxone, has emerged in *S.* Typhimurium (54, 65, 102). The plasmid-mediated *bla*$_{CMY-2}$ was also detected in the recently emergent MDR-AmpC *S.* Newport (77). In addition, alterations in the PBPs, acquisition of a novel PBP insensitive to β-lactams, changes in the outer membrane proteins of Gram-negative organisms, and active efflux which prevent these compounds from reaching their targets can also confer resistance.

Aminoglycosides

Aminoglycosides are a class of drugs which inhibit bacterial protein synthesis by binding irreversibly to the bacterial 30S ribosome subunits. The aminoglycoside-bound bacterial ribosome is unavailable for translation of mRNA during protein synthesis thereby causing bactericidal effects (107). The most common mechanism of aminoglycoside resistance is antibiotic inactivation by plasmid-and transposon- encoded modifying enzymes (189). There are three classes of aminoglycoside-modifying enzymes: acetyltransferases (AAC), adenylytransferases (ANT), and phosphotransferases (APH). AAC enzymes acetylate amino groups, whereas ANT and APH enzymes adenlylate and phosphorylate hydroxyl groups, respectively. An ANT enzyme, AadA, which is encoded in integrons is commonly detected in streptomycin-resistant Gram negative bacteria. In addition, the resistance also is caused by the decreased antibiotic

uptake by outer membrane proteins and the mutation of ribosomal protein S12, which is the target of aminoglycoside antibiotics (96).

Phenicols

Phenicols include chloramphenicol and florfenicol. They inhibit protein synthesis by binding reversibly to the peptidyltransferase component of the 50S ribosomal subunit thus preventing the transpeptidation process of peptide chain elongation (45). Only chloramphenicol is available for clinical use in the United States. Chloramphenicol resistance in bacteria most commonly results from acquisition of plasmids encoding chloramphenicol acetyltransferases (CAT), which enzymatically inactivate the drug. In Gram negative bacteria, three types of CAT enzymes (types I, II, III) have been identified (151). In addition to enzyme resistance is also due to the decreased outer membrane permeability and acquisition of extra chromosomal efflux pumps (20). cmlA is a major facility family transporter based on the amino acid sequence analysis. cmlA can also result in reduced expression of two outer membrane proteins (OmpA and OmpC) and decreased chloramphenicol uptake. Recently, a new gene, *flo*, which shares 65% homology to *cmlA*, was found to confer resistance to both chloramphenicol and florfenicol (101, 228).

Sulfonamides and Trimethoprim

Sulfonamides were the first effective systemic antimicrobial agents used in the United States during the 1930s. They work by competitively inhibiting bacterial modification of para-aminobenzoic acid into dihydrofolate. Trimethoprim is a pyrimidine

analog that inhibits the enzyme dihydrofolate reductase(DHFA), thus interfering with folic acid metabolism. This sequential inhibition of folate metabolism ultimately prevents the synthesis of bacterial DNA (176). The widespread high-level resistance to trimethoprim is likely due to the acquisition of exogenous DNA that specifies a supplementary DHFR which is less sensitive than the chromosomal enzyme to inhibition by trimethoprim (8). To date, at least 16 different DHFRs have been characterized in Gram-negative bacteria. Type I DHFR is most commonly detected in Gram negative bacterial and normally carried by integrons (198). Resistance to sulfonamide is commonly due to acquisition of plasmids that encode a drug-resistant dihydropteroate synthase (DHPS). Two types (type I and II) of resistant DHPS, encoded by the *sulI* and *sulII* genes, respectively, have been identified in Gram-negative organisms (167, 197). The *sulI* gene is often linked to other resistance genes and is located in conserved segments of integrons in Tn21-like elements carried by large conjugative plasmids. The *sulII* gene is frequently linked genetically to a streptomycin resistance gene on broad-host-range plasmids and on small nonconjugative plasmids.

Tetracycline

The tetracyclines act by inhibiting protein synthesis. They enter bacteria by an energy-dependent process and bind reversibly to the 30S ribosomal subunits of the bacteria. This process blocks the access of aminoacyl-tRNA to the RNA-ribosome complex, preventing bacterial polypeptide synthesis (37). Tetracycline resistance is the most common antibiotic resistance encountered in nature. Although it can result from chromosomal mutations affecting outer membrane permeability (44), more commonly it

results from acquisition of exogenous DNA encoding proteins involved in active efflux of tetracycline or in protection of the ribosome (246) (115).

In Gram-negative bacteria, six classes of *tet* efflux pumps including TetA, TetB, TteC, TetD, TetE, TetG, are of clinical importance. These efflux pumps use an antiport mechanism of transport involving the exchange of a proton for tetracycline-cation complex (115). Another mechanism of tetracycline resistance is ribosome protection. Modification of tRNA has been suggested to interfere with ribosomal protection, but its precise role remains to be determined. At least five classes of ribosomal proteins, which can interact with ribosomes such that the protein is unaffected by the presence of antibiotics, have been characterized. tetM is widely disseminated and is found in many Gram-positive and Gram-negative bacteria. Like some other antimicrobial agents, tetracycline resistance is also mediated by decreased membrane permeation and active efflux pumps (246).

Quinolones/ Fluoroquinolones

Quinolones belong to a group of potent antibiotics biochemically related to nalidixic acid, which were developed initially as a urinary antiseptic. Newer quinolones, also known as fluoroquinolones, have been synthesized by adding a fluorine atom attached to the nucleus at position 6. The primary target of quinolones is DNA gyrase, an enzyme essential for DNA replication (82, 89, 237). Their therapeutic index stems from the fact that the clinically useful fluoroquinolones inhibit bacterial DNA gyrase at concentrations far below those required to inhibit mammalian topoisomerases. The mechanisms of fluoroquinolone resistance include target gene mutation and removal

fluoroquinolones by efflux pumps. These mechanisms will be discussed in detail in the following paragraphs(32, 41, 158, 213).

Mechanisms of fluoroquinolone resistance in *E. coli* and *Salmonella*

Bacterial resistance to fluoroquinolones is usually mediated by removing drug by active efflux pumps and development of mutations in fluoroquinolone action targets (bacterial gyrase and topoisomerase IV). It was suggested that enhancing expression of efflux pumps occurs as a first step, allowing the bacteria to survive so that mutations can accumulate in genes encoding target proteins to cause higher level resistance (157). Little is know about the fluoroquinolone resistance in *Salmonella* so far because the reports of fluoroquinolone-resistant cases are rare. However, the resistance to fluoroquinolones in *Salmonella* is increasing (29, 34, 72, 119, 136, 156, 164). This represents substantial risks to human health. Studies are needed to determine the molecular mechanisms of fluoroquinolone resistance in *Salmonella* because understanding the mechanisms of antibiotic resistance should enable us in the future to develop intervention strategies to reduce or arrest the progression of antibiotic resistance and to identify new drug target.

Quinolones and fluoroquinolones

Fluoroquinolones are synthetic antibacterial agents which are basically composed of an 4-oxo-1,8-naphthyridin-3-carboxylic acid nucleus. For two decades, they have become from a relatively small group of drugs used predominantly for urinary track infections (UTIs) to a class that had a worldwide sale of $3.04 billion in 1997. A number of characteristics have led to the wide use of fluoroquinolones including rapid

bactericidal effects against most susceptible organisms, extremely good penetration into tissues and mammalian cells, and broad-spectrum activity to most of Gram-negative and Gram-positive bacteria (10).

The first marketed quinolone, nalidixic acid (1st generation quinolone, patented in 1962), has only modest antimicrobial activity against Gram-negative bacteria and lower oral absorption. High urinary concentration makes it suitable for UTIs. Soon after its introduction into widespread clinical use, it was found that a number of organisms could rapidly develop resistance to nalidixic acid. The real breakthrough in fluoroquinolones came with discovery of 2nd generation quinolon norfloxacin to which a 6-fluorinated compound with a piperazing ring at position 7 were added to quinolones. Although norfloxacin has activity against a broad spectrum of Gram-negative and has Gram-positive activity, the combination of its pharmacokinetic profile and activity are still not adequate for systemic use. One of the most successful and widely used compounds of the class, ciprofloxacin (2nd generation quinolone, patented in 1981) was marketed in 1986 and since then the value of the fluoroquinolones for the treatment of a wide range of infections has become widely recognized. The advantages of this compound are its broad-spectrum activity including Gram-positive as well as Gram-negative pathogens and its good absorption in the gastrointestinal track, providing adequate blood levels to allow its use in systemic infections. However, resistance to ciprofloxacin in bacteria is increasing, especially in Gram-positive bacteria like *Staphylococcu*s. Recently developed fluoroquinolones including levofloxacin and gemifloxacin (3rd and 4th generation quinolones, respectively), have improved activity against Gram-positive, anaerobes and same activity against Gram-negative as ciprofloxacin. These compounds do not select for

resistance at such a high rate as ciprofloxacin and are active against ciprofloxacin-resistant bacteria (10). Several compounds in these classes have been approved for clinical use by FDA in 2002. Growth in the market for fluoroquinolones is likely to continue. However, prudent use of these compounds is necessary to prevent the rapid spread of resistance in bacteria.

Interaction of fluoroquinolones with bacterial gyrase and topoisomerase IV

Fluoroquinolones are potent antibacterial agents that target two related enzymes, DNA gyrase and DNA topoisomerase IV (109). Gyrase, which is composed of two subunits, GyrA and GyrB, is responsible for introducing negative supercoils into DNA and for relieving torsional stress expected to accumulate ahead of transcription and replication complexes. Topoisomerase IV, which is composed of two subunits, ParC and ParE, provides a potent decatenating (unlinking) activity to separate two sets of replicated DNAs. Fluoroquinolones do not simply eliminate enzyme function; they actively kill cells by trapping these two enzymes on DNA as a drug/enzyme/DNA complex in which double-stranded breaks are held together by protein (97, 98). Briefly, when fluoroquinolones bind to an enzyme/DNA complex, the conformation of the protein is changed and the ATPase activity is altered, which results in a distortion in DNA. The distortion causes the break of double-strand DNA. When the DNA is released from cleaved complex, the break of the chromosome leads to cell death. In gram negative bacteria, the gyrase is the primary target of quinolone drugs and mutations in gyrase always happen before those in toposiomerase. The topoisomerase is the secondary target and the mutations in topoisomerase IV always happen after the gyrase mutations.

Fluoroquinolone-resistant mutations of GyrA reduce quinolone binding to the enzyme-DNA complex. The same might be true for mutation in ParC (53).

Quinolone resistance due to altered gyrase and topoisomerase IV

The gene that confers nalidixic acid resistance in early studies became known as *gyrA*. When coexpressed with a wild type (sensitive) *gyrA* allele, resistance is recessive by the dominant presence of wild type allele (79, 192). Similar experiments have been done in *E. coli* to demonstrate the role of *parC* mutations in clinical isolates (84, 105). Sequence analysis of DNA from several bacterial species shows that resistance mutations tend to alter amino acids near the putative active site in the GyrA protein. This region, extending between amino acids 67 and 106, is called the quinolone resistance determining region (QRDR). A similar region is believed to exist in the ParC protein. Within GyrA of *E. coli*, mutations of two codons, serine 83 and aspartic acid 87, are most commonly detected. A single mutation in either site confers resistance to nalidixic acid (226, 242). When both sites are mutated, the level of resistance to nalidixic acid can be three or fourfold higher than that of a single mutations and the cell become resistant to ciprofloxacin (12, 201, 208, 216). Mutations in ParC are only detected in GyrA mutants and always with high fluoroquinolone resistance. Topoisomerase IV mutations do not by themselves confer resistance. It is suggested that GyrA is the primary target in *E. coli* and firstly mutated under fluoroquinolone selective pressure, whereas ParC is as the secondary target and secondly mutated under higher fluoroquinolone selection pressure. Mutations in GyrB could be found in both high and low quinolone resistance isolates. Clinical GyrB mutations are analyzed in two specific sites, Asp426 and Lys 447, in *E.*

coli (240). Recent crystal structure of yeast topoisomerase IV demonstrated a quinolone-binding pocket around the active site for DNA cleavage. GyrB belongs to part of the quinolone-binding pocket. The quinolone resistance mutations in GyrB exist at the distant sites of the gene (83). The homologous gene for topoisomerase IV, ParE, can also display resistance mutations.

In *Salmonella*, single mutations in GyrA, Ser83 or Asp87, have been identified in quinolone-resistant isolates recovered from humans and animals (78, 159, 172). Mutations in both Ser83 and Asp87 in GyrA are detected in ciprofloxacin-resistant *Salmonella* isolates from humans and animals (34, 85, 156). Mutations in GyrB have also been shown, with a substitution of Ser464Tyr revealed in one clinical isolate. A mutation in ParC (Ser80Arg) was only reported recently. The role of ParC mutation in fluoruoquinolone-resistant *Salmonella* was not clear because 1) the ParC mutation was not consistently associated with high fluoroquinolone-resistant *Salmonella* and was only reported recently, and 2) there is no genetic evidence to link the ParC mutation to fluoroquinolone resistance in *Salmonella*. In addition, a novel ParC mutation (Tyr57Ser) was reported in fluoroquinolone-susceptible (ciprofloxacin MIC < 0.06µg/ml) *Salmonella* isolated from Hong Kong (112). A ParE mutation (Ser458Pro) was reported recently in ciprofloxacin-resistant (ciprofloxacin MIC ranges from 16 to 64 µg/ml) *Salmonella* isolates with additional double GyrA mutations and one ParC mutation (112).

Fluoroquinolone resistance and efflux pumps

The Gram-negative cell wall consists of an outer membrane, periplasm, and a cytoplasmic membrane. The outer membrane contains pore-forming proteins (porins) such as OmpF and OmpC that allow solutes, including antibiotics, into the cell(156).

Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including antibiotics) from within cells into the external environment. In the prokaryotic kingdom there are five major superfamilies of active drug efflux pump transporters: ATP-binding cassette (ABC), major facilitator superfamily (MFS), small multidrug resistance (SMR), multi antimicrobial resistance (MAR), resistance nodulation division (RND). Genome sequence analyses of prokaryotic microorganisms with available complete sequence revealed that about 5~10% of all bacterial genes are involved in transport, and that a large proportion of these genes encode efflux pumps. Antibiotic efflux pumps appear a major component of microbial resistance to many classes of antimicrobial agents (210). In many cases, efflux pump genes are within an operon, with a regulatory gene controlling their expression. Increased expression of efflux pump genes is associated with the resistance to substrates including antibiotics. Overexpression of efflux pump genes can result from mutations within local repressor genes or may result from activation of a regulon regulated by a global transcriptional regulator such as MarA or SoxS. For at least four of antibiotics, namely tetracycline, macrolide, chloramphenicol, and fluoroquinolone, antibiotic efflux pumps appear to confer medium or high level of antibiotic resistance, defeating medically applicable treatments of the corresponding infections with these antimicrobial agents (16, 111, 147, 161, 166).

Efflux pumps that contribute to antibiotic resistance have been described from a number of clinically important bacteria other than *Salmonella*, including *Campylobacter* jejuni (CmeABC), *E. coli* (AcrAB-TolC, AcrEF-TolC, EmrB, EmrD), *Pseudomonas aeruginosa* (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM), *Strepotococcus pneumoniae* (PmrA), *Staphylococcus aureus* (NorA) (156). An antibiotic

efflux pump has been described in *Salmonella* is AcrAB-TolC system where AcrB is an efflux protein in the cytoplasmic membrane and AcrA is an accessory protein, linking AcrB with the outer membrane protein TolC (68, 160) (Fig 2). Overexpression of AcrAB-tolC is mediated by the overexpression of transcriptional activators MarA and SoxS, which are regulated by MarR and SoxR proteins (6, 169) and by repressor (AcrR) mutations (116). The overexpression of MarA and SoxS down regulates OmpF, an outer membrane pore forming protein. The net result is that the expression of OmpF is reduced and less drugs is able to enter the cell, that the expression of AcrAB is increased, enhancing efflux from cell. The acrAB-tolC efflux pump plays an important role in fluoruoquinolone resistance in *Salmonella* (16, 30). Other efflux pumps have not yet been studied in *Salmonella*. However, Piddock, L. J et al (160) reported that clinical *Salmonella* isolates that accumulated less ciprofloxacin than pretherapy isolates, did not show any increased expression of AcrAB, MarA, SoxS, suggesting another efflux pumps may contribute to the removal of ciprofloxacin in these *Salmonella* isolates.

Clinical consequence of fluoroquinolone-resistant *Salmonella*

Salmonellosis is an important public health problem in the United States(128) . Most human *Salmonella* infections result in a self-limiting gactrointestinal illness characterized by diarrhea, fever, and abdominal cramps. However *Salmonella* infections that spread to the bloodstream, meningeal linings of the brains, or other deep tissue sites, can result in a severe illness leading to disability or death. Each year, an estimated 1.4 million cases of humans *Salmonella* infections occur in the United States causing 80,000-160,000 persons to seek medical attention, resulting in 16,000 hospitalization, and nearly 600 deaths(128).

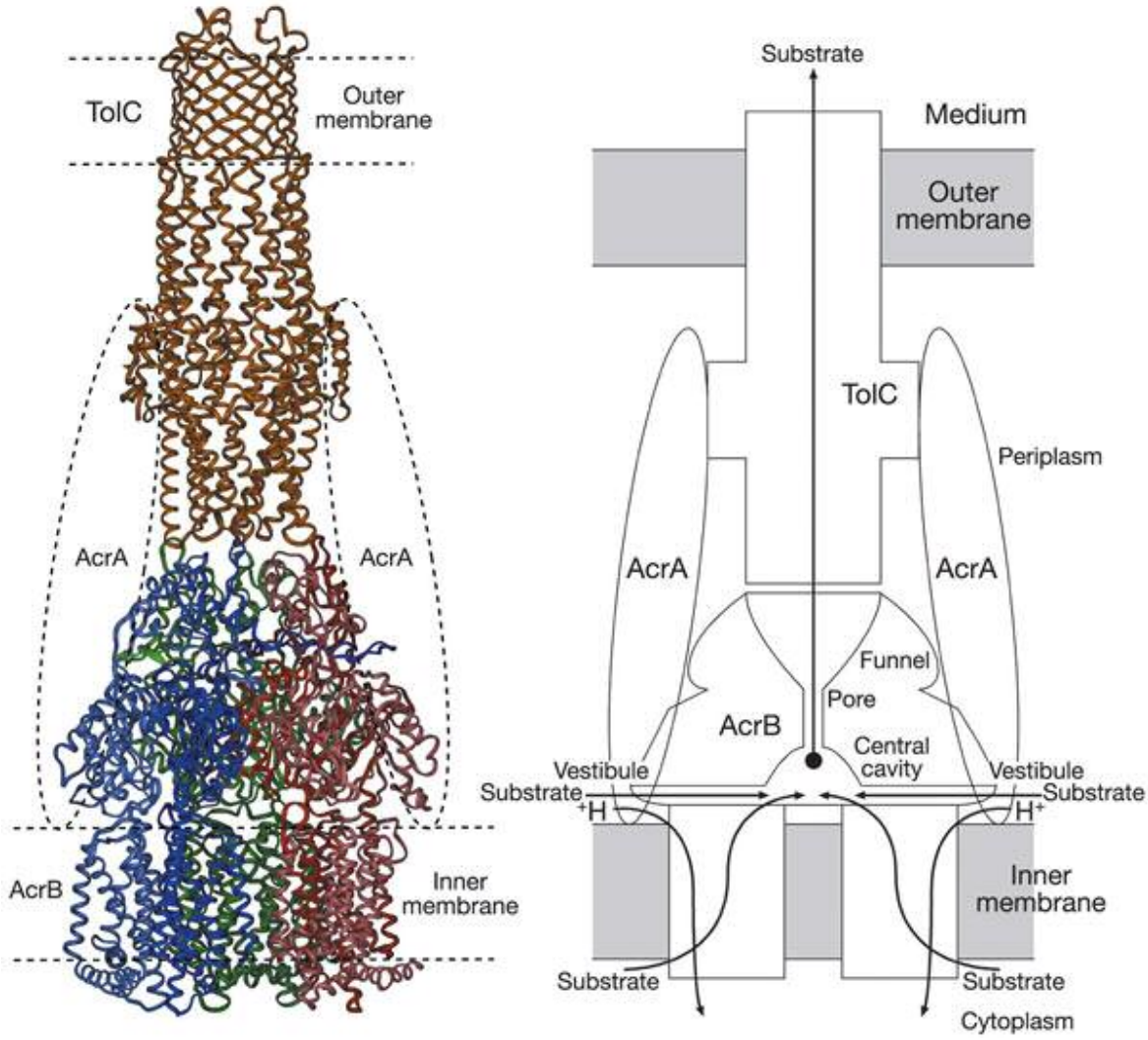

Figure 1-1. Proposed model of the AcrB–AcrA–TolC complex and the schematic mechanism of multidrug export mediated by AcrAB-TolC system. TolC structure[15] is manually docked to AcrB. Dotted ovals indicate AcrA molecules. This figure was produced using Insight II/Discover (Biosym/MSI) (134).

Antimicrobial agents are not essential for the treatment of most *Salmonella* infections, but are commonly prescribed for person who seek medical attention (232). In surveys conducted by Centers for Disease Control (CDC) in 1990 (81) and 1995, 40% of persons with *Salmonella* infections who sought medical attention were treated with antimicrobial agents, suggesting that 32,000-64,000 persons with *Salmonella* infections are treated with antimicrobial agents. Ciprofloxacin was the most commonly prescribed antimicrobial agent for *Salmonella* infections. Ciprofloxacin,was used by approximately 25% of those who received antimicrobial agents in the 1990 survey and 33% in 1995 survey, suggesting >100,000 person with *Salmonella* infections have been treated with ciprofloxacin in the past 10 years in the United States (31).

In contrast to patients with uncomplicated gastroenteritis, effective antimicrobial agents are essential for the treatment of patients with bacteremia, meningitis, or other extraintestinal *Salmonella* infections (232). In approximately 6% of the confirmed cases reported to CDC, *Salmonella*e are isolated from specimens collected from extraintestinal sites, usually from blood (81, 163). Since approximately 40,000 culture-confirmed cases are reported to CDC each year, effective antimicrobial agents are critical and may be life saving for at least 2400 persons a year. The selection of antimicrobial agents for the treatment of invasive infections has become increasly restricted due to increasing antimicrobial resistance in *Salmonella* isolates. In the past, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole were the “treatment of choice” for *Salmonella* infections (36, 104, 209). Among randomly tested *Salmonella* isolates from humans tested at CDC in the National Antimicrobial Resistance Monitoring System in 2001, 17% isolates were resistant to ampicillin, 18% were resistant to trimethoprim-sulfmethoxazole,

and 12% resistant chloramphenicol (165). In contract, only 1% of *Salmonella* isolates are resistant to ceftriaxone or ciprofloxacin. For this reason, and because of clinical response and favorable pharmacodynamic properties, fluoroquinolone and third generation cephalosporin are the current drugs-of –choice for the treatment of invasive *Salmonella* infections in adults and children respectively. Should *Salmonella* develop antimicrobial resistance to these two antimicrobial agents, suitable alternative antimicrobial agents are not currently available and serious adverse human health consequences are expected.

Brief overview of this dissertation

The work that will be described in this dissertation focuses on characterization of antimicrobial resistance in *Salmonella* and *E. coli* isolated from food and animals and the molecular mechanisms of antimicrobial resistance, particular the molecular mechanisms of fluoroquinolone resistance. Chapter 1 is an "overview of antimicrobial resistance, mechanisms of antimicrobial resistance, and the public health concern about the antimicrobial resistance in foodborne pathogens". In Chapter 2 "Characterization of multiple-antimicrobial-resistant *Escherichia coli* isolated from diseased chickens and swine in China", *E. coli* isolates recovered from diseased piglets and chickens in China were characterized as to their O-serogroups, antimicrobial susceptibility phenotypes, presence of virulence genes and class 1 integrons. Additionally, due to the high prevalence of fluoroquinolone resistance, the *gyrA*, *gyrB*, *parC* and *parE* genes from 40 isolates were analyzed for the presence of mutations. The work described in Chapter 2 was submitted to Journal of Clinical Microbiology. In the work described in Chapter 3 "Characterization of Multiple-Antimicrobial-Resistant *Salmonella* isolated from meat

products in the United States and China" identified the phenotypes and genotypes, the presence of class I integrons, and the transfer of the antimicrobial resistance determinants via conjugative plasmid in antimicrobial-resistant in *Salmonella* from the United States and China. The work presented in Chapter 3 was published in the Applied Environmental Microbiology (33). Although genotype characterization can provide useful information about the potential virulence of bacteria, it is time consuming and expensive when using the PCR. Microarray is a powerful tool to analyze hundreds and thousands genes at one time. A DNA-based microarray method is described in Chapter 4 "Identification of Antimicrobial-Resistant and Virulence Genes in *Salmonella* and *E. coli* Using Microarray Analysis". In this work, a microarray was constructed by printing 23 antimicrobial-resistant genes and 22 virulent genes from *E. coli* and *Salmonella* onto microchips. The probes were prepared by random amplifying and DIG labeling of genomic and plasmid DNA. Twenty multiple-antimicrobial-resistant bacterial strains including 12 *Salmonella* and eight *E. coli* were assayed for the presence of antimicrobial resistance and virulence genes using this microarray. Resistance to fluoroquinolone becomes a severe impact on human health. However, the mechanisms of fluroquinolone resistance in bacteria are not clearly understood. Therefore, in the work described in Chapter 5 "The Role of Target Mutation and Efflux in Fluoroquinolone-Resistant *Salmonella*" we studied the molecular mechanisms of fluoruoquinolone resistance including target gene mutation and efflux pumps using gene knockout chromosomal gene replacement techniques.

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Chapter 2 Characterization of Multiple-Antimicrobial-Resistant *Escherichia coli* Isolated from Diseased Chickens and Swine in China

Abstract

Escherichia coli isolates from diseased piglets (n=89) and chickens (n=71) in China were characterized for O-serogroups, virulence genes, antimicrobial susceptibility, class 1 integrons, and mechanisms of fluoroquinolone resistance. O78 was the most common serogroup identified (63%) among the chicken *E. coli* isolates. Most isolates were PCR positive for the increased serum survival gene (ISS) (97%) and the temperature-sensitive hemagglutinin gene (TSH) (93%). The O-serogroups of swine *E. coli* were not those typically associated with pathogenic strains, nor did they posses common characteristic virulence factors. Twenty-three serogroups were identified among the swine isolates, however, 38 % were O non-typeable. Overall, isolates displayed resistance to nalidixic acid (100%), tetracycline (98%), sulfamethoxazole (84%), ampicillin (79%), streptomycin (77%), and trimethoprim-sulfamethoxazole (76%). Among the fluoroquinolones, resistance ranged from 64% to levofloxacin, 79% to ciprofloxacin, and 95% to difloxacin. DNA sequencing of *gyrA*, *gyrB*, *parC*, and *parE* quinolone-resistance determining regions of 39 nalidixic acid-resistant *E. coli* revealed that a single *gyrA* mutation was found in all of the isolates; mutations in *parC* together with double *gyrA* mutations conferred high-level resistance (ciprofloxacin MIC $\geq$4 μg/ml) to fluoroquinolones. Class 1 integrons were identified in 17 (19%) of isolates from swine and 42 (47%) from chickens. The majority of integrons possessed genes

conferring resistance to streptomycin and trimethoprim. These findings suggest that multiple-antimicrobial-resistant *E. coli*, including fluoroquinolone-resistant variants, are commonly present among diseased swine and chickens in China and the need for the introduction of surveillance programs in China to monitor antimicrobial resistance in pathogenic bacteria that can be potentially transmitted to humans from food animals.

Introduction

Although normally commensal in nature, certain strains of *Escherichia coli* are associated with a variety of infections in humans and animals. In swine, pathogenic *E. coli* may cause neonatal and postweaning diarrhea and edema (18, 93). In chickens they may cause infections of the respiratory tract and soft tissues resulting in colibacillosis, air saucculitis, and cellulitis (71). Virulence factors of swine *E. coli* include adhesins and several exotoxins (18, 62, 93). For example, fimbrial types F4 (K88), F5 (K99), F6 (987P), F107, and intimin, an outer membrane protein encoded by *eae* gene, play a role in adhesion to mucosal surfaces. Exotoxins produced by *E. coli* include heat-stable (STa and STb) and heat-labile (LT) enterotoxins, Shiga toxins (Stx1 and Stx2), and cytotoxic necrotizing factors (CNF1 and CNF2). In swine, the most commonly reported *E. coli* serogroups associated with neonatal and postweaning diarrhea and edema belong to a limited number of serogroups including O8, O138, O139, O141, O147, and O157 (62, 63, 93, 135). Avian pathogenic *E. coli* most commonly belong to O1, O2, O78, and typically possess virulence factors such as lipopolysaccharide (LPS), temperature sensitive hemagglutination (TSH), and increased serum survival factor (ISS) (106, 129, 168, 215).

However, the distribution and frequencies of the most prevalent serogroups can vary considerably, both geographically and temporally.

Antimicrobials are valuable tools to treat clinical disease and to maintain healthy and productive animals. However, the treatment of whole herds and flocks with antimicrobials for disease prevention and growth promotion has become a controversial practice (211, 236). When antimicrobial agents are used indiscriminately in animals they may select for resistant bacteria that can eventually contaminate the human food supply. Of particular concern is the emergence of resistance to frontline antimicrobials such as the fluoroquinolones, which because of their low toxicity and relatively broad spectrum coverage, are extremely valuable for treating humans infections. In addition to the human health concerns, antimicrobial-resistant pathogens also pose a severe and costly animal health problem, in that they may prolong illness and decrease productivity through higher morbidity and mortality.

Unfortunately, data on the prevalence of antimicrobial resistant veterinary pathogens are sparse, particularly in developing countries such as China. Such data are urgently needed for science-based risk assessments focusing on the relative risks concerning use of antimicrobials in animal husbandry. The study presented here was undertaken to determine the serogroups, virulence factors, antimicrobial susceptibility profiles, presence of class 1 integrons, and molecular mechanisms of fluoroquinolone resistance in *E. coli* isolated from diseased swine and chickens from farms in China. The overall goal was to further our understanding of both pathogenic and antimicrobial-resistance mechanisms present among *E. coli* in food-producing animals from an area which has not instituted or benefited yet from a national surveillance system.

Materials and Methods

Bacterial isolates. A total of 160 *E. coli* isolates were analyzed in the study. These included 89 isolates recovered from fecal samples of 2 to 10 day-old piglets. The samples were recovered from animals experiencing diarrhea, located on three pig farms in Beijing, China during August 2000. Seventy-one *E. coli* isolates were recovered from the livers of dead chickens from 10 poultry farms in Beijing and Heibei Province from January to October 2000. All *E. coli* were isolated and purified on MacConkey agar (Difco Lab, Detroit, MI), and confirmed as *E. coli* with the API20E bacterial identification system (BioMerieux, Inc., Hazelwood, MO).

Serogroup determination and identification of virulence genes. Serogroups and virulence genes were determined at the Gastroenteric Disease Center of The Pennsylvania State University, State College, PA. PCR was used to detect virulence genes, including LT, STa, STb, Stx1, Stx2, CNF1, CNF2, F4 (K88), F5 (K99), F6(987P), F18(F107), and intimin for swine *E. coli*, and K1, ISS, Tsh, and HlyE for avian *E. coli* using previously published primers and protocols (62, 129).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed according to the National Committee for Clinical Laboratory Standards (NCCLS) (139, 141). Antimicrobial minimum inhibitory concentrations (MICs) of the *E. coli* isolates were determined via broth micro-dilution using the PASCO MIC/ID system (Becton Dickinson, MD) according to manufacturer's instructions. Antimicrobials included in the panels are listed in Table 2-1. In addition, MICs to nine quinolone

Table 2-1. Antimicrobial resistance phenotypes of *E. coli* isolates from diseased chickens and piglets

Class/antimicrobial	Resistance breakpoint [a] (µg/ml)	*Chicken isolates*			*Swine isolates*			*Overall*
		MIC_{50}	*MIC_{90}*	*% Resistance*	*MIC_{50}*	*MIC_{90}*	*% Resistance*	*% Resistance*
Cephalosporins								
Ceftiofur	8	≤0.5	1	0	≤0.5	≤0.5	0	0
Ceftriaxone	64	≤0.025	≤0.025	0	≤0.025	≤0.025	0	0
Cephalothin	32	8	>32	27	16	32	20	23
Penicillins								
Amoxicillin/clavulanic acid	32/16	8/4	8/4	0	8/4	16/8	0	0
Ampicillin	32	>32	>32	77	>32	>32	80	79
Sulfonamides & potentiated sulfonamides								
Sulfamethoxazole	512	>512	>512	79	>512	>512	89	84
Trimethoprim/sulfamethoxazole	4-76	>4/76	>4/76	63	>4/76	>4/76	87	76
Quinolones and fluoroquinolones								
Ciprofloxacin	4	>16	>16	73	16	>16	84	79
Difloxacin	4	>16	>16	91	>16	>16	98	95
Enrofloxacin	2	>16	>16	90	>16	>16	76	83
Gatifloxacin	8	16	>16	67	8	>16	75	72

Levofloxacin	8	16	>16	63	8	>16	65	64
Nalidixic acid	32	>256	>256	99	>256	>256	100	99
Orbifloxacin	8	>16	>16	76	>16	>16	96	87
Sarafloxacin	0.25	>16	>16	100	16	>16	100	100
Phenicols								
Chloramphenicol	32	>32	>32	24	4	>32	63	46
Aminoglycosides								
Gentamicin	16	0.5	>16	30	2	>16	29	29
Streptomycin	64	>256	>256	80	>256	>256	74	77
Tetracycline	16	>32	>32	100	>32	>32	96	98

[a]based on NCCLS standards, with the exception of streptomycin (139, 141).

antimicrobials (Table 2-1) were determined using the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, OH). *E. coli* ATCC 25922 and 35218, *Enterococcus faecalis* ATCC 51299, and *Pseudomonas aeuriginosa* ATCC 27853 were used as quality control organisms in all antimicrobial susceptibility tests.

Detection and sequence analysis of the quinolone resistance-determining regions (QRDR) in *gyrA*, *gyrB*, *parC* and *parE*. Chromosomal DNA was prepared using a Wizard Genomic DNA Purification kit (Promega, Madison WI). The QRDR of *gyrA*, *gyrB*, *parC*, and *parE* were amplified by PCR using previously published primers (Table 2) and protocols (56). The PCR was performed in a 50 µl volume consisting of 0.25mM of each deoxyribonucleotide, 1.5mM $MgCl_2$, 1 U Gold *Taq* DNA polymerase, and 50 pmol each primer. The temperature profile was: 95ºC for 10 min; 30 cycles of 95ºC for 30 s, 55ºC for 45 s, and 72ºC for 45 s; and a final cycle of 72ºC for 7 min.

Predicted polypeptide products were analyzed for amino acid changes by comparison with wild type *E. coli gyrA* (accession number AE000312), *gyrB* (AE000447), *parC* (AE000384), and *parE* (AE000385).

Detection and sequence analysis of class 1 integrons. A PCR assay using 5'CS and 3'CS primers (Table 1) was used to identify class 1 integrons in the *E. coli* isolates, according to the method of Zhao et al (244). Each of the amplified products was purified with a High PCR Purification kit (Roche, Indianapolis, MN) and sequenced at the Center for Agriculture Biotechnology, University of Maryland, College Park, MD. Resultant DNA sequence data were compared to data in the GenBank database via the BLAST algorithm (7).

Results

Serogrouping and virulence genes. Six serogroups consisting of O1, O25, O78, O84, O88, and O160, were identified among the 71 *E. coli* isolates from diseased chickens (Table 2-2). The majority of the chicken isolates (63%) were identified as serogroup O78. Seven isolates were non-typeable. *E. coli* isolates from chickens possessed multiple virulence factors (Table 2-2). Ninety-seven percent of isolates were PCR positive for the increased serum survivial (*iss*) gene and 93% positive for the temperature-sensitive hemagglutinin (*tsh*) gene. None of the isolates contained the *hlyE* gene. Nine isolates carried all three virulence factors K1, ISS, and TSH, including four O25, two O78, two O88, and one non-typeable strain. Two isolates did not have any of the virulence factors examined.

In contrast to the avian *E. coli*, the O-serogroups identified in swine *E. coli* were not those typically associated with pathogenic strains in the U.S. Additionally, the swine isolates did not posses the characteristic toxin and fimbrial virulence factors associated with swine diarrhea (Table 2-3). Twenty-three serogroups were identified among the 89 *E. coli* isolates from swine, however, 38 % were non-typeable. Among the 89 swine isolates, 10% contained Stx1, 7% contained CNF2, and 4 contained either STa, CNF2, K99, or F107. Two *E. coli* isolates possessed two virulence factors: O153 (CNF2, K99) and O171 (Stx1, CNF2). However, most (81%) isolates did not contain any of the virulence factors screened for in this study.

Antimicrobial resistance. Most *E. coli* isolates recovered from diseased chickens and swine were resistant to multiple classes of antimicrobials (Table 2-1). The

Table 2-2. Primer sequences used to amplify class 1 integrons and the quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC* and *parE*

PCR target	Primers	Primer sequence (5' – 3')	Reference
Class I integron	5'CS	GGCATCAAGCACAAGC	(244)
	3'CS	AAGCAGACTTHACTGAT	
QRDR of *gyrA*	*gyrA*-1	ACGTACTAGGCAATGACTGG	(56)
	gyrA-2	AGAAGTCGCCGTCGATAGAA	
QRDR of *gyrB*	*gyrB*-1	TGTATGCGATGTCTGAACTG	(56)
	gyrB-2	CTCAATAGCAGCTCGGAATA	
QRDR of *parC*	*parC*-1	CAGACTGCCAGGAACGCGAT	(56)
	parC-2	AGCCAAGCGCGGTGATAAGC	
QRDR of *parE*	*parE*-1	TACCGAGCTGTTCCTTGTGG	(56)
	parE-2	GGCAATGTGCAGACCATCAG	

Table 2- 3. Serogroups and virulence genes of *E. coli* isolates from diseased chicken and swine

E. coli from chickens						*E. coli* from piglets							
O-group	No. of isolates	K1	ISS	Tsh	HlyE	O-group	No. of isolates	STa	Stx1	CNF1	CNF2	K99	F107
O1	2	0	1	1	0	O1	5	0	1	0	0	0	0
O25	4	4	4	4	0	O21	7	0	0	1	0	0	0
O78	45	2	45	45	0	O101	8	0	1	0	1	1 (O153)	0
O84	6	0	6	6	0	O9, O88, O153, O171	12 (3 each)	0	1 (O9)	0	1 (O153), 1 (O171)	0	0
O88	6	2	6	3	0	O19, O25, O91, O142,O149, O157, O159	14 (2 each)	0	2 (O91)	0	1 (O159)	0	1 (O149)
O160	1	0	0	0	0	O33, O35, O54, O79,O114, O160, X3, X13, X28	9 (1 each)	1 (O114)	1 (X13)	0	0	0	0
NT	7	1	7	7	0	NT	34	0	3	0	2	0	0
Total	71	9	69	66	0	Total	89	1	9	1	6	1	1

K1: capsule antigen K1; ISS: increased serum survival factor; Tsh: temperature sensitive hemagglutination; HlyE: hemolysin E; STa: heat-stable enterotoxins; Stx1: Shiga toxin 1; CNF1 and CNF2: cytotoxic necrotizing factors 1 and 2; K99: fimbrial type 5; F107: fimbrial type 107; X: experimental O group; NT: nontypeable.

majority of isolates were resistant to tetracycline (98%), sulfamethoxazole (84%), ampicillin (79%), streptomycin (77%), and trimethoprim/sulfamethoxazole (76%). Resistance to chloramphenicol among the swine isolates (63%) was significantly higher ($p < 0.05$) than resistance among chicken isolates (24%). Surprisingly, all *E. coli* isolates, regardless of their animal origin, were resistant to the quinolone antimicrobial, nalidixic acid. Due to the high incidence of quinolone resistance, all isolates were also tested against a panel of veterinary and human use fluoroquinolones. Among these isolates, fluoroquinolone resistance ranged from 64% to levofloxacin, 79% to ciprofloxacin, and 95% to difloxacin. *E. coli* isolates of both avian and swine origin displayed elevated levels of resistance to all fluoroquinolones tested (Table 2-3).

With regards to multi-drug resistance profiles, all isolates from swine were resistant to more than 8 of the 19 antimicrobials tested, 86% were resistant to more than 11 antimicrobials, and 2% were resistant to 16 antimicrobials. All isolates recovered from diseased chicken were resistant to at least 3 of the 19 antimicrobial tested. Fifty-six (79%) avian *E. coli* isolates were resistant to more than eight antimicrobials, and two (3%) were resistant to 16 antimicrobials. All *E. coli* isolates from this study were susceptible to ceftiofur, ceftriaxone and, amoxicillin-clavulanic acid.

Topoisomerase point mutations in fluoroquinolone-resistant *E. coli*. All quinolone-resistant *E. coli* isolates were tested for amino acid substitutions in GyrA and 39 representative isolates (20 from swine and 19 from chickens) were also tested for amino acid mutations in GyrB, ParC, and ParE (Table 2-4). All nalidixic acid resistant *E. coli* possessed a mutation at position 83 in GyrA (S83L). Single mutations (S83L) and double mutations (S83L and D87A, D87N, D87G or D87Y) were found in GyrA, whereas no

Table 2-4. Amino acid substitutions in DNA gyrase and topoisomerase IV and corresponding fluoroquinolone resistance profiles among *E. coli* isolated from diseased chickens and swine (n=39).

# of isolates	Amino Acid Substitution[a]			MIC range (μg/ml)			
	GyrA	ParC	ParE	Nalidixic acid	Enrofloxacin	Ciprofloxacin	Gatifloxacin
1	S83L			256	0.5	0.25	0.25
3	S83L	S80R		>256	2-8	1-4	1-4
4	S83L	S80I		>256	1-4	0.5-4	0.5-2
1	S83L D87N	S80I		>256	16	8	4
1	S83L D87G	S80I		>256	16	16	8
1	S83L D87N	E84K		>256	>16	16	8
23	S83L D87N	S80I		>256	16->16	8->16	4->16
2	S83L D87N	S80I	S459A	>256	16->16	8->16	8->16
1	S83L D87Y	S80I		>256	16->16	8->16	8->16
1	S83L D87Y	S80I	S459A	>256	16->16	8->16	8->16
1	S83L D87Y	S80I	N463D	>256	16->16	8->16	8->16

[a]Substituted amino acids, and the position number; e.g. S83L indicates substitution of a leucine for a serine at position 83. Aminoacids: S, serine; L, leucine; D, aspartic acid; N, asparagine ; Y, tyrosine; I, isoleucine; R, arginine; G, glycine; alanine.

mutations in GyrB and only single mutations, S80I, S80R or E84K in ParC, and S459A or N463D in ParE were identified. Among the 38 isolates that possessed mutations in ParC, the most common was S80I (90%), followed by S80R (8%). The most frequent pattern of mutations (n=18) included a double mutation in GryA (S83L, D87N) and a single mutation in ParC (S80I). All 18 isolates exhibited resistance to both enrofloxacin and ciprofloxacin and 94% were resistant to gatifloxacin. Less common mutations included 4 isolates with a mutation in GryA (S83L) and in ParC (S80I) and 4 isolates with a double mutation in GyrA (S83L, D87N), and single mutation in ParC (S80I) (Table 2-4). One isolate possessed only a S83L substitution in GyrA and was nalidixic acid resistant, but susceptible to fluoroquinolones tested with the exception of sarafloxacin (MIC 0.5 μg/ml).

Presence of Class 1 integrons. All veterinary *E. coli* isolates were characterized for the presence of class 1 integrons since a recent study documented plasmid-mediated quinolone resistance in clinical *E. coli* isolates from Shanghai, China, and suggested the possibility that the gene, *qnr*, is possibly located in a class 1 integron (15, 19, 178, 185, 229). Nineteen percent of *E. coli* isolates recovered from swine possessed class 1 integrons, with sizes ranging from ca. 0.7 to 2.0 Kb. Nine different serotypes of swine *E. coli* isolates harbored integrons, however, the majority of serotypes harboring these integrons were non-typeable. Ten isolates carried a 1.5-kb integron containing the *dhfr1* and *aadA1* genes (Table 2-5), which confer resistance to trimethoprim and streptomycin, respectively. Three isolates carried a 2.0-kb integron. In two isolates, the 2.0-kb integron contained *dhfr12* and *aadA2*, and in one strain it contained *dhfr17* and *aadA2*. Two isolates contained a 1.0-kb integron (*aadA1*), with one strain containing an additional

Table 2-5. Integrons and their gene cassettes in swine and avian *E. coli*

Size of Integron (Kb)	No. of Strains		Gene Cassette[a]
	Swine (n=89)	Avian (n=71)	
2.0	2	0	*dhfr 12, aadA2*
2.0	1	0	*dhfr17, aadA2*
1.5	10	33	*dhfr 1, aadA1*
1.5	0	1	*dhfr 17, aadA2*
1.0	3	3	*aadA1*
0.7	0	1	*dhfr13*
1.5, 1.0	1	0	*dhfr17, aadA2; aadA1*
1.5, 0.7	0	4	*dhfr17, aadA2; dhfr13*

[a]*aad*, aminoglycoside adenyltransferase; *dhfr*, dihydrofolate reductase.

1.5-kb integron (*dhfr1*and *aadA1*). The majority (59%) of avian *E. coli* contained class 1 integrons ranging in size from 0.7 kb to 1.5 kb (Table 5). Integrons were found in avian isolates from 4 of the 6 serogroups identified, however, the majority were found within isolates possessing serogroup O78 (35/45). Thirty-four of these isolates contained a 1.5-kb integron with *dhfr1* and *aadA1* and one isolate contained a 1.5-kb integron with *dhfr17*and *aadA2*. Six isolates contained both of 1.5kb integron (*dhfr1 and aadA1*) and 0.7kb integron (*dhfr13*). Two isolates contained 1.0kb integron with *aadA1* gene. No class 1 integrons were found that possessed the plasmid mediated quinolone-resistance gene, *qnr*.

Discussion

More than 160 O-serogroups have been identified in *E. coli*. However, disease-causing *E. coli* typically consists of a relatively few serogroups. In this study, 66% of the *E. coli* isolates from chickens belonged to serogroups (O1 and O78) typically associated with colibacillosis. The ability of these isolates to cause disease in the birds may have been attributable, in part, to the fact that they possessed ISS and TSH - virulence factors known to increase bacterial resistance to serum and colonization of internal organs of infected chickens (106, 129). The finding that most isolates are O78 might prove useful to poultry farms in combating infection and offer an alternative to antibiotics in the form of bacterin vaccines. In contrast to those from chickens, *E. coli* from diseased piglets were found to have O-serogroups different from their counterparts in other regions of the world. These isolates did not possess the virulence factors seen in the serogroups such as

O8, O147, O149, and O157 that are often associated with diarrhea in piglets (181). Thus, while it is possible that other colonization factors or enterotoxins may have contributed to the pathogenicity of these *E. coli*, further study is clearly needed to determine the underlying pathogenicity mechanisms.

Similar to the findings of previous studies (15, 19, 178, 185, 229), most *E. coli* isolates of avian and swine origin described here were resistant to tetracyclines, aminoglycosides, and sulfonamides. A significantly higher number of the swine *E. coli* isolates were resistant to chloramphenicol compared to the chicken isolates. This may be related to the broad use of florfenicol to treat swine diseases in China as compared to its limited use in avian husbandry. Perhaps the most striking finding from this study was the widespread resistance to quinolones and fluoroquinolones. All *E. coli* isolates were resistant to nalidixic acid and sarafloxacin. More than 70% were resistant to ciprofloxacin, and more than 60% were resistant to the newer human fluoroquinolones, gatifloxacin and levofloxacin. Somewhat similar findings have been reported in a recent study of clinical *E. coli* isolates from China, wherein greater than 50% of all isolates were resistant to ciprofloxacin (221). Similar findings were also reported for *E. coli* isolates recovered from chickens and swine in Spain where 90% of chicken isolates and 50% of swine isolates were resistant to ciprofloxacin (178, 222). Fluoroquinolone resistance in these isolates, coupled with the observation of widespread multiple antimicrobial resistance (e.g., 80% of the isolates from this study were resistant to eight or more antibiotics) likely portends further complications in treatment of *E. coli* infections in humans and animals from this region.

The molecular investigations into the underlying quinolone resistance mechanisms revealed that all quinolone resistant isolates possessed the typical mutations in the topoisomerase genes, *gyrA* and *parC*, as reported by other studies (12, 84, 105). Resistance to fluoroquinolones has most frequently been associated with alterations at S83L and D87G in the quinolone resistance-determining region (QRDR) of the DNA gyrase gene *gyrA* (12, 98). Our results are in agreement with previous reports as all quinolone resistant *E. coli* isolates, regardless of swine or avian origin, possessed a point mutation in GyrA of S83L or a double mutation of S83L and either D87A (n=1), D87G (n=1), D87Y (n=3), or D87N (n=26). Our finding that the most common mutation at codon 87 in GyrA resulted in substitution of asparagine for aspartic acid is consistent with other studies (12, 84, 103, 105). With regards to correlation of mutations with decreased susceptibilities to tested fluoroquinolone agents, isolates with single mutations in GyrA had relatively low (≤4 µg/ml) MICs to enrofloxacin and ciprofloxacin (Table 4), whereas many *E. coli* isolates with double GyrA and single ParC mutations had correspondingly high MICs (≥8 µg/mL) to these drugs.

Among the 38 isolates that possessed ParC mutations, almost all possessed a mutation at position 80 resulting in an amino acid substitution of either arginine (S80R, n=3) or isoleucine (S80I, n=34), with the exception of one swine isolate which carried the E84K mutation in ParC. Mutations in *parC* at S80I or E84K have been associated with high-level resistance to fluoroquinolones, and have been detected in clinical strains carrying a *gyrA* mutation (12, 84, 105). As expected, single mutations observed in ParC always coincided with mutations in GyrA among the veterinary *E. coli* isolates in this study. Mutations in *gyrB* and *parE* have been associated with quinolone resistance (83),

however, the mutation frequency is much lower compared to *gryA* and *parC*. Additionally, the ParE mutations reported here have to the best of our knowledge not been reported previously (23, 103). However, it is difficult to determine how much these mutations contribute to decreased fluoroquinolone susceptibility as all isolates that possessed ParE mutations also carried mutations in GyrA and ParC. Other mechanisms of fluoroquinolone resistance exist besides mutations in the genes encoding DNA gyrase and topoisomerase IV. These include decreased production of porin proteins and up-regulation of multidrug resistance efflux pumps (68). Though we did not explore the contribution of these other mechanisms to the fluoroquinolone resistant phenotypes observed in this study, it is more than likely that they play a role in the evolution of fluoroquinolone resistance. The isolates in this study are the subject of future research to determine if efflux mechanisms are also involved in the quinolone resistant phenotypes.

Regarding the genetics of resistance to other antimicrobials in the *E. coli* isolates from this study, integrons were likely important, both in terms of the mechanisms of resistance and in the dissemination of resistance genes (24, 206). Integrons are known to be associated with multiple-drug resistance in enteric organisms, and class 1 integrons specifically, have been shown important in the dissemination of *intI, sulI,* and one or more antimicrobial resistance gene cassettes among Gram-negative bacteria (122, 244). The findings from this study are consistent with most previous data in that varying sizes of integrons were found in the *E. coli* isolates, and they contained genes for resistance to aminoglycosides, trimethoprim, and β-lactams (12, 98). More recently, Wang et al. (222) reported the occurrence of a gene in a class I integron, termed *qnr*, which provides low level quinolone resistance. They found *qnr* in fluoroquinolone resistant humans clinical

E. coli isolates from Shanghai, China, suggesting the possibility that *qnr* was also present in some of our avian and swine strains. None of the integron-positive avian or swine *E. coli* isolates yielded *qnr* (data not shown) or a *qnr* like gene upon DNA sequencing, however, further studies are ongoing on these isolates to determine if *qnr* is indeed present, but not in the class 1 integrons previously identified.

In summary, because trained practitioners are unavailable in many regions of developing countries, regulations on the veterinary use of antibiotics are poorly enforced or absent in many developing countries. Consequently, there is opportunity for the inappropriate use of antibiotics in both humans and veterinary medicine. With concentrated livestock production increasing in developing countries, reliance on antimicrobials will likewise expand. Our data suggest that a lack of restrictions on antimicrobial use in food animals in China has resulted in the dissemination of multiple drug resistant pathogenic *E. coli* isolates, including fluoroquinolone resistant variants. Research is needed to determine the role of antimicrobial use in animal production environments on emergence and spread of resistance in both veterinary and humans medicine worldwide, especially in developing countries. It is also important that antimicrobial prudent use guidelines be developed in conjunction with the establishment of surveillance programs for monitoring antimicrobial resistance in pathogenic bacteria that can be transmitted to humans from food animals. This information should ultimately provide important information for the development of public health policy for use of antimicrobials in food animal production in developing countries.

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Chapter 3 Characterization of Multiple-Antimicrobial-Resistant *Salmonella* Serovars Isolated from Retail Meats

Abstract

One hundred thirty-three *Salmonella* isolates recovered from retail meats purchased in the United States and the People's Republic of China were assayed for antimicrobial susceptibility, the presence of integrons and antimicrobial resistance genes, and horizontal transfer of characterized antimicrobial resistance determinants via conjugation. Seventy three (82%) *Salmonella* isolates were resistant to at least one antimicrobial. Resistance to following antibiotics was common among the U.S. isolates was resistance to tetracycline (68%), streptomycin (61%), sulfamethoxazole (42%), and ampicillin (29%). Eight *Salmonella* isolates (6%) were resistant to ceftriaxone. Fourteen isolates (11%) from China were resistant to nalidixic acid and displayed decreased susceptibility to ciprofloxacin. A total of 19 different antimicrobial resistance genes were identified in 30 multidrug-resistant *Salmonella* isolates. The $bla_{\text{CMY-2}}$ gene, encoding a class A AmpC β-lactamase, was detected in all 10 *Salmonella* isolates resistant to extended spectrum β-lactams. Resistance to ampicillin was most often associated with a *TEM-1* family β-lactamase gene. Six aminoglycoside resistance genes *aadA1, aadA2, aacC2, Kn, aph(3)-IIa, aac(3)-IVa,* were commonly present in the *Salmonella* isolates. Sixteen (54%) of 30 *Salmonella* isolates tested had integrons ranging in size from 0.75 to 2.7kb. Conjugation studies demonstrated plasmid-mediated transfer of genes encoding CMY-2 and TEM1-like β-lactamases. These data indicate that *Salmonella* recovered from retail raw meats are commonly resistant to multiple antimicrobials, including those such as ceftriaxone

used for treating salmonellosis. Genes conferring antimicrobial resistance in *Salmonella* are often carried on integrons and plasmids, and could be transmitted through conjugation. These mobile DNA elements have likely played an important role in transmission and dissemination of antimicrobial resistance determinants among *Salmonella*.

Introduction

The emergence of antimicrobial-resistant bacterial pathogens has become a major public health concern. The use of antimicrobials in any venue, including for disease treatment and growth promotion in domestic livestock, can potentially lead to widespread dissemination of antimicrobial-resistant bacteria (70, 205, 236). In recent years, testing of *Salmonella* isolates from the United States and other countries has shown that an increasing proportion are multidrug-resistant (34, 69, 74, 200). Of particular concern is the isolation of ceftriaxone- and ciprofloxacin-resistant *Salmonella*, because of the importance of these two agents in treating *Salmonella* infections in children and adults (34, 54, 231), respectively.

Resistance to antimicrobial agents in bacteria is mediated by several mechanisms, including 1) changes of bacterial cell wall permeability, 2) energy-dependent removal of antimicrobials via membrane bound efflux pumps, 3) modification of the site of drug action, and 4) destruction or inactivation of antimicrobials (13, 186). Acquired antimicrobial resistance phenotypes most often develop via conjugative transfer of plasmids (59, 66, 73). Plasmids may carry class I integrons, mobile DNA elements

important in the proliferation of bacterial multidrug resistance, especially among the Gram-negative enteric species (11, 50, 162, 214). Integrons primarily have been found located within transposons Tn402 and Tn21, which in turn reside on broad-host-range plasmids or the lncF plasmid (28, 217). By incorporating into transposons and plasmids, integrons participate in the capture and dissemination of resistance genes among bacteria.

Molecular genetic techniques have been used to characterize antimicrobial-resistant *Salmonella*e, especially *S.* Typhimurium DT104 (22, 24, 42, 144, 170). For instance, variant *Salmonella* genomic island 1 (SGI1) multidrug resistance (MDR) regions, consisting of integrons encoding different resistance genes, have been found located in the chromosomal DNA of *S.* Typhimurium DT104 and Agona (22). The formation of these multidrug resistance clusters is hypothesized to favor the expression of a large number of resistance genes and enhance their transfer to other bacteria. Also, because class I integrons have become integrated into the chromosome of *S.* Typhimurium DT104 and Agona, they are able to persist even in the absence of antimicrobial selection (22, 42) with no apparent fitness cost to the cell. This has lead, in the case of DT104, to a stable and widely disseminated clone of multidrug-resistant *S.* Typhimurium.

The objectives of this study were to determine the antimicrobial susceptibility phenotypes of *Salmonella* isolated from retail meats from the Washington D.C. area in the United States and from China, and to characterize the genetic mechanisms underlying antimicrobial-resistant phenotypes observed for the isolates. We also examined select isolates for the ability to donate resistance genes via conjugative transfer of plasmids to *E. coli*. Our goal was to advance understanding of the molecular genetic mechanisms

involved in the emergence and dissemination of antimicrobial-resistant *Salmonella.*

Materials and Methods

***Salmonella* isolates**. A total of 133 *Salmonella* isolates were included in the study. Eighty-nine were recovered from retail ground meat samples of chicken, turkey, pork, and beef purchased in the Washington D.C. area, including 45 from samples purchased between June and September 1998, and 44 from samples purchased between August 1999 to August 2000. The other 44 *Salmonella* isolates were isolated from samples of pork, beef, chicken, and mutton purchased in 10 provinces in China, from October 1999 to December 2000.

All *Salmonella* isolates were recovered from meats using methods described in the 8th edition of the U.S. Food and Drug Administration Bacteriological Analytical Manual (61). The isolates were further identified with API identification kits (BioMerieux, Marcy, France) and serotyped with commercial antiserum (Difco, Detroit, MI) according to manufacturers' instructions.

Antimicrobial susceptibility testing. Antimicrobial minimum inhibitory concentrations (MICs) of the 133 *Salmonella* isolates were determined using the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, OH) and interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS) for microdilution broth methods (138, 140). The 17 antimicrobials used and their recommended resistance breakpoints are presented in Table 3-1. *E. coli* ATCC 25922, 35218, *Enterococcus faecalis* ATCC 29212, *Enterococcus*

faecalis ATCC 29212, *Staphylococcus aureus* ATCC29213, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms.

DNA isolation, PCR, and gene sequence analysis. Based on serotypes and antimicrobial resistance profiles, 30 multidrug-resistant *Salmonella* isolates were selected for further characterization of antimicrobial resistance genes and class 1 integrons. Chromosomal and plasmid DNA of the bacterial isolates were isolated using the Wizard Genomic DNA Purification kit (Promega, Madison WI) and the High Plasmid Purification kit (Roche, Indianapolis IN), respectively. The quantity of the DNA was measured using a Smartspect 3000 spectrophotometer (BioRad, Hercules, CA). Sixty-one pairs of oligonucleotide primers (Table 3-2) were designed to target 61 antimicrobial resistance genes that confer resistance to 6 categories of antimicrobial agents, including β-lactams, aminoglycosides, phenicols, tetracycline, trimethoprim, and sulfonamides. All primers were designed to differentiate the specific gene sequence of interest, except for the $bla_{\text{TEM-1}}$ primers that amplify the entire family of bla_{TEM} genes. The primers were designed using the OLIGO 5.0 software program (National Biosciences, Inc., Plymouth, MN) and synthesized commercially (Invitrogen, Carlsbad, CA). PCR was performed in a total volume of 50 µl distilled H_2O including 0.25mM of each deoxyribonucleotide, 1.5mM $MgCl_2$, 0.2 U Gold *Taq* DNA polymerase, and 50 pmol each primer. The temperature profile included an initial template denaturation step at 95ºC for 10 min, followed by 30 cycles of 95ºC for 30 s, 55ºC for 1 min, and 72ºC for 1 min, and a final step at 72ºC for 7 min (8). The presence of class I integrons among the 30 *Salmonella* isolates was determined by PCR using primers 5’CS (5'-GGCATCCAAGCACAAGC-3')

Table 3-1. Antimicrobial resistance of *Salmonella* isolates from retail meats in the United States and China

Antimicrobials	**Breakpoint**	**US isolates 1998-2000 (n=89)**		**Chinese isolates 1999-2000 (n=44)**	
	(µg/ml)	**% Resistance**	**% Intermediate**	**% Resistance**	**% Intermediate**
β-lactams					
Ampicillin	32	29	0	39	2
Amoxicillin/clavulanate	32	21	9	0	2
Cephalothin	32	24	4	2	0
Ceftiofur	8	19	1	0	0
Ceftriaxone	64	9	10	0	0
Cefoxitin	32	18	0	0	0
Phenicols					
Chloramphenicol	32	11	0	20	2
Tetracycline	16	68	0	43	2
Aminoglycosides					
Amikacin	64	0	0	0	0
Apramycin	32	0	0	0	0
Gentamycin	16	2	0	2	0
Kanamycin	64	6	0	11	0

Streptomycin	64	61	0	27	0
Sulfonamides					
Sulfamethoxazole	512	42	3	16	0
Trimethoprim-sulfamethoxazole	4/76	9	0	9	0
Quinolones and fluoroquinolone					
Nalidixic acid	32	0	0	32	0
Ciprofloxacin	4	0	0	0	0

Tble3-2. Sequences of PCR oligonucleotide primers used for the identification of antimicrobial resistance genes in *Salmonella* isolates

Antimicrobials	Resistance genes	Sequence of olignucleotide primers		Size (bp)	Accession #
		Forward (5' – 3')	Reverse (5' – 3')		
β-lactam					
	bla$_{CMY-2}$	TGG CCG TTG CCG TTA TCT AC	CCC GTT TTA TGC ACC CAT GA	870	X91840
	bla$_{CMY-9}$	TCA GCG AGC AGA CCC TGT TC	CTG GCC GGG ATG GGA TAG TT	874	AB049588
	bla$_{FOX-1}$	CAG CCG ATG CTC AAG GAG TA	CAA CCC AGC CCC TGA GTC AT	761	X77455
	bla$_{DHA-1}$	GCC GGT CAC TGA AAA TAC AC	TAC GGC TGA ACC TGG TTG TC	762	Y16410
	bla$_{MI1}$	AGC GTC GCC AGT TCT GCA TT	GAC CGG CCA GTT GAG CAT CT	858	M37839
	bla$_{SHV-1}$	GGC CGC GTA GGC ATG ATA GA	CCC GGC GAT TTG CTG ATT TC	714	F148850
	bla$_{TEM-1}$	CAG CGG TAA GAT CCT TGA GA	ACT CCC CGT CGT GTA GAT AA	643	AF309824
	bla$_{CTX-M1}$	AAC CGT CAC GCT GTT GTT AG	TTG AGG CTG GGT GAA GTA AG	766	X92506
	bla$_{CTX-M2}$	GGC GTT GCG CTG ATT AAC AC	TTG CCC TTA AGC CAC GTC AC	486	X92507
	bla$_{CTM-M14}$	GCC TGC CGA TCT GGT TAA CT	GCC GGT CGT ATT GCC TTT GA	358	AF252622
	bla$_{VEB-1}$	TAG CCG TTT TGT CTG AGA TA	TTA CCC CAA CAT CAT TAG TG	543	AF205943
	bla$_{OXA-1}$	AAT GGC ACC AGA TTC AAC TT	CTT GGC TTT TAT GCT TGA TG	595	J02976
	bla$_{OXA-2}$	CAA GCC AAA GGC ACG ATA GT	ACG ATT GCC TCC CTC TTG AA	644	X07260
	bla$_{OXA-7}$	GAA GCC GTC AAT GGT GTT TT	ATG CCC TCA CTT GCC ATG AT	686	X75562

	bla$_{PSE-1}$	TGC TTC GCA ACT ATG ACT AC	AGC CTG TGT TTG AGC TAG AT	438	AF153200
	bla$_{IMP-1}$	TGA GGC TTA CCT AAT TGA CA	TCA GGC AAC CAA ACC ACT AC	324	S71932
Aminoglycoside					
	aac(3)-Ia	TGA GGG CTG CTC TTG ATC TT	ATC TCG GCT TGA ACG AAT TG	436	X15852
	aac(3)-IIa	CGG CCT GCT GAA TCA GTT TC	AAA GCC CAC GAC ACC TTC TC	439	X13543
	aacC2	GGCAATAACGGAGGCAATTCGA	CTCGATGGCGACCGAGCTTCA	450	X51534
	aacC4	ACTGAGCATGACCTTGCGATGCTCTA	TACCTTGCCTCTCAAACCCCGCTT	436	AJ009820
	aac(3)-IVa	GAT GGG CCA CCT GGA CTG AT	GCG CTC ACA GCA GTG GTC AT	462	X01385
	aac(6')	TTG GAC GCT GAG ATA TAT GA	GCT CCT TTT CCA GAA TAC TT	476	M18086,
	aph(2")	GAC CGT GTT CTT GAA TTC TA	GCG GGA ATC TTT TAG CAT TA	464	M13771
	ant(3")-Ia	CGC CGA AGT ATC GAC TCA AC	GCG GGA CAA CGT AAG CAC TA	559	X02340
	aadD	F, ATATTGGATAAATATGGGGAT	TCCACCTTCCACTCACCGGTT	161	AF051917
	ant(6)-Ia	GCC GGA GGA TAT GGA ATT AT	TCA GCG GCA TAT GTG CTA TC	666	AF299292
	Kn	ACTGGCTGCTATTGGGCGA	CGTCAAGAAGGCGATAGAAGG	515	U66885
	aph(3')-IIa	TCC GGT GCC CTG AAT GAA CT	ACG GGT AGC CAA CGC TAT GT	519	V00618
	aph(4)-Ia	TCT CGG AGG GCG AAG AAT CT	TTG CCG TCA ACC AAG CTC TG	763	V01499
Tetracycline					
	tetA	GCG CCT TTC CTT TGG GTT CT	CCA CCC GTT CCA CGT TGT TA	831	X00006

	tetB	CCC AGT GCT GTT GTT GTC AT	CCA CCA CCA GCC AAT AAA AT	723	V00611
	tetC	TTG CGG GAT ATC GTC CAT TC	CAT GCC AAC CCG TTC CAT GT	1019	Ab023657
	tetD	CTG GGC AGA TGG TCA GAT AA	TGA CCA GCA CAC CCT GTA GT	832	X65876
	tetE	CGT CGC CCT GTA TTG TTA CT	TGG TCA GCA CCC CTT GTA AT	814	l06940
	tetG	AGC AGG TCG CTG GAC ACT AT	CGC GGT GTT CCA CTG AAA AC	623	Af07155
Trimethoprim					
	dhfrI	CGG TCG TAA CAC GTT CAA GT	CTG GGG ATT TCA GGA AAG TA	220	Af382145
	dhfrII	AGT TTG CGC TTC CCC TGA GT	CTT AGG CCA CAC GTT CAA GTG	194	Af083409
	dhfrIII	ACC TGC CGA TCT GCG TCA T	TCG CAG GCA TAG CTG TTC	387	J03306
	dhfrV	TTG GTT GCG GTC CAC ACA TA	CTC CTT CCG GCT CAA TAT C	330	X12868
	dhfrVI	GTT TCC GAG AAT GGA GTA AT	ACT AAA CGC AAC GCA TAG TA	508	Z86002
	dhfrVII	AGC AAA AGG TGA GCA GTT AC	GTG CTG GAA CGA CTT GTT AG	419	X58425
	dhfrVIII	TTG GGA AGG ACA ACG CAC TT	ACC ATT TCG GCC AGA TCA AC	382	U10186
	dhfrIX	TCA GAT TCC GTG GCA TGA AC	AAT GGT CGG GAC CTC AGA T	400	X57730
	dhfrX	ACC AGA GCA TTC GGT AAT CA	TTG GAT CAC CTA CCC ATA GA	445	l06418
	dhfrXII	AAA TTC CGG GTG AGC AGA AG	CCC GTT GAC GGA ATG GTT AG	429	Z21672
	dhfrXIII	GCA GTC GCC CTA AAA CAA AG	GAT ACG TGT GAC AGC GTT GA	294	Z50802
	dhfrXV	GCC GTG GGT CGA TGT TTG AT	TTC ACC ACC ACC AGA CAC A	395	Z83311

	dhfrXVI	GCT CTC CCA AAT CGA AAG TA	ATT GCA GGC GCT TGT TAA CT	332	Af077008
Sulfornamide					
	sulI	TCA CCG AGG ACT CCT TCT TC	CAG TCC GCC TCA GCA ATA TC	331	X15024
	sulII	CCT GTT TCG TCC GAC ACA GA	GAA GCG CAG CCG CAA TTC AT	435	M36657
Chloramphenicol					
	cat1	CTT GTC GCC TTG CGT ATA AT	ATC CCA ATG GCA TCG TAA AG	508	M64281
	cat2	AAC GGC ATG ATG AAC CTG AA	ATC CCA ATG GCA TCG TAA AG	547	Aj401047
	cat3	ATC GGC ATC GTT TAC CAT GT	ATC CCC TTC TTG CTG ATA TT	531	Ay042185
	cmlA	CGC CAC GGT GTT GTT GTT AT	GCG ACC TGC GTA AAT GTC AC	394	Af078527
	cmlB	ACT CGG CAT GGA CAT GTA CT	ACG GAC TGC GGA ATC CAT AG	840	Af034958
	flo	CTG AGG GTG TCG TCA TCT AC	GCT CCG ACA ATG CTG ACT AT	673	Af252855.

and 3'-CS (5'-AAGCAGACTTGACTGAT-3') as previously described (244).

All PCR products were purified with High PCR Purification kits (Roche, Indianapolis, MN) and sequenced at the University of Maryland Center of Agriculture Biotechnology, College Park, MD. Resultant DNA sequence data were compared to data in the GenBank database using the BLAST algorithm (7) available at the National Center of Biotechnology Information's web site (www.ncbi.nlm.nih.gov).

Conjugation experiments Multidrug-resistant *Salmonella* isolates recovered from retail meats (10 from the Washington DC area and 4 from China) were used as donor strains in conjugation experiments to study antimicrobial resistance gene transfer. Two nalidixic acid-resistant *E. coli* strains (1003 and 1016) were used as recipient strains. Conjugation was performed using the filter mating method as described previously (40). Briefly, donor and recipient cells (1:10) were mixed in Luria-Bertani broth (Difco). The mixture was then collected on a 0.45 µm pore-size filter and incubated on blood agar plates (BAP) at 37°C overnight. The mating mixture was washed from the filter and spread onto BAP containing a combination of nalidixic acid (60µg/ml) and streptomycin (50µg/ml), or nalidixic acid (100µg/ml) and kanamycin (50µg/ml). Bacterial colonies on BAP containing appropriate antibiotics were transferred onto MacConkey agar (Difco) plates and incubated overnight at 37^{o}C. Presumptive *E. coli* transconjugants were confirmed as *E. coli* via the API test, and assayed for susceptibility to 17 antimicrobial agents. Transfer of antimicrobial resistance genes was confirmed by PCR using primers shown in Table 2.

Results

Antimicrobial resistance of *Salmonella* isolates. Seventy three (82%) of the *Salmonella* isolated from retail meats purchased in the Washington D.C area displayed resistance to at least one antimicrobial. Resistance to tetracycline (68%), streptomycin (61%), and sulfamethoxazole (42%) was most often observed whereas resistance to β -lactams was less frequently observed (Table 1). Among β-lactams, resistance was highest to ampicillin (29%) followed by cephalothin (24%), amoxicillin-clavulanate (21%), ceftiofur (19%), cefoxitin (18%), and ceftriaxone (9%). In addition to eight isolates resistant to ceftriaxone, nine isolates (10%) displayed intermediate susceptibility to ceftriaxone. All the *Salmonella* that were intermediately susceptible to ceftriaxone were resistant to other β-lactams tested. The *Salmonella* also exhibited resistance to chloramphenicol (11%), kanamycin (6%), and gentamicin (2%). All *Salmonella* recovered from retail foods in the Washington DC area were susceptible to amikacin, apramycin, ciprofloxacin, and nalidixic acid (Table 3-1).

Twenty eight (64%) *Salmonella* isolates from China displayed resistance to at least one antimicrobial. The highest frequencies of resistance were for tetracycline (43%), ampicillin (39%), and streptomycin (32%). Resistance was also observed, but to a lesser extent, for chloramphenicol (20%), sulfamethoxazole (16%), kanamycin (11%), and trimethoprim (9%) (Table 1). None of the isolates displayed resistance to β-lactams other than ampicillin, except for one isolate that was resistant to cephalothin. In contrast to the US isolates, approximately one third of the isolates from China were quinolone-resistant. Fourteen (32%) of the isolates were resistant to nalidixic acid, concomitant with

increased MICs for ciprofloxacin. The MIC_{90} for ciprofloxacin in the isolates from China was more than 30 times higher (0.5 µg/ml) than that of the isolates from the United States (<0.015) (data not shown).

Antimicrobial resistance genes and class 1 integrons. Among the 30 multiple-antimicrobial-resistant *Salmonella* isolates (defined as resistant to two or more antimicrobials), 19 resistance genes conferring resistant to to six categories of antimicrobials, including β-lactams, aminoglycosides, phenicols, tetracycline, trimethoprim, and sulfonamides, were identified. The PCR results were consistent with the antimicrobial susceptibility phenotypes (Table 3-3). For example, the *sulI* and/or *sulII* genes were detected in each of the sulfonamide-resistant *Salmonella* isolates; the *tetA* and/or *tetB* genes were detected in each of the tetracycline-resistant isolates; and the dihydrofolate reductase genes, *dhfr1, dhfr12, dhfr13,* were detected in each of the trimethoprim-resistant isolates. Either or both of chloramphenicol acetyltransferase genes, *cat1* and *cat2*, were detected in the chloramphenicol-resistant *Salmonella* isolates from China, while the *flo* gene was detected in each of the chloramphenicol-resistant *Salmonella* isolated from the United States.

The distribution of aminoglycoside resistance genes in the *Salmonella* isolates was diverse. Six different resistance genes, *aadA1, aadA2, aacC2, Kn, aph(3)-IIa,* and *aac(3)-IVa,* were detected. The *aadA1* gene was most frequently detected, being present in 17 of the isolates. Three isolates contained *aadA1* and *aadA2*. Isolate CHS31 contained four types of aminoglycoside resistance genes, *aadA1*, *aadA2*, *aacC2*, and *aac(3)-Iva*. A total of 12 antimicrobial resistance genes were amplified from the DNA of this isolate. The *aac(3)-IVa*, *aacC2* genes, (conferring resistance to gentamicin), and the

Table 3-3. Antimicrobial resistance and resistance gene profiles

Strain	Serotypes	Meats	Antimicrobial Resistance Profile[a]	Antimicrobial Resistance Genes	Size (kb) of integron
1083 [b], 1089 [b], 1126 [b]	Agona	Turkey	Amo-Amp-Cef-Cet-Cep-Fox-Str-Sul-Tet-Tri	*bla$_{CMY2}$, bla$_{TEM1}$, aadA1, dhfr1, sulI, sulII, tetA*	1.2kb
1163 [b]	Agona	Turkey	Str-Sul-Tet	*aadA1, sulI, tetB*	1.0kb
1271 [b]	Djugu	Pork	Sul-Tri	*dhfr12, dhfr13, sulI,*	2.0kb
S34 [c]	H:E-2	Chicken	Amo-Amp-Cef-Cep-Fox	*bla$_{CMY2}$,*	
S14 [c], S14[b]	Hadar	Turkey	Sul-Tet	*sulI, sulII, tetA*	
1272 [b]	Heidelberg	Pork	Kan-Str-Sul-Tet	*aadA1, sulI, tetB*	1.0kb
S31 [c]	Infantis	Chicken	Amo-Amp-Cef-Cep-Fox	*bla$_{CMY2}$*	
S33 [c]	Infantis	Chicken	Amo-Amp-Cef-Cep-Fox-Sul	*bla$_{CMY2}$, sulI*	
S16 [c]	Orion	Pork	Sul-Tet	*sulI, sulII, tetA*	
1189 [b]	Typhim.	Chicken	Sul-Tet-Tri	*dhfr12, dhfr13, sulI, tetA, tetB*	0.75kb
S21 [c]	Typhim. DT104	Pork	Amp-Cml-Str-Sul-Tet	*pse-1, flo-1, aadA2, aadA1, sulI, sulII, tetA, tetB*	1.0kb
S27 , S29 [c]	Typhim.	Chicken	Amo-Amp-Cef-Cep-Fox	*bla$_{CMY2}$*	
S44 [c]	Typhim.	Chicken	Amo-Amp-Cef-Cep-Cet-Fox	*bla$_{CMY2}$*	

1275 [b]	Typhim. DT104	Pork	Amp-Cml-Ffc-Str-Sul-Tet	*pse-1, flo-1, aadA2, aadA1, sulI, sulII, tetA, tetB*	1.0kb
S43 [c]	Typhim.	Chicken	Amo-Amp-Cef-Cep-Fox-Str-Sul-Tet	*bla_{CMY2}, aadA1, sulI, tetB*	
1290 [b]	Typhim. DT208	Chicken	Amo-Amp-Cef-Cet-Cep-Cml-Fox-Gen-Kan-Str-Sul-Tet	*bla_{CMY2}, bla_{TEM1}, flo-1, aadA1, sulII, tetA, tetB*	2.7kb
1291 [b]	Typhim. DT208	Chicken	Amo-Amp-Cef-Cep-Cet-Cml-Fox-Gen-Kan-Str-Sul-Tet	*bla_{CMY2}, bla_{TEM1}, flo-1, aadA1, sulII, tetA*	2.7kb
CHS34 [d]	Derby	Pork	Amp-Cml-Tri-Sul-Tet	*bla_{TEM1},cat1, cat2, dhfr1, sulI, tetA*	1.5kb
CHS36[d], CHS38 [d]	Derby	Beef	Amp-Cml-Str-Tri-Sul-Tet	*bla_{TEM1}, cat1, cat2, aadA1, dhfr1, sulI, tetA*	1.5kb
CHS32 [d]	Derby	Pork	Amp-Cml-Kan-Nal-Str-Tet	*bla_{TEM1}, cat2, aph(3)-IIA, aadA1, sulI, tetA*	
CHS5c [d]	Enteritidis	Chicken	Amp-Str-Sul-Tet	*bla_{TEM1}, aadA1, sulII, tetA*	
CHS14 [d]	Enteritidis	Chicken	Nal-Str-Sul-Tet	*aadA1, sulI, sulII, tetA*	
CHS45 [d]	Enteritidis	Chicken	Amp-Cml-Kan-Nal-Str-Tet	*bla_{TEM1}, cat2, aph(3)-IIA, tetA*	
CHS43	Haardt	Chicken	Amp-Cml-Kan-Nal-Str-Tet	*bla_{TEM1}, cat2, aadA1, aph(3)-IIA, tetA*	1.5kb
CHS31 [d]	Typhim.	Beef	Amp-Cef-Cml-Gen-Kan-Nal-Str-Tri-Sul-Tet	*bla_{TEM1}, cat1, cat2, aadA2, aadA1, aac(3)-IVA, aacC2, dhfr12, dhfr13, sulII, tetA, tetB*	2.0kb
CHS46 [d]	Non-typable	Chicken	Amp-Cml-Kan-Nal-Str-Tet	*bla_{TEM1}, cat2, aadA1, aph(3)-IIA, tetA*	

a. Amo, amoxicillin / clavulanic acid; Amp, ampicillin; Cef, ceftiofur; Cet, ceftriaxone; Cep, cephalothin; Fox, cefoxitin; Cml, chloramphenicol; Gen, gentamicin; Kan, kanamycin; Nal, nalidixic acid; Str, streptomycin; Sul, sulfamethoxazole; Tet, tetracycline; Tri, trimethoprim / sulfamethoxazole. b. *Salmonella* isolated in US from Jun. to Spt. 1998. c. *Salmonella* isolated in US from Aug. 1999 to Aug.2000. d. *Salmonella* isolated in China from Oct. 1999 to Dec. 2000

aph(3)-IIa gene conferring resistanct to kanamycin were detected in *Salmonlella* .isolates from China.

Three kinds of β-lactamase genes were detected in the *Salmonella* isolates. The $bla_{\text{CMY-2}}$ gene was detected in 10 ESBL-resistant *Salmonella*, 5 of which also contained a $bla_{\text{TEM-1}}$ like gene. Each of the nine ampicillin-resistant isolates from China contained a $bla_{\text{TEM-1}}$ like gene. Consistent with previous findings (113), the $bla_{\text{PSE-1,}}$ which was located in a 1.0 kb class 1 integron, was amplified in each of two *S.* Typhimurium DT104 isolates with the ACSSuT antibiogram (Table 3-3).

Six integron amplicons, with sizes of 0.75kb, 1kb, 1.2kb, 1.5kb, 2.0kb and 2.7kb, were detected in 16 (54%) of the 30 *Salmonella* isolates (Table 3-3). The most common antimicrobial resistance genes carried by these integrons were *aadA*1 and *aadA*2 conferring resistance to streptomycin, and *dhfr*XII conferring resistance to trimethoprim. A 2.7kb integron in two *S.* Typhimurium DT208 isolates had an *aadA* gene as well as a 1.2kb gene of unknown function (GenBank accession # AY204504). A protein BLAST search revealed that the 1.2kb open reading frame (ORF) shared 56% amino acid homology with a reverse transcriptase from *Serratia marcescens*. No change in antimicrobial susceptibility was observed when this ORF was over-expressed as a cloned copy in *E. coli* (data not shown).

Conjugative transfer of resistance genes. Each of the 10 *Salmonella* isolates from retail meats purchased in the Washington D.C. area transferred their plasmids to *E. coli* at rates ranging from 6.0×10^{-8} to 2.4×10^{-4} transconjugants per recipient cell. Examples of the conjugation study results are shown in Table 4. However, it is important to note that because the experiments were done with an excess of donor to recipient cells,

the efficiency of conjugation may have been constrained by the number recipient cells. Transconjugants 1083/1003 and 1290/1003 acquired resistance to 9 and 11 of the antimicrobial agents tested, respectively. Transfer of $bla_{\text{CMY-2}}$ and $bla_{\text{TEM-1}}$ -like genes to the recipient *E. coli* strain was confirmed by PCR assay. Because antimicrobial resistance genes specifying the ACSSuT resistance phenotype have integrated into the *Salmonella* chromosome (22, 24), the two *S.* Typhimurium DT104 isolates did not transfer this phenotype to the *E. coli* recipient strain (Table 3-4). One of four *Salmonella* from China transferred the ampicillin resistance phenotype to *E. coli* 1016. The transfer of other resistance phenotypes could not be measured because *E. coli* 1016 had these phenotypes prior to the conjugation experiment (Table 3-4).

Discussion

In this study, we examined *Salmonella* isolates recovered from retail meats purchased in the United States and China for antimicrobial susceptibility phenotypes and genotypes. In general, the findings are similar to those described in previous studies, showing that *Salmonella* in retail meats were commonly resistant to multiple antimicrobials, including tetracycline, sulfamethoxazole, and streptomycin (120, 231). Our findings also showed that frequencies of antimicrobial resistance among *Salmonella* isolated from retail meats purchased in China were lower compared to those from the United States. Further studies involving larger sample sizes are required to more precisely determine if differences in antimicrobial resistance exist between *Salmonella* from the two countries.

Table 3-4. Antimicrobial susceptibility profiles of donors, recipients, and transconjugants in the conjugation experiments[a]

Strains	Designation	MIC (μg/ml)															Conj.	Resis.
		Fox	Cml	Tet	Cet	Amo	Cip	Gen	Nal	Cef	Sul	Cep	Tri	Kan	Amp	Str	Rate	Genes
E. coli (1003)	Recipient	4*	8	< 4	<0.25	1/0.5	**>4**	0.5	**>32**	0.25	16	8	0.12	<16	2	32		
1083[b]	Donor	**>16**	< 4	**>32**	**>64**	**>32/16**	< 0.01	1	<4	**8**	**>512**	**>32**	**>4**	<16	**>32**	**>64**		*bla*$_{CMY-2,}$ *bla*$_{TEM-1}$
1083/1003	Transconjugant	**>16**	8	**>32**	16	**>32/16**	**>4**	1	**>32**	**8**	**>512**	**>32**	**>4**	<16	**>32**	**>64**	2.4 x 10^{-4}	*bla*$_{CMY-2}$ *bla*$_{TEM-1}$
DT208[c] (1290)	Donor	**>16**	**>32**	**>32**	32	**>32/16**	< 0.01	**16**	<4	**>8**	**>512**	**>32**	<0.12	**>64**	**>32**	**>64**		*bla*$_{CMY-2,}$ *bla*$_{TEM-1}$
1290/1003	Transconjugant	**>16**	**>32**	**>32**	32	**>32/16**	**>4**	**16**	**>32**	**>8**	**>512**	**>32**	<0.12	**>64**	**>32**	**>64**	6.0 x 10^{-8}	*bla*$_{CMY-2}$ *bla*$_{TEM-1}$
DT104[c] (1275)	Donor	< 0.5	**>32**	**32**	<0.25	16/8	< 0.01	< 0.25	<4	0.25	**>512**	4	0.25	<16	**>32**	**>64**		
1275/1003	Transconjugant	2	2	< 4	<0.25	16/8	**4**	0.5	**>32**	0.25	**>512**	2	0.25	<16	2	**>64**	6.0 x10^{-8}	
DT104[c] (S21)	Donor	2	**>32**	**>32**	<0.25	16/8	< 0.01	< 0.25	<4	0.25	**>512**	4	<0.12	<16	**>32**	**>64**		
S21/1003	Transconjugant	2	2	< 4	<0.25	16/8	4	0.5	>32	0.25	16	2	<0.12	<16	2	**>64**	6.0 x 10^{-8}	
E. coli (1016)	recipient	4	**>32**	**>32**	<0.25	1/5	0.25	**>16**	**>32**	0.25	**>512**	8	**>4**	**>64**	4	**>64**		
CHS5[d]	Donor	2	4	**>32**	<0.25	8/4	< 0.01	< 0.25	<4	0.25	**>512**	8	0.25	<16	**>32**	**>64**		*bla*$_{TEM-1}$
CHS5/1016	Transconjugant	4	**>32**	**>32**	<0.25	8/4	0.25	**>16**	**>32**	0.25	**>512**	16	**>4**	**>64**	**>32**	**>64**	8.0 x 10^{-5}	*bla*$_{TEM-1}$

a. Amp, ampicillin; Cef, ceftiofur; Cet, ceftriaxone; Cep, cephalothin; Cip, ciprofloxacin; Fox, cefoxitin; Cml, chloramphenicol; Gen, gentamicin; Kan, kanamycin; Nal, nalidixic acid; Str, streptomycin; Sul, sulfamethoxazole; Tet, tetracycline; Tri, trimethoprim / sulfamethoxazole. Resistance is indicated by boldface values. b. *Salmonella* Agona. c. *Salmonella* Typhimurium. d. *Salmonella* Enteritidis. *, MIC (μg/ml).

Resistance to ceftriaxone is a concern because of its importance for the treatment of salmonellosis in children. Ceftriaxone resistance in *Salmonella* is largely due to the AmpC β-lactamase (bla_{CMY-2}) gene and has been increasingly reported in the United States (54, 231, 234). Strains of *Salmonella* carrying bla_{CMY-2} were first isolated from humans, animal, and food samples in the United States in 1996 (54, 245) . In this study, 19% of *Salmonella* isolates from retail meats purchased in the United States were resistant or intermediately susceptible to ceftriaxone and harbored the bla_{CMY-2} gene. Conversely, each of the *Salmonella* isolates from China was susceptible to ceftriaxone (and other cephalosporins), and none harbored bla_{CMY-2}. A possible explanation for this observation is that ceftriaxone-resistant *Salmonella* in meats have arisen due to cross-resistance between ceftriaxone and ceftiofur, a cephalosporin used in food animals (205, 234). Ceftiofur, the only cephalosporin approved for therapeutic use in cattle, has been approved for use in the United States since 1988 whereas it was approved for use in China in 2002 (www.agri.gov.cn/blgg/t20021219_36976.htm).

Quinolones and fluoroquinolones have been used in veterinary medicine in China since the 1980s. In contrast, they were not approved for therapeutic use in animals in the United States until 1995. The differences in fluoroquinolone susceptibility between the US and China isolates likely reflect the different approval dates in the two countries. Thirty-two of the *Salmonella* isolates from China were resistant to nalidixic acid and displayed increased MICs for ciprofloxacin, while each of isolates from the United States was susceptible to these drugs. Nevertheless, the relatively high frequency of increased MICs for ciprofloxacin among the isolates from China warrants continued surveillance to detect emerging ciprofloxacin-resistant phenotypes.

Two *S.* Typhimurium DT104 strains (1275 and S21) isolated from pork within a one-year span in the Washington DC area displayed highly similar antimicrobial resistance phenotypes, genotypes, and PFGE patterns. Both of these isolates had the classical ACSSuT resistance phentoype, and accordingly, were found to contain the $bla_{\text{PSE-1}}$, *flo-1*, *aadA2*, *sulI*, and *tetA* genes. These genes are known constituents of the *Salmonella* genomic island 1 (SGI1) multi-drug resistance (MDR) region (22, 144). In addition, three more resistance genes, *sulII*, *aadA1*, and *tetB*, were detected in these isolates, suggesting *Salmonella* may contain multiple genes that specify resistance to similar drugs (5, 9). In DT104, the resistance genes known as constituents of SGI1 were not transferred to *E. coli*, whereas the *aadA1* gene specifying the streptomycin-resistant determinant is encoded in a conjugal plasmid, which can be transferred to *E. coli* by conjugation. In contrast to *S.* Typhimurium DT104, most of the antimicrobial resistance determinants in other *Salmonella* isolates were encoded in a transferable plasmid and could be transferred to *E. coli* by conjugation. Furthermore, the molecular mechanisms of antimicrobial resistance in these isolates were also different from SGI1 MDR in *S.* Typhimurium DT104. The reason for widespread dissemination of SGI1 MDR among *S.* Typhymurium DT104 is at present unclear.

Most of the resistance genes, including $bla_{\text{CMY-2}}$ and those contained in integrons, were located on plasmids in the *Salmonella* isolates in this study. Plasmids carrying the $bla_{\text{CMY-2}}$ resistance were readily transferred under the selective pressure of β-lactam antibiotics; they were also co-transferred by selection with other antibiotics on the same plasmid (e.g., streptomycin). The *E. coli* recipient cells acquired 9 to 11 antimicrobial resistance phenotypes by receiving the plasmid from *S.* Agona and *S.* Typhimurium

DT208 via conjugation. These findings indicated that conjugal plasmids play a significant role in the dissemination of multiple-antimicrobial- resistant bacteria.

Better understanding the molecular mechanisms by which antimicrobial resistance emerges and spreads should enable us in the future to design intervention strategies to reduce its progression. Because antimicrobial-resistant bacteria may be transferred to humans through the food chain (203, 236), selection of novel antimicrobial resistance mechanisms in *Salmonella* in animals (203), which specify resistance to antibiotics used in humans, is troubling. Efforts including further implementation of HACCP programs in food production are needed to reduce the incidence of *Salmonella* in food. The judicious use of antibiotics, including cephalosporins and fluoroquinolones in food animals, is also critical to control the rapid spread of antimicrobial-resistant bacteria.

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Chapter 4 Identification of Virulence and Antimicrobial-Resistant Genes in *Salmonella* and *Escherichia coli* using Microarray Analysis

Abstract

Characterization of antimicrobial resistance and virulence gene profiles provides important information of potential pathogenicity of bacterial pathogens. This information can in many instances be used to facilitate effective treatment of bacterial diseases. We developed and tested a PCR-based microarray analysis for detection of antimicrobial resistance and virulence genes in *Salmonella* and *Escherichia coli*. Twelve *Salmonella* and seven *E. coli* isolates were screened for the presence of 23 antimicrobial-resistance genes and 25 virulence genes. Consistency between antimicrobial resistance phenotype and genotype was observed for each isolate. All isolates exhibiting resistance to third generation cephalosporins harbored bla_{CMY-2} and bla_{TEM-1}. The *aadA*, *tetA*, and *sulI* genes were most commonly detected in bacteria resistant to streptomycin, tetracycline and sulfonamide, respectively. All *S.* Typhimurium DT104 isolates harbored virulence plasmids. *E. coli* O157 isolates harbored virulence genes typical of enterohemorrhagic *E. coli* (EHEC); serotype O126 isolates harbored virulence genes typical to enteropathogenic *E. coli* (EPEC); and serotype O111, O78 and O147 isolates harbored virulence genes typical of enterotoxigenic *E. coli* (ETEC). Microarray analysis is an effective method to rapidly screen pathogenic strains of *Salmonella* and *E. coli* for multiple antimicrobial resistance and virulence genes.

Introduction

Salmonella spp. and diarrheagenic *Escherichia coli* are important causes of food-borne diseases in the United States (128). The virulence of the former are mediated by gene clusters, including *Salmonella* pathogenicity islands SPI1, SPI2 and SPI5 (5), and by virulence plasmids, which facilitate infection of deep tissue (75, 76). In *E.* coli, virulence mechanisms are encoded by various chromosomal and plasmid-borne genes, products of which include the heat-labile and heat-stable toxins (STs), Shiga toxin (Stx1 and Stx2), enterohemolysin, intestinal adherence factors, and pili (39, 137). These virulence factors are reliable indicators of bacterial pathogenesis and, as such, are valuable markers that can characterize clinical isolates (17, 137). PCR assays provide a relatively rapid screen for virulence factors, and they can be used with a variety of samples (38, 150, 188). Further, multiplex PCRs have been devised for detection of more than one gene (58, 220). The drawback of these and similar assays, however, is that they are for the most part limited in the number of genes that can be reasonably screened.

Characterization of antimicrobial resistance genes is also a powerful tool for describing bacterial pathogens. For instance, it can be used to investigate the population dynamics of clinical isolates, including the spatial and temporal distribution of resistance genes (33, 132). Various methods, including DNA sequencing, DNA-DNA hybridization, and PCR arrays, have been used to characterize antimicrobial resistance genes in bacteria (21, 33, 52, 132). Variant *Salmonella* genomic island multi-drug resistance (MDR regions), have been used to characterize clinical isolates

belonging to *Salmonella* serovars Typhimurium DT104 and Agona (21). We recently used PCR technology to screen multiple-antimicrobial-resistant *Salmonella* from retail meats for the presence of 61 different resistance genes (33). Nevertheless, while these methods provide valuable genotypic information, they are time-consuming, expensive, and otherwise ill-suited for analyzing a large number of genes from multiple samples.

DNA microarrays offer a viable alternative to PCR for genotypic characterization of bacteria. Microarrays have been used for detection of antimicrobial resistance genes (26, 227), characterization of virulence and pathogenecity, species determination (35, 100), genome comparisons and detection of genetic polymorphisms (51, 91, 124). Prototype microarrays have been developed to detect erythromycin, tetracycline, and rifampin resistance determinants in clinical isolates (26, 195, 218), as well as virulence factors (35, 227). We describe a PCR product-based DNA microarray for characterization of antimicrobial resistance and virulence genes in *Salmonella* and diarrheagenic *E. coli*.

Materials and methods

Bacterial isolates Isolates investigated in this study are shown in Table 4-1. All isolates were recovered from retail ground meat and food animals using methods described in the U.S. Food and Drug Administration Bacteriological Analytical Manual (1). The isolates were further identified with API identification kits (BioMerieux, Marcy, France) and serotyped with commercial antiserum (Difco, Detroit) according to manufacturers' instructions. Susceptibilities to seventeen antimicrobials used in the

Table 4-1. Bacterial strains, serotypes, antimicrobial resistance profile and their gene hybridizations to microarray

ID	Serotype	Antimicrobial Resistance Profiles	Antimicrobial Resistance Genes Hybridized	Virulence Genes Hybridized
S01	Agona	Amp-Amo-Cef-Cet-Cep-Fox-Str-Tri-Sul-Tet	*bla$_{CMY-2}$, bla$_{TEM-1}$, bla$_{PSE-1}$, aadA, aac(3)-IVA, tetA, dhfr1, sulI, sulII, Int, QacA*	*sipA, sipB, sipC, invA, sop, fliC, pagC, ent*
S02	Agona	Amp-Amo-Cef-Cet-Cep-Fox-Str-Tri-Sul-Tet	*bla$_{CMY-2}$, bla$_{TEM}$-1blaa$_{PSE-1}$, AADa, aac(3)-IVA, tetA, dhfr1, sulI, sulII, Int, QacA*	*sipA, sipB, sipC, invA, sop, fliC, pagC, ent*
S03	Thphimurium DT208	Amp-Amo-Cef-Cet-Cep-Fox-Cml-Gen-Kan-Str-Sul-Tet	*bla$_{CMY-2}$, bla$_{TEM-1}$, aadA, aac(3)-IVA, tetA, tetB, flo, sulI, Int, QacA*	*sipA, sipB, sipC, invA, sop, fliC, pagC, ent*
S04	Thphimurium DT208	Amp-Amo-Cef-Cet-Cep-Fox-Cml-Gen-Kan-Str-Sul-Tet	*bla$_{CMY-2}$, blaPSE-1, aadA, aac(3)-IVA, tetA, flo, sulI, Int, QacA*	*sipA, sipB, sipC, invA, sop, fliC, pagC, ent*
S05	Thphimurium DT104	Amp-Cml-Str-Sul-Tet	*bla$_{PSE-1}$, aadA, aac(3)-IVA, tetA, tetB, flo, cmlA, sulI, sulII, Int, QacA*	*spvR, spvC, sipA, sipB, SipC, invA, sop, fliC, pagC, ent*
S06	Typhimurium DT104	Amp-Cml-Str-Sul-Tet	*bla$_{PSE-1}$, aadA, aac(3)-IVA, flo, tetA, tetB, sulI, sulI, Int, QacA*	*spvR, spvC, sipA, sipB, SipC, invA, sop, fliC, pagC, ent*
S07	Typhimurium DT104	Amp-Cef-Cml-Nal-Gen-Kan-Str-Tri-Sul-Tet	*bla$_{TEM-1}$, bla$_{PSE-1}$, aadA, aac(3)-IVA, aacC2, tetA, tetB, cat1, cat2, dhfr12, dhfr13, sulII, Int, QacA*	*spvR, spvC, sipA, sipB, SipC, invA, sop, fliC, pagC, ent*
S08	Typhimurium	Amp-Fox-Cef-Cep-Amo-Str-Sul-Tet	*bla$_{CMY-2}$, bla$_{TEM-1}$, aadA, aac(3)-IVA, tetA, tetB, flo, sulI, Int, QacA*	*sipA, sipB, sipC, invA, sop, fliC, pagC, ent*
S09	Heidelberg	Kan-Str-Sul-Tet	*bla$_{PSE-1}$, bla$_{TEM-1}$, aadA, aphA, tetB, sulI, Int, QacA*	*spvR, spvC, sipA, sipB, SipC, invA, sop, fliC, pagC, ent*

S10	Infantis	Amp-Fox-Cef-Cep-Amo	*bla$_{CMY-2}$, bla$_{PSE-1}$, aac(3)-IVA, aacC2, sulI, Int, QacA*	*sipA, sipB, sipC, invA, sop, fliC, pagC, ent*
S11	Untypable	Amp-Cml-Nal-Kan-Str-Tet	*bla$_{TEM-1}$, bla$_{PSE-1}$, aadA, aac(3)-IVA, aphA, tetA, cmlA, sulI, Int, QacA*	*sipA, sipB, sipC, invA, sop, fliC, pagC, ent*
S12	Haardt	Amp-Cml-Nal-Kan-Str-Tet	*bla$_{TEM-1}$, aadA, aac(3)-IVA, tetA, cat2, Int, QacA*	*sipA, sipB, sipC, invA, sop, fliC, pagC, ent*
E01	O157	Cef-Cml-Nal	*aac(3)-IvA, cmlA*	*VT1, VT2, hlyA, fliC, rfbE, eafP*
E02	O111	Amp-Fox-Cef-Cep-Amo-Cml-Gen-Str-Tri-Sul-Tet	*bla$_{CMY-2}$, bla$_{TEM-1}$, bla$_{PSE-1}$, aadA, aac(3)-IVA, aacC2, aphA, cmlA, sulII, Int, Qac,*	*LT, Sta, VT1, hlyA,*
E03	O111	Amp-Fox-Cef-Cep-Amo-Cml-Gen-Str-Tri-Sul-Tet	*bla$_{CMY-2}$, bla$_{TEM-1}$, bla$_{PSE-1}$, aadA, aac(3)-IVA, aacC2, aphA, cmlA, sulII, Int, Qac,*	*LT, ST, VT1, hlyA,*
E04	O126	Amp-Cep-Cml-Gen-Str-Sul-Tet	*bla$_{TEM-1}$, aadA, aac(3)-IVA, cat1, tetA, sulII, Int, QacA*	*eafP, bfp, astA,*
E05	O78	Amp-Cml-Cep-Gen-Nal-Cip-Str-Tri-Tet	*bla$_{TEM-1}$, bla$_{PSE-1}$, aadA, aac(3)-IVA, aacC2, tetA, , cmlA, dhfr13, sulII, Int, QacA*	*LT, Sta, VT1, hlyA, fliC,*
E06	O78	Amp-Cml-Cep-Gen-Nal-Cip-Str-Tri-Tet	*bla$_{TEM-1}$, blapse-1, aadA, aac(3)-IVA, tetA, cmlA, dhfr13, sulII*	*LT, Sta, astA,*
E07	O147	Cml-Gen	*aac(3)-IVA ,cmlA, Int, QacA*	*LT, Sta*

S, *Salmonella*; E, *E. coli*; Amo, Amoxicillin / Clavulanic acid; Amp, Ampicillin; Cef, Ceftiofur; Cet, Ceftriaxone; Cep, Cephalothin; Fox, cefoxitin Cml, chloramphenicol; Gen, Gentamicin; Kan, Kanamycin; Str, Streptomycin; Sul, Sulfamethoxazole; Tet, Tetracycline; Tri, Trimethoprim / Sulfamethoxazole Cip, cipro*flo*xacin

National Antimicrobial Resistance Monitoring System (NARMS) were determined via National Committee for Clinical Laboratory Standards (NCCLS) guidelines for broth micro-dilution (139, 141). All susceptibility assays were done with the Sensititre Automated Antimicrobial Susceptibility System (Trek Diagnostic Systems, Westlake, Ohio).

Microchip design and construction The design of the microchips used in this study is diagrammed in Figure 4-1. Genes included on the microchip are listed in Table 4-2. Three general classes of genes were arranged on the microchip: (i) antimicrobial resistance genes, (ii) *Salmonella* virulence genes, and (iii) *E. coli* virulence genes. All these genes were amplified by PCR using forty-seven different primer sets targeting to 23 antimicrobial resistance genes conferring resistance to: (i) β-lactams, (ii) aminoglycosides, (iii) chloramphenicols, (iv) tetracycline, (v) trimethoprim, (vi) macrolides, and (vii) sulfonamides , 12 *E. coli* virulence genes, 10 *Salmonella* virulence genes, and two positive control genes (*E. coli* and *Salmonella gyrA* gene). Briefly, PCR was performed in a total volume of 50 µl distilled H_2O with 0.25mM of each deoxyribonucleotide, 1.5mM $MgCl_2$, 0.2 U Gold *Taq* DNA polymerase, and 50 pmol each primer. The temperature profile included an initial template denaturation step at 95ºC for 10 min, followed by 30 cycles of 95ºC for 30 s, 55ºC for 1 min, and 72ºC for 1 min, and a final step at 72ºC for 7 min (8). The presence of class I integrons among the 30 *Salmonella* isolates was determined by PCR using primers 5’CS (5'-GGCATCCAAGCACAAGC-3') and 3’-CS (5'-AAGCAGACTTGACTGAT-3') as previously described (244). All PCR products were purified with High PCR Purification kits (Roche) and sequenced at the Center of Agriculture Biotechnology, University of

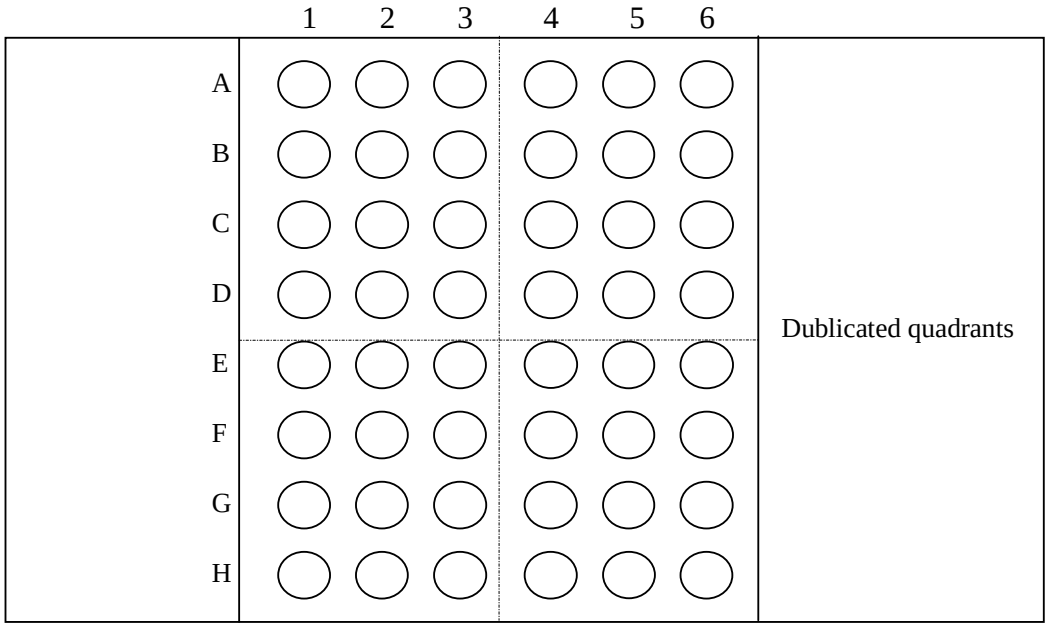


Fig 4-1. Microchip design and construction. Each circle on the microchip represents one gene with four repeated spots. Genes included on the microchip are listed in Table 2. All genes were arranged into four distinct quadrants and these four quadrants were duplicated on the slides. The left two quadrants (Column 1-3, row A-F) contained antimicrobial resistance genes and the right up quadrant (Column 4-6, row A-D)contained *E. coli* virulence genes and right down quadrant (Column 4-6, row E-H) contained *Salmonella* virulence genes.

Table 4-2. Microarray design, gene location, gene functions and the primer sequence of antimicrobial-resistant and virulence genes

GL	Genes	Description	Oligonucleotide sequence	
1A	*bla*$_{TEM-1}$	ß-lactamase gene	CAG CGG TAA GAT CCT TGA GA	ACT CCC CGT CGT GTA GAT AA
2A	*bla*$_{CMY-2}$	cephasporine resistant gene	TGG CCG TTG CCG TTA TCT AC	CCC GTT TTA TGC ACC CAT GA
3A	*bla*$_{PSE-1}$	ß -lactamase gene	TGC TTC GCA ACT ATG ACT AC	AGC CTG TGT TTG AGC TAG AT
1B	*aadA*	striptomycin resistance gene	CGC CGA AGT ATC GAC TCA AC	GCG GGA CAA CGT AAG CAC TA
2B	*aac(3)-IVA*	Gentamicin resistance gene	GAT GGG CCA CCT GGA CTG AT	GCG CTC ACA GCA GTG GTC AT
3B	*aacC2*	Gentamicin resistance gene	GGCAATAACGGAGGCAATTCG A	CTCGATGGCGACCGAGCTTCA
1C	*S gyrA*	Positive control	ACGTATTGGGCAATGACTGG	GGAGTCGCCGTCAATAGAAC
2C	*E gyrA*	Positive control	ACGTACTAGGCAATGACTGG	AGAAGTCGCCGTCGATAGA
3C	*aph(3)IA*	Kanamycin resistance gene	TCT CGG AGG GCG AAG AAT CT	TTG CCG TCA ACC AAG CTC TG
1D	*kn*	Kanamycin resistance gene	ACTGGCTGCTATTGGGCGA	CGTCAAGAAGGCGATAGAAGG
2D	*tetA*	Tetracyclinec resistance gene	GCG CCT TTC CTT TGG GTT CT	CCA CCC GTT CCA CGT TGT TA
3D	*tetB*	Tetracycline resistance gene	CCC AGT GCT GTT GTT GTC AT	CCA CCA CCA GCC AAT AAA AT
1E	*flo*	Chloramphenicol resistance gene	CTG AGG GTG TCG TCA TCT AC	GCT CCG ACA ATG CTG ACT AT
2E	*cmlA*	Chloramphenicol resistance gene	CGC CAC GGT GTT GTT GTT AT	GCG ACC TGC GTA AAT GTC AC
3E	*cat1*	Chloramphenicol resistance gene	CTT GTC GCC TTG CGT ATA AT	ATC CCA ATG GCA TCG TAA AG

1F	*cat2*	Chloramphenicol resistance gene	CTG AGG GTG TCG TCA TCT AC	GCT CCG ACA ATG CTG ACT AT
2F	*dhfr1*	Trimethoprime resistance gene	CGG TCG TAA CAC GTT CAA GT	CTG GGG ATT TCA GGA AAG TA
3F	*dhfr12*	Trimethoprime resistance gene	AAA TTC CGG GTG AGC AGA AG	CCC GTT GAC GGA ATG GTT AG
1G	*dgfr13*	Trimethoprime resistance gene	GCA GTC GCC CTA AAA CAA AG	GAT ACG TGT GAC AGC GTT GA
2G	*dhfr16*	Trimethoprime resistance gene	GCT CTC CCA AAT CGA AAG TA	ATT GCA GGC GCT TGT TAA CT
3G	*sulI*	Sulfornamide resistance gene	TCA CCG AGG ACT CCT TCT TC	CAG TCC GCC TCA GCA ATA TC
1H	*sulII*	Sulfornamide resistance gene	CCT GTT TCG TCC GAC ACA GA	GAA GCG CAG CCG CAA TTC AT
2H	*int*	Intergrase gene	GCGCTGAAAGGTCTGGTCAT	GGCGGCCTTGCTGTTCTTCT
3H	*qac*	Detergent resistance gene	TCTAGCGAGGGCTTTACTAA	AGGAGTCCTCGGTGAGATTC
1A	*LT*	Heat-liable toxin	TGGATTCATCATGCACCACAAG G	CCATTTCTCTTTTGCCTGCCATC
2A	*sta*	Heat-stable toxin a	TTTCCCCTCTTTTAGTCAGTCA ACTG	GGCAGGATTACAACAAAGTTCAC AG
3A	*stb*	Heat-stable toxin b	CCCCCTCTCTTTTGCACTTCTTT CC	TGCTCCAGCAGTACCATCTCTAAC CC
4B	*VT1*	Verotoxin 1	ACGTTACAGCGTGTTGCTGGGA TC	TTGCCACAGACTGCGTCAGTTAG G
5B	*VT2*	Verotoxin 2	TGTGGCTGGGTTCGTTAATACG GC	TCCGTTGTCATGGAAACCGTTGTC
6B	*hlyA*	hemolysin	GGTGCAGCAGAAAAAGTTGTA G	TCTCGCCTGATAGTGTTTGGTA
4C	*fliC*	H7 antigen	GCGCTGTCGAGTTCTATCGAGC	CAACGGTGACTTATCGCCATTCC

5C	*rfbE*	Probable perosamine synthetase	TGTCCATTTATACGGACATCCATG	CCTATAACGTCATGCCAATATTGCC
6C	*eae*	Attaching and effacing	GGCGAATTCCGCATGAGCGGCTG	ATTGAATTCATAGGCGCGAGCCGTCAC
4D	*eafP*	Attaching and effacing plasmid	CAGGGTAAAAGAAAGATGATAA	TATGGGGACCATGTATTATCA
5D	*bfp*	Bundle-formingpilus	AATGGTGCTTGCGCTTGCTGC	GCCGCTTTATCCAACCTGGTA
6D	*astA*	EAST1, homologous to ST	GGGCGAAGTTCTGGCTCAAT	GCGGATGTCCGTTGGATAAG
4E	*spvR*	Virulence plasmid	CGGAAGAATGGCACTCTTAT	GAATGCAGGGTTGGTAATAC
5E	*spvC*	Virulence plasmid	ACTCCTTGCACAACCAAATGCGGA	TGTCTTCTGCATTTCGCCACCATCA
6E	*sipA*	SPI1 component	TACCCCTGCTGCTACGTAAT	CTCCAGGGCTTTACGTATCA
4F	*sipB*	SPI1 component	TGGCAGGCGATGATTGAGTC	CCCATAATGCGGTTCGTTTC
5F	*SipC*	SPI1 component	TGCCCTGGCAAATAATGTCA	CATCGATTCGGGTCATATCC
6F	*invA*	Invasion gene SPI1	ACAGTGCTCGTTTACGACCTGAAT	AGACGACTGGTACTGATCGATAAT
4G	*sop*	SPI5 component	GCTCGCCCGGAAATTATTGT	CCCGCTAAGGCTTTGTTAAG
5G	*fliC*	Flagella filament protein	TACGCTGAATGTGCAACAAA	TACCGTCATCTGCAGTGTAT
6G	*pagC*	Survivor promoter	TGTTGCACAGGCCGATACTA	GCAAATCCCGTTTTCCTTGA
4H	*mpha*	Macrolide resistance gene	AGC CCC TCT TCA CCA AAG AC	GCC GAT ACC TCC CAA CTG TA
5H	*ent*	*Salmonella* entrotoxin	CATCGCACAGTACCAGATCA	GATGCCCAAAGCAGAGAGAT
6H	*negative*	negative control		

* Gene Location in microchips.

Maryland, College Park, MD. Resultant DNA sequence data were compared to data in the GenBank database using the BLAST algorithm (7) via the National Center of Biotechnology Information's web site (www.ncbi.nlm.nih.gov).

PCR products with the sizes ranging from 400 ~ 800bp were then quantified and diluted to a final concentration of 10 mg/ml after which 50 ul of each product were transferred to 96-well plates and printed onto CAST slides (Schleicher & Schuell, Keene, NH) using the Microcaster MicroArray system (Schleicher & Schuell). Antimicrobial resistance genes were spotted in duplicate on the left-most two quadrants of the chips and virulence genes from *Salmonella* and *E. coli* were spotted in duplicate on the two right-most.The average spot size was 500 µm. Printed slides were put on a sheet of GB002 paper saturated with 0.4N NaOH/3M NaCl (to denature the DNA) and neutralized by placement on GB002 paper saturated with 0.5M Tris/1.5M NaCl. The slides were cross-linked to be immobilized under 20 mjoules/cm^2 UV light using GS GENE LINKER^{TM} UV Chamber (Bio-Rad). They were then dipped in distilled water for 5 min, dried at room temperature, and stored at 4^{o}C until further use.

Detection of antimicrobiał resistant and virulence genes using microchip

Chromosomal and plasmid DNA from *Salmonella* and *E. coli* were isolated using Wizard Genomic DNA Purification kits (Promega, Madison WI) and High Plasmid Purification kits (Roche, Indianapolis IN), respectively. Chromosomal and plasmid DNA were randomly amplified and labeled with digoxigenin (DIG) using the DIG-High Prime Kit (Roche) according to manufacturer's instructions.

Labeled DNA was hybridized to microchips using a microhybridization kit (Schleicher & Schuell) according to manufacture's instructions. Hybridized

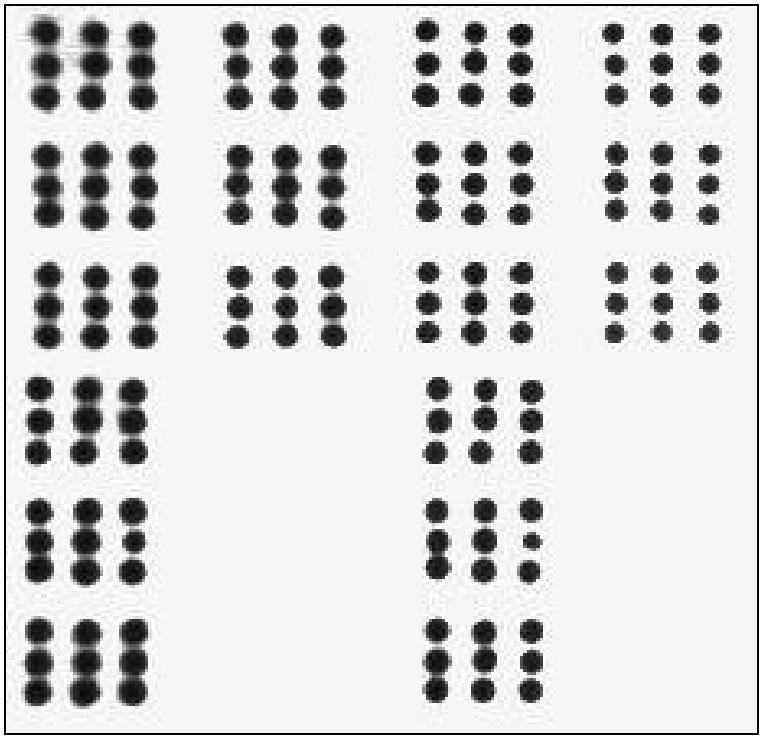
microchips were then detected using the DIG Nucleic Acid Detection Kit (Roche). Briefly, after three post-hybridization washes, microchips were incubated in 100 ml blocking buffer solution at room temperature for 1 h. The microchips were then transferred to 20 ml blocking buffer supplemented with anti-DIG-AP conjugate and incubated for 1 h at room temperature. Next, the microchips were washed in buffer three times for 15 min at 42ºC. Lastly, microchips were incubated in 10 ml detection buffer supplemented with 4 ul AP substrate nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche) for 5 min. Reactions were stopped by washing the slides the slides in distilled water, followed by storage in distilled water for two to three months.

After hybridization, colorimetric images of processed microarrays were generated using the Gel-Doc 1000 system (Bio-Rad). DNA spots with densities that differed from the negative control at a statistical level of $P<0.01$ were considered positive for hybridization.

Experimental controls Six genes, *bla*$_{TEM-1}$, *aadA*, *tetA*, *cat1*, *dhfrI*, and *LT* (Fig 1), which were amplified from *E. coli*CH4, and the *hylA* gene, which could not be amplified from CH4, were printed to CAST slides. All six genes that could be amplified by PCR resulted in positive hybridization. The gene which could not be amplified by PCR resulted in negative hybridization (Fig 4-2).

Results and discussion

Each of the 19 multiple-antimicrobial-resistant bacterial isolates examined here (12 *Salmonella* and 7 *E. coli*) were successfully differentiated by their patterns of hybridization to virulence genes (Fig 4-3A, 4-3B). DNA from each of the isolates



bla$_{TEM-1}$	*aadA*	*tetA*	*cat1*
dhfrI	*hylA*	*LT*	Negative Control

Fig 4-2. Evaluation of the validity of Microarray methodology. The microarray for experimental control was constructed by printing *bla*$_{TEM-1}$, *aadA*, *tetA*, *cat1*, *dhfrI*, and LT genes which were amplified from strain CHA4 and *hlyA* gene which could not be amplified from this isolate onto CAST slides. Each gene has three vertically-arranged repeated areas with nigh spots each area. The arrangement of the genes on slide was seen in the following table. CHA 4 showed positive hybridization to all genes which were successfully amplified in CHA4 by PCR and negative hybridization to *hlyA* which was not amplified by PCR and negative control.

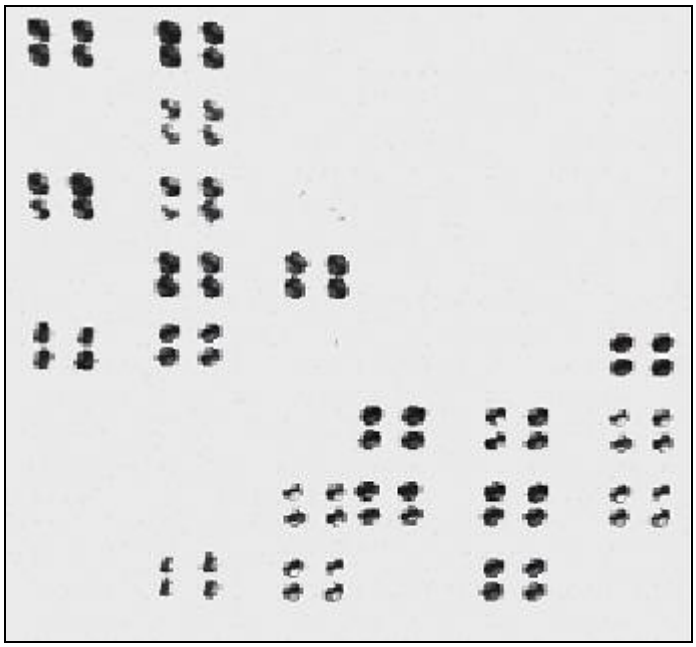


Fig 4-3A. The hybridization of S04 to microarray, The figure presented half of the slides without duplication. The microarray was designed as seen in Fig 1 and table 2. S04 showed positive hybridization to *bla*$_{CMY-2}$, *bla*$_{TEM-1}$, *aac(3)-Iva, Salmonella gyrA, E. coli gyrA, tetA, tetB, flo, cmlA, sulI, int, qacA, sipA, sipB, SipC, invA, sop, fliC, pag, ent.*

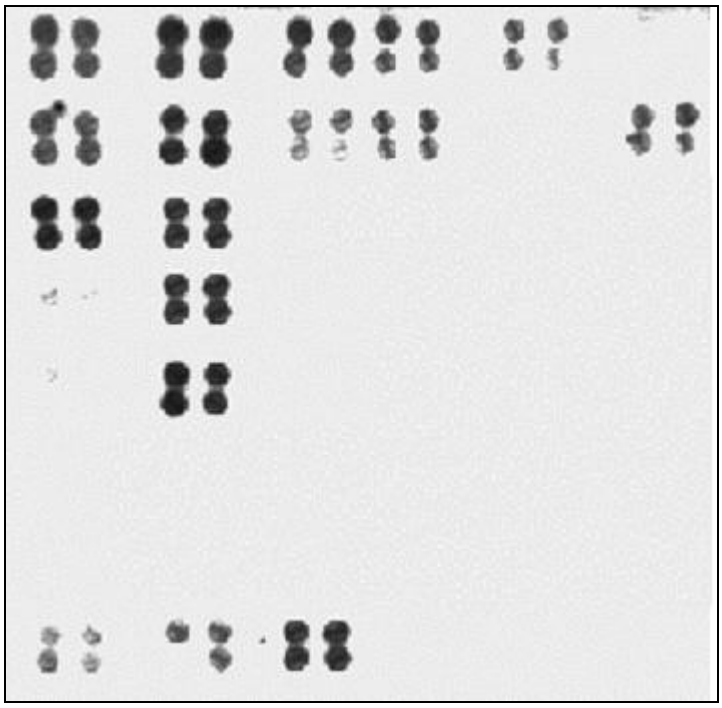


Fig 4-3B. The hybridization of E03 to microarray, The figure showed half of the slides without duplication. The microarray was designed as seen in Fig 1 and table 2. E03 showed positive hybridization to *bla*$_{CMY-2}$, *bla*$_{TEM-1}$, *bla*$_{PSE-1}$, *aadA, AAC(3)-IvA, aacC2, Salmonella gyrA, E. coli gryA, tetA, cmlA, sulII, int, qac, LT, ST, VT1, hlyA,*

positively hybridized to the *gyrA* genes which were positive control for all *Salmonella* and Ecoli isolates. DNA from the *Salmonella* isolates hybridized to *Salmonella*-specific virulence genes whereas DNA from the *E. coli* isolates hybridized to *E. coli*-specific virulence genes (Table 4-1). Four of 12 *Salmonella* isolates, including all of three *S.* Typhimurium DT104 and one *S.* Heidelberg showed positive hybridization to *spvR* and *spvC*These two genes were carried on the *Salmonella* virulence plasmid, which is known to be required for the efficient infection of deep tissues by this pathogen (75, 76). Each of the *Salmonella* isolates identified in this study harbored virulence genes encoding SPI1, SPI5, and other virulence factors such as *pagC*. These genes encoded *Salmonella* major virulence factors which enable *Salmonella* to invade host cells, induce enteropathogenicity (diarrhea), and promote survival in macrophage (4, 179, 199) .

S. Typhimurium DT104 is recognized as a serious public health concern. *S.* Typhimurium DT104 can cause severe illness and is usually resistant to ampicillin, chloramphenicol, streptomycin, sulfornamides, and tetracycline (175). The number of cases of infection with serotype typhimurium DT104 has increased in many countries (27, 95, 121). Each of the three *S.* Typhimurium DT104 isolates identified in this study showed positive hybridization to *spv*C and *spvR* genes of virulence plasmids, whereas none of S.Typhimurium DT208 isolates showed positive hybridization to these genes (Table 4-1). Furthermore, each of the *S.*Typhinurium DT104 isolates were also found to harbor *sipA, sipB, SipC* genes (which are SPI1 components), *invA* (responsible for *Salmonella* invasion of host tissue), *sop* (a component of SPI5), *fliC* (the flagella filament protein), *pagC* (the survivor promoter) and *ent* (*Salmonella* enterotoxin) (5). Although the number of strains studied was relatively few, these results give preliminary evidence suggesting the

presence of virulence genes and plasmids may be a valuable marker for rapid identification of *S.* Typhimurium DT104.

The microarray assay also proved useful for differentiating pathogenic strains of *E. coli*. Isolates of serotype O157 harbored virulence genes characteristic of EHEC, including stx*1, stx2, hylA, eseA, fliC* and *rfbE*. Those of serotypes O111, O78 and O147 showed positive hybridization genes encoding LT and ST, which are the typical virulence products associated with ETEC. Serotype O78 was avian pathogenic *E. coli* and typically possessed virulence factors such as temperature sensitive hemagglutination (TSH), and increased serum survival factor (ISS). In addition to these virulence factors, O78 possessed virulence factors typical of EHEC and ETEC, which may contribute the pathogenicity of O78 (57, 130). Serotype O126 hybridized to *aefP, bfp* and *astA*, the typical virulence genes of EPEC (Table 4-1). In addition to typical ETEC virulence genes, isolates of serotypes O111 and O78 possesed s*tx1* and *hlyA* genes, which are major virulence factors found in EHEC. Several studies have reported that some classical EPEC and ETEC carried s*tx* gene suggesting that EHEC groups of pathogenic *E. coli* have evolved from other pathogenic *E coli* through acquiring of s*tx* system (184). Nevertheless, these data suggest microarray analysis was a useful tool for rapidly identifying and differentiating diarrheagenic *E. coli* into pathogenic subgroups, which in turn could be used to help guide treatment choices.

It is well understood that continued emergence of antimicrobial resistance among bacterial pathogens constitutes a serious public health threat. Data presented here indicate microarray analysis can be used to rapidly identify the genetic underpinnings of resistant strains. We observed that the most of theantimicrobial resistance phenotypes of the bacteria identified in this study correlated with their

hybridization genotypes (Table 4-1). For example, each of the isolates observed to be resistant to third-generation cephalosporins also harbored the bla_{CMY-2} and bla_{TEM-1} genes. In the United States, the former of these two genes has been implicated in emergence of *S.*Typhimurium strains resistant to expanded-spectrum β-lactams, as well as AmpC-multi-drug-resistant strains of *S.* Newport (55, 143, 171, 230).

Among the β-lactam-resistant isolates, two *E. coli* isolates, E03 and E04, and two *Salmonella* isolates, S01 and S02, harbored bla_{PSE-1} other than bla_{CMY-2} and bla_{TEM-1} genes. *Salmonella* isolate S09, which was susceptible to ß-lactam antibiotics, showed hybridization to bla_{PSE-1}; however, PCR with bla_{PSE-1}-specific primers failed to amplify bla_{PSE-1} in this isolate (data not shown). We thus reasoned that either this isolate contained either (i) a gene with high homology to bal_{PSE-1} or (ii) a silent ß-lactamase gene. *E. coli* isolate E01 was found resistant to cephalothin, but it did not hybridize to any of the three ß-lactamase genes tested. It is reasonable to suggest that this isolate may have contained a ß-lactamase different from those included in this study.

All streptomycin-resistant isolates harbored the *aadA* gene; all tetracycline-resistant isolates harbored *tetA*, *tetB*, or both; and all sulfonamide-resistant isolates harbored the *sulI*and/or *sulII* genes. Consistent with recent findings (33) the *cat1* and *flo* genes were most frequently detected in chloramphenicol-resistant *E. coli* and the *cmlA* gene was most frequently detected in chloramphenicol-resistant *Salmonella*.

The microarray was also useful for identifying integrons among *Salmonella* and *E. coli*. The *int* and *qac* gene cassettes are hallmark features of class I integrons (177). With the exception of two *Salmonella* isolates, each of the *int*- and *qac*-

positive isolates contained class I integrons ranging in size from 1 to 2.7 kb. The presence of class I integrons among these isolates suggests they may have played a role in the dissemination of antimicrobial resistance genes. Regarding the two *Salmonella* isolates that contained the *int* and *qac* cassettes, but in which class I integrons were not detected, we offer two explanations. First, the isolates may have contained integrons of a size that could not be amplified by regular PCR. For instance, long-range PCR is known to be more effective than regular PCR for amplification of integron cassettes longer than 3 kb (117). Second, the isolates may have contained integrons devoid of gene cassettes. Further investigation is underway.

Two *S.*Thyphimurium DT104 strains with classical ACSSuT resistant phenotypes harbored the bla_{PSE-1}, *flo, aadA, sulI,* and *tetA* genes. These genes are known constituents of the SGI1 MDR region of *S.*Typhimurium DT104 (21, 52). In addition, these two isolates contained *tetB* and *sulII* genes as well. Another *S.*Typhimurium DT104 showed positive hybridization to twelve different antimicrobial-resistant genes. The *aac(3)-IVA* gene, previously shown to confer resistance to gentamicin (180), was hybridized by all the bacterial isolates, regardless of whether gentamicin-resistant or susceptible; the PCR assay specific to *aac(3)-IVA* failed, however, to amplify the gene from all of isolates. The positive hybridization of *aac(3) IVA* to all the isolates indicated that there may have some *Salmonella* and *E. coli* chromosomal genes which showed high homology to *aac(3)-IVA.* The failure to amplify *aac(3) IVA* from gentamicin-resistant bacteria suggested that resistance determinants other than those included on the microchips were responsible for the observed gentamicin-resistant phenotypes.

We conclude that the PCR-based microarray can be used as an efficient and

rapid method to screen the genotypes *Salmonella* and diarrheagenic *E. coli* for multiple antimicrobial resistance and virulence genes. Moreover, this method can be used to screen a large number of isolates. The method developed here has potential clinical significance in that by rapidly and accurately providing information about virulence mechanisms and antimicrobial resistance patterns, it can help guide disease treatment for infections caused by these important foodborne pathogens.

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Chapter 5 The Role of Target Gene Mutation and Efflux Pump in Fluoroquinolone-Resistant *Salmonella*

Abstract

Increasing resistance to antimicrobial agents that are important in the treatment of human diseases, such as fluoroquinolones (FQ) for the treatment of *Salmonella*, has significant public health implications. The mechanisms involved in fluoroquinolone resistance in *Salmonella* include target alterations and overexpression of efflux pumps. The present study evaluated known and predicted efflux pumps that are associated with FQ resistance and the interactive role that target gene mutations and efflux pumps play in FQ resistance. *S.* Typhimurium strains S21 and PY1 were in vitro induced to be FQ-resistant via step wise selection on increasing concentrations of ciprofloxacin. The wild type *Salmonella* isolate and four different level fluoroquinolone-resistant mutants with ciprofloxacin minimum inhibitory concentration (MIC) = 0.25, 4, 32, 256 ug/ml were selected. The expression of efflux pump genes *acrA, acrB, acrE, acrF, emrA, emrB* and *tolC* were significantly increased among the FQ-resistant mutants using quantitative RT-PCR. The expression of *yegO, yegN,* and *emrD* was also detected among mutants with higher ciprofloxacin MICs. Deletion of *acrAB* or *tolC* in *Salmonella* S21, Spy1 and their mutants resulted in increased susceptibility to FQ and other antimicrobial classes including chloramphenicol, tetracycline, β-lactams and trimethoprim. The replacement of mutated *gyrA* of all FQ-resistant mutants with wild type allele *gyrA+* also significantly increased the susceptibility to FQ, whereas the replacement of *parC* with *parC+* did not. Similar results were also observed among field isolates of

ciprofloxacin- or nalidixic acid-resistant *Salmonell*a when *acrAB* or *tolC* was deleted, or *gyrA* or *parC* was replaced. Our findings show that the combination of mutations in *gyrA* and upregulation of the AcrAB efflux pump were the primary mechanisms associated with fluoroquinolone resistance in *Salmonella*. It appears that overexpression of the AcrAB-TolC efflux pump enables *Salmonella* to be tolerant to low levels of FQ and target gene mutations occur subsequently under further FQ selective pressure. The continuous overexpression of AcrAB-tolC efflux conferred high FQ resistance in *Salmonella*.

Introduction

Quinolone-resistant *Salmonella* has emerged in humans and animals in the past few years and spread worldwide. However, high-level fluoroquinolone resistance is not frequently encountered in *Salmonella* spp, although it was suggested that fluoroquinolone-resistant *Salmonella* strains could be counterselected in the field (67). High-level fluoroquinolone-resistance *Salmonella* has been firstly reported in *S.* Typhimurium DT204 during the early 1990s in Germany (86). More recently, three nosocomial outbreaks of fluoroquinolone-resistant *Salmonella* infections have been reported in the United States, Taiwan and Japan (34, 149). The resistance to fluoroquinolone in *Salmonella* is increasing recently, based on the report from National Antimicrobial Resistance Monitoring System (NARMS). The emergence of fluoroquinolone resistance in *Salmonella* threatens human health because fluoroquinolones are commonly used to treat invasive *Salmonella* infections in humans (145, 224). It is very important and really needed to study the mechanisms of fluoroquinolone resistance in *Salmonella* because the understanding of molecular

mechanisms by which the antimicrobial resistance emerges and spreads should enable us to design strategies to intervene or stop or arrest the progress.

Bacterial resistance to fluoroquinolone is usually mediated by removing the drug by active efflux pump and development of mutations in the target of fluoroquinolone action (bacterial gyrase and topoisomerase IV). Within GyrA of *E. coli*, mutations of two amino acids, serine 83 and aspartic acid 87 are most commonly detected. A single mutation in each site confers resistance to nalidixic acid (226, 242). When both sites are mutated, the level of resistance can be three or fourfold higher than a single mutation, which can confer the resistance to ciprofloxacin (12, 201, 208, 216). Mutations in ParC are only detected in GyrA mutants and always with high fluoroquinolone resistance. Topoisomerase IV mutations do not by themselves confer resistance. These findings suggested that GyrA is the primary target in *E. coli*, whereas ParC is as the secondly target. Mutations in GyrB could be found in both high and low quinolone resistance isolates. GyrB mutations in clinical isolates are analyzed in two specific sites, Asp426 and Lys 447 in *E. coli* (240). Recent crystal structure of yeast topoisomerase IV demonstrated a quinolone-binding pocket around the active site for DNA cleavage. GyrB might be part of the quinolone-binding pocket. The homologous gene for topoisomerase IV, ParE , can also display resistance mutations.

In *Salmonella*, single mutations in GyrA, Ser83 or Asp87, are identified in quinolone-resistant isolates recovered from humans and animals (78, 159, 172). Mutations in both Ser83 and Asp87 in GyrA are detected in ciprofloxacin-resistant *Salmonella* isolates from humans and animals (34, 85, 156). Mutations in ParC, Ser80 or Gly78, has recently reported in ciprofloxacine-resistant human isolate and laboratory induced isolates. Mutations in GyrB have also been found, with a

substitution of Ser464 revealed in one clinical isolate. Introducing into the respective mutants the corresponding plasmid-coded quinolone-susceptible allele of either *E. coli gyrA*, *gyrB*, or *parC* resulted in reduction of quinolone resistance, indicating a role for these mutations in quinolone resistance. No mutations in ParE have yet been described in quinolone-resistant *Salmonella* strains (88).

Efflux pumps are membrane transport proteins involved in the extrusion of toxic substrates (including antibiotics) from within cells into the external environment. In the prokaryotic kingdom there are five major superfamilies of active drug efflux pump transporters: ATP-binding cassette (ABC), major facilitator superfamily (MFS), small multidrug resistance (SMR), multi antimicrobial resistance (MAR), resistance nodulation division (RND) and multidrug and toxic compound extrusion (MATE) (210). Genome sequences analyses of prokaryotic microorganisms with available complete sequence revealed that about 5~10% of all bacterial genes are involved in transport, and that a large proportion of these genes encode efflux pumps. Antibiotic efflux pumps appear a major component of microbial resistance to many classes of antimicrobial agents (210). In many cases, efflux pump genes are within an operon, with a regulatory gene controlling their expression. Increased expression of efflux pump genes is associated with the resistance to substrates including antibiotics. Overexpression of efflux pump genes can result from mutations within local repressor gene or may result from activation of a regulon regulated by a global transcriptional regulator such as MarA or Sox (127, 169). For at least four of antibiotics, namely tetracycline, macrolide, chloramphenicol, and fluoroquinolone, antibiotic efflux pumps appear to confer medium or high level of antibiotic resistance, defeating medically applicable treatments of the corresponding infections with these antimicrobial agents (16, 111, 147, 161, 166).

Efflux pumps that contribute to antibiotic resistance have been described from a number of clinically important bacteria other than *Salmonella*including *Campylobacter jejuni* (CmeABC), *E. coli* (AcrAB-TolC, AcrEF-TolC, EmrB, EmrD), *Pseudomonas aeruginosa* (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM), *Strepotococcus pneumoniae* (PmrA), *Staphylococcus aureus* (NorA) (156). An antibiotic efflux pump thathas been described in *Salmonella* is an AcrAB-TolC system where AcrB is an efflux protein in the cytoplasmic membrane and AcrA is an accessory protein, linking AcrB with the outer membrane protein TolC (68, 160). Overexpression of AcrAB-tolC is mediated by the overexpression of transcriptional activators MarA and SoxS, which are regulated by MarR and SoxR proteins (6, 169). The overexpression of MarA and SoxS adversely regulates OmpF, an outer membrane pore forming protein. The net result is that the OmpF is less expressed and less drugs is able to enter the cell, that the AcrAB is overexpressed and more drugs were effluxed from cell. Other efflux pumps have not yet been studied in *Salmonella*. The objectives of this study are to determine the molecular mechanisms, including efflux pumps and gyrase and topoisomerase IV gene mutations, of fluoroquinolone resistance in *Salmonella*

Materials and methods

Bacterial strains *Salmonella* Typhimurium S.py1 (14028s) was an ATCC standard strain and *Salmonella* Typhimurium S21 was a field strain isolated from meat product. *S.* Typhimurium CS1, CS3, CS9 isolated from diseased animals were resistant to ciprofloxacin and *S.* Typhimurium CHS14, CHS18, CHS38 isolated from meat products were resistant to nalidixic acid but susceptible to ciprofloxacin.

Selection of nalidixic acid and ciprofloxacin-resistant mutants *in vitro* The selection of nalidixic acid and ciprofloxacin-resistant *Salmonella* mutants was performed as described by Heisig (84) with modification. Briefly, *Salmonella* strains susceptible to nalidixic acid and ciprofloxacin were grown in brain heart infusion (BHI) broth (Difico Detroit, MI) at 37°C overnight. The overnight culture was spread on Mueller Hinton agar (MHA) plates (Difico) supplemented with 4μg/ml nalidixic acid and incubated for 24-48h at 37°C. Single colonies were selected and incubated in BHI supplemented with the same concentration of the antibiotic as in MHA plates. The overnight culture was spread on MHA plates supplemented with 2X concentration (8μg/ml) of nalidixic acid as that in BHI broth. Similar selections were repeated in 4X, 8X and higher concentrations of antibiotics. Mutants selected from 128μg/ml nalidixic acid plates were then exposed to ciprofloxacin at concentrations ranging from 0.5μg/ml to 128μg/ml. The *in vitro* selection procedures were similar as those for nalidixic acid resistance. The mutants from each stage were stored in –80°C freezer until further study.

Identification of multiple-drug resistance efflux pump genes In order to conduct a comprehensive investigation of the role of multidrug efflux pump on susceptibility to fluoroquinolone in *Salmonella*, 12 multidrug efflux pump genes belonging to super families of ABC, MFS, RND, SMR, and MATE were selected for use (Table 5-1). These genes included known and predicted efflux pump that were identified by bioinformatics analysis as described by Paulsen et al (152, 153). Except for AcrAB, all other efflux pump genes were predicted or had been identified to encode a fluoroquinolone or multidrug efflux pumps in otherGram negative organisms such as *E. coli*.

Table 5-1. Known and predicted *Salmonella* effux pumps and regulatory genes

Salmonella Efflux pumps	Assigned families	Genebank #	Functions	Genetic Context
MarA		ae008766	regulation	*marA*
SoxS		ae008750	regulation	*soxS*
TolC	OMP	ae008846	putative out membrane protein	*tolC*
MdlB	ABC	ae008717	MDR like ATP-binding protein	*mdlB*
EmrB	MF	ae008828	translocase substance of light hydrophobicity	*emrB*
EmrD	MFS	ae008877	multidrug pump	*emrD*
AcrA	MFS	ae008717	transmembrane	*acrA*
AcrE	MFS	ae008856	membrane	*acrE*
EmrA	MFS	ae008828	MDR secetion protein	*emrA*
YdhE	MATE	ae008762	putatative transport protein	*ydhE*
AcrB	RND	ae008717	multidrug efflux pump	*acrB*
AcrF	RND	ae008856	envelop formation	*acrF*
YegN	RND	ae008794	acrifluvin resistance protein D	*yegN*
YegO	RND	ae008794	acrifluvin resistance protein E	*yegO*

ABC, ATP-binding cassette; MFS,major facilitator superfamily; SMR, small multidrug resistance; MAR, multi antimicrobial resistance; RND, resistance nodulation division; MATE, multidrug and toxic compound extrusion.

Determination of efflux pump gene expression level The efflux pump gene expression level was determined by RT-PCR. Briefly, total RNA was extracted from *Salmonella* using the RNeasy Kit (Qiagen Inc, Valencia CA) and then treated with DNA-free kit (Ambion, Austin TX) to remove DNA contamination according to the manufacturer's instructions. The concentration of the RNA was measured using Smartspect 300 (BioRad, Hercules, CA) and 1µg of the total RNA was used in RT-PCR reactions. The RT-PCR was performed using Access RT-PCR system (Promega, Madison, WI) following the manufacturer's instructions. The RT-PCR reaction was carried out in a 50µl reaction containing 10× RT-PCR buffer, 2.0mM $MgSO_4$, 0.2mM each dNTP, 50pmol each primer (Table 5-2), each of 0.1u/µl *Tfl* DNA polymerase and AMV reverse transcriptase. One-step RT-PCR was performed in thermal cycler (GenAmp PCR System 9600, Perkin Elmer) by incubating at 48ºC for 45min, then denaturing at 94ºC for 2min, following by 25 cycles at 94ºC for 30sec, 60ºC for 1min, 68ºC for 2min, then incubating at 68ºC for 7min. RT-PCR products were quantified in Agilent 2100 bioanalyzer (Agilent Technologies Palo Alto, CA) utilizing DNA 1000 LabChip® kit following the manufacture's instructions. The ratio of efflux pumps/house keeping gene was determined by dividing the concentration of target gene to that of 16SrRNA. The target/ house keeping gene ratio was assumed to be the relative expression level of efflux pump genes in bacteria (239). RT-PCR was repeated three times and the mean expression levels of certain efflux pump in different level fluoroquinolone-resistant *Salmonella* mutants were compared using SAS CONTRASTS statements. The $P < 0.01$ was considered as significant difference.

Deletion of efflux pump genes in *Salmonella* The deletion of efflux pump genes from *Salmonella* was done by one step PCR gene knockout technique described by Datsenko K. A. et (49) (Fig 5-1). Briefly, PCR primer sets A (Table 5-2) were

Table 5-2, Oligonucleotide for efflux RT-PCR, gyrase, topoisomerase IV and regulatory gene amplification, efflux pump gene knock out and verification, and *gyrA* and *parC* gene replacement.

Genes/ Primer sets	**Oligonucletide Sequence (Forward Primer)**	**Oligonucleotide Sequence (Reverse Primer)**
Oligonucleotide for RT-PCR		
16SrRNA	AAAGCGTGGGGAGCAAACAG	CCGCTGGCAACAAAGGATAA
marA	ATCCGCAGCCGTAAAATGAC	TGGTTCAGCGGCAGCATATA
soxS	AAATCGGGCTACTCCAAGTG	CTACAGGCGGTGACGGTAAT
tolC	CAGACGCTGATCCTCAATAC	TGCTGATGGAGGCGTTAATA
mdlB	GCTACAGCACGCCGATTGTA	TGCCCGACGGACAGCGTATT
emrB	ACCGAACGGCGACGTATTGA	GTTGGTGCGTCATCCCTAAG
emrD	ATCCTCGTCGGCATGTCTAT	GTCATCAGCAATCCCAGACT
acrA	CGGTCGTTCTGATGCTCTCA	GCCCTGTTGTGGAACCAGTA
acrE	TGCTGGCCGGTTGTAATGAC	CGTGCCTTTAATCGGGTAGA
emrA	GCAGCAACCGGCTAAGAAGA	ATCCAGACCGACGACTTTAC
ydhE	ATGTTTACGGGGATCGTACA	TTAGCCAATGTCGGCCTGAT
acrB	AAGAGCACGCATCACTACAC	CGCTTCGGACATCACGTAAA
acrF	GAGCTTGGCGGTGAAAACTA	AACGCAACCAGAACGGATAG
yegN	CCGAATCCGCCGATTTACAG	GCCATCAGCGTCAGGTTATT
yegO	GCCGTTCCCGTTTCGCTCAT	ATCCTGTACCGCCATCAGAA
Oligonucleotide for gyrase, topoisomerase and regulatory gene amplification		
gyrA	ACGTATTGGGCAATGACTGG	GGAGTCGCCGTCAATAGAAC
gyrB	CAAACTGGCGGACTGTCAGG	AGCCCAGCGCGGTGATCAGC
parC	CGTCTATGCGATGTCAGAGC	TAACAGCAGCTCGGCGTATT
parE	GTCAATGTGCGGCATTTGTT	ATCCCCTTCCACAAGGAACA
marR	TGCGTCTGGACATCGTCATA	CGGACGCCTTTTAGCAAATC
AcrR	ATGGCACGAAAAACCAAACA	TTATCAGGGGGAGCCGTTGA
Oligonucleotides for gene knockout		
mrA,B (A)	AGCGCAAATGCGGAGATCCA AACCCCGCAGCAACCGGCTA AGAAGTGTAGGCTGGAGCTG CTTC	AGAAGATTTCGTTGGCGGAA ATAATCAGCCCCTGATTCGTG ATTATGGGAATTAGCCATGGT CC

(B)	TCCGCCTCAGCATCATTGTC	GGCGGTTTGGCGAACCACAC
acrEF		
(A)	CCAGGTTTTCACTCCTGCCCTCATTCATCATATTCTCTGCTGCGGTGTAGGCTGGAGCTGCTTC	ATCGGCGTTTTACAACAACGAAGAATACCGGCACGAAGAAGATAATGGGAATTAGCCATGGTCC
(B)	CGCACAATATCGCCAAATCA	ACCGCTGCGAAAACGAGAGT
yegOMN		
(A)	TCCGCTGGGCAATAGCGATTGGGTTGTAGTGGCCGCCGCCGCAGTGTAGGCTGGAGCTGCTTC	GGTTTGCTATTTTTACGCGAAAAACGTAGCCGCAGACGATCGAAATGGGAATTAGCCATGGTCC
(B)	CTCCCCCTGGTGTCTTAGTA	ACAGCGAACCCAGCGTGAAC
mdlAB		
(A)	CTTGGCGCAGTGGCCCTGCTTATGCTTATTGCGATGCTACAGCTGTGTAGGCTGGAGCTGCTTC	CGTGTACGCTGGCGGCTAACTCCTCGCCAACTAACTGTAATTGAATGGGAATTAGCCATGGTCC
(B)	AAACTGGGGCTGGGATTAAC	AGCTGGCGAGCGAGGAAAAG
acrAB		
(A)	GTAAAAAAGGCCGCTTGCGCGGCCTTATCAACAGTGAGCAAATCAGGTGTAGGCTGGAGCTGCTTC	CCTCGAGTGTCCGATTTCAAATTGGTCAATGGTCAAAGGTCCTATGGGAATTAGCCATGGTCC
(B)	CAGGAGAAAATAGCCAGGAA	AGCGACACAGAAAATGTCCA
tolC		
(A)	GCCCTGCTAGCAATAATGATTAAATGATGAATTTCAAGGGTGGTGTGTAGGCTGGAGCTGCTTC	GCGCCGCGCTTACCAGACCTACAAGGGCACAGGTCTGATAAGCGATGGGAATTAGCCATGGTCC
(B)	GATCTGCTGGCTTGAACACA	AAATCAGCGACGCAATCTT
Kan	TCATAGCCGAATAGCCTCTC	CGGTGCCCTGAATGAACTGC
Oligonucleotides for gene replacement		
gyrA+		
(A1)	CGAATAAAGCATTGTCTGGCTGCATTCCGTTTACCAGTACGTGTAGGCTGGAGCTGCTTC	CCCGGATTCAAAGGTCGCAAATTATAACACATTCGCCCACATGGGAATTAGCCATGGTCC
(B1)	CACTGGCGAGCGTTCCTACA	GAACAGCGCTTGCGCTAACC

parC		
(A1)	TTTGTGACTCAGACCGTGAGAGGATCCTCAATCTGCATGCTG TAGGGTATTATCTGCGGCAGT GTAGGCTGGAGCTGCTTC	ACCGTTCGGACGGCAATATAT GGGAATTAGCCATGGTCC
(B1)[e]	TCACCGGCAGTTTACCACCT	ATCCGGATAAGGTCAACGCC

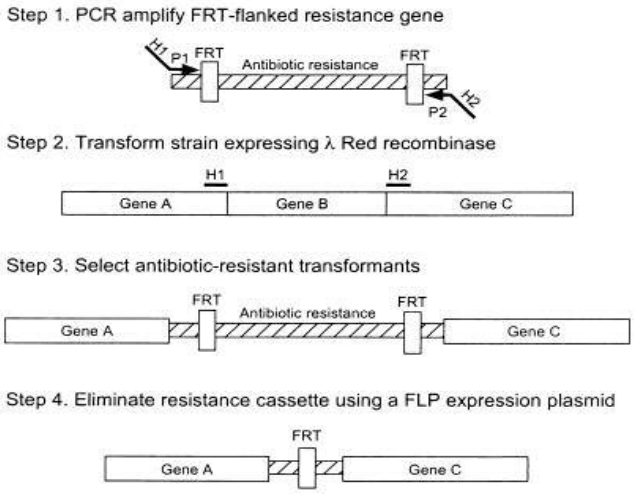

Figure 5-1. A simple four-step gene deletion strategy. H1 and H2 refer to the homology extensions or regions. P1 and P2 refer to priming sites (49).

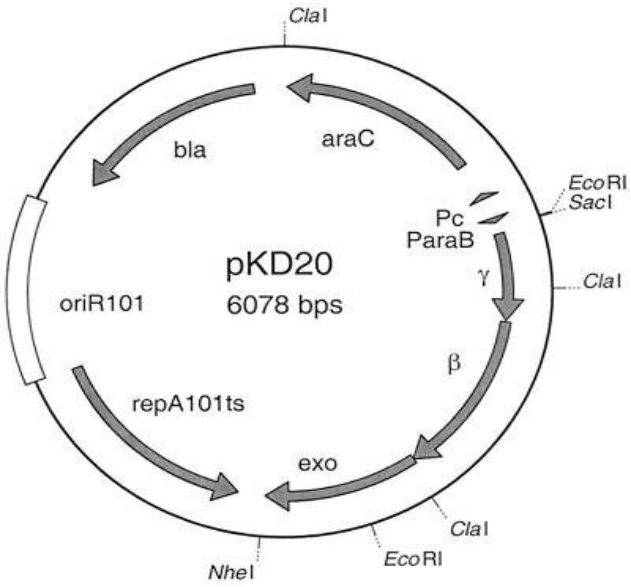

Figure 5-2. Red recombinase expression plasmids. pKD20 and pKD46 (not shown) include 1,894 nt (31348–33241) and 2,154 nt (31088–33241) of phage λ (GenBank accession no. J02459), respectively (49).

designed to amplify the FRT (Flp recognition target)-flanked resistance gene (Km^R) from template plasmid pKD46. The Primers A and B were composed of 3' 20bp sequence homologous to the KmR gene, and 44bp 5' extensions, which were homologous to the nearby genes at both sides of the target gene. Electrocompetent cells were prepared from *Salmonella* S.Py1 strain by concentrating 100-fold of mid-log culture and washing three times with ice-cold 10% glycerol and transformed with Red helper plasmid pKD20 (Fig 5-2). Transformants carrying a Red helper plasmid was made electrocompetent cell again. PCR products, which contain a kanamycin resistance gene (Km^R), FRT site, and homologous segments of nearby genes, were gel-purified, digested with *Dpn*I, repurified, and suspended in elution buffer (10 mM Tris, pH 8.0). Electroporation was done by using a Micropulser electroporator (Bio-Rad Hercules, CA) and 0.1-cm chambers according to the manufacturer's instructions using 40 µl of cells and 10–100 ng (2µl) PCR product. Shocked cells were added to 1-ml SOC, incubated 1 h at 37°C, and then one-half was spread onto agar plates to select Cm^R or Km^R transformants. After primary selection, mutants were maintained on medium without an antibiotic. They were colony-purified once nonselectively at 37°C because the helper plasmid pKD20 is a temperature sensitive plasmid. The colony was tested for ampicillin sensitivity to test for loss of helper plasmid.

PCR assays were used to verify the correct deletion of target gene in *Salmonella* chromosome. Several freshly isolated colonies were suspended in 100-µl water and heated for 10min at 100°C. Five-µl portions were used in 50-µl PCRs as described previously. Common test primers include: K1, K2 inside Km^R and two nearby locus-specific primer sets B (table 5-2), which targeted to both of the nearby genes of the target gene. Three PCR reactions were used to verify the correct deletion using forward B and K1, K2 and reverse B, primer set B.

Phage transduction mediated by P22/H105 was used to conduct similar mutations in other level mutants and field strains as described in bacterial laboratory manual. The deletion was verified to be correct by three PCR reactions using forward B and K1, K2 and reverse B, and primer set B as described previously. In addition, the primers, which are specific to the individual efflux pump gene (Table 5-2) were used to test the loss of efflux pump gene in deletion mutants.

Determination of drug target and efflux pump regulatory gene mutations in *Salmonella* mutants Amplifications of *gyrA*, *gyrB*, *parC*, *parE*, *marR* and *acrR* were performed using primers listed in Table 5-2. PCR was performed in 50µl distilled water with 0.25mM dNTP, 1.5mM MgCl2, 0.2u Tag enzyme and 50pmol each primer. DNA fragments *gyrA*, *gyrB*, *parC* and *parE* were amplified by using the following temperature profiles: incubating at 95 ºC for 10min; 90ºC 30s, 55ºC 45s and 72ºC 45s for 30 cycles, and one final cycle of 72ºC for 7min.

Predicted polypeptide products were analyzed for amino acid changes by comparison with wild type *gyrA* (#AE008801), *gyrB* (AE008878), *parC* (#AE008846), *parE*(#AE008846) , *marR* (AE008766) and *acrR* (AE008717) and amino acid of these genes.

Replacement of mutated *gyrA* and *parC* with wild type alleles in chromosome

In order to identify the role of *gyrA* and *parC* mutations on fluoroquinolone resistance in *Salmonella*, the *gyrA* and *parC* of fluoroquinolone-resistant mutants were replaced with their wild type alleles. The basic strategy of gene replacement is to insert an antimicrobial selective marker upstream of wild type *gyrA* gene in the bacterial chromosome. The DNA fragment containing wild type *gyrA* gene and a selective marker along with flanking DNA was packed in phage P22 and transformed into recipient mutant cell to replace the mutated *gyrA* gene by homologous

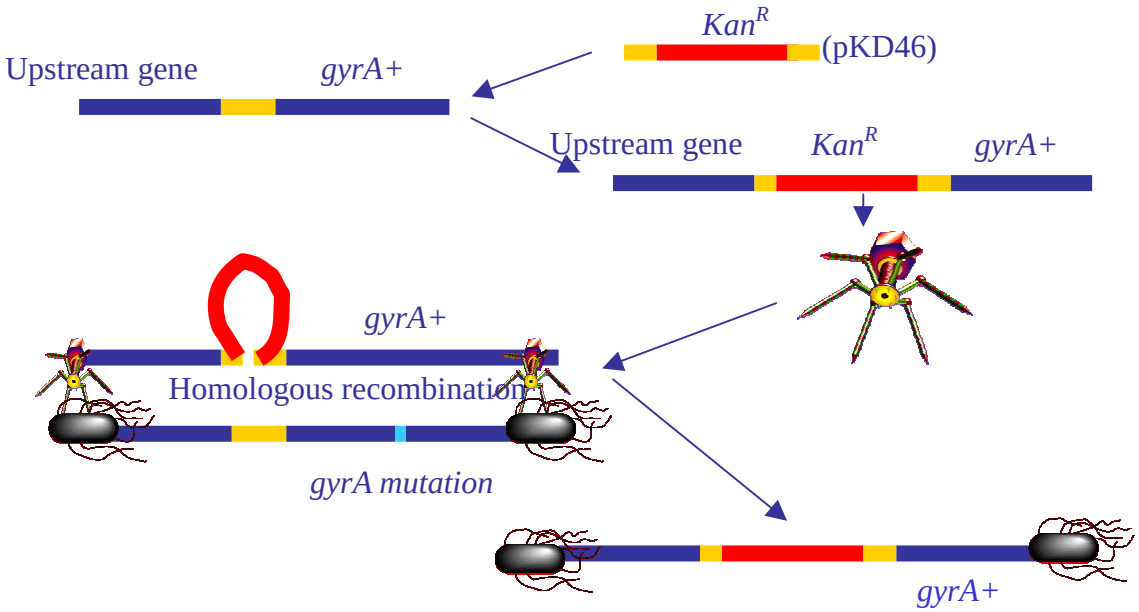

Fig 5-3. The procedure for gene replacement. A kanamycin resistance gene was inserted into upstream of a wild type *gyrA*+ gene. The DNA fragment containing wild type *gyrA* and selective marker was transferred to fluroquinolone-resistant *Salmonella* with mutations in *gyrA* through phage transduction. The wild type *gyrA*+ will replace mutated *gyrA* via homologous recombination.

recombination (Fig 5-3). Briefly, Primer set A1 and B1 were designed to PCR amplify the FRT -flanked Km^R from template plasmid pKD46. Each primer A1 and B1 were composed of 3' 20bp, which were targeted to the KmR gene, and 40bp 5' extensions, which was homologous to the upstream sequences of *gyrA* or *parC* genes. These two 40bp extension sequences of primers A1 and B1 were homologous to the 80bp sequence in the upstream of *gyrA* or *parC* gene. PCR products amplified using these primers were transformed to *Salmonella* S.py1 strain containing the Red helper plasmid. The transformants were selected on knanmycine plates and purified as previously described. The replacement of *gyrA* or *parC* of fluoroquinolone-resistant *Salmonella* with wild type *gyrA* or *parC* gene by *Salmonella* S.Py1 was conducted by phage transduction as before. The replacement of mutated *gyrA* or *parC* with wild type allele was confirmed by sequencing the QRDR of *gyrA* and *parC*.

Antimicrobial susceptibility testing All *Salmonella* strains including S21, S.py1, *Salmonella* field strains CS1, 3, 9, CHS 14, 18, 38 , induction mutants, deletion mutants, and *gyrA* or *parC* replacement mutants of these strains were assayed for the susceptibility to antimicrobial agents including cefoxitin, ceftiofur, ceftriaxone, cephalothin, amoxicillin-clavulanic, ampicillin, sulfamethoxazole, trimethoprime, ciprofloxacin, difloxacin, enrofloxacin, levofloxacin, gatifloxacin, nalidixic acid, orbifloxacin, sarafloxacin, danafloxacin, choramphenicol, gentamycin, streptomycin, amikacin, tetracycline. Minimal inhibitory concentrations were determined by the broth-microdilution method with the use of Sentitre system (Trek Diagnostic Systems, Westlake, Ohio). The seventeen antimicrobial agents used and their recommended resistance breakpoints are presented in Table 1. The strains were also assayed for the susceptibility to fluoroquinolones using fluoroquinolone sensititre system (Trek Diagnostic Syatem, Westlake, Ohio). The susceptibility to ciprofloxacin was also

determined by the use of agar dilution method to cover high concentration of ciprofloxacin. The dilution range of ciprofloxacin agar dilution method was from 0.0075µg/ml to128 ug/ml. All testing followed guidelines set forth by the National Committee Clinical Laboratory Standards (NCCLS) (90, 238). *Escherichia coli* ATCC 25922, 35218, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms.

Results

Efflux pump gene expression level and deletion of efflux pump genes *Salmonella* S.py1, S21 and their induction mutants (table 5-3), which showed different susceptibility to ciprofloxacin and nalidixic acid were screened for the expression of 12 efflux pumps and two regulatory genes (table 5-3). Independent of genetic background, the efflux pump genes showedhigher level of expression after the induction of *Salmonella* isolates to be resistant to fluoroquinolones. However, the increase of the expression of individual efflux pump genes in S.py1 and S21 was different from each other. In S21, the expression level of *mdl, acrE* and *acrF,* which were undetectable in wild type, was significantly increased in S21-3 and S21-4 mutants ($P<0.01$) (Fig 5-4 A). The expression level of *acrA, acrB,* and *tolC, marA, emrA* and *emrB* were significantly, consistently increased from S21 to S21-4 ($P<0.01$). The expression level of *yegO, yegN,* and *emrD* which were undetectable in S21, S21-1, and S21-2 showed, although in low level, significantly increased in S21-3 and S21-4 ($P<0.01$). Nearly no difference of *ydhE* expression was detected in all level mutants (Fig. 5-4 A). In Spy1, the expression of all efflux pump genes could be detected. Similar as S21, the expression level of *acrE, acrF* was significantly increased in S. py1-3 and S. py1-4, and the expression level of *acrA, acrB, tolC, emrA* and *emrB* were significantly, consistently increased form S.py1 to S.py1-4 ($P<0.01$). In contrast to S21, the expression level of *mdlB* didn't not show significantly increased in S.py1-3 and S.py1-4. The *marA, soxS* and *ydhE* efflux pump genes did not show significantly overexpressed. (Fig 5-4 B).

In order to further characterize the function of efflux pump on fluoroquinolone resistance in *Salmonella*, efflux pumps which showed increased expression with the

Table 5-3. Quinolone MICs of different fluoroquinolone-resistant *Salmonella* mutants, and mutations of their gyrase, topoisomerase IV and regulatory genes

Mutants	MIC (NA) μg/ml	MIC (CIP) μg/ml	GyrA	GyrB	ParC	ParE	*marR*	*acrR*
S21	<4	<0.0015	-*	-	-	-	-	-
S21-1	64	0.5	S83F	-	-	-	-	-
S21-2	>256	4	S83F	-	-	-	-	-
S21-3	>256	32	S83F	-	-	-	-	-
S21-4	>256	256	S83F	-	-	-	-	-
S.py1	<4	<0.0015	-	-	-	-	-	-
S.py1-1	64	0.5	S87Y	-	-	-	-	-
S.py1-2	>256	4	S87Y	-	S80I	-	-	-
S.py1-3	>256	32	S87Y	-	S80I-	-	-	-
S.py1-4	>256	256	S87Y	-	S80I-	-	-	-
CS1	>256	16	S83F, S87N	-	S80I	-	-	-
CS3	>256	16	S83F, S87N	-	S80I	-	-	-
CS9	>256	16	S83F, S87N	-	S80I	-	-	-
CHS14	128	0.12	S87G	-	-	-	-	-
CHS18	256	0.12	S83F	-	-	-	-	-
CHS38	32	0.06	S87G	-	-	-	-	-

* No mutations

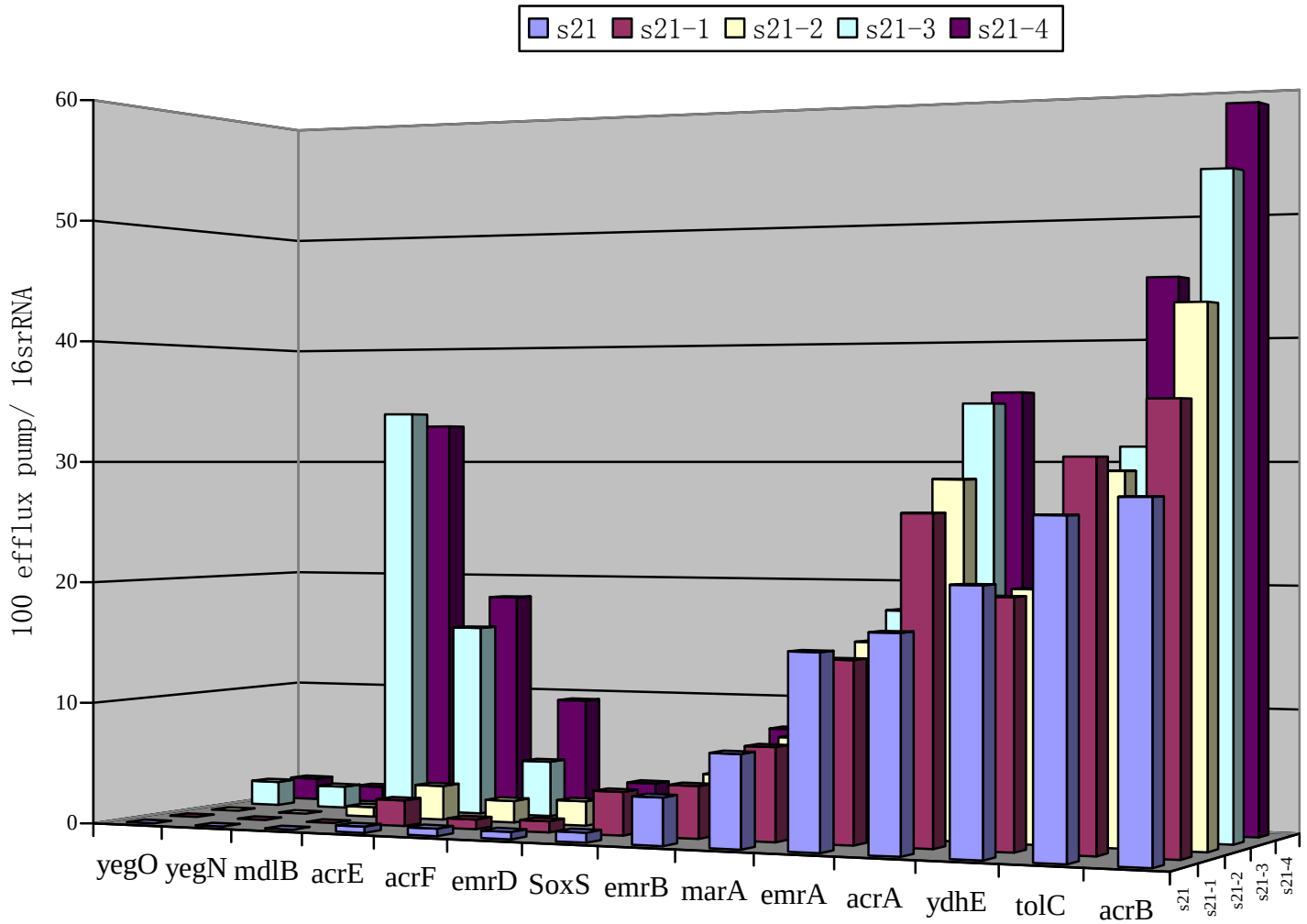

Fig 5-4 (A). Expression level of different efflux pump and regulatory genes in *Salmonella* S21 and its fluoroquinolone-resistant mutants

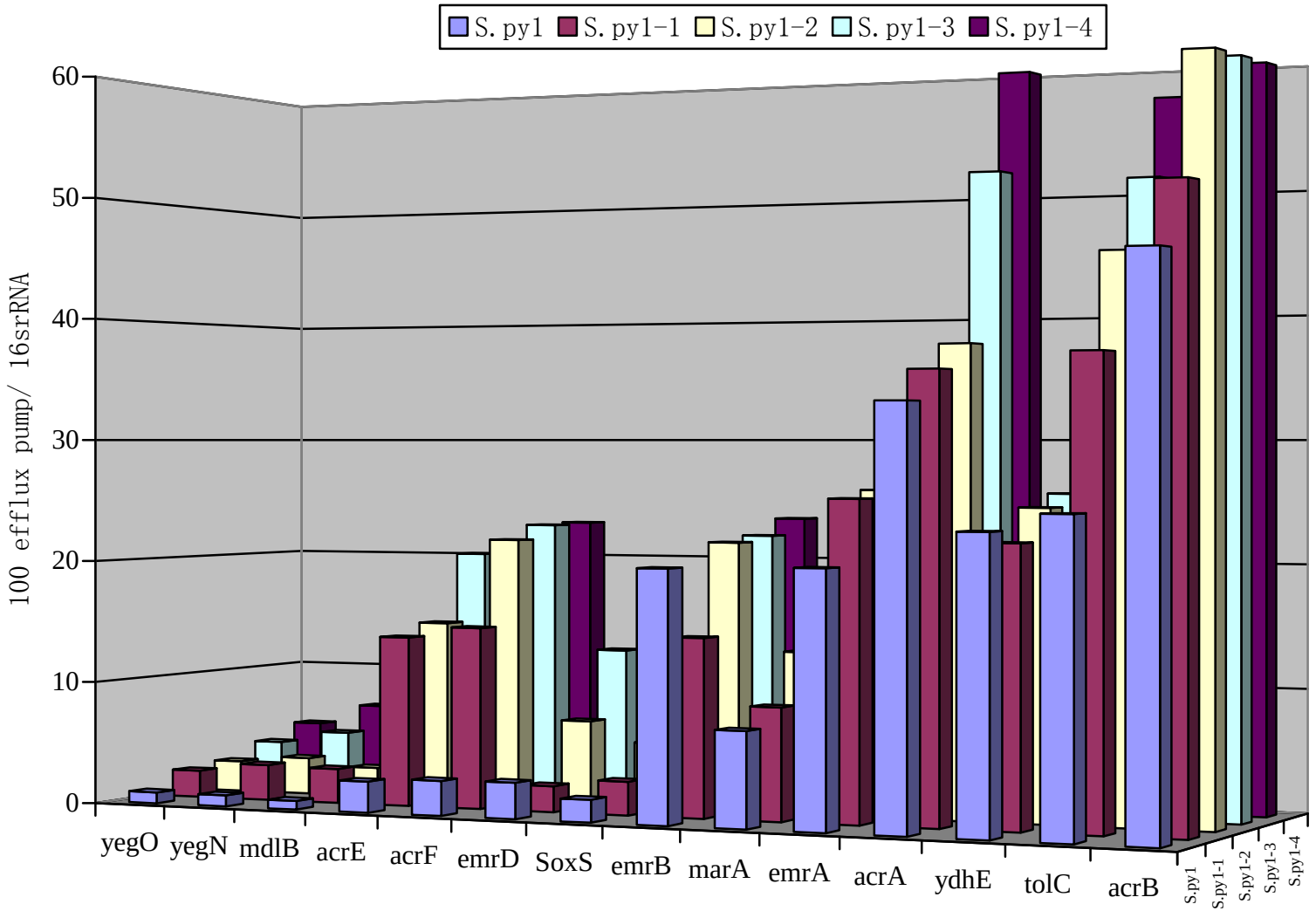

Fig 5-4 (B). Expression level different efflux pump and regulatory genes in *Salmonella* S.py1 and its fluoroquinolone-resistant mutants

increase of resistance to ciprofloxacin were deleted as previously described. Contiguous MDR efflux pump genes oriented in the same direction can constitute a multidrug efflux operon. We choose to delete the contiguous MDR pump genes, which might associate to form the same MDR efflux pump system. Therefore, six unique deletions representing 12 individual genes were made in *Salmonella* S.py1, S21 and their mutants using the gene knockout technique and phage transduction technique described previously. Firstly, each of the deletions was conducted in *Salmonella* S.py1 strain. All deletions were verified as correct by analyzing the chromosomal locus by PCR amplification using the common verification primers forward B and K1, K2 and reverse B, B primer set. The deletions were termed *acrAB*::Kan, *tolC*::Kan, *acrE*F::Kan, *yegMNO*::Kan, *emrAB*::Kan, *mdlAB*Kan. The deletion of efflux pump genes of S21, all level mutants of S21 and S.py1, and ciprofloxacin-resistant and nalidixic acid-resistant field strains were done by phage P22/H105 transduction as described in Materials and Methods. All transductants were confirmed to be lysogens by testing their sensitivity to phage H5. A further PCR assay was used to test the loss of the deleted gene. All the strains without deletion had the amplification with the same size as predicted, while no amplification was shown in deletion strains, providing that the deletion of efflux pump by phage transduction was successful.

Target mutations and replacement The mutations in GyrA, GyrB, ParC, and ParE of S.py1, S21, and their fluoroquinolone resistance mutants were investigated. No mutations were detected in the QRDR of *gyrA, gyrB, parC, parE* genes of wild type strains S.py1 and S21 (table 5-3). Different topoisomerase gene mutations were detected in the mutants of S.py1 and S21 after induction with cirofloxacin. A single GyrA mutation with the substitution of S83F was detected in all

fluoroquinolone resistance mutants of S21. No mutations in GyrB, ParC and ParE were detected in those mutants. In contrast to S21, in addition to a single mutation with the substitution of S87Y was present in all fluoroquinolone resistance mutants of S.py1, a single S80I mutation in ParC was detected in S.py1-2, S.py1-3, and S.py1-4 . No mutations were detected in GyrB and ParE in all fluoroquinolone resistance mutants of S.py1 (table 5-3). The topoisomerase gene mutations were also detected in fluoroquinolone-resistant field strains. Double GyrA mutations, S83D and S87Y, and single ParC mutation, S80I, were detected in CS1, CS3, and CS9 which were resistant to ciprofloxacin. Different single GyrA mutation, S87G, S83F and S87G, were found in CH14, CH18 and CH38 respectively. No mutations in GyrA, ParE, MarR and AcrR genes were detected (Table 5-3).

The construction of *Salmonella* S.py1 mutants by insertion of Km^R-FRT upstream of *gyrA* and *parC* gene was performed as described above. A Km^R with FRT flanking sequence was successfully inserted into upstream of *gyrA* or *parC* of *Salmonella* S.py1. The replacement of mutated *gyrA* and *parC* in fluroquinolone-resistant mutants and field strains with wild type alleles from *Salmonella* S.py1 was performed by phage transduction as described above. Each of the four fluoroquinolone-resistant mutants of *Salmonella* S21 and S.py1 and six of the field strains, which showed mutation in *gyrA* gene was replaced with wild type *gyrA* allele from S.py1. The replacement mutants were confirmed to be lysogens and further confirmed by sequencing the QRDR of the *gyrA* gene. All the strains with *gyrA* gene replacement failed to show any mutation in the QRDR of their *gyrA* gene. The wild type *parC* allele from *Salmonella* S.py1 was used to replace the mutated *parC* in S.py1-2, S.py1-3, S.py1-4 and three field strains CS1, CS3, CS9 as done in *gyrA* replacement.

Antimicrobial susceptibility testing results All *Salmonella* strains including S21, S.py1, *Salmonella* field strains CS1, 3, 9, CHS 14, 18, 38, induction mutants, deletion mutants, and *gyrA*/*parC* replacement mutants of these strains (table 5-4) were assayed for their susceptibility to 24 different antimicrobials, as described in Materials and Methods. The exposureof *Salmonella* S21 to different concentrations of ciprofloxacin caused the strain resistant to not only fluoroquinolone but also other classes of drugs including tetracycline, chloramphenicol, trimethoprim, and ß-lactam. First level mutant S21-1 was resistant to nalidixic acid as well as tetracycline, chloramphenicol, and ampicillin. In addition to the resistance to these drug, S21-2 and upper level mutants were resistant to cephalothin and cefoxitin and showed moderate resistance to amoxicillin-clauvulanic (MIC=16/8µg/ml) and ceftiofur (MIC=4 µg/ml). The MIC to ceftriaxone, gentamycin, and amikacin didn't change in all level mutants. The change of MIC to sulfamethoxazole and streptomycin couldn't be addressed because S21 was resistant to these two drugs. Similar to S21, the induction of S.py1 with ciprofloxacin changed the susceptibility to previously described drugs. All level mutants of S.py1 acquired resistance to chloramphenicol, cefoxitin and cephalothin. However, the mutants only showed moderate resistance to tetracycline, ampicillin, and ceftiofur. The MIC to trimethoprim only increased four fold. No change was detected in other drugs including streptomycin.

The *acrAB* or *tolC* deletion mutants dramatically decreased the MIC of fluoroquinolone as well as other classes of drugs. Deletion of *acrAB* or *tolC* of wild type strains S21 and S.py1 made these two strains to be hypersusceptible to fluoroquinolones (MIC < 0.015µg/ml) and other class of drugs including

Table 5-4. Antimicrobial susceptibility of deletion and replacement mutants of *Salmonella* S21

ID	GAT	SAR	ENR	CIP	DIF	DAN	LEV	ORB	NAL	FOX	CHL	TET	AMO	TIO	COT	CEP	KAN	AMP
S21	0.03	0.03	0.06	<0.015	0.25	0.03	0.03	0.12	4	4	8	<4	<1/05	0.5	0.25/4.75	<2	<8	2
S21 *tolC*::Kan	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	1	1	<2	<4	<1/0.5	<0.12	<0.12/2.38	<2	>64	<1
S21 *acrAB*::Kan	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	1	1	<2	<4	<1/0.5	<0.12	<0.12/2.38	<2	>64	<1
S21gyrA+	0.03	0.03	0.06	<0.015	0.25	0.03	0.03	0.12	4	4	8	<4	<1/05	0.5	0.25/4.75	<2	>64	2
S21parC+	0.03	0.03	0.06	<0.015	0.25	0.03	0.03	0.12	4	4	8	<4	<1/05	0.5	0.25/4.75	<2	>64	2
S21-1	0.5	1	0.5	0.25	4	0.5	0.5	2	256	2	>32	32	16/8	0.5	0.25/4.75	8	<8	>32
S21-1*tolC*::Kan	0.03	0.12	0.06	0.06	0.25	0.06	0.06	0.12	32	1	<2	<4	16/8	<0.12	<0.12/2.38	<2	>64	>32
S21-1 *acrAB*::Kan	0.03	0.12	0.06	0.06	0.25	0.06	0.06	0.12	32	1	<2	<4	16/8	<0.12	<0.12/2.38	<2	>64	>32
S21-1 *gyrA*+	0.03	0.03	0.06	0.015	0.25	0.03	0.03	0.12	4	8	>32	>32	4/2	1	0.25/4.75	8	>64	>32
S21-1parC+	0.5	1	0.5	0.25	4	0.5	0.5	2	256	2	>32	32	16/8	0.5	0.25/4.75	8	>64	>32
S21-2	1	8	8	4	>16	4	1	16	>256	>16	>32	>32	16/8	4	2/38	>32	<8	>32
S21-2*tolC*::*Kan*	0.06	0.5	0.12	0.12	0.25	0.12	0.06	0.25	64	2	<2	<4	16/8	<0.12	<0.12/2.38	8	>64	>32
S21- 2 *acrAB*::*Kan*	0.06	0.5	0.12	0.12	0.25	0.12	0.12	0.25	64	2	<2	<4	16/8	<0.12	<0.12/2.38	8	>64	>32
S21-2*gyrA*+	0.25	0.25	1	0.25	2	1	0.25	2	64	>16	>32	>32	8/4	4	>4/76	32	>64	>32

S21-2*parC*+	1	8	8	4	>16	4	1	16	>256	>16	>32	>32	16/8	2	>4/76	32	>64	>32
S21-3	8	>16	>16	32	>16	>16	>16	16	>256	>16	>32	>32	16/8	4	>4/76	>32	<8	>32
S21-3*tolC::Kan*	0.06	0.5	0.12	0.25	0.5	0.25	0.12	0.5	32	1	<2	<4	16/8	<0.12	<0.12/2.38	8	>64	>32
S21-3 *acrAB::Kan*	0.06	0.5	0.25	0.25	1	0.25	0.12	0.5	64	1	<2	<4	16/8	<0.12	<0.12/2.38	8	>64	>32
S21-3*gyrA*+	1	1	2	1	4	2	1	2	>256	>16	>32	>32	8/4	2	>4/76	>32	>64	>32
S21-3*parC*+	16	>16	>16	32	>16	>16	16	>16	>256	>16	>32	>32	8/4	4	>4/76	32	>64	>32
S21-4	16	>16	>16	256	>16	>16	>16	16	>256	>16	>32	>32	16/8	4	>4/76	>32	<8	>32
S21-4 *tol::KanC*	0.25	1	0.5	0.5	4	0.5	0.5	1	128	2	32	8	16/8	<0.12	<0.12/2.38	8	>64	>32
S21-4 *acrAB::Kan*	0.06	0.5	0.12	0.25	1	0.25	0.25	0.12	64	1	<2	<4	16/8	<0.12	<0.12/2.38	8	>64	>32
S21-4*gyrA*+	2	2	4	2	4	2	2	8	128	>16	>32	>32	8/4	2	>4/76	>32	>64	>32
S21-4*parC*+	16	>16	>16	128	>16	>16	16	>16	>256	>16	>32	>32	8/4	4	>4/76	32	>64	>32

GAT, Gatifloxacin; SAR, Sarafloxacin; ENR, Enrofloxacin; CIP, Ciprofloxacin; DIF, Difloxacin; DAN, Danofloxacin; LEV, Levofloxacin; ORB, Orbifloxacin, NAL, Nalidixic Acid, FOX, Cefoxitin; CHL, Choramphenicol; TET, Tetracyclin; AMO, Amoxicillin-clavulanic; TIO, Ceftiofur; COT, Trimethoprim-sulfamethoxazole; CEP, Cephalothin; KAN, Kanamycin; AMP, Ampicillin.

Table 5-5. Antimicrobial susceptibility of deletion and replacement mutants of *Salmonella* field strains

ID	GAT	SAR	ENR	CIP	DIF	DAN	LEV	ORB	NAL	FOX	CHL	TET	AMO	TIO	COT	CEP	KAN	AMP
CS1	4	16	16	16	>16	>16	8	>16	>256	1	>32	>32	32/16	1	>4/76	16	<8	>32
CS1*tolC;;Kan*	0.5	4	4	2	4	4	1	4	256	1	>32	32	32/16	0.5	2/38	8	>64	>32
CS1*acrAB::Kan*	0.5	4	4	4	16	4	2	4	>256	1	>32	>32	32/16	0.5	2/38	8	>64	>32
CS1*parC+*	8	>16	>16	16	>16	>16	8	>16	>256	1	>32	>32	32/16	1	>4/76	16	>64	>32
CS1*gyrA+*	0.06	0.06	0.06	0.03	0.5	0.06	0.06	0.25	4	2	>32	>32	32/16	1	>4/76	8	>64	>32
CS3	4	16	16	16	>16	>16	8	>16	>256	1	>32	>32	32/16	1	>4/76	16	<8	>32
CS3*tolC::Kan*	1	4	2	2	4	4	1	4	256	1	>32	32	32/16	0.5	2/38	8	>64	>32
CS3*acrAB::Kan*	0.5	4	4	4	4	4	1	4	>256	1	>32	>32	32/16	0.5	2/38	8	>64	>32
CS3*parC+*	4	16	16	16	>16	>16	8	>16	>256	1	>32	>32	32/16	1	>4/76	16	>64	>32
CS3*gyrA+*	0.06	0.06	0.06	0.03	0.25	0.06	0.06	0.12	4	2	>32	>32	32/16	1	>4/76	8	>64	>32
CS9	8	>16	>16	16	>16	>16	8	>16	>256	1	>32	>32	32/16	1	>4/76	16	<8	>32
CS9*tolC;;Kan*	0.5	4	4	2	4	4	1	>16	256	1	>32	32	32/16	0.5	2/38	8	>64	>32
CS9*acrAB::Kan*	0.5	4	4	2	8	4	1	4	256	1	>32	>32	32/16	0.5	2/38	8	>64	>32
CS9*parC+*	8	>16	>16	16	>16	>16	8	>16	>256	1	>32	>32	32/16	1	>4/76	16	>64	>32
CS9*gyrA+*	0.03	0.03	0.06	0.03	0.25	0.06	0.06	0.12	4	2	>32	>32	32/16	1	>4/76	8	>64	>32
CHS14	0.25	0.25	1	0.12	1	0.25	0.25	1	128	4	8	>32	2/1	1	0.25/4.75	4	<8	<1

CHS14*tolC::Kan*	<0.015	<0.015	<0.015	<0.015	0.03	0.03	<0.015	0.03	16	1	<2	>32	2/1	<0.12	<0.12/2.38	<2	>64	<1
CHS14*acrAB::Kan*	<0.015	<0.015	0.03	<0.015	0.03	0.03	0.03	0.03	8	1	<2	>32	2/1	<0.12	<0.12/2.38	<2	>64	<1
CHS14*gyrA+*	0.03	0.03	0.06	<0.015	0.5	0.03	0.06	0.12	4	2	8	>32	2/1	1	0.25/4.75	<2	>64	<1
CHS18	0.25	0.25	0.5	0.12	2	0.5	0.25	1	256	2	8	4	1/0.5	1	0.12/2.38	4	8	2
CHS18*tolC::Kan*	0.03	0.03	0.03	0.03	0.06	0.06	0.03	0.06	32	1	2	4	1/0.5	0.12	0.12/2.38	2	>64	1
CHS18*acrAB::Kan*	0.12	0.03	0.25	0.03	1	0.03	0.03	0.5	16	1	2	4	1/0.5	0.12	0.12/2.38	2	>64	1
CHS18*gyrA+*	0.06	0.06	0.12	0.06	0.25	0.06	0.06	0.12	32	2	4	4	1/0.5	1	0.12/2.38	4	>64	2
CHS38	0.12	0.12	0.12	0.06	1	0.25	0.12	0.5	32	4	32	32	1/0.5	0.5	>4/76	8	8	>32
CHS38*tolC::Kan*	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.15	0.5	0.5	4	16	1/0.5	0.12	>4/76	8	>64	>32
CHS38*acrAB::Kan*	<0.015	0.03	0.03	0.03	0.06	0.03	0.03	0.06	1	0.5	4	16	1/0.5	0.12	>4/76	8	>64	>32
CHS38*gyrA+*	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.15	8	4	32	32	1/0.5	0.5	>4/76	16	>64	>32

GAT, Gatifloxacin; SAR, Sarafloxacin; ENR, Enrofloxacin; CIP, Ciprofloxacin; DIF, Difloxacin; DAN, Danofloxacin; LEV, Levofloxacin; ORB, Orbifloxacin, NAL, Nalidixic Acid, FOX, Cefoxitin; CHL, Choramphenicol; TET, Tetracyclin; AMO, Amoxicillin-clavulanic; TIO, Ceftiofur; COT, Trimethoprim-sulfamethoxazole; CEP, Cephalothin; KAN, Kanamycin; AMP, Ampicillin.

chloramphenicol, tetracycline, trimethoprim, ampicillin, cefoxitin, and ß-lactam (Table 5-4). The ciprofloxacin MIC reduced up to 11 log2 dilutions when the *acrAB* or *tolC* was deleted in these mutants of S21 and Spy1. All deletion mutants for *acrAB* and *tolC* became susceptible to all fluoroquinolones. High level fluoroquinolone resistance mutants remained, although lower MIC than breakpoint, higher MIC after the deletion of *acrAB* or *tolC* in these mutants (Table 5-4). The deletion of *acrAB* and *tolC* made all mutants more susceptible than wild type to chloramphenicol, tetracycline, ceftioufur, cefoxitin, amoxicillin/clavulance, trimethoprim/sulfamethoxazol. However, *acrAB* or *tolC* deletion mutants of S21-2, S21-3, and S21-4 remained resistant to ampicillin (MIC>32µg/ml) and cephalothin (MIC=8µg/ml) (Table 5-4). It is suggested that efflux pumps other than *acrAB-tolC*, which was induced by fluoroquinolone, accounted for the resistance to ampicillin and decreased susceptibility to cephalothin. The deletion of *acrE*F, *emrAB*, *mdlAB*, *yegMNO* in S21, S.py1, and ifluoroquinolone resistance mutants didn't affect the MIC to any antimicrobials other than fluoroquinolones tested (table 5-4).

The replacement of mutated *gyrA/parC* with wild type alleles in S21 and S.py1 didn't change the susceptibility to any antimicobials other than fluoroquinolones tested. However, replacement of mutated gyrA with its wild type allele gyrA+ reduced the fluoroquinolone MICs of fluoroquinolone resistance mutants up to eight log2 dilutions, while not to other antimicrobial MICs. High level fluoroquinolone resistance mutants remained, although lower MIC than breakpoint, higher MIC after the replacement of mutated gyrA with its wild type allele gyrA+ in these mutants (Table 5-4). Replacement of mutated *parC* with its wild type allele in fluoroquinolone resistance mutants of S.Py1 didn't affect the susceptibility to any of the drugs tested.

The mechanisms of fluoroquinolone resistance in *Salmonella* field strains were similar to what was found in the laboratory-induced strains. The deletion of *acrAB* or *tolC* or replacement with a wild type *gyrA* allele in field strains significantly reduced fluoruoquinolone MICs (Table 5-5). The deletion of *acrAB* and *tolC* in field strains CS1, CS3, and CS9 did not reduced fluoroquinolone MICs to a level as low as in laboratory induced strains. For instance, S21-2 and CS1, CS3, CS9 have same ciprofloxacin MIC of 8µg/ml. The deletion of *acrAB* and *tolC* in S21-2 reduced the ciprofloxacin MIC to 0.06µg/m, while deletion of *acrAB* and *tolC* in CS1, CS3, CS9 reduced the ciprofloxacin MIC to 0.5µg/ml. Replacement of mutated *gyrA* of CS1, CS3, and CS9 with wild type allele *gyrA*+ reduced fluoroquinolone MIC to lower level than in laboratory induced mutants. For instance, replacement of mutated *gyrA* with wild type allele in S21-2 reduced ciprofloxacin MIC to 0.25µg/ml, while replacement of mutated *gyrA* with wild type allele in CS1, CS3, CS9 reduced the ciprofloxacin to 0.03µg/ml. Deletion of *acrAB* or *tolC* or replacement of *gyrA* of CHS14, CHS18, and CHS38 with wild type allele reduced the MIC to a very low level. In contrast to laboratory-induced mutants, the deletion of *acrAB* or *tolC* of CS1, CS3, and CS9 failed to reduce chloramphenicol, tetracycline, and ß-lactam MIC. The maintenance of the resistance to these drugs was due to the presence of other mechanisms of resistance in these strains. These strains were detected to contain the *cat1* and *cat2* genes conferring resistance to chloramphenicol; the *tetB* and *tetA* genes conferring resistance to tetracycline; a *dhfrI* gene conferring resistance to trimethoprim; and a bla_{OXA1} conferring resistance to ampicillin and showing decreased susceptibility to cephalothin and cefoxitin. The maintenance of tetracycline resistance in CH14 and tetracycline, trimethoprim and ampicillin resistance in CH38

strains was due to the presence of *tetA* gene in both strains and bla_{TEM-1} and *dhfrI* in CHS38. Like laboratory-induced strains, the deletion of *acrEF, emrAB, mdlAB, yegMNO* in these field strains failed to change MIC of all drugs tested. The replacement of *parC* gene in CS1, CS3, and CS9 failed to reduce the MIC of all drugs tested.

Discussion

The mechanism of nalidixic acid resistance in *Salmonella* has been attributed to a single mutation in the *gyrA* gene, leading to an amino acid substitution either at position 83 or 87. These *Salmonella* strains were nalidixic acid-resistant and showed decreased susceptibility to fluoroquinolone. In high-level fluoroquinolone-resistant *Salmonella*, double mutations leading to an amino acid substitution in both of the 83 and 87 positions were detected in the *gyrA* gene. No mutations in *parC* gene were detected in high level fluoroquinolone-resistant *Salmonella* until recently. A substitution at position Ser80 in *parC* has been reported in animal and human isolates. The role of target gene mutations in quinolone resistance has been studied in *E. coli* by introducing the mutation experimentally in the absence of quinolone selection. Introducing a single *gyrA* S83 or S87 mutation, double *gyrA* mutations, or single *parC* S80 didn't result in ciprofloxacin resistance (MIC<4µg/ml). However, the combination of three mutations (*gyrA* S83, D87, and *parC* S80) was associated with higher MIC of CIP (>64 µg/ml)(12). In addition, the introducing a plasmid-coded allele of *gyrA*+ or *parC*+ respectively reduced the MICs of ciprofloxacin, which indicted that both types of mutations contributed to the expression of quinolone resistance (84, 105). The role of *gryA* or *parC* mutations on fluoroquinolone

resistance in *Salmonella* is different from that in *E. coli*. In this study, *parC* mutation was detected in S.py1-2, S.py1-3, S.py1-4, while no *parC* mutation was detected all mutants of S21. Independent of *parC* mutation, the replacement of *gyrA* S83, S87 or the combination of these two mutations with wild type *gyrA* allele significantly reduced fluoroquinolone MICs in both laboratory-induced mutants and field strains. The replacement of mutated *parC* with wild type allele in S.py1 mutants and field strains failed to reduce fluoroquinolone MICs in these strains. No mutation was detected in *gyrB* and *parE* in induction mutants of S21 and S.py1. Although mutation in *Salmonella* gyrB was reported (80, 112), it is suggested that the *gyrB* and *parE* in *Salmonella* were not associated with fluoroquinolone resistance. It was concluded that mutation in *gyrA* while not the mutation in other topoisamerase genes was associated with fluoroquinolone resistance in *Salmonella*.

Our results are consistent with the phenomenon of *gyrA* and *parC* mutations in fluoroquinolone resistance *Salmonella*. The *gyrA* mutation is always detected in fluoroquinolone-resistant *Salmonella*, while the *parC* mutation is not always associated with high level fluoruoquinolone resistance in *Salmonella* and only reported recently. The inconsistency between the *parC* mutation and fluoroquinolone resistance in *Salmonella* may indicated that *Salmonella parC* was not a secondly target of fluoroquinolone. In contrast to *Salmonella*, the *parC* mutation in *E. coli* is always detected in high level fluoroquinolone-resistance *E. coli* and was suggested as secondly target of fluoroquinolone (84). The *E. coli parC* as a secondly target of fluoroquinolone was supported by testing the role of *parC* mutation in fluoroquinolone-resistant *E. coli*. The

introducing of wild type *parC* into fluoroqinolone-resistant *E. coli* reduced the fluoroquinolone MICs in this *E coli* strain.

Our study disagreed with a recent report of the role of *parC* mutation in fluoroquinolone-resistant *Salmonella* (80). The difference between these two studies might be due to the use of deficient methods in their study. First, in their study, a wild type *parC* gene from *E. coli* while not from *Salmonella* carried by a high copy cloning vector was transformed into fluoroquinolone-resistant *Salmonella*. The dominance of *E. coli parC* gene in *Salmonella* can not address of the role of *Salmonella parC* mutation in fluoroquinolone resistance. Second, the dominance test which lacked the power to directly identify the role of topoisomerase gene mutations on fluoroquinolone resistance was used in their study to address the role of target mutation on fluoroquinolone resistance. In this study, we directly replace the mutated *parC* of *Salmonella* with wild type allele in bacterial chromosome. The results from our study directly demonstrate the role of topoisomerase gene mutation in fluoroquinolone resistance in bacteria. Our methodology is extremely useful in the study of the role of gene mutations on fluoroquinolone resistance in *Salmonella, E. coli* or other Gram-positive or Gram-negative organisms.

Efflux pump is a known mechanism of antimicrobial resistance. Among more than 20 multiple drug resistant efflux pumps studied in *E. coli*, at least three representing *acrAB, yegM*ON, *norm* are associated with fluoroquinolone resistance (148, 241). *acrAB* appears to affect fluoroquinolone susceptibility most significantly (148, 196, 241). The deletion of *acrAB* in *E. coli* including strains with target gene mutations, resulted in fluoroquinolone hypersusceptibility (MIC<0.06μg/ml) and increased susceptibility to

other classes of drugs (196, 241). In order to examine the role of efflux pumps in fluoroquinolone resistance in *Salmonella*, RT-PCR was first used to screen for the overexpessed efflux pump gene in fluoroquinolone-resistant *Salmonella* strains. The expression level of several efflux pumps is consistently increased according to the decrease of the susceptibility to fluoroquinolone. However, some efflux pumps such as MdlB, which was dramatically overexpressed in high level mutants of S21, was not significantly changed in mutants of S.py1. It is suggested that different systems might be stimulated in these two isolates to respond to the fluoroquinolone selection pressure. In our study, only *acrAB* was associated with the fluoroquinolone resistance in *Salmonella*. Deletion of *acrAB* in all *Salmonella* strains dramatically increased the susceptibility to fluoroquinolone, while deletion of *acrE*F, *emrAB, yegMNO, mdlAB* had no effect. Overexpression of these efflux pumps in *Salmonella* may be due to the overexpression of some regulatory gene such as *marA* and *soxS*, whose overexpression can lead to the overexpression of *acrAB* and *tolC*. The overexpression of *marA* and *soxS* can also cause the overexpression of other efflux pump genes in *Salmonella* (169). Deletion of *tolC* caused the same effect as deletion of *acrAB* in *Salmonella*, which agreed with the *acrAB-tolC* efflux pump model (134). The key role of *acrAB-tolC* in fluoroquinolone resistance in *Salmonella* agreed with the resent report in which the deletion of *acrAB* in fluoroquinolone-resistant *Salmonella* enterica Typhimurium DT204 resulted in a fluoroquinolone susceptible phenotype for the ciprofloxacin (MIC=2µg/ml) (196). In contrast to fluoroquinolone-resistant efflux pumps in *E. coli*, the efflux pump YegMNO is not associated with fluoroquinolone resistance in *Salmonella*. Although the YdhE efflux pump is not further studied by gene deletion, the expression level of YdhE efflux

pump didn't change in all level mutants suggesting that YdhE efflux pump might not be related to the fluoroquinolone resistance in *Salmonella*.

In conclusion, *gyrA* mutation and upregulation of *acrAB-tolC*, are implicated in the fluoroquinolone resistance of *Salmonella*. Lower level expression of *acrAB* was associated with bacterial survival in lower level fluoroquinolone. The deletion of *acrAB* in *Salmonella* S.py1 and S21 made the cell hyperrsusceptible to fluoroquinolone (MIC<0.015µg/ml). Mutations in *gyrA* can confer bacteria resistant to quinolone (nalidixic acid) and showed decreased susceptibility to fluoroquinolone. The increased expression of *acrAB-tolC*, which can pump more and more fluoroquinolone out of bacteria plasma, can confer the bacteria to be resistant to higher level of fluoroquinolone. Independent of target gene mutation, the increased expression of *acrAB-tolC* in different *Salmonella* mutants caused the bacteria to be resistant to higher and higher concentrations of fluoroquinolone (ciprofloxacin MIC = 32 and 256 µg/ml). The highly fluoroquinolone resistance in *Salmonella* relied on the combination of these two mechanisms. The deletion of *acrAB-tolC* or replacement of mutated *gyrA* made the high level fluoroquinolone-resistant mutant (ciprofloxacin MIC = 256 µg/ml)) susceptible all fluoroquinolones tested.

The difference of mechanisms of fluoroquinolone resistance between laboratory-induced and field-selected fluoroquinolone-resistant *Salmonella* was the different roles the target gene mutation and efflux played in these *Salmonella* isolates. Overexpression of efflux pump played a major role in laboratory-induced fluoroquinolone-resistant *Salmonella*. Deletion of *acrAB* or *tolC* in S21-3 or S.py1-3 (ciprofloxacin MIC = 32 µg/ml) reduced MIC to 0.25 µg/ml, while replacement mutated *gyrA* (single mutation

S87) with wild type allele reduced the MIC to 1 µg/ml. In contract to laboratory-induced strains, target mutations played major role in fluoroqunolone resistance in field *Salmonella* strains. Double mutations in *gyrA* (S83 or S87) were found in field selected fluoroquinolone-resistant *Salmonella* strains (ciprofloxacin MIC = 16 µg/ml). The replacement of mutated gyrA with wild type allele reduced the MIC to 0.03 µg/ml, while the deletion of *acrAB* or *tolC* only reduced the MIC to 4 µg/ml. It can be concluded that long-term low concentrations selection (field selection) tented to select more mutations in target gene which can confer higher level resistance than single target gene mutation and short term high concentrations of drugs tented to upregulate efflux expression which can confer high level resistance.

Independent of target mutations, the high level fluoroquinolone-resistant mutants displayed higher level fluouroquinolone MICs after replacement of mutated target gene with wild type allele. It is easy to understand because the expression level of efflux pump increased in different level fluoroquinoloen-resistant mutants. The more efflux pump was expressed in high level fluoroquinolone mutants can maintain higher MIC when *gyrA* mutation was converted to wild type allele. Independent of efflux pump, the high level fluoroquinolone-resistant mutants displayed higher fluoroquinolone MICs after the deletion of *acrAB* or *tolC*. It is suggested that this difference may due to the following reasons: 1) the overexpression of *acrAB* in *Salmonella* mutants was regulated by the overexpression of *marA*, which in turn decreased the expression of outer membrane protein ompF. The decreased expression of ompF would decrease the intake of fluoroquinolone to bacterial plasm. 2) some other efflux pumps, which can also be

induced by fluoroquinolone, can slightly effect the fluoroquinolone resistance in *Salmonella*.

Understanding the mechanisms of fluoroquinolone resistance in *Salmonella* provide guidance in fluoroquinolone chemotherapy in the future. The combination use of fluoroquinolone and an efflux pump inhibitor, particularly the *acrAB-tolC* efflux pump inhibitor such as 7-nitro-8-methyl-4-[2'-(piperidino)ethyl]-aminoquinoline, could 1) make fluoroquinolone efficient to intermediate resistant or resistant *Salmonella*, 2) decrease the chance of target gene mutation, 3) prolong the serve life of fluoroquinolone drugs in the future (118, 173, 174). However, the inhibition of acrAB-tolC might stimulate other backup systems (other efflux pumps) to confer resistance to fluoroquinolone (196). Such a strategy will require the development of a broad efflux pump inhibitor in the future.

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Chapter 6 Summary

Bacterial antimicrobial resistance in both the medical and agricultural fields has become a serious problem worldwide. The use of antimicrobial agents in animals, particular use antimicrobial agents as prophylaxis and growth promoter, favors the selection of antimicrobial resistance in bacteria. These resistant bacteria may subsequently transmit to humans through food chain or human and animal interactions. The main thrust of this work was to characterize and explore the molecular mechanisms of antimicrobial resistance in foodborne pathogens.

Salmonella is a leading cause of foodborne illness in the United States. Each year, an estimated of 1.4 million cases of salmonellosis occour in the US which causes16,000 hospitalization and 600 deaths. In recent years, the portion of *Salmonella* which are resistant to clinical important drugs such as ciprofloxacin and ceftriaxone are increasing. The emergence of resistance to front line drugs in Salmonella has caused public health consequence. The antimicrobial resistance phenotypes of Salmonella isolated from food samples were characterized in this study to evaluate the safety of food products because most of *Salmonella* infections resulted from the ingestion of contaminated meat products. Of the most important are the detection of ceftriaxone-resistant *Salmonella* from the US meat products and the fluoroquinolone-resistant *Salmonella* from Chinese meat products. Genotype characterization of these multiple antimicrobial-resistant *Salmonella* revealed that ß-lactamase gene $bla_{CMY\text{-}2}$ was widely present in ceftriaxone-resistant *Salmonella* and conferred resistance to most of the ß-lactams tested. Most of the antimicrobial-resistant determinants were encoded in self-transmissible plasmid and can be transferred between

different organisms. The class I integron also played an important role on transmission of antimicrobial resistance determinants in bacteria.

Characterization of antimicrobial resistance genes is a powerful tool for describing bacterial pathogens. For instance, it can be used to investigate the population dynamics of clinical isolates, including the spatial and temporal distribution of resistance genes. The drawback of using PCR and similar assays to characterize the antimicrobial resistance genes, however, is that they are for the most part limited in the number of genes that can be reasonably screened. Microarray is a powerful tool to analyze hundreds and thousands of genes on a microchip and could be a good tool for characterization of antimicrobial resistance genes in bacteria. Testing of antimicrobial-resistant and virulence genes from *E. coli* and *Salmonella* using DNA-based microarray demonstrated that Microarray analysis is an effective method to rapidly screen pathogenic strains of *Salmonella* and *E. coli* for multiple antimicrobial resistance and virulence genes. This method could also be useful to identify the potential antimicrobial resistance genes which failed to show any phenotype in bacteria but would be easily inducted under antibiotic selective pressure.

Although the *Salmonella* isolates which were resistant to fluoruoquinolone were rare, the *Salmonella* that showed decreased susceptibility to fluoroquinolone are increasing. The emergence of fluoruoquinolone resistance in *Salmonella* caused significant public health consequence because fluoroquinolone is a choice in the treatment of *Salmonella* invasive infections in the US. It is important to study the molecular mechanisms of antimicrobial resistance particularly the resistance to front line drugs because the understanding of the molecular mechanisms by which

antimicrobial resistance emerges and spreads should enable us in the future to develop effective intervention strategies to reduce or stop its progression. Our work has shown that when *Salmonella* are exposed to fluoroquinolones, certain efflux pumps are overexpressed in tandem with particular mutations in topoisomerase genes (*gyrA* and *parC*). Using deletion studies, it appears that the most relevant genes with regards to the selection of fluoroquinolone resistance phenotypes among Salmonella are the AcrAB efflux pump and the *gyrA* portion of DNA gyrase. Understanding the mechanisms of fluoroquinolone resistance in *Salmonella* can give guidance in fluoroquinolone chemotherapy in the future. Combination use of fluoroquinolone and efflux pump inhibitor, particularly *acrAB-tolC* efflux pump inhibitor such as 7-nitro-8-methyl-4-[2'-(piperidino)ethyl]-aminoquinoline, can 1) make fluoroquinolone efficient to intermediate resistant or resistant *Salmonella*, 2) decrease the chance of target gene mutation, 3) prolong the serve life of fluoroquinolone drugs in the future.

Pathogenic bacteria, such as *Salmonella*, are not the only concern when considering antimicrobial resistance in bacteria with food animal reservoirs. Commensal bacteria, which are naturally occurring host flora, constitute an enormous potential reservoir of resistance genes for pathogenic bacteria. The prevalence of antibiotic resistance in the commensal bacteria of humans and animals is considered to be a good indicator of the selective pressure of antibiotic usage and reflects the potential for resistance in future infection. Characterization of *E. coli* isolates from diseased swine and chicken revealed that multiple-antimicrobial-resistant *E. coli*, including fluoroquinolone-resistant variants, are commonly present among diseased swine and chickens in China It was suggested the need for the introduction of surveillance programs in China to monitor antimicrobial

resistance in pathogenic bacteria that can be potentially transmitted to humans from food animals.

In the future, we will keep monitoring the prevalence of the resistance to novel drugs in foodborne pathogens and to study the molecular mechanisms of these resistances. From our work, the efflux pumps played an important role in the resistance to many different antimicrobials. The study of efflux pump and antimicrobial resistance is another important aspect in this field. In the future, we will propose to develop a microarray which contains all membrane transporters of bacteria. This microarray could be used to screen for the associated efflux pump which can coffer resistance to different antimicrobials. This efflux pumps could be further characterized using the deletion and RNAi techniques. The doness of these studies may provide useful information on how to control the antimicrobial resistance in bacteria in the future.

Chapter 7 Reference

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