THE PENNSYLVANIA STATE UNIVERSITY
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ANTIBIOTIC RESISTANCE PATTERNS OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS STRAINS ISOLATED FROM BROILER CHICKENS

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ABSTRACT

Salmonella enterica, a group of rod-shaped, Gram-negative bacteria is a leading cause of foodborne illness in humans. Although more than 2,600 serovars of the bacteria exist, *Salmonella enterica* serovar Enteritidis is one of the most common causes of foodborne salmonellosis in humans primarily spread through contaminated shell eggs, egg products, and other poultry products. Antimicrobial resistance among foodborne bacterial pathogens like *Salmonella* species has become an ongoing public health issue. In order to assess the degree of antibiotic resistance in *Salmonella* Enteritidis (SE), this study sought to analyze antibiotic resistance patterns and several genes known to be involved in the expression of antibiotic resistance in SE. A total of eighty nine SE isolates recovered from diseased broiler chickens submitted to The Pennsylvania State University's Animal Diagnostic Laboratory were examined for their susceptibility to 26 antibiotics using Kirby Bauer disc diffusion test and for the presence of the *marRAB* operon (Multiple Antibiotic Resistance) genes, which are known to regulate resistant gene expression in some bacteria, by polymerase chain reaction. The tested isolates were also subjected to pulsed-field gel electrophoresis (PFGE) to determine if there is any association between PFGE fingerprint patterns and antimicrobial resistance profiles of SE. Although all the isolates contained the *marRAB* operon genes, only very few isolates (4.49%) showed any resistance to the antibiotics used in the study. Furthermore, no correlation was made between antibiotic resistant profiles and PFGE types. Regardless of the lack of relationships found, the study showed that only a small amount of isolates displayed resistance to antibiotics despite the growing threat of antibiotic resistance. Thus, although antibiotic resistance is a

prevalent issue in food animal production, the results obtained showed no significant role of resistance in clinical isolates of SE isolated from broiler chickens.

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ABBREVIATIONS

CLSI	Clinical Laboratory Standards Institute
marRAB	Multiple Antibiotic Resistance Operon
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field Gel Electrophoresis
SE	*Salmonella enterica* serovar Enteritidis

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Chapter 1

Introduction

Salmonella, a genus of Gram-negative bacilli in the family *Enterobacteriaceae*, causes salmonellosis in humans and a broad range of animals. Infection can occur through direct or indirect contact with infected humans and other species and often occurs through consumption of contaminated food products or via contact with the feces from infected animals (1). Humans primarily become infected through the improper handling and undercooking of contaminated meat, poultry, eggs, and produce (2). Once infected, people with salmonellosis typically develop gastroenteritis with clinical signs including nausea, diarrhea, fever, abdominal cramps, and occasional vomiting 12 to 72 hours post-infection (1). The infection usually resolves after four to seven days without treatment but can be life threatening (3, 4). The elderly, infants, and people who are immune compromised or impaired are most susceptible to severe infection (5).

The genus *Salmonella* is divided into two species, *Salmonella enterica* and *Salmonella bongori*, based on phenotype (6). *Salmonella enterica* is further divided into six subspecies. Serotyping is used to differentiate isolates of *Salmonella* beyond the subspecies level, and more than 2,600 serotypes of *Salmonella* have been designated to date. *Salmonella* can be classified into two groups based on clinical manifestations and serovar classification: typhoidal and nontyphoidal. Typhoidal serovars, which are only adapted to human hosts and are responsible for causing typhoid fever or paratyphoid fever, include Typhi, Paratyphi A, Paratyphi B, and Paratyphi C. Nontyphoidal *Salmonella* includes all other serovars and can infect humans, as well as other species (1, 7). In the United States, nontyphoidal *Salmonella* species account for over

1.2 million illnesses, 23,000 hospitalizations, and 450 deaths every year (4, 5). These numbers make it unsurprising that it is the leading pathogen resulting in hospitalizations and deaths from foodborne illness (8).

Specifically, *Salmonella enterica* is a leading cause of gastroenteritis both in humans and in animals and is the primary cause of salmonellosis in warm-blooded vertebrates (9, 10). Although over 2,600 serovars of *Salmonella enterica* exist, *Salmonella enterica* serovar Enteritidis (SE) is one of the most common serovars with a broad host range, infecting both human and animal hosts (1, 11). It accounted for 39.5% of salmonellosis cases in the European Union in 2013 and 14.5% in the United States in 2012 (12). Additionally, *Salmonella* Enteritidis is a major source of enteric disease in horses and poultry (10).

Considered the primary source of salmonellosis, contaminated poultry are an especially important source of SE in humans (12). Both laying hens and broiler chickens are susceptible to infection from the bacteria, thus allowing a mode of entry into the human food supply. Transmission of SE to broiler chickens can occur via horizontal transfer in which birds acquire bacteria after oviposition from the environment through contaminated food or water or via vertical transmission in which the egg is contaminated while it travels through the reproductive tract of the infected hen (13).

As consumption of red meat in the United States has decreased in the past several decades, the consumption of poultry and poultry products has increased. While the total consumption of red meat per capita has decreased from 133.9 pounds in 1965 to a projected 105.7 pounds in 2016, per capita consumption of broiler chickens in the United States has nearly tripled from 32.1 pounds in 1966 to a projected 90.6 pounds in 2016 (14). Likewise, the country's consumption of eggs has also increased. The average consumption per capita of eggs

has increased from 252.8 in 2001 to 263 in 2014. Thus, both laying hens and broiler chickens play a critical role in the nation's food supply (15).

A growing problem that threatens animal food production including poultry is antibiotic resistance. With the overuse and inappropriate use of antibiotics, bacteria have acquired different mechanisms to become resistant to these drugs. Bacteria may obtain such mechanisms by acquiring genes from plasmids, transposons, integrons, and bacteriophages; through mutations of genes; or, through a combination of these methods (16). According to the Centers for Disease Control and Prevention (CDC), antibiotic resistance causes over 2 million illnesses and is responsible for about 23,000 deaths annually (17).

One operon that plays a role in antibiotic resistance in bacteria is the multiple antibiotic resistance operon (*marRAB*) and is found in bacteria within the family *Enterobacteriaceae*, of which *Salmonella* is a member. The *marRAB* operon is responsible for encoding for several proteins, including a repressor MarR, a transcriptional activator MarA, and MarB which has an unknown function. While MarR typically represses the operon by binding to the operator *marO*, in cases that it is unable to do so, greater resistance may result (18). The MarR may be unable to bind to *marO* if there is a mutation in the *marR* gene or if the bacterium is exposed to a compound that is able to reduce repression via MarR binding. If MarR is unable to bind to *marO* through one of these mechanisms, *marRAB* expression is then induced, thus potentially increasing resistance to antibiotic drugs. Past research has shown that the activation of *marRAB* operon results in increased resistance to antibiotics like chloramphenicol, cephalosporins, nalidixic acid, fluoroquinolones, penicillins, puromycin, rifampicin, and tetracyclines (19).

The *marRAB* operon is able to successfully increase antibiotic resistance by influencing the expression of a variety of other chromosomal genes. While the operon influences over 60

other genes, the ones of specific importance are those that place a role in influx and efflux. Three specific genes that the operon influences are *acrA*, *acrB*, and *micF*. The *marRAB* operon increases antibiotic resistance by increasing efflux through the upregulation of *acrA* and *acrB* and by decreasing influx by upregulating *micF*, which plays a role in the regulation of the Outer Membrane Protein F (OmpF) porin. Overall, the *marRAB* operon and the genes it affects have been found to play a role in low-level antibiotic resistance to a variety of different antibiotics (19).

Based on the growing threat of antibiotic resistance on the United States' food supply, this study seeks to examine the role of the *marRAB* operon in SE in conferring resistance to a variety of antibiotics. It was the goal of this research to observe if the *marRAB* operon plays a role in resistance phenotypes and, if so, in what capacity. The following experiments seek to identify broiler isolates containing the *marRAB* operon, to assess those isolates' resistance patterns and genomic profiles, and to determine if any relationships between these findings exist.

Chapter 2

Materials and Methods

Selection and Growth of Bacterial Isolates

Eighty nine isolates of SE, labeled B1-B89, were studied and their sources are shown in Table 1. All isolates were taken from broiler chickens or their surrounding environment and isolated at The Pennsylvania State University's Animal Diagnostic Lab (ADL). All bacteria were maintained at ADL on trypticase soy agar slants kept at room temperature (Remel, Lenexa, KS). For experiments, bacteria were grown on Luria-Bertani agar (LBA) (BD Diagnostics, Franklin Lakes, NJ) at 37°C.

Table 1 Bacterial Strains Used
Eight nine SE isolates taken from diseased broiler chickens or their surrounding environment and submitted to The Pennsylvania State University's Animal Diagnostic Lab were studied. Here, the source of each isolated is listed.

Strain	***Source***	***Strain***	***Source***	***Strain***	***Source***	***Strain***	***Source***
B1	Liver	B24	Embryo	B46	Embryo	B68	Liver
B2	Tissue	B25	Embryo	B47	Embryo	B69	Liver
B3	Embryo	B26	Embryo	B48	Embryo	B70	Pericardium
B4	Environment	B27	Embryo	B49	Embryo	B71	Liver
B5	Embryo	B28	Embryo	B50	Embryo	B72	Jejunum
B6	Embryo	B29	Embryo	B51	Embryo	B73	Pericardium
B7	Embryo	B30	Embryo	B52	Embryo	B74	Dead-in-shell
B8	Environment	B31	Embryo	B53	Egg	B75	Hock joint
B9	Embryo	B32	Embryo	B54	Embryo	B76	Pericardium
B10	Embryo	B33	Liver	B55	Yolk	B77	Intestine
B11	Embryo	B34	Yolk	B56	Pericardium	B78	Liver
B12	Embryo	B35	Embryo	B57	Liver	B79	Intestine
B13	Tissue	B36	Joint	B58	Liver	B80	Pericardium

B14	Embryo	B37	Embryo	B59	Yolk	B81	Joint
B15	Embryo	B38	Embryo	B60	Unknown	B82	Chick room
B16	Embryo	B39	Embryo	B61	Unknown	B83	Embryo
B17	Embryo	B40	Embryo	B62	Pericardium	B84	Liver
B18	Embryo	B41	Embryo	B63	Liver	B85	Yolk
B19	Yolk	B42	Embryo	B64	Pericardium	B86	Liver
B20	Pericardium	B43	Embryo	B65	Air Sac	B87	Cecal tonsil
B21	Yolk	B44	Embryo	B66	Egg	B88	Pericardium
B22	Yolk	B45	Embryo	B67	Yolk	B89	Liver
B23	Embryo						

DNA Preparation

Genomic DNA was extracted from the SE isolates by the rapid boiling method. With this method, an isolated colony was suspended in 200 μL of distilled water and heated at 100°C for 10 minutes. The bacterial suspension was then vortexed for 30 seconds and centrifuged at 6000 rpm for 5 minutes. The resulting supernatant containing genomic DNA was collected and stored at -20°C until use.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was utilized to amplify and screen for the *marRAB* operon, *acrA*, and *acrB*. Reactions were run on the Mastercycler® pro (Eppendorf, Hauppauge, NY). Each reaction contained a no template control containing all reagents except genomic DNA and at least one positive control containing genomic DNA from one of two SE isolates from eggs, deemed SEE1 and SEE2. Primers were designed using Primer3 software, version 0.4.0, (Untergasser et al.) and obtained from Integrated DNA Technologies (Coralville, Iowa). Primer sequences can be found in Table 2.

Table 2 Primer Designs for PCR Screening
The following nucleotide sequences were used as primers for PCR reactions to screen for antibiotic resistance genes.

Gene	*Primer Sequences*	*Melting Temperature*	*Expected Product Size*
***marRAB* operon (18)**			
Forward Primer	5' – GGG AAC AGG TTT CCG GCA GAC GAA – 3'	63.4°C	2300 bp
Reverse Primer	5' – GCT GGC GAG CGC CGC GGT GGT GTT AC – 3'	61.2°C	
acrA			
Forward Primer	5' – TAA CAG GAT GTG ACG ACA AAC A – 3'	54.2°C	939 bp
Reverse Primer	5' – GGG TTT CCA CTT TGT TAT CAG C – 3'	54.3°C	
acrB			
Forward Primer	5' – ATT ATC CTC GTG TTC CTG GTG A – 3'	55.6°C	543 bp
Reverse Primer	5' – GGT GTA GTG ATG CGT GCT CTT A – 3'	56.9°C	

PCR products were separated on agarose gels using agarose from Denville Scientific, Inc. (Holliston, MA) and visualized using ethidium bromide under UV light using AlphaImager HP software, version 3.4.0.0 (ProteinSimple, San Jose, CA).

Screening of marRAB Operon

The LongRange PCR Kit from Qiagen (Valencia, CA) was used to amplify the *marRAB* operon, which is 2.3 kb in size, PCR. The reaction consisted of 1 U of LongRange PCR enzyme mix, 0.4 μM of both the forward and reverse primers, 500 μM of each deoxynucleoside triphosphate, and 1X LongRange PCR buffer (containing 25 mM Mg^{2+}) in a final volume of 25 μL. Cycling conditions for screening of the *marRAB* operon were an initial denaturation step at

93°C for 3 minutes; 35 cycles of 93°C for 15 seconds, 62°C for 30 seconds, and 68°C for 2 minutes and 18 seconds; and a final extension of 72°C for 10 minutes. The resulting PCR product was separated by electrophoresis with Promega 1kb DNA ladder (Madison, WI) on a 1.5% agarose gel at 160 V for 120 minutes.

Screening of acrA

Taq Polymerase and 10X buffer (G-Biosciences, St. Louis, MO) were used to screen for *acrA* amplification. The reaction consisted of 1X buffer (120 mM Tris-HCl, 500 mM KCl, 1% Triton® X-100, 100 mM lysine, and 25 mM $MgSO_4$), 50 µM of each deoxynucleoside triphosphate, 0.2 µM of both the forward and reverse primers, and 0.625 U Taq in a final volume of 25 µL. Cycling conditions for screening of *acrA* were an initial denaturation step at 95°C for 1 minute; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3 minutes and 15 seconds; and a final extension of 72°C for 5 minutes. The resulting PCR product was separated by electrophoresis with Omega 1 kb DNA ladder (Norcross, GA) on a 1% agarose gel at 120 V for 120 minutes.

Screening of acrB

Taq Polymerase and 10X buffer (Fisher BioReagants, Leicestershire, United Kingdom) were used to screen for *acrB* amplification. The reaction consisted of 1X buffer (100 mM Tris-HCl, 500 mM KCl, and 15 mM MgCl2), 50 µM of each deoxynucleoside triphosphate, 0.2 µM of both the forward and reverse primers, and 0.625 U Taq in a final volume of 25 µL. Cycling conditions for screening of *acrB* were an initial denaturation step at 94°C for 3 minutes; 30

cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension of 72°C for 5 minutes. The resulting PCR product was separated by electrophoresis with Bionexus H-Lo ladder (Oakland, CA) on a 1% agarose gel at 120 volts for 120 minutes.

Gel Extraction of *marRAB* Operon

PCR product from *marRAB* was extracted and purified using QIAGEN's MiniElute Gel Extraction Kit. DNA fragments were cut out of the gel and weighed. Three volumes of Buffer QG were added for every one volume of gel (i.e. 300 µL of Buffer QG was added for every 100 mg of gel). The gel buffer mixture was then incubated at 50°C or until the gel had completely melted in the buffer. One gel volume of isopropanol was added and mixed by inverting the tube several times. 800 µL of the mixture was transferred to a MiniElute column within a 2 mL collection tube and centrifuged for one minute and flow-through was discarded. For volumes larger than 800 µL, transfer of the mixture to the MiniElute column and centrifugation for one minute was continued until all of the mixture had been centrifuged, and flow-through was discarded. 500 µL of Buffer QG was then added to the column and centrifuged again, and flow-through was once again discarded. 750 µL of Buffer PE with ethanol was added to the column. The column was left for five minutes before centrifuging for one minute, and flow-through was again discarded. The column was centrifuged for an additional minute. The column was then transferred to a 1.5 mL microcentrifuge tube, and 10 µL was added to the center of the column. The tube was allowed to sit for one minute before being centrifuged for one minute.

DNA Sequencing

The purified PCR products were kept at -4°C until being sent out for sequencing. Sequencing was completed using Sanger sequencing at the Genomics Core Facility at Penn State's Huck Institutes of the Life Sciences.

Antibiotic Resistance Assay

Kirby-Bauer disc diffusion antimicrobial susceptibility assay was used to determine the susceptibility of all 89 isolates to 26 antibiotics representing a variety of antibiotic classes. The assay was conducted according to the guidelines of the Clinical Laboratory Standards Institute (CLSI). Briefly, the isolates were grown overnight on LBA at 37°C and suspended in sterile normal saline (0.85% sodium chloride) to obtain a turbidity equivalent to 0.5 McFarland standard. With a sterile cotton swab, bacterial suspensions were streaked onto Mueller-Hinton II agar (Becton, Dickinson, and Company, Franklin Lakes, NJ), covering the surface of the plate three times. The cotton swab was dipped into the inoculation tube before the first two times, and the plate was rotated 60 degrees between each streaking to obtain an even distribution of the inoculum on the agar plate. The swab was then circled around the rim of the agar plate before allowing the plate to dry for 5 minutes. Antibiotic discs (Becton, Dickinson, and Company, Franklin Lakes, NJ) were then applied using a disc applicator, and plates were incubated overnight at 35°C. After 24 hours of incubation, the zones around the antibiotic discs were measured and compared to the CLSI Performance Standards for Antimicrobial Susceptibility Testing (24th Informational Supplement, 2014). Table 3 displays the antibiotics used and their respective classes.

Table 3 Antibiotics Used for Kirby Bauer Assays

SE isolates were tested against the following antibiotics whose antibiotics classes and concentrations are listed. Twenty six antibiotics from fourteen antibiotic classes were used in the Kirby Bauer assays.

Antimicrobial Category	*Antimicrobial Agent*	*Disc Concentration*
Aminoglycosides	Gentamicin (GM)	10 μg
	Amikacin (AN)	30 μg
	Tobramycin (NN)	10 μg
	Netilimicin (NET)	30 μg
Antipseudomonal penicillins + β-lactamase inhibitors	Ticarcillin-clavulanic acid (TIM)	75/10 μg
	Piperacillin-tazobactam (TZP)	100/10 μg
Carbapenems	Imipenem (IPM)	10 μg
	Meropenem (MEM)	10 μg
Non-extended spectrum cephalosporins; 1st and 2nd generation cephalosporins	Cefazolin (CZ)	30 μg
	Cefuroxime (CXM)	30 μg
Non-extended spectrum cephalosporins; 3rd and 4th generation cephalosporins	Ceftazidime (CAZ)	30 μg
	Cefotaxime (CTX)	30 μg
Cephamycins	Cefoxitin (FOX)	30 μg
	Cefotetan (CTT)	30 μg
Fluoroquinolones	Ciprofloxacin (CIP)	5 μg
Quinolones	Nalidixic Acid (NA)	30 μg
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75 μg
Monobactams	Aztreonam (ATM)	30 μg
Penicillins	Ampicillin (AM)	10 μg
	Ticarcillin (TIC)	75 μg
Penicillins + β-lactamase inhibitors	Amoxicillin-clavulanic acid (AMC)	20/10 μg
	Ampicillin-sulbactam (SAM)	10/10 μg
Phenicols	Chloramphenicol (C)	30 μg
Tetracyclines	Tetracycline (TE)	30 μg
	Doxycycline (D)	30 μg
	Minocycline (MI)	30 μg

Pulsed-field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was utilized for genetic profiling as it is considered PulseNet's "gold standard" of fingerprinting methods (20). PFGE was used to genotype all 89 isolates, using *Salmonella* serovar Braenderup strain H9812 (ATCC® BAA664™, Manassas, VA) as a standard for normalization of the gels. Plugs were made by first suspending a full loop of bacterial colonies grown overnight on LBA at 37°C into 200 µL of cell suspension buffer (100mM Tris:100mM EDTA, pH 8.0). The bacterial suspension was then added to 1.0 mL of the cell suspension buffer until the new suspension had a 0.8 ± 0.05 absorbance. 200 µL of this new suspension was then combined and mixed with 10 µL of proteinase K, followed by 200 µL of InCert agarose (1.6% InCert:1% sodium dodecyl sulfate agarose in TE buffer) kept at 55°C until use. Immediately upon mixing the InCert agarose into the suspension with proteinase K, 200 µL of the mixture was added to a well of a plug mold. Two plugs were made for each isolate. Plugs were incubated in 5.0 mL of cell lysis buffer (50mM Tris:50mM EDTA, pH 8.0 + 1% sarcosine) and 40 µL of proteinase K in a 54°C shaker water bath for one hour.

After incubating the plugs for an hour, they were washed. Plugs were first washed with 10 mL of 50°C sterile water and incubated for 15 minutes in a 50°C shaker water bath. Four washes with 5 mL of 50°C TE buffer (10mM Tris:1 mM EDTA, pH 8.0) for 15 minutes in a 50°C shaker water bath followed the first wash with water. Upon completion of all five washes, plugs were sliced and underwent a restriction endonuclease digestion. Plugs were incubated for 2 hours at 37°C in the following 50 µL per isolate xbaI enzyme master mix: 43 µL sterile water, 5 µL 10X buffer, 0.5 µL BSA, and 1.5 µL of xbaI enzyme. Upon completion of the enzyme digestion, the master mix was removed and 200 µL of 0.5X TBE was added to the plugs. Plugs

either remained in the TBE buffer overnight or were incubated in the TBE buffer for 5 minutes at room temperature if the enzyme digestion was run in the same day as the gel was run.

The gel was made by combining and heating 100 mL of 0.5X TBE buffer with 1.0 gram of SeaKem Gold agarose (Lonza, Allendale, NJ) until the agarose had dissolved. The agarose was kept at 55°C in a water bath until used. Meanwhile, the plug slices were removed from the TBE buffer and aligned and allowed to dry on the teeth of the comb. The comb was then placed in the gel casting mold, and the agarose was poured in the mold (leaving 2-3 mL of agarose, kept in the 55°C water bath, to later seal the wells). Once the agarose had hardened after approximately 30 minutes, the comb was removed gently and the agarose set aside was used to seal the wells. Once the gel had completely dried, it was run using the CHEF DRII system (Bio-Rad, Marnes-la-Coquette, France) for 19 hours (low MW: 30 kb, high MW: 700 kb, initial switch time: 2.16 seconds, final switch time: 63.8 seconds). Once the gel had finished running, it was stained with ethidium bromide in 500 mL of distilled water on a rotator for 15 minutes, followed by two 10 minute washes with 500 mL of distilled water only.

Gel images were analyzed using Bionumerics software, version 4.0 (Applied Maths, Austin, Texas). Dendrograms were completed using the Dice similarity coefficient and unweighted pair-grouping (UPGMA) with 1.6% position tolerance. The software divided isolates' PFGE results into distinct groups called pulsotypes.

Chapter 3

Results

Polymerase Chain Reaction

Screening of marRAB Operon

Once genomic DNA was successfully collected from all 89 isolates, the *marRAB* operon primers as described in Table 2 were used to amplify the *marRAB* operon in all 89 isolates. SEE2, noted as S2 in the below gel images, was used as a positive control. Gel electrophoresis showed that all 89 isolates contain a 2.3 kb product, as expected.

Figure 1 *marRAB* Screening in Isolates B1-B20
PCR products from the screening of the *marRAB* operon were separated by electrophoresis on a 1.5% agarose gel at 160 V for 120 minutes. Products from broiler isolates B1-B20 contained the expected 2.3 kb product.

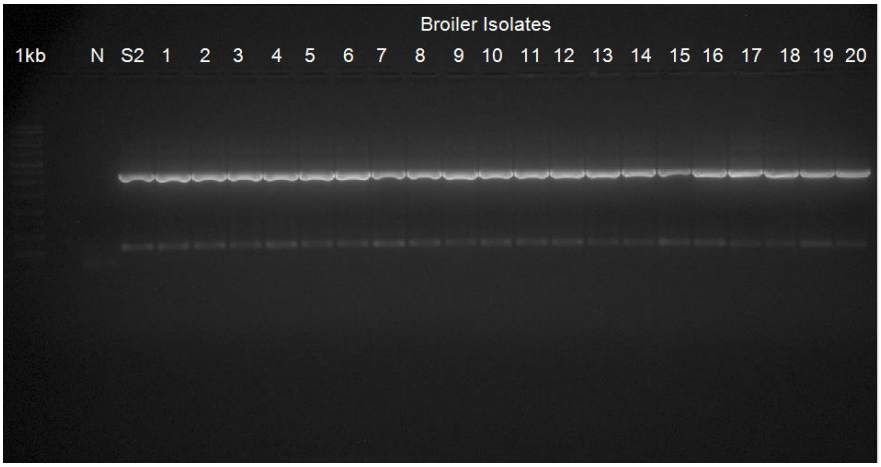


Figure 2 *marRAB* Screening in Isolates B21-B40
PCR products from the screening of the *marRAB* operon were separated by electrophoresis on a 1.5% agarose gel at 160 V for 120 minutes. Products from broiler isolates B21-B40 contained the expected 2.3 kb product.

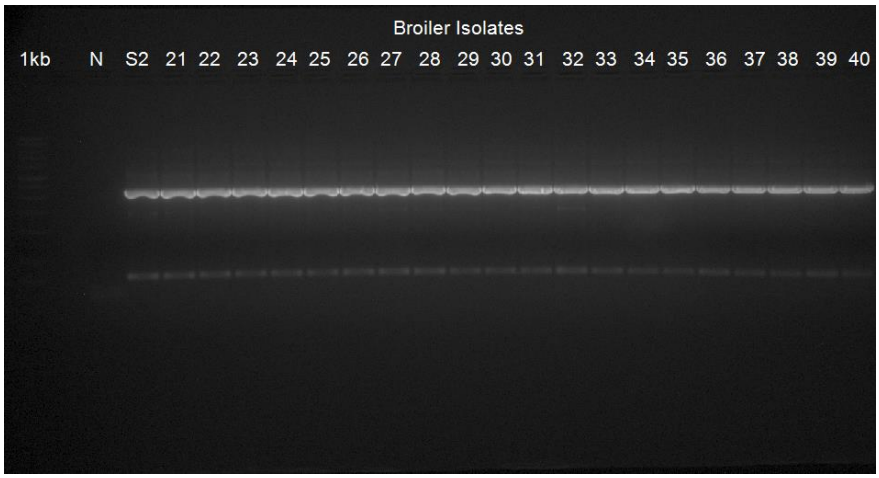


Figure 3 *marRAB* Screening in Isolates B41-B60
PCR products from the screening of the *marRAB* operon were separated by electrophoresis on a 1.5% agarose gel at 160 V for 120 minutes. Products from broiler isolates B41-B60 contained the expected 2.3 kb product.

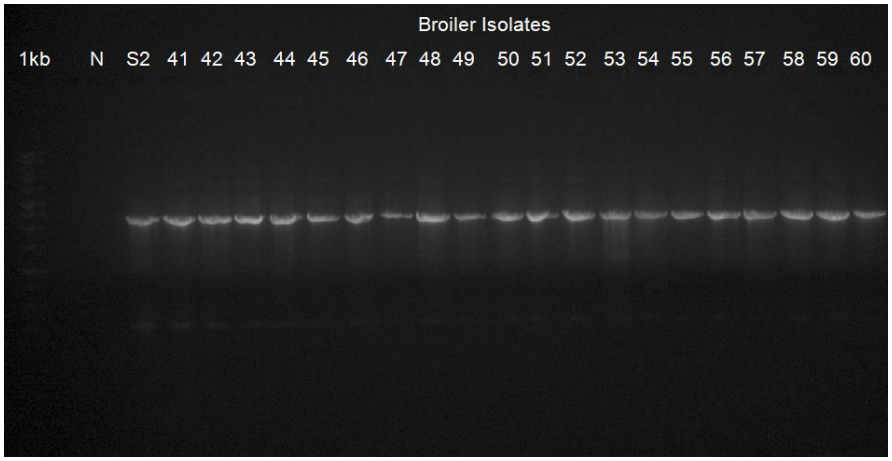


Figure 4 *marRAB* Screening in Isolates B61-74
PCR products from the screening of the *marRAB* operon were separated by electrophoresis on a 1.5% agarose gel at 160 V for 120 minutes. Products from broiler isolates B61-B74 contained the expected 2.3 kb product.

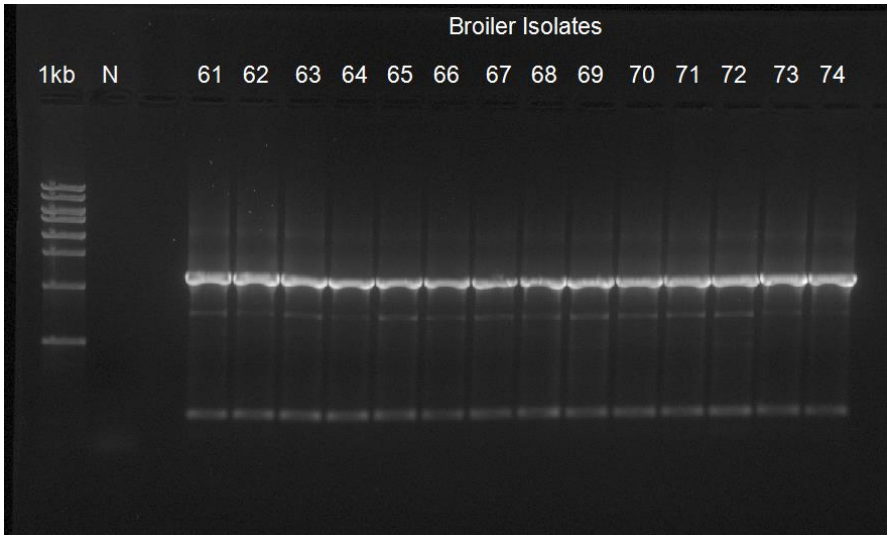
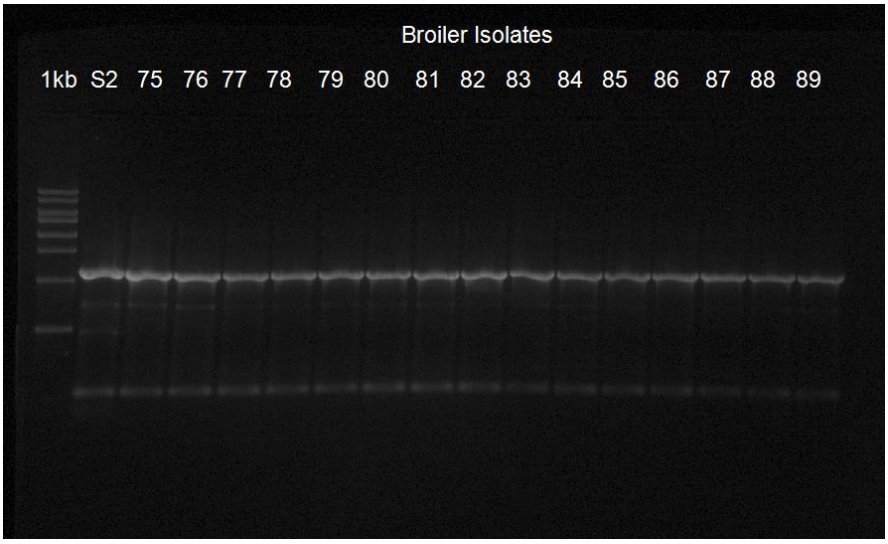


Figure 5 *marRAB* Screening in Isolates B75-B89
PCR products from the screening of the *marRAB* operon were separated by electrophoresis on a 1.5% agarose gel at 160 V for 120 minutes. Products from broiler isolates B75-B89 contained the expected 2.3 kb product.



Screening of acrA

The *acrA* primers as described in Table 2 were used to amplify *acrA* in all 89 isolates. Either SEE1 (noted as SE1) or SEE2 (noted as SE2) were used as positive controls in the gel images shown below. Gel electrophoresis showed that all 89 isolates contain a 939 bp product, as expected.

Figure 6 *acrA* Screening in Isolates B1-B18
PCR products from the screening of *acrA* were separated by electrophoresis on a 1% agarose gel at 120 V for 120 minutes. Products from broiler isolates B1-B18 contained the expected 939 bp product.

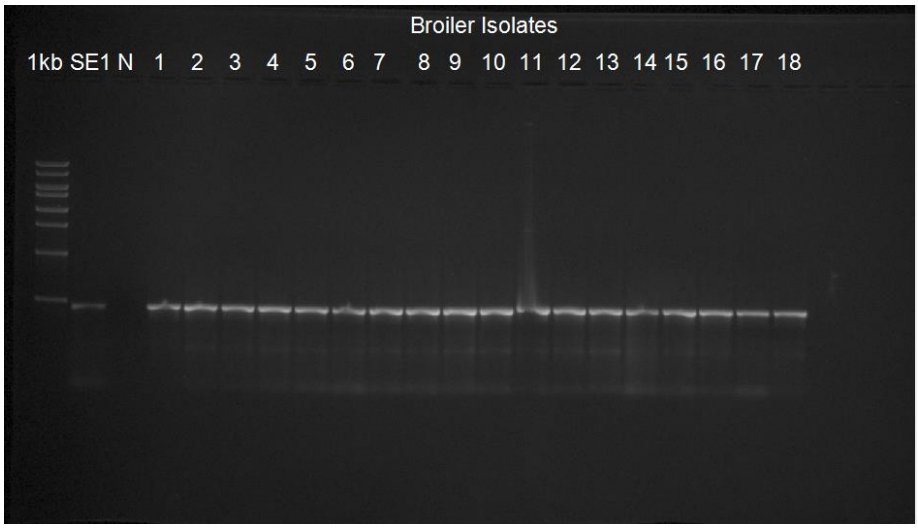


Figure 7 *acrA* Screening in Isolates B19-B35
PCR products from the screening of *acrA* were separated by electrophoresis on a 1% agarose gel at 120 V for 120 minutes. Products from broiler isolates B19-B35 contained the expected 939 bp product.

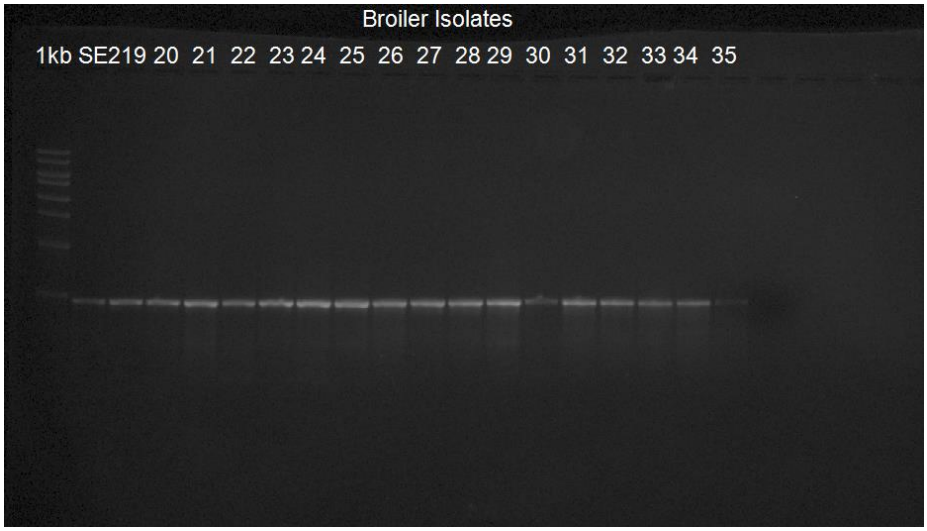


Figure 8 *acrA* Screening in Isolates B36-B53
PCR products from the screening of *acrA* were separated by electrophoresis on a 1% agarose gel at 120 V for 120 minutes. Products from broiler isolates B36-B53 contained the expected 939 bp product.

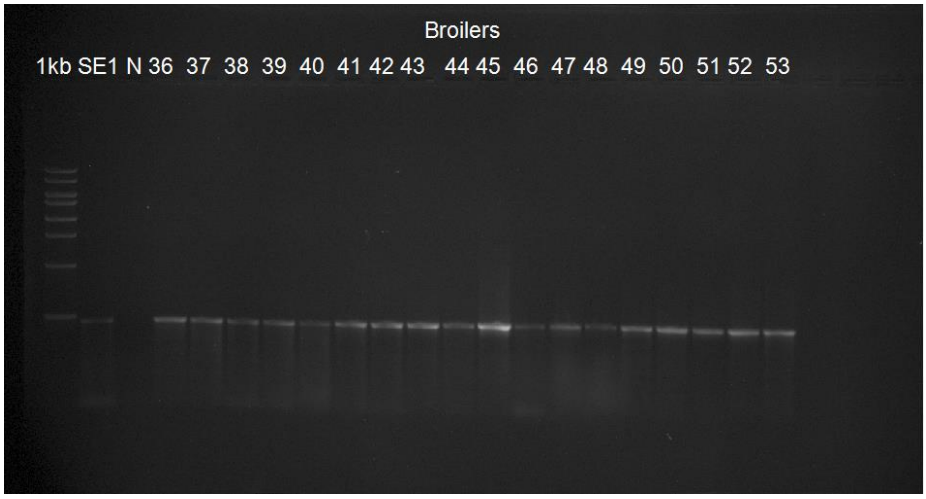


Figure 9 *acrA* Screening in Isolates B54-B72
PCR products from the screening of *acrA* were separated by electrophoresis on a 1% agarose gel at 120 V for 120 minutes. Products from broiler isolates B54-B72 contained the expected 939 bp product.

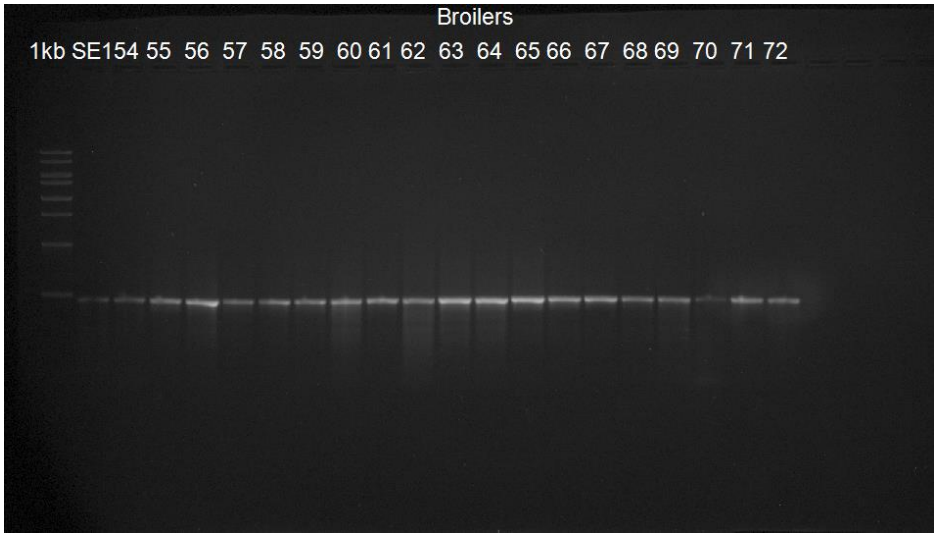
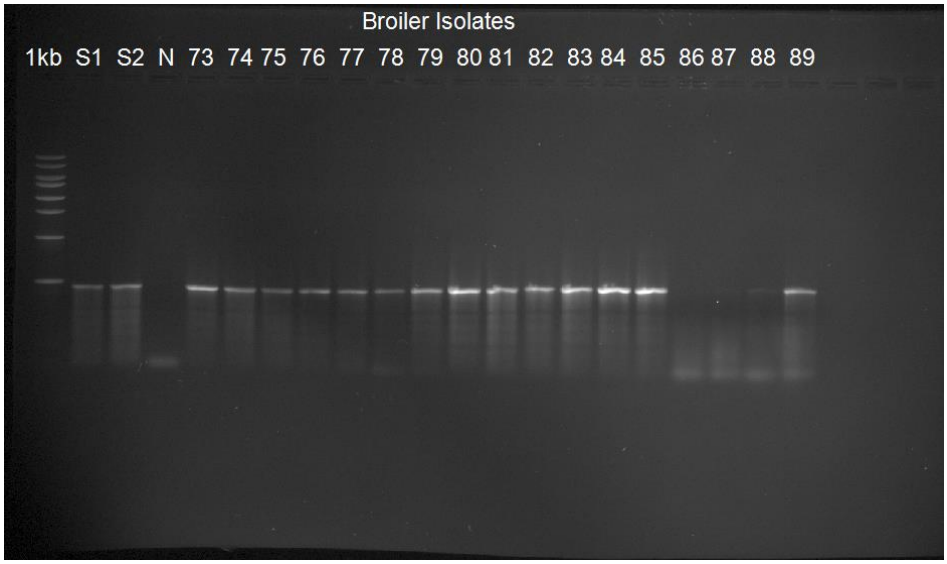


Figure 10 *acrA* Screening in Isolates B73-B89
PCR products from the screening of *acrA* were separated by electrophoresis on a 1% agarose gel at 120 V for 120 minutes. Products from broiler isolates B54-B72 contained the expected 939 bp product.



Screening of acrB

The *acrB* primers as described in Table 2 were used to amplify *acrB* in all 89 isolates. Either SEE1 or SSE2 were used as positive controls in the gel images shown below. In initial screening of *acrB*, the SEE1 and SEE2 controls did not produce bands. Thus, PCR was repeated on these two positive controls with those isolates that did not contain bands for *acrB*. Upon this final screening of *acrB*, gel electrophoresis showed that all 89 isolates contain a 543 bp product, as expected.

Figure 11 *acrB* Screening in Isolates B1-B46
PCR products from the screening of *acrB* were separated by electrophoresis on a 1% agarose gel at 120 V for 120 minutes. The isolates in the below gel contain the expected 543 bp product. Given the SEE1 positive control did not contain the expected band in this gel though, PCR was repeated on the SEE1 control with B39, B40, and B44. Figure 13 shows that these isolates all contain the expected 543 bp product.

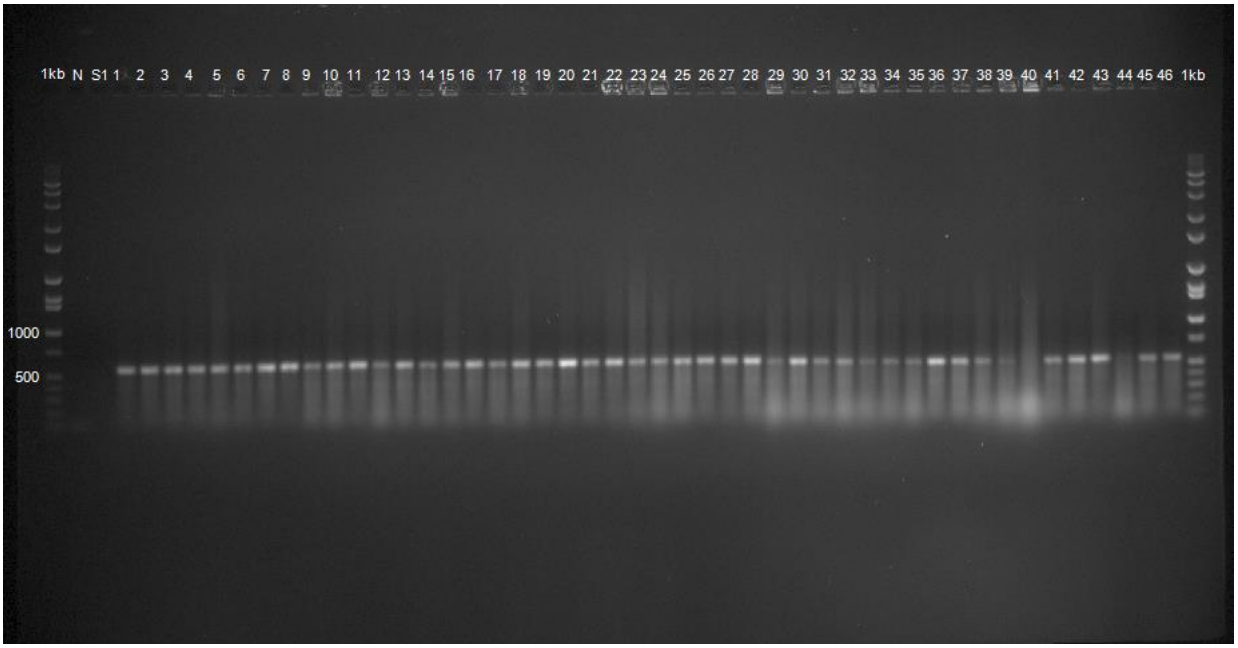


Figure 12 *acrB* Screening in Isolates B47-B89

PCR products from the screening of *acrB* were separated by electrophoresis on a 1% agarose gel at 120 V for 120 minutes. The isolates in the below gel contain the expected 543 bp product. Given the SEE2 positive control did not contain the expected band in this gel though, PCR was repeated on the SEE2 control with B49, B50, B51, B52, B57, B60, B64, B65, B66, B67, B70, B74, B75, B76, B77, B78, B79, B80, B81, B82, B83, B84, B85, B86, B87, B88, and B89. Figure 13 shows that these isolates all contain the expected 543 bp product.

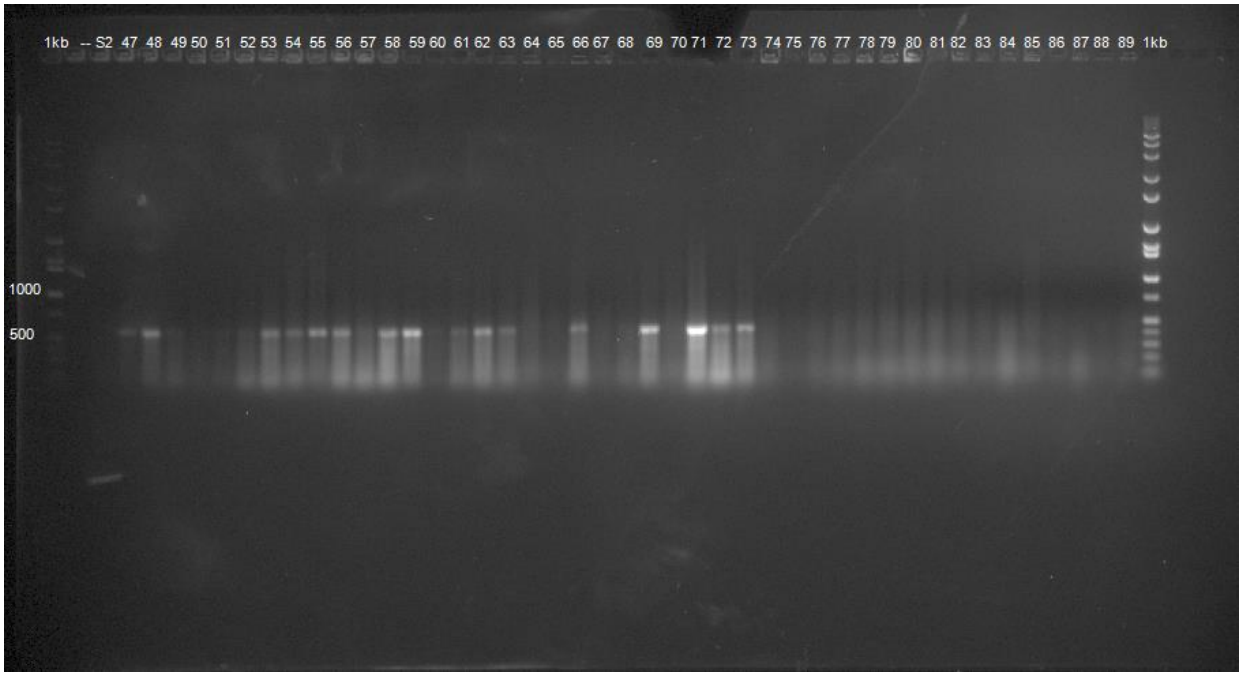
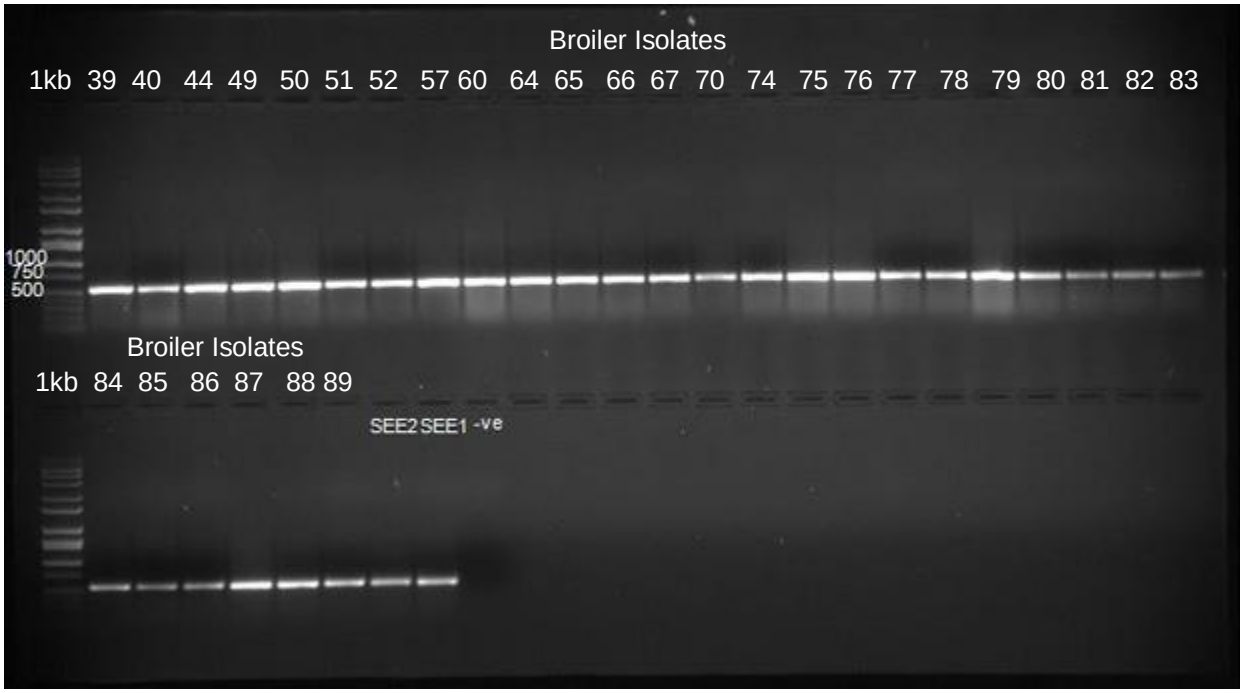


Figure 13 Repeated *acrB* Screening in Isolates
PCR was repeated on positive controls SEE1 and SEE2, along with isolates from Figures 11 and 12 that did not produce bands. PCR products from the screening of *acrB* were separated by electrophoresis on a 1% agarose gel at 120 V for 120 minutes. The isolates in the below gel contain the expected 543 bp product. Thus, all 89 isolates contain the expected product.



DNA Sequencing

Sequencing of the *marRAB* region was completed on isolates B2, B3, B42, B48, B49, and B84. Isolates B2 and B3, which were susceptible to all antibiotics tested, were sequenced as a comparison against the other four isolates, which were resistant to at least one antibiotic. Sequencing did not yield the *marR* region of the genome, but, of the areas of the genome obtained, there was no difference in sequences among isolates sequenced.

Antibiotic Resistance Assay

After completing the Kirby Bauer method on all 89 isolates, four isolates were found to be resistant to at least one antibiotic. B42 was resistant to amoxicillin-clavulanic acid (AMC), B48 was resistant to tetracycline (TE), B49 was resistant to cefuroxime (CXM) and nalidixic acid (NA), and B84 was resistant to ampicillin-sulbactam (SAM). The tables in Appendix A show complete results for all antibiotics and isolates. Diameter measurements are in millimeters.

Pulsed-field Gel Electrophoresis

XbaI endonuclease digestion of the 89 isolates generated 10-14 fragments. Overall, seven different pulsotypes were observed from the 89 isolates. Of these seven pulsotypes, four isolates (B56, B66, B70, and B80) had unique fingerprints. B56 belongs to pulsotype 6, B66 belongs to pulsotype 7, B70 belongs to pulsotype 2, and B80 belongs to pulsotype 4. Additionally, two isolates (B57 and B58) shared the same unique pulsotype, pulsotype 5. All other isolates were in pulsotypes 1 or 3. Appendix B contains a dendrogram outlining the PFGE fingerprints obtained based on Dice similarity coefficient and unweighted pair-grouping with 1.6% position tolerance.

Chapter 4

Discussion

The data obtained in this study showed no relationship between the antimicrobial resistance profiles of clinical broiler isolates of SE and occurrence of the *marRAB* operon genes. Primarily, all 89 isolates contained the *marRAB* operon, *acrA*, and *acrB*, though only four isolates expressed resistance to one of the antibiotics used in the study. Furthermore, no relationship existed between PFGE type and resistance patterns observed. Thus, no concrete results were obtained to definitively show either that the *marRAB* operon plays a role in conferring resistance or that there is a relationship between PFGE type and resistance patterns.

Out of the 89 isolates, only four isolates expressed resistance to at least one antibiotic. B42 and B84 were resistant to amoxicillin-clavulanic acid (AMC) and ampicillin-sulbactam (SAM) respectively, both of which consist of a penicillin and β-lactamase inhibitor. Meanwhile, B48 was resistant to tetracycline (TE), and B49 was resistant to cefuroxime (CXM), a non-extended spectrum cephalosporin, and nalidixic acid (NA), a quinolone. When the *marRAB* operon is expressed, bacteria may express resistance to chloramphenicol, cephalosporins, nalidixic acid and fluoroquinolones, penicillins, puromycin, rifampicin, and tetracycline (19). Thus, the antibiotics that the four isolates were resistant to are characteristic of the Mar positive phenotype.

Upon screening for the *marRAB* operon, all 89 isolates, in addition to SEE1 and SEE2 isolated and used as positive control strains, contained the *marRAB* operon. The repressor protein MarR is responsible for binding to the *marO* operator region of the operon to negatively regulate the expression of *marRAB*. However, if MarR is inhibited from binding to *marO* due to a mutation in *marR* or exposure to a compound that is able to reduce repression, *marRAB*

expression is induced, thus potentially increasing resistance (19). Although no differences existed in the sequences obtained, isolates B42, B48, B49, and B84, which all were resistant to at least one antibiotic, may have had a mutation in *marR*, leading to the resistance observed. Activation of *marRAB* can also be achieved by overexpression of SoxS and RobA. Along with MarA, they activate promoters resulting in the Mar phenotype (21). Expression of either of these proteins may have been responsible for the antibiotic resistance seen in isolates B42, B48, B49, and B84.

Further, given that all the isolates contained the *marRAB* operon, the isolates were then screened for genes regulated by the *marRAB* operon. The operon increases efflux by upregulating *acrA* and *acrB* and decreases influx by upregulating *micF* (19). Thus, isolates were screened for *acrA* and *acrB*, and all isolates contained these genes as well. Because all isolates contained *marRAB*, *acrA* and *acrB*, either none played a role in the resistance patterns observed or one, two, or all of them were not actually expressed. Additionally, *marRAB* upregulates *micF*, which encodes an antisense RNA involved in inhibiting synthesis of Outer Membrane Protein F (OmpF) (21). If the isolates that expressed resistance contained *micF* or if this gene was expressed in these isolates and not in the others, this could be another explanation for the observed resistance patterns.

To determine whether the *marRAB* operon played a role in conferring resistance in these 89 isolates, further research should be conducted to examine the *marRAB* operon and the genes it regulates in the isolates used. The *marRAB* operon, specifically the *marR* region of the operon, of all isolates should be sequenced to determine if there is a mutation in *marR* accounting for the resistance patterns observed. By knocking out the *marRAB* operon from B42, B48, B49, and B84, it can also be determined if and how significant of a role the *marRAB* operon had in

conferring resistance. Additionally, the role of SoxS and RobA should be assessed to conclude whether or not they played a role in the resistant isolates. Finally, *micF* should also be screened for to determine if it is present and expressed in the isolates to determine its role in isolates B42, B48, B49, and B84.

Of the seven pulsotypes observed, none of the isolates that exhibited resistance to one or more antibiotics had unique PFGE fingerprint patterns. Isolates B42 (resistant to amoxicillin-clavulanic acid) and B84 (resistant to ampicillin-sulbactam) belonged to pulsotype 1. Both amoxicillin-clavulanic acid and ampicillin-sulbactam consist of a penicillin and β-lactamase inhibitor; thus, because both isolates that were resistant to this antibiotic class belonged to the same pulsotype, this pulsotype may be responsible for generating strains resistant to a penicillin and β-lactamase inhibitor. Testing the other isolates in pulsotype 1 against other penicillin and β-lactamase inhibitors may aid in identifying the clonality of SE resistant to this group of drugs.

Additionally, B48 (resistant to tetracycline) and B49 (resistant to cefuroxime and nalidixic acid) belonged to pulsotype 3. Tetracycline belongs to the tetracycline class, cefuroxime belongs to the non-extended spectrum 1st and 2nd generation cephalosporin class, and nalidixic acid belongs to the quinolone class. Although none of these antibiotics belong to the same class, pulsotype 3 may represent SE resistant to these antibiotic classes.

Overall, more research needs to be conducted to determine the role of *marRAB* in antibiotic resistance among SE. By further analyzing *marRAB* and the genes it regulates in these isolates, perhaps a stronger understanding could be developed of the operon's specific role in each isolate tested. This study offers a good basis on which further research can be completed to assess the resistance effects of the *marRAB* operon. Additional insight into expression of the *marRAB* operon may also help in determining its role in antibiotic resistance. Nonetheless,

despite the lack of gained understanding of the *marRAB* operon, the broiler isolates did show only minimal resistance to antibiotics even though antibiotic resistance has become a greater threat to human and animal health.

Chapter 5

Conclusion

Overall, the goal of this study was to determine the role of *marRAB* operon of SE in conferring resistance to a variety of antibiotics from different classes. Although only four of the 89 broiler isolates tested expressed resistance to at least one antibiotic, each isolate did exhibit resistance typical of a positive Mar phenotype. Although, because no concrete relationships were established among presence of the *marRAB* operon or any of its related genes, antibiotic resistance profiles, and PFGE pulsotype, more research should be conducted to determine whether or not the *marRAB* operon was responsible for producing the resistance patterns observed. Upon better understanding the role that the *marRAB* operon plays in antibiotic resistance, a more clinical approach can be taken to target the operon in resistant bacteria. With future research, a more in-depth appreciation of the *marRAB* operon and its role can be developed. This study also demonstrated that, despite the concern of foodborne bacteria as a source of antibiotic resistance bacteria to humans, most of the clinical isolates of broiler chicken SE examined were sensitive to the antibiotics included in the study.

Appendix A

Complete Kirby Bauer Results

The zones around the antibiotic discs were measured and compared to the CLSI Performance Standards for Antimicrobial Susceptibility Testing (24th Informational Supplement, 2014). If bacteria were susceptible to an antibiotic, there was an area, known as the zone of inhibition, with no bacterial growth surrounding that antibiotic disc. If bacteria grew completely around the antibiotic disc, the bacteria could be considered resistant to that antibiotic. Measurements of zones of inhibition are displayed in millimeters. The following tables include all results from the Kirby Bauer assays.

Table 4 Kirby Bauer Assay Results
Eighty nine SE isolates were tested against a total of 26 antibiotics using Kirby Bauer assays. The following table lists the zones of inhibition for isolates against gentamicin (GM), tobramycin (NN), amikacin (AN), netilmicin (NET), ticarcillin-clavulanic acid (TIM), piperacillin-tazobactam (TZP), imipenem (IPM), meropenem (MEM), cefazolin (CZ), cefuroxime (CXM), cefotaxime (CTX), ceftazidime (CAZ), and cefoxitin (FOX).

Isolate	*GM*	*NN*	*AN*	*NET*	*TIM*	*TZP*	*IPM*	*MEM*	*CZ*	*CXM*	*CTX*	*CAZ*	*FOX*
B1	29	24	27	32	32	31	35	32	29	26	38	34	27
B2	25	21	25	30	26	27	30	30	25	23	35	31	24
B3	28	28	21	32	29	27	32	31	26	25	36	32	28
B4	26	20	27	32	26	26	31	31	25	23	32	30	24
B5	26	23	24	28	27	27	31	35	27	25	35	30	27
B6	22	20	25	31	29	30	34	32	22	25	36	32	29
B7	32	24	31	37	32	31	31	32	26	24	38	32	25
B8	28	25	27	30	30	32	32	32	28	25	37	32	30
B9	29	23	25	30	31	29	34	31	27	25	28	35	28
B10	29	22	27	29	23	27	34	31	26	24	34	33	28
B11	30	22	28	31	27	27	34	28	24	22	31	28	23
B12	26	23	25	30	31	29	33	36	28	25	35	31	28
B13	29	21	27	31	26	27	32	31	26	21	35	30	26

B14	26	21	25	31	26	27	30	30	24	24	31	29	26
B15	27	23	24	27	27	27	32	29	28	23	35	32	26
B16	28	23	29	32	30	29	30	33	27	26	35	32	27
B17	27	22	26	32	29	28	34	32	26	25	31	26	24
B18	26	21	26	30	26	25	30	29	23	21	30	29	25
B19	26	23	27	32	32	32	34	34	29	27	40	36	30
B20	25	26	27	28	30	31	36	34	31	27	38	36	32
B21	30	24	29	33	30	29	42	34	30	27	42	34	32
B22	27	22	27	33	30	29	32	32	28	26	36	32	30
B23	25	26	31	29	30	32	39	36	30	28	40	35	31
B24	28	27	30	33	36	33	38	37	36	37	46	43	34
B25	29	25	29	32	30	31	36	33	31	22	38	35	29
B26	27	24	30	32	30	29	38	32	30	26	39	36	32
B27	27	25	26	30	29	30	38	35	31	27	37	34	32
B28	28	23	28	30	30	28	36	32	30	29	40	34	31
B29	29	24	29	35	27	27	38	30	30	26	32	36	29
B30	27	23	27	30	30	30	35	35	28	25	39	38	27
B31	29	26	30	33	31	29	36	34	30	27	38	34	30
B32	30	25	30	35	29	30	41	34	31	27	41	33	28
B33	29	24	30	34	32	31	36	34	33	31	38	35	32
B34	29	22	29	31	31	34	35	35	30	28	40	34	31
B35	30	22	25	30	30	28	38	38	26	27	44	33	27
B36	28	24	28	34	37	35	40	36	36	34	40	36	38
B37	28	23	27	33	30	30	42	36	27	27	40	34	28
B38	27	24	28	35	30	30	40	38	29	28	38	34	28
B39	28	24	28	33	31	31	36	39	29	29	40	33	28
B40	29	24	28	32	30	29	32	35	30	28	38	33	31
B41	27	24	29	31	27	32	35	34	30	28	40	31	29
B42	30	26	30	31	33	33	38	34	30	27	39	35	31
B43	26	24	28	32	29	29	34	32	29	28	37	32	30
B44	29	29	26	31	29	30	37	36	31	28	40	35	31
B45	30	24	30	39	29	31	37	35	30	29	40	32	30
B46	29	23	27	31	30	30	35	33	30	25	40	37	30
B47	28	25	27	33	31	33	37	35	30	25	37	35	30
B48	24	23	24	28	27	25	32	32	30	29	35	35	29
B49	28	23	29	32	29	30	35	36	32	0	37	33	28
B50	28	25	28	34	35	31	37	32	25	26	39	31	32
B51	24	24	25	30	35	30	36	32	31	28	30	35	41
B52	30	26	30	33	34	34	38	37	32	28	28	34	29
B53	32	25	27	33	33	34	39	34	32	29	34	34	30
B54	29	23	28	32	33	32	37	34	31	26	38	34	32
B55	26	23	27	30	34	30	36	42	30	25	38	35	35
B56	31	24	31	35	36	34	38	37	31	30	42	36	32
B57	29	23	27	31	33	32	34	35	30	28	36	34	27
B58	29	23	28	34	37	30	38	37	30	25	35	33	32

B59	30	26	31	37	38	35	40	42	34	33	44	33	30
B60	30	25	30	34	36	34	37	42	30	31	40	35	30
B61	29	24	28	34	34	35	37	40	31	21	40	35	32
B62	30	24	30	31	32	32	36	37	29	26	38	34	29
B63	25	22	27	31	33	31	35	36	31	28	40	34	33
B64	31	25	31	35	34	32	37	42	30	27	37	35	30
B65	29	23	28	33	33	33	37	40	32	27	38	34	32
B66	28	22	28	32	35	33	36	37	33	28	40	36	30
B67	28	23	27	32	33	32	36	40	32	29	39	34	33
B68	29	23	29	32	34	32	36	39	31	29	41	35	31
B69	28	23	29	34	34	33	35	38	32	27	39	35	25
B70	32	25	32	35	36	34	38	41	32	29	40	39	32
B71	31	25	31	37	36	32	42	43	32	27	39	33	33
B72	28	23	27	33	34	32	41	39	29	26	40	35	28
B73	26	22	28	27	33	32	31	36	31	26	40	34	32
B74	28	24	28	32	33	32	37	39	32	27	30	33	29
B75	29	25	29	34	33	35	37	40	34	29	43	36	34
B76	27	22	27	30	33	32	31	36	30	30	40	36	33
B77	29	24	29	34	34	33	38	40	30	26	40	35	29
B78	31	25	32	36	37	35	37	38	28	28	39	34	32
B79	28	24	29	32	32	30	34	36	29	25	35	31	30
B80	26	24	27	30	31	29	35	34	31	28	38	35	30
B81	30	23	30	34	35	30	37	38	30	27	37	34	30
B82	30	24	30	33	35	31	36	40	29	27	39	33	32
B83	28	24	28	32	32	33	34	37	31	28	40	35	30
B84	30	24	29	34	35	30	35	38	30	26	35	31	28
B85	32	25	31	35	37	31	35	37	30	26	38	32	26
B86	30	24	30	36	36	34	36	38	30	26	38	34	31
B87	26	22	28	31	30	31	33	35	29	26	38	35	31
B88	28	23	29	33	33	30	36	39	30	27	38	33	32
B89	27	21	28	31	32	30	34	35	28	25	38	32	30

Table 5 Kirby Bauer Assay Results (cont.)
Eighty nine SE isolates were tested against a total of 26 antibiotics using Kirby Bauer assays. The following table lists the zones of inhibition for isolates against cefotetan (CTT), nalidixic acid (NA), ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (SXT), aztreonam (ATM), ampicillin (AM), ticarcillin (TIC), amoxicillin-clavulanic acid (AMC), ampicillin-sulbactam (SAM), chloramphenicol (C), tetracycline (TE), doxycycline (D), and minocycline (MI).

Isolate	*CTT*	*NA*	*CIP*	*SXT*	*ATM*	*AM*	*TIC*	*AMC*	*SAM*	*C*	*TE*	*D*	*MI*
B1	37	25	39	31	40	29	31	29	25	29	21	21	21
B2	32	21	32	25	30	26	23	27	25	30	25	21	21
B3	37	26	36	28	34	28	30	29	25	27	22	19	23
B4	31	21	35	26	32	25	21	24	22	28	20	17	17
B5	32	24	36	28	33	27	27	27	23	26	24	15	21
B6	36	27	34	30	36	30	28	29	26	29	26	25	24
B7	35	25	37	29	35	27	29	29	24	29	22	17	20
B8	36	25	37	29	36	29	31	30	29	29	26	21	24
B9	39	25	36	29	39	30	30	30	23	29	25	22	22
B10	33	25	39	30	33	27	30	28	25	30	20	20	20
B11	36	21	37	26	38	22	29	30	24	30	26	20	18
B12	37	22	39	31	35	28	32	27	26	28	24	21	21
B13	36	24	35	28	35	25	30	27	25	27	20	21	20
B14	35	24	35	27	32	26	28	28	25	31	26	22	22
B15	33	23	38	29	33	25	29	26	22	27	20	15	18
B16	39	25	32	29	37	29	30	29	26	34	26	25	24
B17	34	25	35	28	36	26	29	28	24	29	26	23	23
B18	33	23	39	28	35	25	31	25	20	28	22	16	17
B19	41	28	41	34	42	31	34	33	30	33	28	23	26
B20	40	29	39	35	39	30	31	34	28	30	30	28	27
B21	40	28	38	35	39	30	30	35	30	31	28	24	25
B22	39	25	43	32	40	31	30	31	29	31	28	25	23
B23	41	30	38	34	40	30	31	34	28	29	31	29	28
B24	45	34	42	40	48	34	39	36	34	36	32	24	29
B25	38	28	39	34	39	31	30	34	28	33	32	25	25
B26	40	39	45	37	40	31	31	30	28	30	22	24	23
B27	38	28	43	35	41	32	30	32	28	30	28	29	22
B28	40	29	40	34	39	30	31	32	27	27	29	28	26
B29	40	31	44	34	37	32	31	34	30	33	31	27	26
B30	38	26	38	31	38	30	30	33	29	34	27	24	24
B31	39	30	40	32	38	30	33	33	29	29	27	25	33
B32	38	28	39	35	37	30	30	30	27	31	26	23	20
B33	42	32	42	35	39	33	31	35	29	34	31	28	28
B34	39	28	39	32	42	33	32	35	32	33	30	26	27
B35	38	26	40	30	34	28	31	30	27	32	27	25	25
B36	44	28	40	31	42	33	36	37	32	31	27	23	28

B37	38	27	41	35	37	29	28	30	27	32	27	25	22
B38	36	26	43	33	38	29	30	31	26	32	26	20	22
B39	37	26	41	35	37	30	30	30	30	32	27	24	22
B40	40	29	44	29	38	31	34	33	27	30	28	26	27
B41	31	28	33	32	39	31	31	30	27	32	28	27	29
B42	39	24	42	33	41	31	34	0	27	31	25	23	24
B43	39	28	43	33	39	28	31	30	26	29	29	25	28
B44	38	27	40	34	40	29	33	26	27	31	27	24	19
B45	35	29	40	32	40	31	33	33	29	30	27	26	25
B46	38	29	40	30	40	31	23	33	22	32	27	27	27
B47	37	21	39	31	41	31	32	31	27	26	25	22	24
B48	39	29	40	32	42	31	22	34	28	31	0	27	27
B49	41	0	43	33	39	33	19	31	26	32	27	28	27
B50	36	32	40	32	39	33	20	28	32	30	30	28	30
B51	38	25	44	34	28	25	22	25	30	30	26	23	24
B52	40	22	37	30	38	29	23	32	24	31	28	20	19
B53	33	28	44	35	39	32	25	33	32	33	26	21	22
B54	40	29	37	32	39	30	28	32	30	30	29	29	25
B55	39	30	40	34	36	32	24	35	28	32	28	23	24
B56	38	29	42	35	38	29	23	33	35	37	30	25	23
B57	39	28	39	36	38	29	24	33	27	31	26	24	22
B58	40	28	41	33	35	30	22	33	32	32	26	24	26
B59	42	26	46	35	35	30	27	32	30	32	25	22	22
B60	41	15	41	32	28	30	25	34	27	31	25	20	14
B61	40	25	40	32	40	30	23	31	26	35	27	22	22
B62	38	25	39	32	38	27	24	30	30	33	29	23	24
B63	37	25	43	30	38	30	24	33	32	32	26	22	22
B64	36	24	42	34	38	26	22	31	22	33	22	21	24
B65	40	27	47	33	38	28	20	34	30	35	26	23	24
B66	39	30	49	35	38	31	25	32	32	30	25	20	24
B67	39	26	39	34	40	29	22	31	30	36	26	22	22
B68	39	25	43	32	40	29	26	34	32	33	26	19	22
B69	37	26	44	31	41	30	25	33	32	34	26	21	25
B70	44	30	47	35	41	28	27	36	34	34	29	23	23
B71	38	30	47	37	38	31	22	34	30	34	26	23	25
B72	38	22	41	32	42	31	23	34	31	32	28	22	21
B73	38	30	41	31	38	30	24	34	26	32	26	22	17
B74	37	25	46	32	37	26	22	29	28	29	27	23	25
B75	42	28	47	36	41	31	24	34	30	35	28	23	27
B76	40	26	44	34	40	30	24	34	32	36	28	23	24
B77	37	24	42	32	37	29	24	32	33	32	26	22	24
B78	37	26	42	32	38	30	24	32	31	36	24	22	24
B79	34	26	42	34	37	28	21	31	29	33	25	23	24
B80	38	22	37	34	43	31	30	30	32	25	25	20	21
B81	38	24	42	32	36	28	25	31	28	31	25	22	23

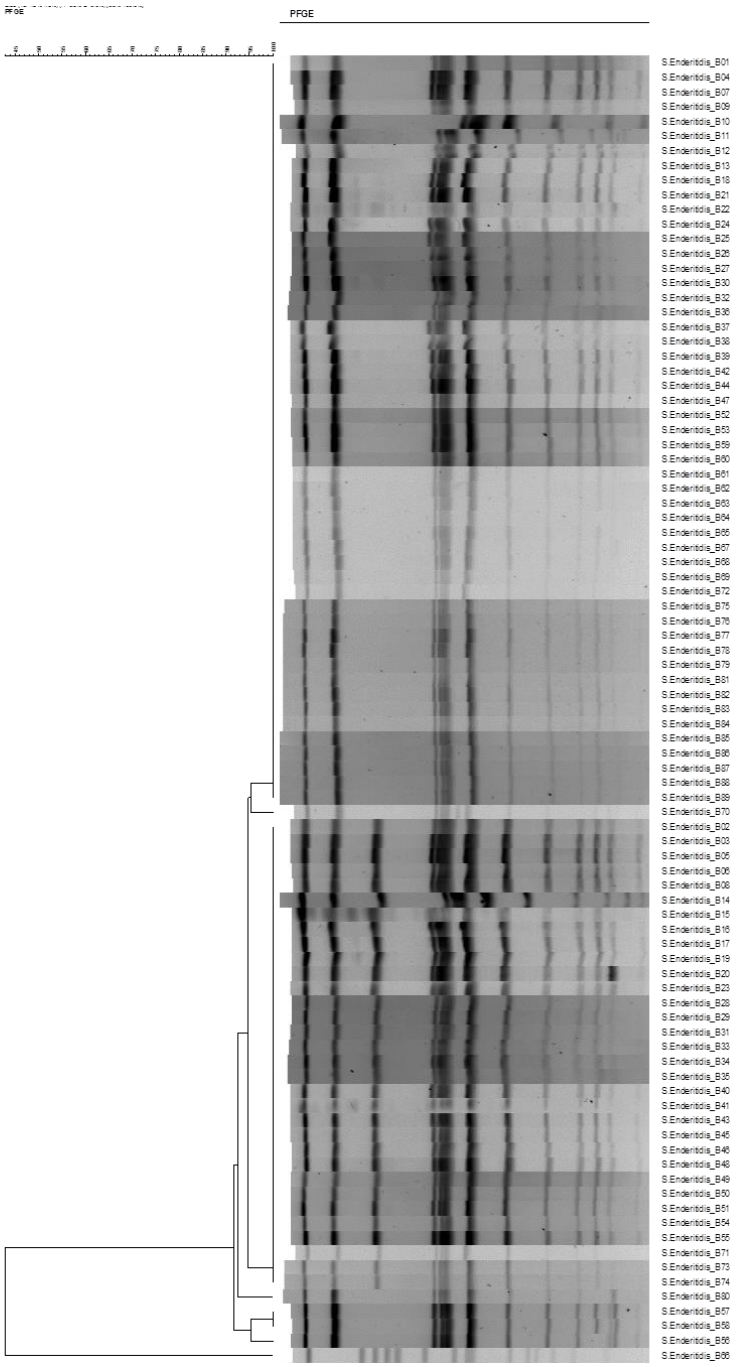
B82	38	23	42	32	39	30	23	32	29	34	28	23	25
B83	40	23	42	34	38	29	25	31	28	34	27	23	24
B84	37	24	45	31	34	27	21	30	0	32	25	21	22
B85	38	24	40	32	35	27	20	30	26	30	27	23	24
B86	37	25	43	31	36	25	23	30	31	30	25	22	25
B87	37	26	38	31	37	30	25	32	31	33	27	21	23
B88	35	25	42	32	38	30	25	31	31	30	26	22	23
B89	35	25	40	30	39	28	31	31	28	30	25	22	23

Appendix B

PFGE Dendrogram

Figure 14 PFGE Dendrogram

The following dendrogram displays the seven pulsotypes obtained through PFGE.



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