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Occurrence of tylosin-resistant enterococci in swine manure and tile drainage systems under no-till management

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Occurrence of tylosin-resistant enterococci in swine manure and tile drainage systems under no-till management

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LIST OF ABBREVIATIONS

Abbreviation	Fullname
ATCC	American Type Culture Collection
Bp	base pair
Cfu	colony forming units
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
H	hour
Min	minute
°C	degrees Celsius
g	grams
d	day
mM	millimolar
mL	millilitre
Mol	mole
s	seconds
PCR	Polymerase Chain Reaction
Q-PCR	Quantitative Polymerase Chain Reaction
MIC	Minimum Inhibitory Concentration
µg	microgram
µL	microlitre
FAO	Food and Agriculture Organization
WHO	World Health Organization
FDA	Food and Drug Administration

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ABSTRACT

Antibiotics are widely used by the swine industry as an important tool to treat and prevent diseases, protect animal welfare, improve growth rates, and improve efficiency of feed utilization. The presence of antibiotics in animal gastrointestinal tracts could result in the development of antibiotic resistant bacteria (Chee-Sanford et al., 2009). Livestock waste entering the environment when manure is applied to agriculture fields may result in possible exposure of humans and animals to antibiotic residues and antibiotic resistant bacteria. Transport of these bacteria through soils into tile drainage lines and possibly even groundwater is a serious threat to public health. Understanding of occurrence and transport mechanism of antibiotic resistant bacteria through tile systems under agricultural fields amended swine manure is very important to improve management of tile drain water quality and reduce the health risk to humans. However, very little is known about the transport of antibiotic resistant bacteria from swine manure through no-till soils and tile drainage lines in agricultural fields receiving swine manure. No hydraulic link has been clearly established between the source of resistant bacteria and their presence in samples collected in nearby soil and waters. Instead, this is often inferred due to the farm (Koike et al., 2007) or sampling locations (Chee-Sanford et al., 2001). The objectives of this study were (1) to detect and quantify the occurrence of tylosin-resistant enterococci in manure from swine feeding tylosin at subtherapeutic doses, in no-till soils amended with swine waste, and in tile drain flow from swine waste amended agricultural fields, (2) to assess the effects of tile flow and total suspended solid on the prevalence of total enterococci and resistant enterococci in the tile water, and (3) to test enterococci isolates for known macrolide resistant genes.

The field study was conducted at a farm near Nashua, IA in April (spring simulation) and November (fall simulation) of 2009. Liquid swine slurry from an operation feeding tylosin at sub-therapeutic levels was injected into no-till field plots. Resistance to tylosin in manure, soil and tile water was investigated by a phenotype-based method and polymerase chain reaction (PCR) using previously published primers (*ermA, ermB, ermC, ermF, ermT, ermX, mefA, msrA*).

Enterococci isolates were recovered from swine manure, soil amended with manure and tile water samples by membrane filtration technique and a culture-based method on m*Enterococcus* media with and without tylosin to detect total and resistant enterococci respectively. Antibiotic resistance genes were identified from the isolates using the PCR method. All enterococci in manure samples were resistant to tylosin in spring, whereas about 68% of total enterococci in fall were resistant to tylosin. Average concentrations of total and tylosin-resistant enterococci in soil samples after manure application over the two experiments were 8.8×10^6 cfu/g of soil and 8.9×10^6 cfu/g of soil, respectively, where as the concentration before manure application was zero or minimal. Total and tylosin-resistant cfus (colony forming units) in tile water were significantly different in both experiments ($P < 0.05$). In drainage water, total enterococci ranged from 1.3×10^1 cfu/100 mL (fall) to 5.0×10^3 cfu/100 mL (spring) while tylosin-resistant enterococci ranged from 1.3×10^1 cfu/100 mL (fall) to 1.2×10^3 cfu/100 mL (spring). The greatest concentration of tylosin-resistant enterococci in manure and smallest in drainage water suggest that enterococci lose resistance as selective pressure from antibiotic residues decreases or die-off/attachment during movement through soil and drainage water. Greater enterococci concentrations were observed in the spring than in the fall, whereas higher tile flows with presence of base-flow during fall experiments indicates that there is relationship between tile flow and enterococci concentration in drainage water. A higher range of total suspended solid (TSS) also have been found in the spring versus the fall suggesting relationship between TSS and tile flow and enterococci concentrations ($R^2 > 0.65$). A total of 200 enterococci isolates from liquid swine manure, soil amended with swine manure and tile water (64, 26, 120 respectively) were tested for macrolide resistant genes. Five genes (*ermB, ermC, ermF, ermT and msrA*) were detected with high frequency. On average, most isolates (> 97%) harbored *msrA*, while only 9.5% of total isolates contains *ermT* gene and 9% contain *ermC* gene. The *ermF* gene was found in 156 out of 200 isolates (78%) and *ermB* gene was detected in 138 isolates (69%). Only four strains representing 2.0% of the total strains contains all five genes. In general, percentages of isolates with genes detected over the total isolates tested in fall were greater than those in spring for every single gene. The *ermX* gene was detected in two enterococci isolates from tile water in spring and the *ermA* gene was detected in only one

isolates from soil in spring. PCR amplifications were negative to *mefA* gene. The tylosin resistant enterococci and resistant genes from a known source (swine manure) links tylosin resistant enterococci and resistant genes in soils and tile water respectively. The results clearly show that the presence of antibiotic resistant genes do not always result in phenotypic expression of the resistance and vice versa. Although resistant enterococci were confirmed by growth on media infused with tylosin, tylosin resistant genes did not show up in strains extracted from them. Only eight genes were tested in this study for confirmation of the occurrence of tylosin resistant enterococci in swine manure, soil and tile water, while there are 32 erm gene classes have been identified previously (Roberts, 2004). Additional genes could be tested in the future to provide a more complete analysis of which genes encoded for tylosin resistance.

CHAPTER 1 GENERAL INTRODUCTION

1. 1 Introduction

Subtherapeutic use of antibiotics in animal production is believed to provide selective pressure for the development and proliferation of antibiotic resistant bacteria. Antibiotic resistant bacteria selection occurs among gastrointestinal bacteria in animal tracts like *E.coli* or enterococci, which are also excreted in manure. The soil and water environment will be exposed to antibiotic resistant bacteria via land application of animal waste following rainfall event. Occurrence of antibiotic resistant bacteria in drainage water from tile-drained fields receiving swine waste is an emerging water quality problem. Drainage water raises a concern about the transport of antibiotic resistant bacteria into open surface water bodies and threatens human health after exposure to contaminated water via swimming or other recreation activities. Among the Great Lakes and Corn Belt states in the upper midwest, Iowa is one of the most heavily drained. In Iowa, subsurface drainage tiles are an important part of farming systems to improve yields in poorly drained soils. It is estimated that about 25-35% of all cropland in Iowa is drained by pile line systems (Zucker and Brown, 1998). While subsurface drainage systems are important and essential for crop production, it is important to understand the associated water quality effects and methods to minimize potential consequences on downstream water bodies as well as protect human health. Moreover, Iowa has the largest swine production industry in the U.S. with 17,300,000 pigs as of December 2006 (USDA, 2007), and tylosin is typically used as a feed additive by swine producers to enhance growth and protect from diseases. Swine manure is typically disposed of through land application, serving as a major source of nutrients and organics for soils and crops in the state. This may increase the risk of antibiotic resistant bacteria entering nearby soil, and surface water and groundwater systems.

Previous studies have identified the occurrence of antibiotic resistant bacteria in swine feces, in soil, and in ground water near swine operations (Campagnolo et al., 2002); however, a clear hydrologic link between the facility and the surface and groundwater has not been made. Instead relationships have been inferred due to farm and sampling locations. Very limited understanding about the release and transport from tile-drained fields receiving

swine-waste application is known. Tile-drainage has the potential to significantly facilitate the transport of these organisms to surface water (Jamieson et al., 2002), and may cause a critical threat to human health through exposure by swimming or other recreational activities.

There are several implications related to antibiotic resistance on human health. As reported by CDC (1998), antibiotic resistant bacteria caused 2 million deaths each year particularly among individuals with compromised immune systems like children and the elderly. Antibiotic resistant infections result in longer hospital stays and require higher cost to treat, indicating that in addition to human heath effects, antibiotic resistance may have economic impacts. Recent studies have reported direct spread of resistant commensal enteric bacteria (e.g. *Escherichia coli, enterococci*), and zoonotic enteropathogens (e.g. *Salmonella* and *Campylobacter*) from animals to human via food chain or direct contact (McEwen and Fedorka-Cray, 2002). Some other studies found similar resistant patterns in isolates from human and animals. Apramycin resistance genes were found in human strains of *Salmonella* and *E.coli* even though apramycine has not been used in human therapy, indicating the transfer of resistant gene from animals to human (Barton, 2000). The threat of diseases caused by antibiotic resistant microorganisms become more complicated because antibiotics used in human medicine have similar structures to those used by the agricultural industry (Mathew et al., 2003).

Results from this study will further our understanding of the occurrence and transport of antibiotic-resistant bacteria through tile-drained lands from agricultural fields receiving swine waste. Furthermore, this will aid in assessment of the impacts of subsurface drainage on the movement of antibiotic-resistant bacteria into surface waters.

1. 2 Goal and objectives

The general aim of the study was to detect the occurrence of resistant enterococci in Iowa tile water and to study the transport of these bacteria through soil and water system under no-till management. The following specific objectives were pursued:

- To detect and quantify the occurrence of tylosin-resistant enterococci in manure from swine feeding tylosin at subtherapeutic doses, in soils amended with swine waste, and in tile drain flow from swine waste amended agricultural fields.

- To assess the effects of tile flow and total suspended solid on the prevalence of total enterococci and resistant enterococci in the tile water
- To test enterococci isolates for known macrolide resistant genes.

1. 3 Hypothesis

The following hypotheses were tested:

- Tylosin resistant enterococci are present in manure from farms feeding at sub-therapeutic levels, soils amended with swine waste, and tile drain flow from swine waste amended no-till fields.
- Tylosin resistant enterococci concentrations are correlated with tile drainage flow and total suspended solid.
- Tylosin resistant determinants are present among enterococci isolates collected from swine manure, soil and tile drainage water samples.

1. 4 Thesis organization

The objective of this research was obtained via two components: laboratory and field studies. Chapter 1 consists of the literature review on antibiotics uses, the development of antibiotic resistant bacteria, previous studies on transport of antibiotic resistant bacteria into the environment and detection methods. Chapter 2 presents the paper prepared for submision to a peer review journal. Chapter 3 provides overall conclusions, recommendation for further research and implication of the study.

CHAPTER 2 LITERATURE REVIEW

To study the transport of tylosin resistant enterococci from swine manure through no-till subsurface systems, it is necessary to understand how antibiotics are used in swine production, how bacteria become resistant and spread their resistance to other bacteria, species, the environment, and what existing methods for detection of antibiotic resistant bacteria. Literature review provides information regarding the aforementioned subjects; relevant information is presented in the following sections.

2. 1 Antibiotic use in animal production

2.1.1 Antibiotics and classification

Antibiotics are one of the greatest and most important discorveres for humans in treatment of infection and diseases. Antibiotics are subset of antimicrobial compounds that kill or inhibit the growth of bacteria. Antibiotics target bacterial functions or growth processes. The degree of effect depends on the specific mechanism of actions of the antibiotics. Most selective antibiotics are those affecting the chemical structure (e.g. cell wall) or functions (e.g. folic acid synthesis). The less selective agents are those affecting protein or nucleic acid (DNA, RNA) synthesis. into Antibiotics are divided into two groups, namely bactericide and bacteriostatic agents based on their effect on bacteria. Bactericide are antibiotics that kill bacteria while bacteriostatic antibiotics only impair bacterial growth. Antibiotics that target the bacterial cell wall, or cell membrane, or interfere with essential bacterial enzymes are usually bactericidal in nature. Those that target protein synthesis are usually bacteriostatic.

Antibiotics are classified based on either their chemical structure, mechanisms of action or spectrum of activity. They are group of chemicals that can be divided into classes such as beta-lactams (including penicilins, cephalosporins, and carbapenems), tetracylines, aminoglycosides, sulfonamides, glycopeptides, quinolones, oxazolidinones, macrolides (Kümmerer, 2009). The metabolism of an actively dividing cells is defined by the production of new cell wall components, DNA, RNA, proteins and cell membrane. Consequently, antimicrobial agents are divided into categories base on which of these

metabolic targets they affect (Fig. 1). These categories include (1) inhibition of cell wall synthesis (e.g. penicillins and vancomycin), (2) inhibition of nucleic acid (RNA, DNA) structure and function (e.g. quinolones and rifampicin), (3) inhibition of protein synthesis (e.g. aminoglycosides, tetracyclines, chloramphenicol, lincosamides and macrolides), (4) inhibition of folic acid synthesis, or (5) interference with cell membrane structure or function (e.g. polymixins). These categories are not completely discrete and some effects can overlap (Cowan, 2008).

Antibiotics can also be classified by their range of effectiveness. Broad-spectrum antibiotics include compounds effective against both Gram-positive and Gram-negative bacteria like quinolones and tetracyclines. Intermediate spectrum antibiotics generally include substances with reduced activity against some Gram-negative bacterial species (e.g. ampicillin and amoxicillin) while narrow spectrum antibiotics are only effective against limited number of bacteria species.

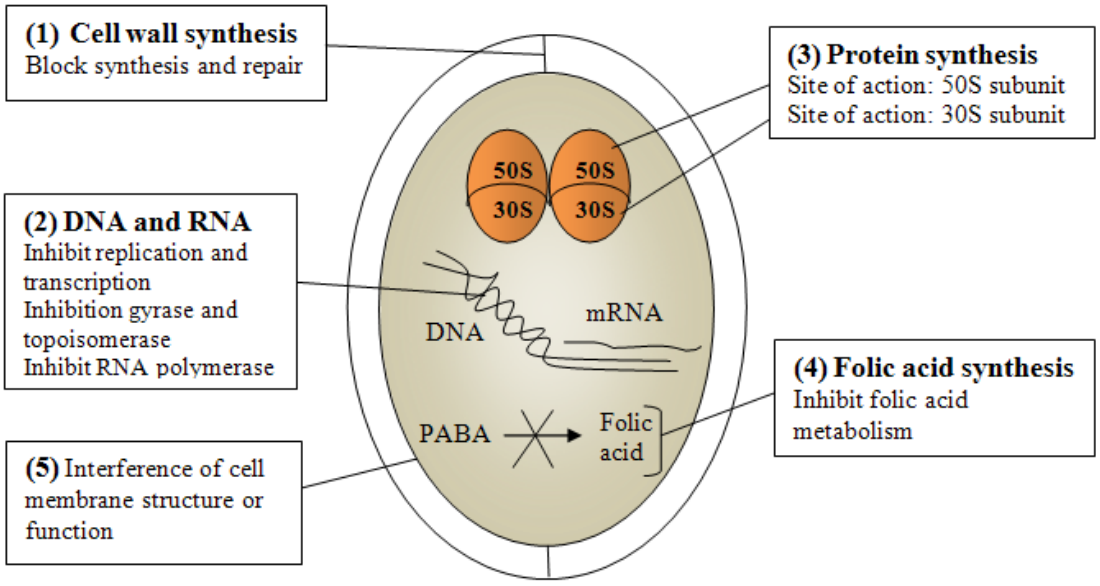

Figure 1- Primary site of actions of antibiotics on bacterial cells

(modified from Cowan, 2008)

2.1.2 Sub-therapeutic usage of antibiotics in swine production and residues

Since the early 1940s, antibiotics began to be used in animal production for disease treatment, and growth promotion purposes (Barton, 2000). At therapeutic level, antibiotics are administered in drinking water or feed to treat sick animals. Antimicrobial agents are also

used in animal production to promote growth and efficiency of feed utilization and control endemic diseases (Cromwell, 2002). When used for this purpose, low doses of antimicrobial agents are added to the feed of healthy animals to boost daily growth and reduce animal death rate, consequently enhancing overall production efficiency and increasing profitability. The term “non-therapeutic”or “subtherapeutic” is used in these case.

A major proportion of the antibiotics in animal production are used for growth-promotion purposes (Khachatourians, 1998). As reported by Union of Concerned Scentists (2001), nontherapeutic use in livestock sectors (cattle, swine and poultry) accounts for 78% of the total annual use of antimicrobial agents in the United States (Ketherine, 2004), in which, the swine industry is one of the most important users. Antibiotics are added to the feed of swine at every stage from birth to growing - finishing phases (Cromwell, 2002). It is estimated that the percentage of feeds containing antibiotics is 90% in nursery phase, 70% in growing phase, 50% in finishing phase, and 20% for sows. Mellon (2001) estimated that over 10.4 million pounds of antibiotics were administered nontherapeutically to swine in the United States in the late 1990. In which, about 70% of these antibiotics were dispensed in the 90-day finishing stage representing 7.3 million pounds (Mellon et al., 2001).

The benefits of using antibiotics in swine at subtherapeutic levels have been well documented (Cromwell, 2002; Gustafson and Bowen, 1997). As reported by Cromwell (2002), antibiotics are most effective in improving growth rate and feed utilization efficency and reducing mortality and morbidity in young pigs compared to older pigs because young pigs are more vulnerable to diseases, but antibiotics are quite effective during the entire life cycle of pigs. Antibiotics improved growth rate by average 16.4% in weaning pigs, 10.6% in growing pigs and 4.2% in finishing pigs. Efficiency of feed utilization was increased by 6.9%, 4.5%, and 2.2% in the three phases of growth, respectively. Addition of antibiotics in swine feeds contributed to decrease mortality and morbidity in pigs. Antibiotics also plays an improtant role in reproductive performance, especially at the time of breeding (Cromwell, 2002). Through comparisons the improvements from the same antibiotics in the period from 1950 to 1977 and the period since 1977, Cromwell (2002) found that the overall effectiveness of antibiotics over the two periods are similar and antibiotics have not lost their effectiveness over time.

Data about the use of feed additives in swine feeds from the United States National Swine Survey showed that up to 57% of 699 examined feeds used by the swine industry contain antibiotic levels three to four time higher than recommended levels (Dewey et al., 1997). Only a fraction of antibiotics fed to swine are metabolized by them, the non-metabolized antibiotics or residues may remain unchanged through the animal digestion system and they are excreted in animal waste (Gustafson and Bowen, 1997; Onan and LaPara, 2003). Up to 90% of fed antibiotics have been found to be released with urine and feces (Chander et al., 2006; Dolliver and Gupta, 2008). Table 1 lists all antimicrobial agents used in swine feeds.

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LaPara, 2003). Up to 90% of fed antibiotics have been found to be released with urine and feces (Chander et al., 2006; Dolliver and Gupta, 2008).

Table 1 - List of antibiotics approved for uses in swine feed by FDA

Antimicrobial	Class	Subtherapeutic rate	Trade name
Antibiotics			
Apramycin	Aminoglycoside	150 g/ton	
Bacitracin Methylene disalicylate	Bacitracin	45-90 g/ton	
Bacitracin zinc	Bacitracin	10-50 g/ton	Albac
Bambermycin	Bambermycin	2-4 g/ton	Flavomycin
Chlortetracycline	Tetracycline	10-50 g/ton	Aureomycin
Lincomycin	Lincosamide	20 g/ton	Lincomix
Neomycin	Aminoglycoside	NA	Unknown
Oxytetracycline	Tetracycline	10-50 g/ton	Terramycin
Penicillin	B-lactam	10-50 g/ton	
Tiamulin	Diterpene	10 g/ton	Tiamutin
Tylosin	Macrolide	20-100 g/ton (starter) 20-40 g/ton (grower) 10-20 g/ton (finisher)	Tylan
Virginiamycin	streptogrammin	5-10 g/ton	Stafac
Chemotherapeutics			
Arsanilic acid	arsenical	10-30 g/ton	
Carbadox	Quinoxaline	10-25 g/ton	Mecadox
Roxarsone	Arsenical	22.7-34.1 g/ton	
Sulfamethazine	Sulfonamide	100 g/ton	
Sulfathiazole	sulfonamide	100 g/ton in combination with Chlortetracycline	

Antibiotic residues have been introduced into the environment via land application of animal waste (Kümmerer, 2009). A recent study by Hoese et al. (2009) revealed that percentage of chlortetracycline residues in manure can be recovered from runoff ranged from 0.9 to 3.5% of the amount applied whereas tylosin ranged from 8.4 to 12%. Antibiotics can also enter the environment via disposal of unused drugs and antibiotic containers. Antibiotics released in environments can transport either in dissolved phase or absorbed onto soil particles into surface or groundwater. Understanding the absorption of antibiotics to soils is very important to improve understanding of the fate and transport of antibiotics residues. Agricultural land under drainage management may directly transport antibiotics to streams,

contaminating surface water bodies, or infiltrate through karst areas contaminating groundwater.

2.1.3 Tylosin use in swine production and persistence of tylosin residues in environment

Currently, 19 antimicrobial agents are approved for uses in swine feed by the Food and Drug Administration, including 13 antibiotics (naturally occurring agents) and 6 chemotherapeutics (chemically synthesized agents). Most of these compounds have been used for many years, and only a few agents have been introduced during the past ten years. Among those agents, tylosin is one of the most frequently used antibiotics in swine production for grow promoter purpose in grower/finisher farms (Jindal et al., 2006; McEwen and Fedorka-Cray, 2002). As reported in Swine 2006 by the National Animal Health Monitoring System (USDA, 2007), the most common antibiotics administered via feed to grower/finisher pigs were chlortetracycline (52.6% of sites), tylosin (44.2% of sites), and bacitracin (29.1% of sites).

Tylosin is a macrolide consisting of a 16-membered lactone ring active mostly against gram-positive bacteria including *Enterococcus* and certain gram-negative bacteria. Like other macrolides, tylosin has a bacteriostatic effect on susceptible organisms through binding to the 50S subunit of the bacterial ribosome, eventually resulting in inhibition of protein synthesis (Retsema and Fu, 2001). Tylosin and erythromycin are the most popular macrolides and share many common characteristics. Both are naturally occurring antibiotics which are synthesized by actinomycete bacteria. Tylosin is known as a mixture of 4 components produced by a strain of *Streptomyces fradiae*, namely tylosin A, B, C, D, in which tylosin A is the main component of the mixture (> 80%) (Loke et al., 2000). Tylosin B, tylosin C and tylosin D may also be present. All of these four components contribute to the potency of tylosin. The majority (94%-99%) of the metabolic residue of tylosin was excreted in the faeces and only 1%-6% in the urine. Of the excreted tylosin residues, 33% was tylosin D and 6% was tylosin A (Lewicki, 2006).

Previous researches have determined that tylosin can enter the environment in significant concentrations and land-applied tylosin in manure can sorb to the soil or move to surface and groundwater (Allaire et al., 2006; Clay et al., 2005; Dolliver and Gupta, 2008; Kolpin et al.,

2002; Kolz et al., 2005; Loke et al., 2002). Tylosin has high potential to transport with sediment during runoff events due to its sorption characteristics to manure and soil particles (Davis et al., 2006; Loke et al., 2002). A study conducted with swine manure in South Dakota by Clay et al. (2005) showed that sorption coefficient values of tylosin in manure ranged between 175 - 840 ml/g. The mobility of tylosin in soil depends on the soil type. As shown by Rabølle and Spliid (2000), partition coefficients between soil and solution of tylosin (K*d*) is 8.3 ml/g in a loamy sand soil. Tylosin can move downward in soil column up to 25m in sandy soil and 5m in sandy loam soil because sorption coefficients of sandy soil is 11 (ml/g) which is much smaller than sorption coefficients of sandy loam soil of 128 (ml/g). No tylosin was detected in the leachate of any of the soil types. Even with the high sorption to both soil and manure, movement of tylosin to offsite locations in runoff still may still occur. Tylosin was found at concentrations of 1.2µg/L and 6.0 µg/L in leachate and runoff from field receiving liquid swine manure, respectively (Dolliver and Gupta, 2008).

2. 2 Enterococci

Most bacteria falls into one of two categories, gram- possitive and gram-negative (the "gram" designations refer to the reaction of the bacteria when stained with the gram stain) based on differences in the cell wall structure of bacterial cells. Enterococci are gram-positive cocci that occur singly, in pairs or as short chain (Murray, 1990). At this time, there are more than 32 distinct enterococcal species. In which, *Enterococcus faecalis* and *Enterococcus faecium* are the most commonly isolated species. According to data from National Nosocomial Infections Surveillance system (NNIS system), 12% of nosocomial infections in the US from 1986 to 1989 was caused by enterococci (Emori and Gaynes, 1993) They are facultative anaerobic organisms that can grow between 10^{o}C and 45^{o}C with the optimum growth temperature of 35^{o}C. They can survive in broth at extreme conditions such as high salt concentration (6.5% NaCl), high pH (up to 10) or high temperature (up to 60^{o}C).

Enterococci were recommended for use as an indicator of fecal contamination in water systems in the U.S in 1986 by the U.S. Environmental Protection Agency (USEPA, 1986). Studies demonstrated that enterococci had a strong direct relationship to swimming-associated illness in both marine water (Moore et al., 2008) and freshwater (USEPA, 1984)

environments. They are now the third most common organism detected in nosocomial infections. They are commonly found in gastrointestinal tracts of many livestocks, in the feces of humans and other warm-blooded animals (Jett et al., 1994). Enterococci have been detected in surface water, ground water near swine feeding operations (Sapkota et al., 2007), and in runoff from land receiving animal-waste applications (Soupir et al., 2006). Other sources of enterococci in the environment include pets and wildlife, septic-tank discharges, and effluents from municipal wastewater treatment plants. Cools (2001) found that enterococci can survive up to 54 days at 25^{o}C and 80 days at moiture contents of 100% field capacity. The length of their survival depends on soil moisture, incubation temperature and soil texture. They can survive best in the loamy soil with low incubation temperature and high soil moisture content. These characteristics my contribute to the virulence and resistance to wide range of antibiotics.

As reported by Jett et al. (1994), enterococci possess typical properties of human disease causing pathogens. They can naturally acquire, accumulate, and share extra-chromosomal elements encoding virulence traits or antibiotic resistance genes. The study also listed four main features of enterococcal virulence: (i) adherence to host tissues, (ii) invasion and abscess formation, (iii) factors potentially relevant to modulation of host inflammatory responses, and (iv) potentially toxic secreted products. These understandings are very helpful in explaining their survival under unusual environmental stresses as well as their increasing importance as nosocomial pathogens.

2. 3 Antibiotic resistance in enterococci

Antibiotic resistance is a growing international problem affecting both current and future generations. The addition of antibiotics to the livestock feed creates selective pressure in favor of resistant bacteria and thus contributes to the development and the proliferation of antibiotic resistant bacteria. However, antibiotics resistance is not a recent phenonmenon. Resistance to penicillin in some strains of staphylococci was recognized almost immediately after mass-production of the drug was approved in 1943 (Grossman, 2008).

Enterococci are potential pathogens and they are becoming resistant and have acquired resistant genes to withstand the effects of antibiotics (Aarestrup et al., 2000; Butaye et al.,

2001; Portillo et al., 2000; Thal et al., 1995; Yuri. et al., 2005). Enterococci have a number of both inherent and acquired resistance traits to different types of antibiotics such as macrolides (erythromycin and tylosin); clindamycin, tiamulin and virginiamycin; aminoglycosides (streptomycin, gentamicin, apramycin); spectinomycin, tetracycline and monensin (Barton, 2000). As reported in Swine Studies conducted by National Animal Health Monitoring System in 2006, of the 857 *Enterococcus* isolates tested, 90.6 % are resistant to lincomycine, 81.9% are resistant to tetracyline and 52.6% are resistant to tylosin. While it is agreed that most resistant genomes were present before the antibiotic in question was used, the use of antibiotics will create selective pressure in favor of antibiotic resistance and contribute to the proliferation of antibiotic resistant bacteria in the animals (Aarestrup and Carstensen, 1998).

2.3.1 Natural selection and antibiotic resistance

Studies have shown that plasmids encoded with antibiotic resistance are naturally present in microorganisms without exposing to the antibiotics (Jindal et al., 2006; Storteboom et al., 2007). As long as the antibiotics is not present in the habitat, the concentration of these resistant genes will be very small because they have no particular favourable conditions to propagate. If the population of bacteria is exposed to antibiotics, sensitive individuals are inhibited and resistant ones will survive and proliferate. Chromosomal genes and plasmids containing DNA codes for antibiotic resistance are replicated and inherited by all subsequent offspring. In time, the resistant forms will become completely resistant and dominate in the general microbial population. Exposure to antibiotics therefore provides selective pressure for bacteria to become resistant. Even low quantities of antibiotics can encourage the selection of antibiotic resistant bacteria, called natural selection of antibiotic resistance. This is a common phenonmenon in various natural environments, laboratories and medical environments and it also occurs in bodies of humans and animals during drug therapy.

2.3.2 Development of antibiotic resistance

Antibiotic resistance is the ability of a microorganism to withstand the effects of antibiotics. According to Levy (1998), when an antibiotic is sub-therapeutically administered to a group of bacteria, the most susceptible cells will die while those that survive via

mutation or exchange of genes will proliferate. Antibiotic resistance evolves via natural selection acting upon random mutation in the pathogen genome or can be acquired from another bacterium. Enterococci have a large number of both inherent and acquired resistance traits which can be transferred to other enterococci (Gimore, 2002).

To respond to the action of antibiotics, bacteria begin to develop mechanisms to tolerate the amount of antibotics that would be ordinarily inhibitory. Bacteria can evolve resistance to antibiotics in several different ways. The most common mechanisms of resistance are those that enhance the production of enzymes that degrade or inactivate the antibiotic, so that they can not reach the target site (Wilke et al., 2005). The second mechanism is acquiring a mutation that modifies the target site of an antibiotic so that the target is no longer sensitive (Tenover, 2006). And the third mechanism involve in a change in the permeability of the cell membrane which either prevents the entry of the antibiotic into the cell or causes the antibiotic to be pumped out of the cell (Del Grosso et al., 2002).

There are two ways that bacteria can acquire resistance to antibiotics. (i) spontaneous mutations in critical chromosomal genes in a population of cells or (ii) acquisition acquisition of DNA that codes for resistance from another bacterium in the environment through a process called Horizontal Gene Transfer. In Horizontal Gene Transfer a gene or genes can be exchanged between different microbial species. Therefore, antibiotic resistance can be either intrinsic or acquired (Ochman et al., 2000).

2.3.3 Mutational resistance

Some bacteria are resistant to specific antibiotics via mutation and have not acquired antibiotic resistance from another organism. Mutations are are spontaneous changes of the bacteria's genetic material in bacterial genome. There are many of genes involved in antibiotic resistance either because there are several different target, access, or protection pathways for the antibiotic in the bacterial cell or because each pathway requires the expression of several genes (Martinez and Baquero, 2000). They determined three types of intrinsic genes which pre-exist in the genome of the susceptible population that are responsible for the occurrence of antibiotic resistant mutants:

(i) Genes involve in the synthesis and cell positioning of the antibiotic target - mutations in these genes can be named target-structural mutations;

(ii) Genes involved in the access of the antibiotics to the target (including those required for activation of the formerly inactive antibiotics), which are needed for the biochemical access of the antibiotics - mutations in these genes are named target access mutations; and

(iii) Genes involved in the protection target from the antibiotics, including detoxification by antibiotic-modifying enzymes or efflux of the antibiotics - mutations which activate the expression of those genes are named target-protection mutations.

Occurence of mutation depends on the structure and the number of genes in which mutations can produce a selectable phenotype (Martinez and Baquero, 2000). Different genetic mutations yield different types of resistance as following (Fig. 2):

(i) Some mutations enable the bacteria to decreasing the ability of the antibiotic to permeate the cell;

(ii) Some mutations allow the bacteria to produce new potent chemicals (enzymes) that inactivate antibiotics ;

(iii) Some mutations alter the target sites of bacteria that the antibiotic attacks;

(iv) Some mutations manufacture pumping mechanisms to actively efflux the antibiotic out of the cell, so it never reaches its target.

The frequences of chromosomal mutations leading to antibiotic resistance depend on concentration of given antibiotic during selection (Martinez and Baquero, 2000). In the presence of antibiotics, the process of natural selection will occur, favoring the survival and reproduction of the mutant bacteria. Natural selection relate to vertical gene transfer where bacteria receives resistant gene from its ancestor. Thus, the antibiotic action against bacteria can be seen as a selective antibiotic pressure for resistant bacteria to that antibiotic.

Depending on specific antibiotic-bacterium interaction at a given concetration of antibiotics, antibiotic resistance can be resulted from a single gene mutations or a cooperative mutation of several genes. Resistance to some antibiotics such as ß-lactams, streptomycins, aminoglycosides are most typical examples of intrinsic resistance in enterococci (Murray, 1990).

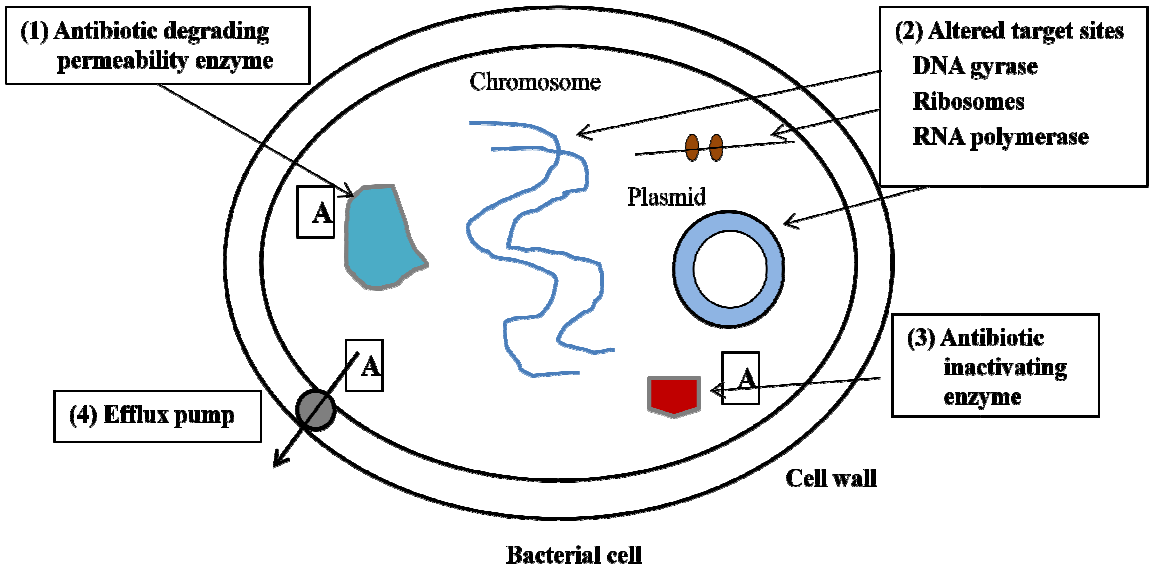

Figure 2 - Mechanism of resistance to antibiotics in a bacterial cell

(modified from Cowan, 2008)

2.3.4 Acquired resistance by horizontal gene transfer

Genetically, antibiotic resistance spreads through bacteria populations both "vertically," when new generations inherit antibiotic resistance genes, and "horizontally," when bacteria share or exchange sections of genetic material with other bacteria. In horizontal transfer, bacteria become antibiotic resistant by gaining resistant genes from other bacteria. Bacteria acquire genes encoding proteins that protect them from the effects of the antibiotic via horizontal gene transfer. Horizontal gene transfer is thought to be a significant cause of dissemination of antibiotic resistant genes among bacterial pathogens in human and animals (de la Cruz and Davies, 2000). Resistance genes are frequently carried on plasmids, which are loops of DNA that are separate from the chromosomal DNA, thus, easily transfered from one bacterium to another or even from one species of bacterium to another (Flint et al. 1987). Bacteria also can transfer genes conferring resistance to antibiotics by transposons which are DNA fragments that can move around to different positions within the genome of a single cell. Both plasmids and transposon are mobile genetic elements and they provide the means for genes to persist in new host cells: plasmids by their ability to replicate independently as replicons, and transposons by providing a mechanism for insertion of genes into the main

chromosome. This is a very rapid adaptation of bacterial populations to a strong selective pressure. Bacteria can also become infected with viruses (i.e., bacteriophage) that pick up antibiotic resistance genes and transfer them during the infection of other bacteria.

There are three common mechanisms for horizontal gene transfer have been reported in enterococci: conjunction, transformation, and transduction (Ochman et al., 2000). Conjuction is believed to be the most important mode of transfer within the gastrointestinal microbiota (Mazel and Davies, 1999). Most plasmid-encoded and chromosome-located resistant genes can be transferred via conjugation. In this process, bacterial cells transfer genetic material which is resistant DNA to another cell by direct cell-to-cell contact or through a bridge-like connection between two cells by conjugal plasmids or conjugal transposons. Conjugative plasmids are self-transmissible due to the presence of the origin of transfer gene and transfer genes (Cowan and Marjorie, 2009). Conjugative transposons are mobile DNA elements that possess the genetic machinary to facilitate their own transfer between cells e.g. from plasmid to plasmid or from chromosome to plasmid and vice versa by conjugation. Conjugative transposons are very common in enterococci and they play an important role in the dissemination of antibiotics resistance genes in enterococci.

Horizontal gene transfer can also occur by transformation and transduction (Ochman et al., 2000). Transformation is the process in which competent bacteria uptake and express foreign genetic material, resulting in the genetic alteration. This mode of horizontal gene transfer can mediate the exchange of any part of a chromosome. Transduction is the process in which bacterial DNA is moved from one bacterium to another by a bacterial virus called a bacteriophage, or phage. Phages consist of an outer protein capsid enclosing genetic material (RNA, DNA). This mechanism requires that the donor and recipient share cell surface receptors for phage binding and thus is usually limited to closely related bacteria. These two mechanisms have a less broad of host gene transfer range than conjugation.

Any bacteria that acquires resistance genes, whether by spontaneous mutation or genetic exchange with other bacteria, has the ability to resist one or more antibiotics. The DNA that codes for resistance can be grouped in a single easily transferable package. Bacteria can become resistant to many different groups of antibiotics because of the transfer of one piece of DNA and collection of multiple resistance traits over time.

2.3.5 Resistance to macrolide and tylosin

The macrolides are a group of antibiotics whose structure includes a macrolide ring and a large macrocyclic lactone ring with attachment of one or more deoxy sugars (usually cladinose and desosamine). Common antibiotic macrolides are known as erythromycin, Spiramycin and tylosin. Activity of macrolides is related to protein synthesis inhibition (Portillo et al., 2000).

To resist macrolides, gram-positive bacteria have collected mobile elements that help them evade the lethal effects of antibiotics. There are three different mechanisms associated with the acquired resistance to macrolide antibiotics in gram-positive bacteria: (i) modification of the antibiotic target by methylation or mutation that prevent antibiotic binding to its ribosomal target, (ii) active efflux of the antibiotics which prevent them to reach the ribosome, and (iii) inactivation of the antibiotics. (Leclercq and Courvalin, 2002). In which, the most common acquired mechanism of macrolide resistance development among enterococci is target site modification.

Ribosomal modification by methylation was the first mechanism of resistant to erythcomycin discovered. In this mechanism, an *erm* gene (erythcomycin ribosome methylase) encodes a ribosomal methylase which specially methylates nucleotide A2058 in the 23S rRNA of the 50S ribosomal subunit. Research have shown a large number of bacteria that are targets for macloride express *erm* genes (Bean and Klena, 2002; Chen et al., 2007; De Leener et al., 2004; Jensen et al., 2000; Jost et al., 2004; Sutcliffe et al., 1996b). Currently, there are nearly 40 *erm* genes which are mostly transmitted by plasmids and transposons have been reported (Roberts, 2004a) . The presence of 4 major *erm* classes, *erm(A), erm(B), erm(C), erm(F))* in different *Enterococcus* spieces have been described in recent studies (Jost et al., 2004; Luna et al., 1999; Portillo et al., 2000). As claimed by Portillo et al. (2000), the most commonly *erm* class found in streptococci and enterococci is *erm(B)* class, followed by *erm (TR),* a subset of *erm(A)* class.

Acquired resistance to macrolides by active efflux has been detected in various bacteria including streptococci and staphylococci (Bean and Klena, 2002; Del Grosso et al., 2002; Descheemaeker et al., 2000; Luna et al., 1999). In gram-positive bacteria, acquisition of resistant bacteria by active efflux is caused by 2 classes of pumps: ATP-binding-cassette

(ABC) transporter and major facilitator (MFS), encoded by two efflux gene classes *msr(A),* and *mef(A)* respectively. The erythromycin efflux *mef(A)* gene have been first described in *Streptococcus pyogenes* by Clancy et al. (1996). The presence of *mef* genes (*mefA/E*) have been reported resistant to macrolides in *Enterococcus spp.* in a study with thirty four erythromycin resistant isolates of *Steptococcus pneumonia* collected from three different sites (Luna et al., 1999). The *msr(A)* gene in *Enterococcus ssp.* Appears to be on a conjugative element and mediates a lower level of erythromicin resistance (MIC 2 to 16 µg/mL) than erm(B) gene (>32 µg/ml). Another emerging macrolide resistant gene *mphB* was found conferring resistance in *Enterococcus faecalis* to spiramycine (Achard et al., 2008). The *msr(A)* gene confers resistance to macrolide antibiotics via an ATP binding transporter protein and has been detected in *E.feacium* clinical isolates (Roberts et al., 1999; Wierzbowski et al., 2005).

2. 4 Transport of antibiotic resistant enterococci in the environment

Land application of animal waste is an important route to spread enteric bacteria as well as resistant bacteria into environment, (Descheemaeker et al., 2000) making this become a very complex problem. Recent studies showed that numerous bacterial pathogen has become resistance to antibiotics (Heuer and Smalla, 2007; M. Kólar, 2002; Portillo et al., 2000) due to the overuse and misuse of antibiotics in the community, in the hospital and on the farm (Amyes et al., 2001). Recently, there has been growing concern about transmission of antibiotic resistant genes from animals to humans through food –related pathogens or recreation activities in contaminated water bodies (McMahon et al., 2007; Teuber, 1999). Halling - Sørensen (1998) discribed some curcumstances in which the presence of antibiotics in water and sediments have facilitated bacterial flora to develop resistance to those particular agents.

2.4.1 Antibiotics resistant bacteria in manure

Antibiotic resistant selection occurs in animal gastrointestinal tracts (Aminov et al., 2001) and is introduced into the environment via excretion in animal wastes. Swine waste is often stored in collection pits and lagoons and periodically disposed of through land application. A number of studies have reported the occurrence of antibiotic resistant bacteria in animal feces

and manure. In a study conducted by Koike et al. (2007) in Illinois, tetracycline resistance genes was detected in manure samples in lagoons under two swine confinement operations. Genes resistant to tetracyline have also been detected in horse manure, beef feetlot manure and dairy manure (Chander et al., 2006; Storteboom et al., 2007). The occurrence of antibiotic resistant bacteria in feces is closely related to the addition of antibiotic in feed (Yuri. et al., 2005). As reported by Storteboom et al. (2007), feeding antibiotics subtherapeutically to animals results in greater levels of antibiotic resistant genes in manure than administering therapeutic dose of antibiotics for disease treatment. Cotta et al. (2003) found that up to 32% of the bacteria in swine manure collected at 3 ft depths in the manure storage pits was resistant to tylosin.

2.4.2 Transport of antibiotics resistant bacteria into soil and water

Once resistance genes are introduced into the environment, they are also exposed to selective pressure due to the presence of antibiotics produced by indigenous antibiotic producers in soil. However, selection can occur in the environment without antibiotic selective pressure (Alonso et al., 2001). This means that antibiotic resistance genes might be transferred and preserved in the environment with or without antibiotic selective pressure.

Tylosin resistant bacteria were found higher in three agricultural soils amended with manure associated with antibiotic uses at subtherapeutic levels than those in soils unaffected by subtherapeutic use of antibiotics (Onan and LaPara, 2003). The study results imply a relationship between subtherapeutic use of antibiotics and the development of antibiotic resistant bacteria in soil, however the study did not take into account the soil types and impact of temperature variation.

Resistant bacteria as well as other waterborne pathogens that came from animal wastes can migrate from manure to the land and water and become a non-point source of polution (Smith et al., 1972). The major mode of pathogen and bacteria movement from manure to soils and ground water is through infitration water. Accoding to Rysz and Alvarez (2006), infiltration process may attenuate bacteria and DNA migration due to interception, sorption, and sedimentation. Many studies have been conducted to simulate the transport of bacteria (Abu-Ashour et al., 1993; Jiang et al., 2007). The physical processes controlling microbial

movement through porous media are convection, advection, or hydrodynamic dispersion. However, transport via macropore flow is considered to be one of driving forces of microorganism transport (Beven and Germann, 1982). Microorganisms in the soil may move in the vertical or horizontal direction. The horizontal movement may occur when the soil is saturated or impermeable in nature (Mawdsley et al., 1995). According to Gerba et al (1975), migration distance of coliforms in sand – gravel media is up to 759 m and this distance varies with the type of the medium. Gagliardi and Karns (2000) studied the movement of *E.coli* from manure through three different types of soils (silt loam, clay loam and sandy loam) with tilled and no-till treatment and found that *E.coli* concentrations in leachate and soil from no-till soil were higher than those in tilled soil. Studies showed that irrigation or rainfall over the land surface after manure application provides favorable conditions for bacteria to disperse into surface or groundwater (Chee-Sanford et al., 2009).

Land application of manure may affect the spead of antibiotic resistant bacteria through the way manure is applied into soils. Surface application is likely to be the most efficient method in managing pathogens and resistant bacteria due to desiccation when manure is exposed to ultraviolet solar radiation (Hoerter et al., 2005). Injection method is preferred from the nutrient management standpoint, but this method transfers bacteria and resistant genes directly into the soils and increases bacteria sorption to soil particles (Unc and Goss, 2004) and survival. Unc and Goss (2004) summarized 4 major factors affecting the movement of bacteria in soils including (i) characteristic of flow which closely relate to properties of the porous media, (ii) soil macropores, (iii) saturation of organic material, and (iv) absorption and adhesion of bacterial cells on soil particles.

Liquid manure applied to tile drained agricultural fields can easily penetrate through the soil and contaminate tile water. Application of animals waste into soil can change soil structure, soil texture, other physical and chemical properties of soils resulting in promotion of bacteria movement (Jamieson et al., 2002). Survival and attachment of bacteria onto agricultural soils have been reported in recent studies (Jamieson et al., 2002; Unc and Goss, 2004). Dean and Foran (1992) reported the application of liquid manures to tile drained fields resulted in elevated levels of nutrients and bacteria compared to tile discharges from unmanured sites.

Jamieson et al. (2002) confirmed the significant transport of bacteria in the tile drainage systems, thus, a similar situation can be expected for antibiotic resistant bacteria. Preferential transport processes can move significant quantities of contaminants very rapidly, with little alteration, to the shallow groundwater and then to tiles, subsequently into receiving water bodies. The occurrence of bacterial resistance recorded for tetracyline, cephalothin and sulfafurazole were 51%, 41% and 32% respectively. Two sample sets of water and sand were taken and analyzed for antibiotic resistant enterococci in two recreational beaches in Southeastern Brazil (Oliveira and Pinhata, 2008). The study results revealed high leve of pollution correlated with high percentage of resistant strains in two water samples (31.25% and 61.5%). Antibiotic resistant gene were able to transfer a significant distance into groundwater and can be detected 200m downstream of a swine waste lagoon (Chee-Sanford et al., 2001).

2. 5 Detection of enterococci and resistant enterococci

In microbiology, one of the most pressing and important question is how to identify the unknown bacteria from samples in nature. Detection methods are effective tools to determine and detect the occurrence of bacteria. Currently, there is no method considered to be the best for all applications. According to levels of genus and species of bacterial identification, bacterial detecting methods can be categorized as either phenotypic methods or genotypic methods (Cowan and Marjorie, 2009). To confirm the results, we can use non-molecular methods to validate molecular methods.

2.5.1 Phenotypic methods

Phenotypic methods are the conventional approach to detect enterococcus bacteria. They are based on morphology (microscopic and macroscopic) as well as bacteria physiology and biochemistry. They rely on culturing on a medium that selectively permits the growth of bacteria. Phenotypic identification methods access microbe appearance and growth characteristics. Traits that can be used for diagnosing bacteria are appearance of colonies, patterns of growth in agar and broth, cell shape and size, gram stain reaction, acid fast reaction, and special structures such as endospores, granules, and capsules. Phenotypes result from the expression of an organism's genes as well as the influence of environmental factors

and the interactions between the two. Not all organisms with the same genetic material look or act the same way because appearance and behavior are modified by environmental and developmental conditions. Similarly, not all organisms that look alike necessarily have the same genes.

Enterococci bacteria can grow well on tripticase-soy 5% sheep blood agar, brain heart infusion 5% sheep blood agar, mEnterococcus agar or any blood agar base containing 5% animal blood. They are coccoid-shaped bacteria, stained purple and typically form short chains or are arranged in pairs. Gram-positive enterococci can be differentiated from other catalase-negative gram-positive cocci by their ability to hydrolyze esculin in the presence of 40% bile salts (turn Bile Esculin Azide Agar into brown or black), grow in 6.5% sodium chloride at 45°C.

2.5.2.1. Agar and broth dilution method

Agar and broth dilution, membrane filtration and gram staining are the most popular techniques have been used for phenotypic assessment of the antibiotic sesceptibility of bacteria. Agar dilution and broth dilution are the most commonly used techniques to determine the minimal inhibitory concentration (MIC) of antibiotics (Benning and Mathers, 1999). This can be achieved by dilution of antimicrobial in either agar or broth media.

MIC is the lowest concentration of the antibiotic that inhibits the visible growth of the bacterium of interest under defined test conditions. MIC values are used to determine susceptibilities of bacteria to antibiotics and also to evaluate the activity of new antimicrobial agents.

Agar dilutions are most often prepared in petri dishes and have advantage that it is possible to test several organisms on each plate. The agar dilution method, however, is not convenient for testing a few isolates at a time (Gaudreau et al., 2007). In agar dilution, antibiotics are incorporated into the nutrient agar plates at different concentrations. A standard concentration of organisms is inoculated onto the surface of this medium contained in Petri dishes. After incubation, the presence of bacterial colonies on the plates indicates growth of the organism. The antibiotic concentration of the first plate showing more than 99% inhibition is taken as the MIC for the organism.

Broth dilution uses liquid growth medium containing geometrically increasing concentrations of the antibiotics which is inoculated with a defined number of bacterial cells. Two type of broth dilution methods are macrodilution and microdilution which is classified based on the final volume of the test is 2 ml or less than 500 µl per well of microtiter plates. After incubation, the presence of turbidity or a sediment indicates growth of the organism.

In both the agar and the broth dilution approaches, the MIC is defined as the lowest concentration (in mg/l) of the antibiotics that prevents visible growth of a microorganism under defined conditions. This protocol applies only to aerobic bacteria and can be used for monitoring the development of antibiotic resistance.

2.5.2.2. Membrane Filtration Technique

Membrane Filtration (known as Method 1600) is an Environmental Protection Agency (EPA) certified method for the detection and enumeration of the enterococci bacteria in water (USEPA, 2006). The membrane filtration (MF) technique provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter (Messer and Dufour, 1998). This method is highly reproducible, can be used to test relatively large sample volumes, and usually yields numerical results more rapidly. The existing method 1600 a single-step method that is a modification of EPA Method 1106.1. After water samples are filtered through a membrane, the membrane is placed on the mEnterococcus (mE) agar and incubated at 41±0.5°C for 48 hours. To detect the occurrence of resistant bacteria over the total population, it is necessary to prepare agar supplemented with the antibiotics of interest at MIC level. All colonies greater than or equal to 0.5 mm in diameter (regardless of color) with a blue halo are recorded as enterococci colonies (USEPA, 2006). The MF technique is extremely useful in monitoring drinking water and groundwater (Salem et al., 2008; Shirey and Bissonnette, 1991). However, when testing waters with high turbidity or large numbers of noncoliform (background) bacteria, the MF technique is not accurate because sediment blocks spores of filter papers (Halls and Ayres, 1974).

2.5.2.3. Gram staining Technique

This technique that is used to make bacteria in specimens more visible, so that the existance of bacteria of interest can be recognized through their morphology. The gram-

staining technique consists of timed, subsequential applications of crystal violet (the primary dye), gram's iodin (the mordant), an alcohol (the decolorizer), and safranin (counterstain). This protocol is based on Hucker modification of the original stain method.

Gram staining is used to distinguish between Gram-positive and Gram-negative bacteria, based on the chemical and physical properties of their cell walls (Cowan and Marjorie, 2009). Gram-positive bacteria have a thick cell wall stains purple while Gram-negative bacteria have a thinner layer which stains red. Gram-positive and Gram-negative bacteria are both stained by the Crystal violet. The addition of the iodine leads to the formation of a crystal violet-iodine complex within the cell wall. Because of thicker cell wall in Gram-positive cells, the entrapment of the dye is far more extensive in them than in the Gram-negative cells. Application of alcohol in the third step dissolves lipid in the outer membrane and remove dye from the cell wall of Gram-negative bacteria. By contrast, the crystals of dye tightly embedded in the cell wall of gram positive bacteria are relative inaccessible and resistant to the removal. Because of the increase in porosity, the safranin counter stain is able to permeate the cell wall of the Gram-negative bacteria and make them visible in the final step.

The Gram stain is almost always the first step in the identification of a bacterial organism. While Gram staining is a valuable diagnostic tool in bacteria identification, not all bacteria can be definitively classified by this technique. Some organisms may stain either negative or positive which are called Gram-variable. Some organisms are not susceptible to any stain in the Gram staining technique.

2.5.2 Genotypic methods

Although useful, phenotype-based detection methods are limited in several ways. Some organisms are unculturable, fastidious, slow or difficult to propagate. Thus, a large proportion of them could not be seen on the culture dish. Survival characteristics of some bacteria in the laboratory have determined that traditional enumerative techniques, based on selective culture, do not detect all viable bacteria present (Lewis et al., 2002). For resistance detection in particular, examination from behavior perspective just provides information

about the conditions needed for proper expression of a number of resistant determinants but very little or nothing about the mechanisms involved (Cowan and Marjorie, 2009) .

Phenotypic methods are usually slow and have high potential of contamination during experimental process. There are many advantages of genotypic methods over phenotypic methods, such as that they can rapidly detect specific antibiotic resistant genes and thus contribute to the understanding of transport and genetics of acquired enterococcal resistance. Non-culture dependent tools have revealed vast number of species that could never be observed based on phenotypic techniques (Lewis et al., 2002). A major advantage of genotypic-based resistant detection methods is that it does not generally require growth of the target organism. These groups of techniques target the genetic basis of the fundamental mechanisms responsible for resistant, and disgards unrelated genes and nonspecific bacterial properties. These methods are also rapid, cost saving, reliable so that improvements or adjustments can be provided during experiment in a timely fashion.

Existing molecular techniques are derived from basic principles such as hibridization, amplification. Based on these principles, following methods have been developed and used widely to detect resistant encoding genes, namely probes, polymerase chain reaction (PCR) and DNA microarrays. In which, PCR amplification is now widely used to detect and quantify low levels of target sequences, and has become key procedures in the detection and identification of bacteria and genes from a variety of environments including soil, water, and fecal materials.

2.5.2.4. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is now widely used to enable detection of low levels of target sequences, and it has become a key procedure in the detection and identification of bacteria and genes from a variety of environments including soil, water, and fecal materials (Bockelmann et al., 2009; Lucena et al., 2006; Sapkota et al., 2007). The employment of PCR in detecting resistant genes have been well documented (Baele et al., 2000; Bockelmann et al., 2009; Chen et al., 2007; Dutka-Malen et al., 1995; Kariyama et al., 2000; Ke et al., 1999; Luthje and Schwarz, 2007; Sutcliffe et al., 1996b).

Developed in the mid 1980s by Kary Mullis of the Cetus Cooperation (Bartlett and Stirling, 2003), PCR is now becoming a common and indispensable technique in microbiology related researcher. This method can amplify DNA present in samples, even in trace amount, to produce a quantity sufficient to investigate using conventional laboratory methods.

PCR method is based on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA (Wax et al., 2008). The PCR experiment requires 3 basic components: (i) DNA template that contains the DNA fragment (target) to be amplified, (ii) Two primers (Forward and Reverse) that are complementary to the 3' (three prime) ends of each of the strand of the DNA target, and (iii) Taq polymerase or another DNA polymerase (an enzyme to synthesize a new DNA strand complementary to the DNA template strand). The PCR procedure classically involves three steps that make up one cycle.

- Step 1. Denaturation: DNA template is heated to a high temperature (~ 95^{o}C) to break the hydrogen bonds between the complementary bases, resulting in a single strand of DNA.
- Step 2. Annealing: Temperature is lowered to 50^{o}C – 60^{o}C so that primers can bind to the complementary sequence on the single-stranded template by forming hydrogen bonds between bases.
- Step 3. Extension: The temperature is raised to 72^{o}C. The Tag polymerase attaches at each priming site and extends (synthesizes) a new DNA strand.

As cycling continues, the number of DNA strands doubles each cycle, resulting in an exponential increase in the number of sequences. Depending on the amount of input DNA, that is the number of copies of the target DNA, number of cycles can change from 20 to 40 cycles.

Recently, a number of variations of PCR have been developed (Wax et al., 2008). The most common is real-time PCR or quantitative PCR, in which the amplication process can be monitored when a flourescent dye or a florescently labelled probe is added to the PCR reaction (Bockelmann et al., 2009; Chen et al., 2007). Real-time PCR enables both detection and quantification of one or more specific sequences in a DNA sample.

Although the PCR may yield a detectable fragment, the fragment identity should be confirmed, for examples, based on the length of the product. Because PCR results are affected by a number of parameters including magnesium, template DNA, primer concentration, and annealing temperature during amplification, appropriate controls should be included in PCR reaction. Negative controls are used to check for contamination and positive controls for both DNA template purification and amplification.

2.5.3 Summary

Althrough genetic methods are now becoming a powerful tool in detection antibiotic resistant genes, they still have the following limitations. Firstly, there can be several genetic mechanisms for resistance to a single antibiotics, the test of a single mechanism does not mean that the screening has been comprehensive as in the case of phenotype-based methods. Secondly, because the genes are not always expressed, detection of a gene may or may not have clinical meaning in terms of expression potential and relevance. Finally, this group of methods are heavily technology intensive which limits their accessibility and appropriateness for a number of settings. Therefore, genotypic methods should be accompanied by phenotypic methods, so that they can support each other in detection and quantitation of resistant genes and bacteria (Wax et al., 2008) .

CHAPTER 3 OCCURRENCE OF TYLOSIN RESISTANT ENTEROCOCCI IN SWINE MANURE AND TILE WATER UNDER NO-TILL MANAGEMENT

A paper to be submitted to *Water Research* Journal

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Abstract. The sub-therapeutically use of tylosin by the swine industry provides selective pressure for the development of antibiotic resistance in gastrointestinal bacteria. Land application of swine manure into drained agricultural fields might accelerate the transport and dissemination of these microorganisms through soils, into tile drainage lines, and ultimately into surface waters; however, little is known about the hydrologic link between sources of antibiotic resistant bacteria and their transport in tile drainage systems. The objective of this study was to investigate the occurrence and transport of tylosin-resistant enterococci from tile-drained agricultural fields receiving semi-annual swine-waste applications. A field study was conducted at a farm near Nashua, IA in April (spring) and November (fall) of 2009. Liquid swine slurry from an operation feeding tylosin at sub-therapeutic levels was injected into no-till field plots. Resistance to tylosin in manure, soil and tile water was investigated by a phenotype-based method and polymerase chain reaction (PCR) using previously published primers, *ermA, ermB, ermC, ermF, ermT, ermX, mefA, msrA.* All enterococci in manure samples were resistant to tylosin in the spring, whereas about 68% of total enterococci in fall were resistant to tylosin. Average concentrations of total and tylosin-resistant enterococci in soil samples over the two experiments were 8.8×10^6 cfu/g of soil and 8.9×10^6 cfu/g of soil, respectively. Total and tylosin-resistant concentrations in tile water were significantly different during both the spring and fall

experiments ($P < 0.05$). In drainage water, total enterococci ranged from 1.3×10^1 cfu/100 mL (fall) to 5.0×10^3 cfu/100 mL (spring) while tylosin-resistant enterococci ranged from 1.3×10^1 cfu/100 mL (fall) to 1.2×10^3 cfu/100 mL (spring). The greatest concentration of tylosin-resistant enterococci in manure and lowest in drainage water suggest that enterococci lose resistance as selective pressure from antibiotic residues decrease. Five macrolide-resistance genes namely *ermB*, *ermC*, *ermF, ermT, msrA* were detected in greater than 9% of the samples. On average, most isolates (97%) harbored *msrA*, while only 9.5% of total isolates contains *ermT* gene and 9% contain *ermC* gene. The *ermF* gene was found in 156 out of 200 isolates (78%) and *ermB* gene was detected in 138 isolates (69%). Only four strains representing 2.0% of the total strains contains all five genes. The study results confirmed the hydrologic connection between the tylosin resistant enterococci and macrolide resistant genes in swine manure and their presence in tile water when manure is applied to agricultural fields followed by rainfall event. This work suggests that application of liquid manure from swine facilities administering sub-therapeutic doses of tylosin to tile-drained lands will promote the transport of antibiotic resistant bacteria through soil and tile lines, thus contaminating nearby soils and waters.

Keywords: tylosin resistant enterococci, microbial transport, swine waste, antibiotics, no-till, tile drainage.

3.1 Introduction

Approximately 1/3 of the land area in Iowa is under subsurface drainage management (Zucker and Brown, 1998b). Subsurface tile drainage systems remove excess water from the land and improve the crop production in areas with hydric soils; however, these systems also have the potential to convey pollutants directly to nearby aquatic systems. Iowa also leads the United States in swine production with more than 19.3 million pigs produced in 2007 (Chee-Sanford et al., 2001; Koike et al., 2007; Oliveira and Pinhata, 2008; USDA, 2009). Antibiotics such as tylosin, penicillin, tetracycline, and chlortetracycline are typically administered at sub-therapeutic levels to improve growth rates and feed utilization efficiency of swine (Cromwell, 2002). Only a fraction of the antibiotics are utilized by the animals; the non-metabolized antibiotics or metabolites may remain unchanged through the animal

digestive system, with up to 90% of administered antibiotics being excreted in urine and feces (Onan and LaPara, 2003). Swine waste is often stored in collection pits and periodically disposed through land application, serving as a major nutrient source to crops; however, this practice introduces large quantities of antibiotics into the environment (Chee-Sanford et al., 2009). Antibiotics applied to soils are transported to surface and groundwater systems where they contribute to the development and spread of antibiotic resistance in the environment via mutation or horizontal gene transfer (Dolliver and Gupta, 2008).

The gram-positive enterococci, recommended as an indicator of fecal contamination in water systems in the U.S. (USEPA, 1986), are commonly found in the feces of humans and other warm-blooded animals (Jett et al., 1994). Enterococci have been detected in surface water and ground water near feeding operations (Sapkota et al., 2007), and in runoff from land receiving animal-waste applications (Soupir et al., 2006). Enterococci are potential pathogens and have acquired antibiotic resistance genes to withstand the effects of different types of antibiotics including tylosin, vancomycin, avilamycin, avoparcin, quinupristin, dalfopristin, tetracycline, gentamicin (Aarestrup and Carstensen, 1998; Barton, 2000; Butaye et al., 2001; Thal et al., 1995). Recently, two mechanisms of resistance to macrolide antibiotics have been recognized in enterococci, including target site modification and active efflux (Pechere, 2001). Target site modification is mediated by an erythromycin resistant methylase (*erm)* which prevents macrolide binding and allows synthesis of bacterial proteins to continue. The active efflux mechanism produces transporters to discharge macrolide antibiotics out of the cell before reaching the target (Luna et al., 1999).

Rainfall facilitates the movement of pathogens through the soil, and into surface and groundwater (Auckenthaler et al., 2002). Antibiotic-resistant bacteria from manure sources can move into soil and contaminate water via preferential flow through soil macropores such as cracks, holes formed by plant roots or earthworms or other voids in the soil (Shipitalo and Gibbs, 2000), and fractures in karsts areas (Auckenthaler et al., 2002) Transport via macropores is considered one of the main pathways for bacteria to move into subsurface waters and possibly groundwater (Abu-Ashour et al., 1998; Beven and Germann, 1982). Macropore continuity resulted in greater bacteria concentrations in no-till soils than in tilled

soils (Gagliardi and Karns, 2000) and the occurrence of bacteria below the crop root zone and ground waters.

Although the occurrence of antibiotic-resistant bacteria in soils and waters near swine operations has been reported widely (Graham et al., 2009; Jindal et al., 2006; Koike et al., 2007), relationships between the source and the resistant microorganism in the stream typically are inferred due to farm and sample location (Campagnolo et al., 2002) instead of a clearly established hydrologic link. Much research has been done to improve understanding of the fate and transport of resistant genes through soil and water systems (Chee-Sanford et al., 2009). However, understanding of the release and transport of antibiotic-resistant bacteria from tile-drained fields receiving manure application is limited. If resistant microorganisms are transported through macropores and into tile lines, the tile lines will quickly facilitate the transport of these organisms to surface waters, creating a risk to human health due to potential exposure through swimming and recreational activities. The objectives of this study were to (1) detect and quantify the occurrence of tylosin-resistant enterococci in manure from swine facilities feeding tylosin at sub-therapeutic doses, soils amended with swine waste, and tile drain flow from swine waste amended agricultural fields; (2) assess the effects of flow and total suspended solids transport on the prevalence of total and tylosin-resistant enterococci in tile water; and (3) test enterococci isolates for known macrolide resistant genes. Results will further understanding of the occurrence and transport of enterococci expressing tylosin resistance and the mechanisms of resistance from tile-drained lands receiving swine waste applications.

3.2 Materials and Methods

3.2.1 Study site and sample collection

Field experiments were conducted at the Iowa State University Research Farm near Nashua, IA, where there are 36 plots, each 67 m by 58.5 m, which have been managed with consistent tillage practices under corn-soybean crop rotation since 1978 (Bakhsh et al., 2005). Two plots (plot 20 and plot 25) were selected for this study because (1) these plots are under no-till management, (2) the planting crop in the corn-soybean rotation was corn, which allowed for nitrogen (N)-based manure application, and (3) the plot locations had convenient

access to irrigation water and power to operate a rainfall simulator. Soils at the site are classified as poorly to moderately well drained with Floyd loam in plot 20 and Kenyon loam in plot 25 (USDA and NRCS, 1995). During the study period, the water table varied from one to two m below the ground surface. Each plot is drained separately with subsurface drainage pipes of 10 cm in diameter installed at 1.2 m below ground surface and located in the center of the plot. Tile lines are spaced 28.5 m apart which includes border tiles to prevent flow across plot borders (Kanwar et al., 1999). The tile lines are connected to sumps for measuring tile drainage flow and collecting water samples as described by Kanwar (2006).

Swine manure was collected from a manure pit under a growing/finishing facility that administers tylosin to swine feed at an approximate rate of 40 µg/g. A sub-area of 30.48 m by 30.48 m (929 m2) containing a tile line in the center was selected within each plot and swine manure was applied in the spring before planting and in the fall after harvest at a rate of 168 kg N per ha (Kanwar, 2006). Half of the N was applied to the experimental areas in April (spring) and the other half in November (fall) prior to each rainfall simulation at a rate of 18,662 L/ha and 13,478 L/ha respectively. Injection knives placed the manure in a concentrated, vertical band below the soil surface with 76.2 cm spacing between bands. A manure sample was collected from the injector during application and two composite soil samples were collected before and after manure application using a soil probe. A composite soil sample for each plot was created by mixing three replicate soil cores of 10 cm in depth from three locations within the plot. Manure and soil samples were transported to the laboratory on ice and stored for less than 24 h at 4˚ C prior to analysis.

A boom, liner-moving rainfall simulator was used to apply groundwater to the soil surface of the test area. The boom was powered by a water turbine which carried water to rotating nozzles. The nozzles (Nelson Irrigation Corporation, Walla Walla, WA) with orifice diameters of 8.7 mm were fixed every 3 m on the boom and were directed vertically downward, spraying water within a 9-m-radius disk. In a stationary state, a rainfall intensity of 5.6 cm/h was obtained; however, under field conditions the effects of wind and motion reduced the intensity to an average of 5.1 cm/h. The speed set for the irrigation system

determined the duration of the rainfall simulation. At 25% speed, the rainfall simulation moved over the length of 30.5 m of test area in approximately one hour. For both simulations, the rainfall simulator moved back and forth over the test area for 90 min with two runs: The first run lasted for 1 h (25% speed) and the second run 30 min (50% speed). A data logger was connected to the water flow meter at the tile drain outlet to record the time and cumulative flows in volumetric increments of 14.2 L of drained water.

In the spring simulation, water sampling began at the onset of flow in the tile drain 53 min after the initiation of the rainfall simulation. During the fall simulation, base flow was present prior to the start of the experiment, so water sampling was initiated at the beginning of the rainfall simulation. Grab samples of drain flow were collected every 4 to 7 min in the first hour after the start of flow. After the first hour, samples were collected every 15 to 60 minutes. All samples were collected in 150-mL sterile polypropylene bottles, transported to the laboratory on ice, and stored for less than 24 h at 4˚ C prior to analysis.

3.2.2 Sample Analysis

Manure, soil, and water samples were assayed for enterococci and enterococci-resistant to tylosin by the membrane filtration method (Greenberg et al., 1998). Soil and manure samples were diluted by phosphate-buffered water prior to filtration. Concentrations of total enterococci and tylosin-resistant enterococci were determined by enumerating colony forming units (cfu) present on m *Enterococcus* (mE) agar (Difco, Detroit, MI) without tylosin (control) and infused with tylosin at 35 mg/L following the procedures described by Kaukas et al. (1988). Agar was infused with a stock solution made from tylosin tartrate (Sigma-Aldrich, St. Louis, MO) by dissolving the tylosin in 10 mL methanol and adjusting the pH to 7.9 with 0.1 M phosphate-buffer. After filtration, the samples were placed onto the media and incubated at 35 ± 0.5°C for 48 hours. All samples were analyzed in triplicate. Colonies enumerated on control media accounted for the total enterococci population and colonies enumerated on tylosin infused media accounted for tylosin-resistant enterococci. Water samples also were analyzed for total suspended solids (TSS) by filtering samples through a 0.45-µm glass fiber filter (Pall Life Sciences, Ann Arbor, MI) following EPA method 160.2 (USEPA, 1999).

The presence of enterococci in the samples was phenotypically verified by using EPA method 1600 (USEPA, 2002). Typical and atypical enterococci colonies were selected from mE agar of swine manure, soil and tile water. Gram-positive cocci that grow and hydrolyze esculin on Bile Esculin Agar (EMD Chemicals, Gibbstown, NJ), in brain heart infusion broth (BHIB) with 6.5% sodium chloride (EMD Chemicals, Gibbstown, NJ) at 35°C ± 0.5°C, and BHIB at 45°C ± 0.5°C are confirmed as enterococci. All isolates were also confirmed by a gram stain and microscopic analysis (Nova, Portland, OR).

Tylosin in manure samples from both simulations were extracted using ultrasonic extraction followed by solid phase partitioning and positive ion electrospray on a Thermofinnigan LCQ ion trap LC/MS/MS as described by Snow et al. (2003). All isolates used for PCR amplifications were grown on mE agar supplemented with tylosin at 35 mg/L.

3.2.3 Detection of tylosin resistant genes by PCR

The identification of tylosin resistant genes present in enterococci was confirmed by PCR (Bockelmann et al., 2009; Sutcliffe et al., 1996b). Colonies isolated on mE agar from swine manure, soil, tile water samples in each simulation were grown on BHI Agar slants before extraction for PCR analysis. The number of isolates preserved differed in the spring and the fall because of available enterococci colonies on mE agar (Table 2). Isolates were inoculated into 1 mL of BHIB and incubated at 37°C for 24 h. The broth culture was then extracted for genomic DNA using DNeasy Blood & Tissue Kits (QIAGEN, Valencia, CA). The extracted DNA was amplified with primers specific for macrolide resistance genes (*ermA, ermB, ermC, ermF, ermT, ermX, mefA,* and *msrA*) (Portillo et al., 2000). The sequences related to the six *erm* genes coding for rRNA **e**rythromycin **r**ibosomal **m**ethylases and the two genes encoding macrolide efflux (*mefA* and *msrA*) have been determined previously (Chen et al., 2007; Sutcliffe et al., 1996a; Sutcliffe et al., 1996b). Forward and reverse primers were purchased from Eurofin MWG Operon (Huntsville, AL). *E coli* strains with *erm* resistance genes cloned into plasmids were obtained from Dr. Robert (University of Washington) and used as positive controls (Table 3), except for *msrA* which was carried on a plasmid in *Staphylococcus aureas*. The negative control was *Enterococcus feacalis* ATCC 29212, purchased from American Type Culture Collection (Manassas, VA).

Table 2 - Number of enterococci isolates from swine manure, soil and tile water samples collected in spring and fall simulations used for PCR amplifications

Type of samples	Number of enterococci isolates	
	Spring simulation	Fall simulation
Manure	40	24
Soil	18	8
Water	50	60

Each PCR reaction was carried out in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) with total reaction volume of 20 µL containing 0.5 µL DNA solution, 10 µL Taq PCR Master Mix (QIAGEN, Valencia, CA), 0.2 µL of each primer, and 9.1 µL DNA free water. The PCR conditions for all genes consisted of an initial denaturation of 95°C for 5 minutes; followed by 35 cycles of one minute of denaturation at 95°C, one minute of annealing at the temperature specified in Table 3, and one minute of extension at 72°C; followed by a final extension at 72°C for 7 minutes. Both positive and negative controls were run with each assay. Following PCR amplification, PCR products were run on 1% or 1.2% 1xTAE-agarose

Table 3 – Primers, annealing temperature, and positive controls used for PCR amplification of macrolide resistant genes.

Target gene	Primers	Positive control	Sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
ermA	ermA-F ermA-R	*pEM9592*	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAGT	~ 645	53	Sutcliffe et al. 1996
ermB	ermB-F ermB-R	*pJIR229*	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTT TAC	~ 639	55	Sutcliffe et al. 1996
ermC	erm C-F ermC-R	*pBR328:RV*	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAAT	~ 642	47	Sutcliffe et al. 1996
ermF	ermF-F ermF-R	*pVA831*	GAGATCGGRCCAGGAAGC GTGTGCACCATCGCCTGA	~ 309	59	Chen et al., 2007
ermT	ermT-F ermT-R	p121BS	CATATAAATGAAATTTTGAG ACGATTTGTATTTAGCAACC	~ 369	51	Chen et al., 2007
ermX	ermX-F ermX-R	*pNG2*	CGACACAGCTTTGGTTGAAC GGACCTACCTCATAGACAAG	~ 488	58	Chen et al., 2007
mefA	mef A-F mefA-R	*pMR970*	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	~ 348	54	Sutcliffe et al. 1996
msrA	msrA-F msrA-R	*pAT10*	GCAAATGGTGTAGGTAAGACAACT ATCATGTGATGTAAACAAAAT	~ 399	52	Sutcliffe et al. 1996

gel (Fisher Scientific, Fair Lawn, NJ) and visualized by SYBR Safe staining under UV light using a Syngene UGenius Gel documentation system (Syngene, Frederick, MD). The PCR product size was estimated by standard molecular weight markers (1kb Plus DNA Ladder from USB Cooperation, Cleveland, OH). Gels were analyzed using GeneTools software (Syngene, Cambridge, UK) to confirm the size of the PCR products.

3.2.4 Calculations and Statistical Analysis

The percent of resistant enterococci in any sample was calculated as the ratio between tylosin-resistant concentration and total enterococci multiplied by 100. Loads were calculated for total enterococci and tylosin-resistant enterocci in each experiment using accumulated drainage flow and enterococci concentrations. The flow-weighted enterococci loads per ha (cfu/ha) were determined by multiplying the enterococci concentrations by the flow volume and then divided by the area (ha) receiving swine waste application. Statistical analysis of data was performed using R project software (version 2.8.1). A t - test was conducted to test for the difference between total enterococci and tylosin-resistant enterococci concentrations and loadings during the two simulations. Significance was determined at the $p < 0.05$ level. The null hypothesis was that there would be no difference in the concentrations or loadings of enterococci in tile water among the treatments. Correlation analysis was used to measure the linear relationship between enterococci concentrations and TSS content in tile water. A value of correlation coefficient near 1 indicates a high level of correlation.

3.3 Results and discussions

3.3.1 Occurrence of tylosin-resistant enterococci in manure, soil, and tile water

Enterococci concentrations were greatest in swine manure, averaging 8.8×10^5 cfu/100 mL for total enterococci and 8.9×10^5 cfu/100 mL for resistant enterococci over the two simulations. Greater enterococci concentrations were detected in soil samples collected in the spring. After manure application to the no-till plots, enterococci concentrations in soil averaged 9.8×10^6 cfu/g of soil for total enterococci and 7.5×10^6 cfu/g of soil for tylosin resistant enterococci, but concentrations were zero or

minimal prior to application (Table 4). The fraction of enterococci that was resistant differed between experiments. During the spring simulation, all enterococci were resistant in the manure samples, but after manure application, 76% of the enterococci in soil were resistant to tylosin. However, during the fall simulation, only 68% of total enterococci in the manure sample were resistant while 77% of enterococci in soil after manure application were resistant to tylosin.

Table 4- Concentrations of total enterococci and tylosin-resistant enterococci[a] in swine manure and soil[b] samples during spring and fall simulations.

	Enterococci concentrations		
	Swine manure (cfu/100mL±SD)	Soil before manure application (cfu/g±SD)	Soil after manure application (cfu/g±SD)
Spring simulation			
Total	1.2×10^{8} ($\pm 0.7\times10^{8}$)	3.3 ($\pm 1.9\times10^{3}$)	6.6×10^{6} ($\pm 6.0\times10^{6}$)
Resistant	1.4×10^{8} ($\pm 0.9\times10^{8}$)	0	5.0×10^{6}($\pm 1.3\times10^{6}$)
Percent of resistance (%)	100	0	76
Fall simulation			
Total	5.6×10^{7} ($\pm 1.2\times10^{7}$)	0	1.3×10^{7} ($\pm 0.2\times10^{7}$)
Resistant	3.8×10^{7} ($\pm 0.6\times10^{7}$)	0	1×10^{7}
Percent of resistance (%)	68	0	77

[a] Enterococci concentrations are the mean of samples analyzed in triplicate.
[b] Soil samples include two composite samples before and after manure application

The response of total enterococci and tylosin-resistant enterococci in water samples during each simulation followed similar trends (Fig. 3). During the spring simulation, concentrations peaked at the outlet of the tile drain 80 min after initiation of flow. Peak concentrations for total and tylosin-resistant enterococci were 5.0×10^{3} cfu/100 mL and 1.2×10^{3} cfu/100 mL, respectively. Concentrations decreased rapidly during the 170 minutes following the peak, and then total enterococci concentrations stabilized while tylosin-resistant enterococci concentrations became nearly non-detectable. Enterococci concentrations ranged from 3.3×10^{1} to 5.0×10^{3} cfu/100 mL for total enterococci and 6.7×10^{1} to 1.17×10^{3} cfu/100 mL for resistant enterococci (Fig. 3A). No enterococci were found in the base-flow before manure application in the fall. During the fall simulation, bacteria concentrations peaked twice (Fig. 3B). The first peak occurred 45 min after the start of

sampling, and the second after 110 min. The second peak was approximately one-half the concentration of the first. The range of enterococci concentrations in drain flow was 1.3×10^1 to 2.6×10^3cfu/100 mL for total enterococci and 1.3×10^1 to 1.4×10^3 cfu/100 mL for resistant enterococci. The differences between total enterococci and tylosin-resistant enterococci concentrations were statistically significant for both experiments ($P \leq 0.0001$). In general, enterococci concentrations in spring were much greater than in fall for all three type of samples This is associated with higher application rate swine manure in spring (18,662 L/ha) than in fall (13,487 L/h).

Similar to the findings from this study, Warnemuende and Kanwar (2002), determined that enterococci concentrations in leachate from soil columns receiving manure had a close relationship with manure application rates and timing: higher manure application rates caused elevated enterococci concentrations in drainage water and more enterococci were observed during the spring than the fall under the same application rate. Our results are also consistent with previous findings on the occurrence of antibiotic-resistant bacteria in manure, soil and tile water. The use of tylosin at sub-therapeutic concentrations will select and increase resistance to macrolides in enterococci living in the intestinal tract of pigs (Aarestrup and Carstensen, 1998). Jindal (2006) found that up to 70% of total fecal streptococci detected in swine lagoons were resistant to tylosin. Onan and LaPara (2003) found that an increased proportion of tylosin-resistant bacteria were detected in fields amended with cattle, swine and chicken manure associated with subtherapeutic use of antibiotic when compared with fields where organic manure was applied. The study showed that proportion of the tylosin-resistant population to the total bacterial population in three soils affected by antibiotics ranged from 7.2 to 16.5% while only 0.7-2.5% of total bacteria were resistant to tylosin in control soils.

Although the occurrence of antibiotic resistant bacteria have been well documented in surface or ground water (Chee-Sanford et al., 2001; Koike et al., 2007; Oliveira and Pinhata, 2008; Watkinson et al., 2007), very little research has been conducted to detect the presence of antibiotic resistant bacteria in tile drainage water. Previous studies have not clearly established a hydrologic link between the source and the resistant microorganisms in waters;

instead the sources of resistant bacteria is often inferred due to swine operations proximity to sampling points (Koike et al., 2007) or water sampling points located down gradient of swine farms (Sapkota et al., 2007). This work provides the first data tracking the pathway of tylosin-resistant enterococci from a specific source through a subsurface drainage system. Our results showed that tylosin-resistant enterococci were present in swine manure, soil amended with swine waste, and tile-drainage water. The fractions of tylosin-resistant enterococci were greatest in manure samples, lower in soil samples containing manure, and lowest in drainage-water samples (Table 4, Fig. 3), suggesting that enterococci may lose resistance as selective pressure from antimicrobial residues decreases (Monroe and Polk, 2000) because of die-off during movement through soil and drainage water (Jamieson et al., 2002).

Studies showed that the tylosin concentration in runoff from manure-applied fields is not high enough for development of resistant enterococci. The tylosin load in surface runoff has ranged from 8.4% to 12% of total tylosin applied to the fields (Hoese et al., 2009). Tylosin concentrations in spring manure was found at 122 µg/L, over three timesgreater than that in the fall (38 µg/L) but they are very low compared to the tylosin MIC for Enterococcus (>32 µg/mL) (NARMS, 2005). Tylosin in surface runoff were observed at a concentration of 6.0 µg/L as reported by Dolliver and Gupta (2008) or nondetectable in soil and drainage water from lands receiving pig slurry (Kay et al., 2004). This implies that the tylosin concentration in these pools are not high enough to favor development of resistant bacteria, so the tylosin resistant enterococci detected in this study is likely due to the presence of antibiotics in the swine manure. However, many researchers have found tylosin resistant bacteria released from soils where no antibiotics have been used (Jackson et al., 2004). Selection of antibiotic resistance is not always a consequence of antibiotic selective pressure, selection without antibiotic pressure might also occur (Alonso et al., 2001).

3.3.2 Relationship between enterococci concentration and tile flow

Due to high flows with the presence of base-flow during the fall simulation, the magnitude of enterococci concentrations in drainage-water samples were nearly one-half of the concentrations observed during the spring simulations. In the spring, drain flow peaked

22 min after the onset of flow at the outlet of the tile line, 58 min before the peak enterococci concentration (Fig. 3A). After the peak, the flow decreased gradually while enterococci concentrations continued to increase until 80 min after the initiation of flow. Flow in the tile drain occurred only after artificial rainfall and the observed range was between 11.2 and 44.7 L/h. During the fall simulation, a delay of 25 min was observed between the peak enterococci concentrations and peak flow rate (Fig. 3B). Drain flow varied from 0 to 291 L/h. The second peak flow occurred 135 min after the beginning of the rainfall simulation at 291 L/h and was nearly four times greater than the magnitude of first peak (76.3 L/h) with a lower concentration of enterococci. Higher flows correlate with lower enterococci concentrations because of dilution by drainage water.

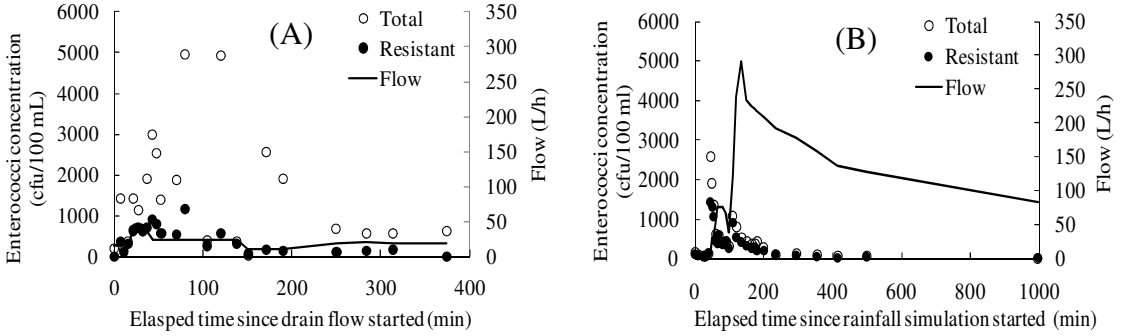

Figure 3 - Concentrations of total enterococci and tylosin-resistant enterococci and drain flow in drainage water during (A) spring and (B) fall simulations.

In the drainage water, we detected a greater range of bacteria concentrations during the spring simulation when compared to the fall (Fig. 3). This can be explained by higher flows, primarily due to the presence of base flow of 29.9 L/h during fall, and perhaps other field conditions that differ between the two plots such as soil type, macropore continuity in soil system and antecedent soil moisture content (Shipitalo and Gibbs, 2000). Both soils of the two plots are classified as a loam, however, the Kenyon soil in plot 25 (spring simulation) usually occurs on convex ridge crests or side slopes which may result in low moisture while the Floyd soil in plot 20 (fall simulation) are often found in the lower sides of slopes (USDA and NRCS, 1995). Application of slurry manure will contribute to the increase of earthworm population and thus increase macropore density in soil (Friend and Chan, 1995). Macropores, created by deep-burrowing earthworms, have the potential to hydraulically connect the soil

surface to subsurface drains. This hydraulic connection leads to rapid movement of antibiotic resistant microorganisms to receiving waters as they bypass the bulk of the soil matrix. During the fall simulation, fresh macropores from worm activity and plant growth during the previous growing season in combination with higher soil moisture content following the rainy season resulted in higher macropore continuity. Previous studies have found that no-till fields with increased macropore connectivity form worm holes and root channels when compared to conventionally tilled fields, have greater movement of water through the soil profile (Shipitalo et al., 2000). Formation of cracks due to dry soil conditions may provide a direct vertical route to subsurface drains for bacterial transport (Harris et al., 1994) and may also play a similar role in increasing bacteria transport. During the spring simulation, the macropores or their interconnectivity may have been impaired or filled with soil over the winter resulting in lower flows (McIntosh and Sharratt, 2003) and potentially decreased bacterial transport. A greater number of macropores was observed during the fall experiment than in the spring (Chi Hoang, personal communication, Iowa State University, 27 April 2010, data not shown).

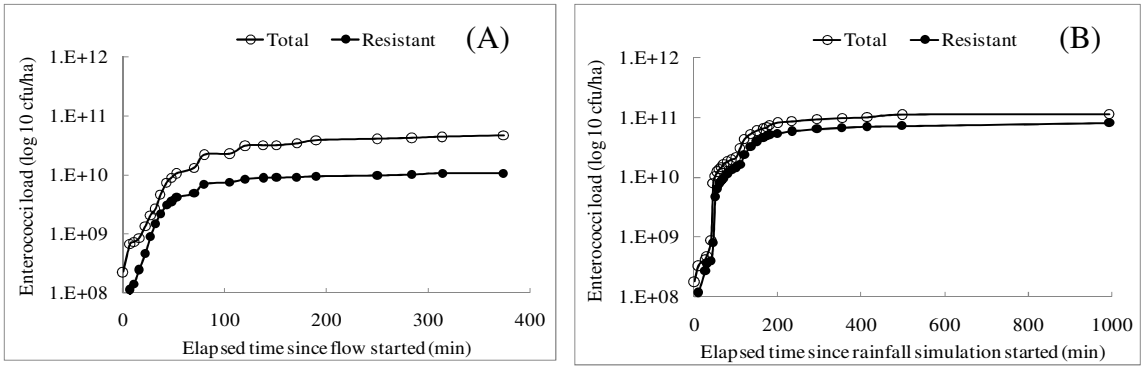

Figure 4 - Total and tylosin-resistant enterococci loadings in tile drainage water during (A) spring and (B) fall experiments.

The enterococci load per hectare considers the impacts of tile flow on microorganism transport. Greater tile flow in the fall with the presence of base-flow during experiment resulted in greater magnitude of enterococci loadings in fall than in spring (Fig. 4). In spring, 23% of accumulate enterococci load per hectare were tylosin resistant while in fall, 73% was resistant. Statistic analysis found significant differences between total and resistant enterococci loads ($p < 0.00001$).

Several other factors should be considered when studying the leaching of resistant bacteria from waste-amended soils including waste characteristics, manure storage and application methods. The physical and chemical properties of animal manure vary with animal species as does the method of storage (e.g. liquid or solid, storage time). The higher concentrations and more diverse bacterial species in manure, the more likely that a fraction will be transported through the soil column (Unc and Goss, 2004). Injection of liquid manure into soil can contribute to the rapid movement of bacteria through soil in a short time period after manure application (Guber et al., 2005). Other possible causal factors include bacterial decay; bacterial attachment to soil particles during the process of flow migration in macropores (Beven and Germann, 1982); filtering and retention of microorganisms by the soil during matrix flow; and tillage treatment and soil moisture before manure application. Transport and die-off of bacteria are impacted by their association with soils and sediments. Attachment of antibiotic-resistant bacteria onto soil particles retains and attenuates their transport to tile-drainage waters (Cook and Baker, 2001; Rysz and Alvarez, 2006).

3.3.3 Relationship between enterococci concentration and TSS

Our results suggest a relationship between TSS and enterococci concentrations in the subsurface drainage water. The range of TSS concentrations during the fall simulation was much smaller than during the spring simulation. Total suspended solids concentrations during the fall ranged from 0 to 370 mg/L, whereas in spring, the TSS concentration varied from 0 to 580 mg/L. The TSS concentration curve was similar to the bacterial concentration curves during the fall but different during the spring (Fig. 5). During the spring simulation, TSS first peaked at 7 minutes after drain flow began because of sediment accumulation in the lines. Subsequently, TSS concentration decreased while enterococci concentrations increased and peaked 80 min after tile flow began. After enterococci concentrations peaked, the TSS in water samples increased and reached a second peak that coincided with the total enterococci concentration peak, but lagged behind the peak of tylosin-resistant enterococci concentration by 25 minutes. During the fall, the pattern of TSS concentrations was the same as the pattern of enterococci concentration with two peaks occurring at the same time (45 and 110 min after

the start of sampling). The first enterococci peak was greater than the second peak while the first TSS peak was less than the second.

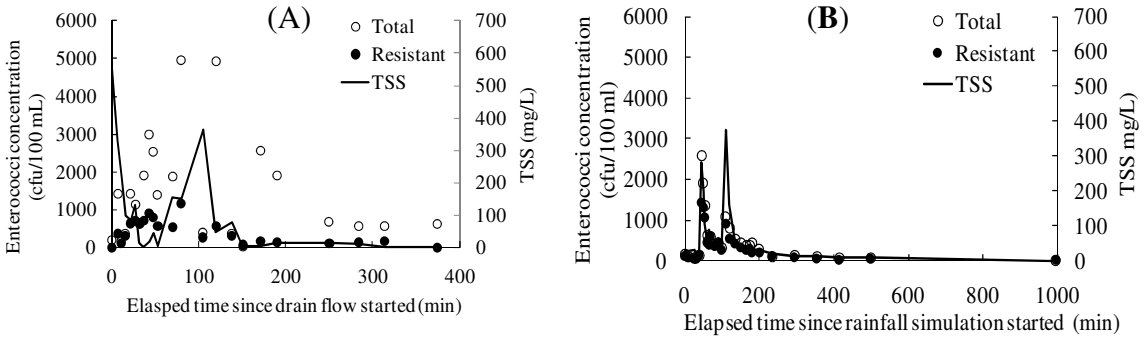

Figure 5 - Concentrations of total enterococci and tylosin-resistant enterococci and TSS in drainage water during (A) spring and (B) fall simulations.

TSS concentrations during the fall experiment were much lower than those during the spring experiment because of the presence of base flow during the fall. The shape of the TSS curve matched the hydrograph of drain flow and bacterial concentration curves (Fig. 5). A good correlation ($R^2 > 0.65$) between TSS concentration and total enterococci and resistant concentrations during the fall experiment indicated that with the presence of high drainage flow, bacteria concentrations are potentially related to sediment content in the water. Poor correlation was found between TSS and enterococci concentrations in spring simulation likely because of the absence of base flow. As discussed previously, tile flows in spring only occurred after the rainfall simulator applied water to the plot surface. Associations between TSS and enterococci transport could imply particulate mediated transport through macropores (Boxall et al., 2002), which has also been observed in recent surface runoff studies (Soupir et al., 2010). Sediments in streams provide an environment suitable for the extended survival and possible growth of fecal microorganisms (Davies et al., 1995; Sherer et al., 1992). However, little is known about the relationship between TSS in subsurface drainage water and bacteria transport. This study observed a similar relationship between sediment concentration in tile drain lines and bacterial concentrations at tile outlets (Fig. 6).

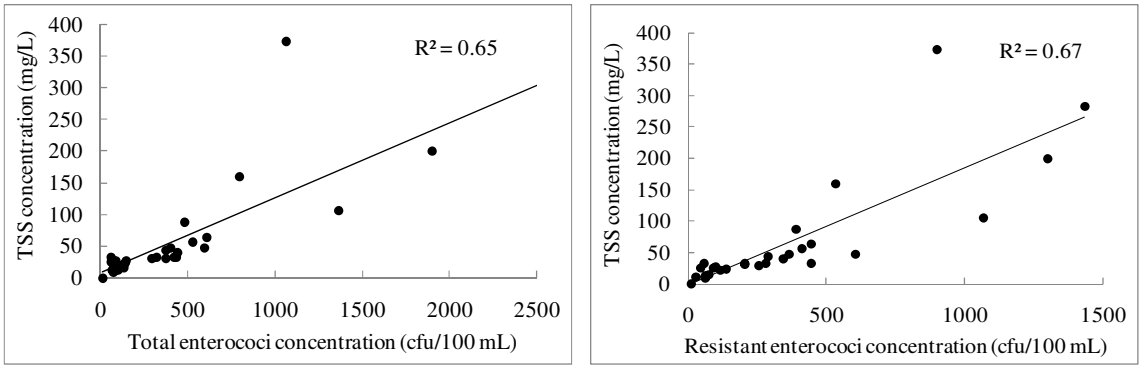

Figure 6. Correlation between TSS concentration and (A) total enterococci and (B) resistant enterococci concentration in tile water in the fall.

3.3.4 Detection of macrolide resistant genes in manure, soil and tile water isolates

Six *erm* genes and one efflux pump gene, *ermA*, *ermB, ermC, ermF, ermT, ermX* and *msrA* were present in all three types of samples (swine manure, soil and tile water) at different prevalence levels (Fig. 7). Five macrolide-resistance genes namely *ermB*, *ermC*, *ermF, ermT,* and *msrA* were detected in all three sample types greater than 9% of the time. Positive PCR amplifications of *msrA* were obtained in 194 of 200 enterococci isolates (97%). The *erm(F)* gene was detected in 156 (78%) isolates and the *ermB* gene in 138 (69%) isolates. Nineteen isolates representing 9.5% were positive for *ermT* PCR amplifications and only 18 isolates (9%) contained *ermC*. Only two enterococci isolates from tile water in spring were positive for *ermX* and one isolate from spring manure harboured *ermA*. The *mefA* were not detected in any of enterococci isolates. Portillo et al. (2000) reported the similar results and suggested that the different geographical distribution of *mef* genes in *Enterococcus* might be the reason. Four strains representing 2.0% of the total strains tested contained all five genes with high frequency. In general, the percentage of isolates containing a resistant gene was greater in the fall than the spring for every single gene. The results showed that most of isolates contained *msrA* gene (up to 95% in spring and 99% in fall) which was not relevant to their phenotypic results (Fig.3). This can be explained by almost all enterococci strains used for PCRs were resistant to tylosin including those were taken from the control plates because they all grew in tylosin-infused media (data not shown). However, the level of resistance or the concentration of resistant genes in each strain was unknown.

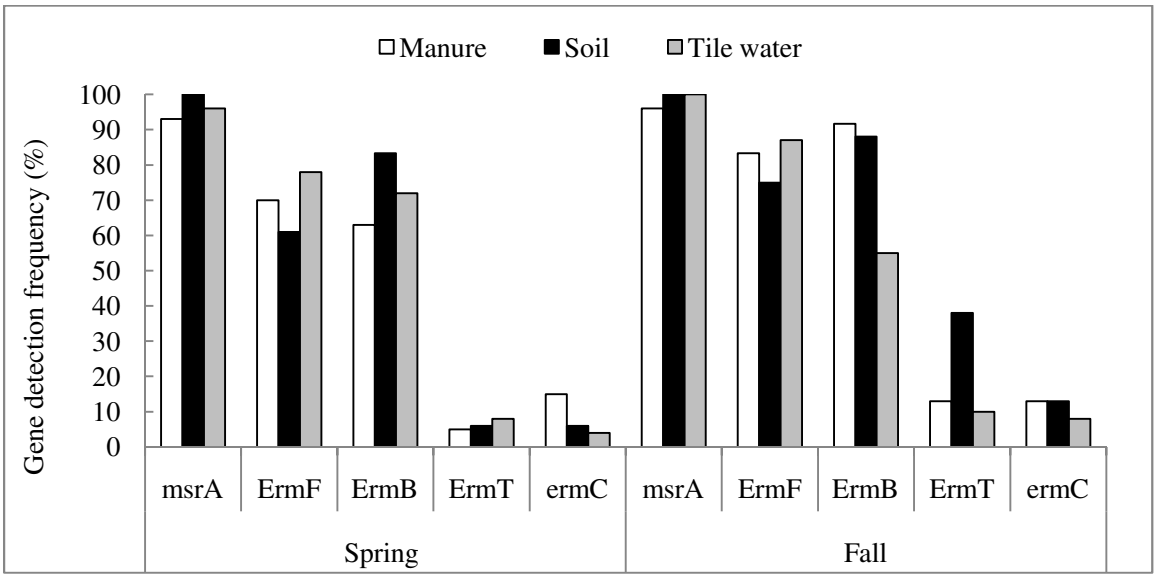

Figure 7 - Resistant gene detection frequencies of enterococci isolates from manure, soil and tile water samples in spring and fall simulations

Resistant genes detected in manure, soil and tile water confirmed the transport of resistant genes in enterococci from swine waste through soil and into tile drainage systems. Our findings indicated that both resistance mechanisms to tylosin occurred in enterococci, which is consistent with previous studies (Chen et al., 2007; Sutcliffe et al., 1996b). Efflux mechanism coded by *msrA* gene (Roberts et al., 1999) was detected in 103 of 108 strains (95%) in spring and 91 of 92 strains (99%) in fall. Negative results were obtained in all isolates for *mef(A).* The *erm(B)* gene, described by Chen (2007) and Graham (2009) as the most prevalent macrolide resistant gene in enterococci occurred in approximately 69% of the total isolates tested. The prevalence of *ermF* was found slightly more frequently than that of *ermB* which differed from results previously report by Chen (2007) where *ermF* was only slightly lower than that of *ermB.*

3.4 Conclusions

This research broadens our knowledge about the release of tylosin-resistant bacteria from swine manure through tile-drained fields under no-tile operations by detecting the occurrence of tylosin-resistant enterococci in manure administering tylosin at subtherapeutic doses, in soil receiving swine manure and in tile water in two spring and fall 2009. In manure, all enterococci were resistant to tylosin in the spring, whereas about 68% of total

enterococci in fall were resistant to tylosin. Concentration of enterococci in soils was zero to minimal in both simulations before swine manure was injected into the field. But after manure application, 76% of the enterococci in soil were resistant to tylosin in spring and 77% of enterococci were resistant to tylosin in fall. Accumulate loadings per hectare of tylosin resistant enterococci were 23% in spring and 73% in fall. This research also provide a potential trend between sediment content and enterococci transport in tile water via a good correlation between TSS and enterococci concentrations ($R^2 > 0.65$).

Our data provide quantitative evidence that higher manure application rate resulted in greater enterococci and tylosin resistant enterococci concentrations in tile water and the macropore connectivity in soil during experiments play important role in the release and transport of resistant bacteria from manure through soil and into tile lines. Enterococci concentrations are also associated with manure application timing that cause greater concentrations in spring than in fall.

The resistant enterococci harbored several different genes encoding for tylosin resistance including *ermA*, *ermB, ermC, ermF, ermT, ermX* and *msrA*. The *msrA* gene was the most frequently detected resistant gene over the two simulations with 97% detection, followed by *ermF* (79%) and *ermB* (69%) while only 9.5% of total isolates contains *ermT* gene and 9% contain *ermC* gene. Only 2 of 200 enterococci isolates were positive for PCR amplification of the ermX, and *ermA* was detected in only one isolate. All enterococcal strains gave negative results by *mefA* PCRs. At least one gene was present in each enterococci isolate indicating that resistance to tylosin in enterococci can be encoded by several macrolide resistant genes encoding several mechanisms. The homology in resistance of selected isolates might be the reason for highly detection of *msrA* in PCR amplifications which was irrespective to the phenotypic results. Quantitative PCR is recommended in future in order to measure concentration of resistant genes in each isolate tested.

The study examined only eight macrolide resistant genes to confirm the occurrence of tylosin resistant enterococci in swine manure, soil and tile water, while there are 32 *erm* gene classes other efflux pump determinants have been identified previously (Portillo et al., 2000; Roberts, 2004b). Additional genes could be tested in the future to provide a more complete analysis of which genes encoded for tylosin resistance. The results of this study strongly

demonstrated that to provide a comprehensive evaluation of resistant bacteria in samples, it is necessary to combine phenotypic-based methods and genotypic techniques.

3.5 Acknowledgement

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CHAPTER 4 GENERAL CONCLUSIONS

4. 1 General discussion and conclusions

A combination of field and laboratory experiment was conducted to detect and quantify the transport of tylosin resistant enterococci from swine manure, through no-till soil and tile drainage system. The goal of this study was to improve understanding of occurrence and transport tylosin resistant enterococci from swine manure applied to tile-drained lands through no-till soils and tile drainage systems. First, the presence and transport of tylosin resistant enterococci in swine manure, soil amended with swine manure and tile drainage water were examined and confirmed by membrane filtration technique and culture-based methods in the laboratory. Secondly, occurrence of tylosin resistant enterococci in tile water were quantified and analyzed for the relationship to tile drain flow and total suspended solid concentration in water. Lastly, 200 enterococci isolates extracted from swine manure, soil and water samples were analyzed to detect resistant genes that coded for tylosin resistance in enterococci.

The study demonstrates that:

- Tylosin-resistant enterococci occurring in gastrointestinal tracts swine can survive and reach natural aquatic environments via manure excretion.
- Tylosin-resistant enterococci occurring in swine manure can transport to soils and water environment and maintain resistant properties following introduction into natural aquatic habitats by manure application. The greatest concentration of tylosin-resistant enterococci in manure and smallest in drainage water suggest that enterococci lose resistance as selective pressure from antibiotic residues decreases or die-off/attachment during movement through soil and drainage water.
- Tylosin resistant enterococci concentration in tile water has relationship with tile flow and TSS concentration. Greater enterococci concentrations were observed in the spring than in the fall, whereas higher tile flows with presence of base-flow during fall experiments. A higher range of total suspended solid (TSS) also have been found in the spring versus the fall suggesting relationship between TSS and tile flow and enterococci concentrations (R^2 >0.65).

- Manure application rate and timing and macropore connectivity in soil during experiments play important role in the release and transport of resistant bacteria from manure through soil and into tile lines. Higher manure application rates resulted in greater enterococci and tylosin resistant enterococci concentrations in tile water. Enterococci concentrations are also associated with manure application timing that cause greater concentrations in spring than in fall.
- Resistant genes to macrolide antibiotics were present in isolates from manure, soil and tile water collected from the fields including *ermA, ermB, ermC, ermF, ermT*, *ermX, msrA*. The *mefA* genes were not dectected in PCR amplifications. Only two enterococci isolates from tile water in spring of 200 isolates tested positive for *ermX* and only one isolate from manure in spring harbored *ermA*. The *msrA* gene coded for efflux mechanism was most prevalence, followed by *ermF, ermB, erm T* and *ermC*.
- At least one gene was present in each enterococci isolate indicating that resistance to tylosin in enterococci can be encoded by several macrolide resistant genes encoding several mechanisms.

4. 2 Implications and Recommendation for future research

The database developed from tylosin resistant enterococci and resistant genes from the known source (swine manure) were able to associate with tylosin resistant enterococci and resistant genes in soils and tile water respectively. The results clearly show that the presence of antibiotic resistant genes does not always result in phenotypic expression of the resistance and vice versa. Although resistant enterococci were confirmed by growth on media infused with tylosin, tylosin resistant genes did not show up in strain extracted from them. There are 32 *erm* genes have been identified up to now, more genes need to be tested to make a complete picture of what resistant genes present in total DNA extracted from samples in each environmental pools.

The results from our investigations underline the need to assess the impact of swine manure containing resistant bacteria on dissemination of antibiotic-resistant bacteria. Future studies monitoring the environmental impact of swine manure/subsurface drainage on the

spread of antibiotic resistance should focus attention on multiple-resistant bacteria rather than bacteria resistant to single antibiotics.

The results will increase awareness on the widespread of antibiotic resistance in both agricultural systems and environmental pools within confined swine farms. Even though good management practices for swine waste management (anaerobic lagoon systems) are effectively mitigating fecal pollution, there is a need for prudent and responsible use of antibiotics especially with those prone to induce resistance.

Furthermore, multiple-resistant strains occurring in swine manure should be investigated for their ability to transfer resistance genes to aquatic microbial communities under *in vivo* conditions. Similarly, the fate of resistant bacteria and resistance genes occurring in sewage sludge intended for agricultural use should be studied following their introduction into natural soil habitats. Finally, the relative importance and contribution of resistant bacteria in the aquatic environment and the consequent risk for resistance problems in veterinary and human medicine should be assessed.

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APPENDIX A. FIELD EXPERIMENTS

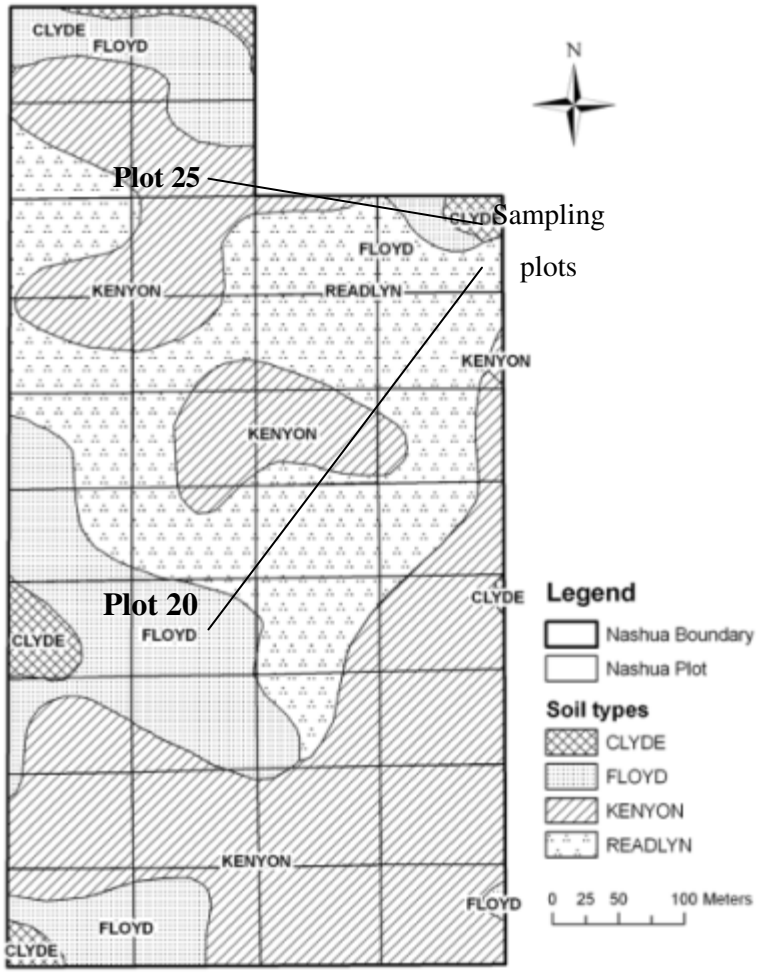

Figure 8 - Soil types and locations of the two experimental plots at Iowa State University's Experiment Station Research Farm, Nashua, Iowa.

Manure sampling

Soil sampling

Water sampling

Figure 9 - Sampling at Iowa State University's Experiment Station Research Farm, Nashua, Iowa.

Figure 10 - Rainfall simulation at Iowa State University's Experiment Station Research Farm, Nashua, Iowa.

APPENDIX B. BACTERIAL RESULTS

Table 5 - Concentrations of total and resistant enterococci in tile drainage water samples since tile flow started in Spring 2009 (plot 25)

Sample ID	Sampling time	Minutes since flow started (min)	Total enterococci (cfu/100 mL)	Tylosin resistant enterococci (35 mg/L) (cfu/100mL)
1	11:18	0	200	0
2	11:25	7	1433	367
3	11:29	11	167	100
4	11:34	16	367	300
5	11:40	22	1433	633
6	11:45	27	1133	700
7	11:50	32	633	633
8	11:55	37	1900	700
9	12:01	43	3000	900
10	12:06	48	2533	800
11	12:11	53	1400	567
13	12:28	70	1867	533
14	12:38	80	4967	1167
15	13:03	105	400	267
16	13:18	120	4933	567
17	13:36	138	367	300
18	13:49	151	33	67
19	14:09	171	2567	167
20	14:28	190	1900	133
22	15:28	250	667	100
23	16:02	284	567	133
24	16:32	314	567	167
25	17:32	374	633	0

Table 6 - Concentrations of total and resistant enterococci in tile drainage water samples since rainfall simulation started in Fall 2009 (Plot 20)

Sample ID	Sampling time	Minutes since flow started (min)	Total enterococci (cfu/100 mL)	Tylosin resistant enterococci (35 mg/L) (cfu/100mL)
1	15:59	0	150	100
2	16:09	10	87	90
3	16:25	26	63	60
4	16:29	30	63	47
5	16:39	40	143	140
6	16:44	45	2567	1433
7	16:49	50	1900	1300
8	16:54	55	1367	1067
9	16:59	60	610	447
10	17:04	65	483	390
11	17:09	70	600	603
12	17:19	80	400	363
13	17:29	90	423	443
14	17:39	100	320	280
15	17:49	110	1067	900
16	17:59	120	800	533
17	18:14	135	533	413
18	18:29	150	443	343
19	18:44	165	377	257
20	18:53	174	377	290
21	18:59	180	433	207
22	19:19	200	297	207
23	19:53	234	97	115
24	20:53	294	137	73
25	21:53	354	100	63
26	22:53	414	67	30
27	0:17	498	77	63
28	08:33	994	13	13

Table 7 - Tile flow and total suspended solid (TSS) concentration in tile drainage water since flow started in Spring 2009

Sample ID	Sampling Time	Minutes since sampling starts (min)	TSS (mg/L)	Flow (L/h)
1	11:18	0	43	
2	11:25	7	577	15
3	11:29	11	331	15
4	11:34	16	217	15
5	11:40	22	97	15
6	11:45	27	85	45
7	11:50	32	129	45
8	11:55	37	15	45
9	12:01	43	1	37
10	12:06	48	17	24
11	12:11	53	43	24
13	12:28	70	3	24
14	12:38	80	153	24
15	13:03	105	149	24
16	13:18	120	363	24
17	13:36	138	48	24
18	13:49	151	77	24
19	14:09	171	6	11
20	14:28	190	4	11
22	15:28	250	13	11
23	16:02	284	16	19
24	16:32	314	12	20
25	17:32	374	1	18

Table 8 - Tile flow and total suspended solid (TSS) concentration in tile drainage water since flow started in Fall 2009

Sample ID	Sampling Time	Minutes since sampling starts (min)	TSS (mg/L)	Flow (L/h)
1	15:59	0	27.5	
2	16:09	10	26.2	0.0
3	16:25	26	32.5	4.1
4	16:29	30	25.0	4.1
5	16:39	40	23.7	4.1
6	16:44	45	282.0	4.1
7	16:49	50	200.0	30.8
8	16:54	55	106.0	30.8
9	16:59	60	63.7	49.0
10	17:04	65	86.7	76.3
11	17:09	70	47.1	76.3
12	17:19	80	47.5	76.3
13	17:29	90	32.5	66.9
14	17:39	100	32.5	38.4
15	17:49	110	373.3	123.7
16	17:59	120	160.0	239.1
17	18:14	135	57.1	291.0
18	18:29	150	40.0	234.4
19	18:44	165	30.0	225.0
20	18:53	174	43.8	221.4
21	18:59	180	32.5	217.9
22	19:19	200	31.2	208.0
23	19:53	234	21.3	192.5
24	20:53	294	15.0	179.0
25	21:53	354	12.5	159.1
26	22:53	414	11.2	137.8
7	00:17	498	8.7	127.4
28	08:33	994	0.0	84.3

APPENDIX C. PCR AMPLIFICATION OF MACROLIDE RESISTANT GENES

C.1. PCR results for *ermA* gene

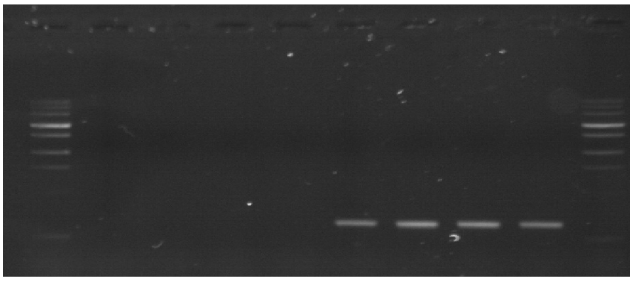

Figure 11 – Gradient PCR results of *ermA*

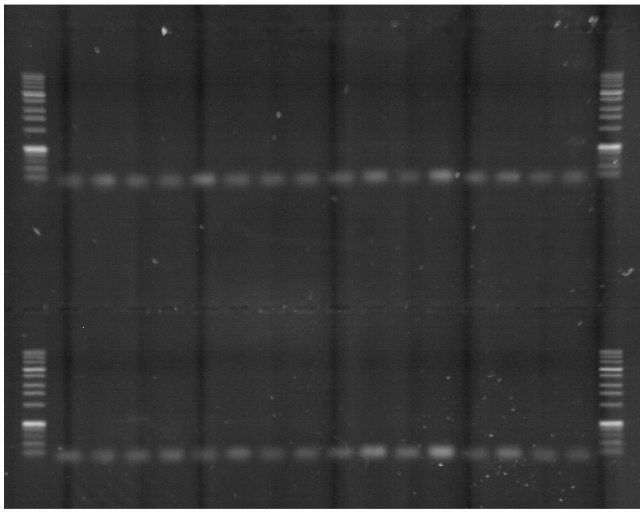

Figure 12 - PCR results of *ermA* (1)

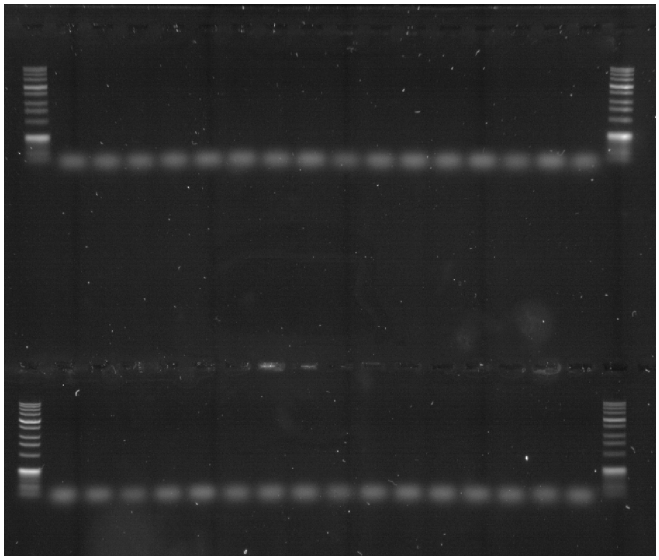

Figure 13 - PCR results of *ermA* (2)

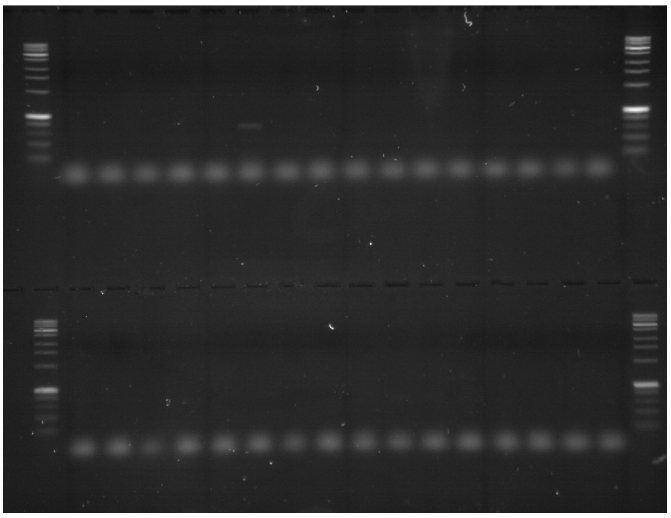

Figure 14 - PCR results of *ermA* (3)

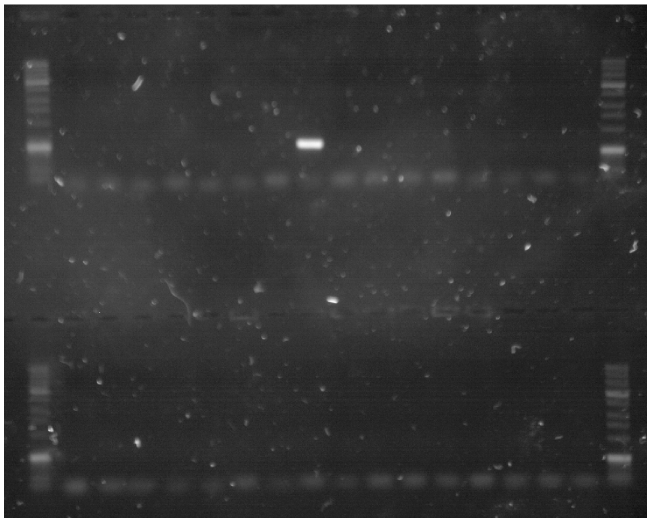

Figure 15 - PCR results of *ermA* (4)

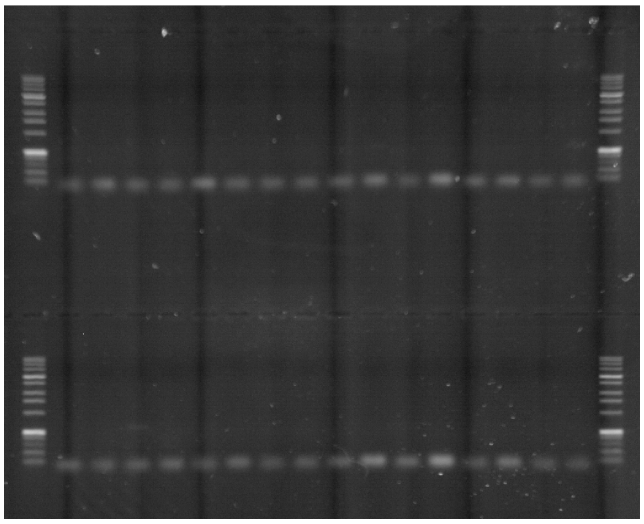

Figure 16 - PCR results of *ermA* (5)

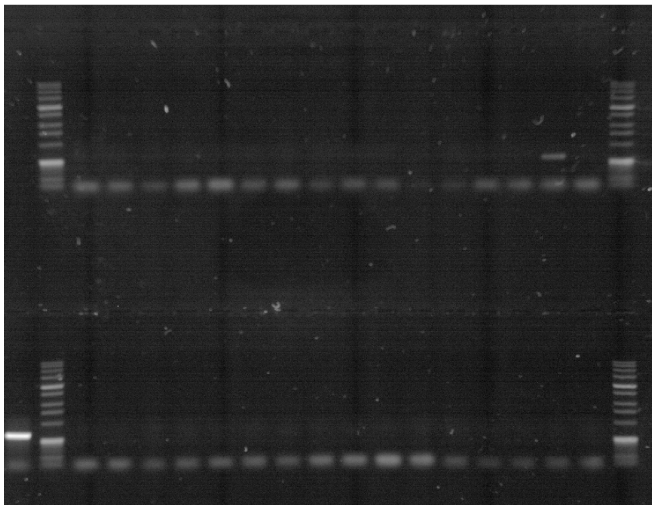

Figure 17 - PCR results of *ermA* (6)

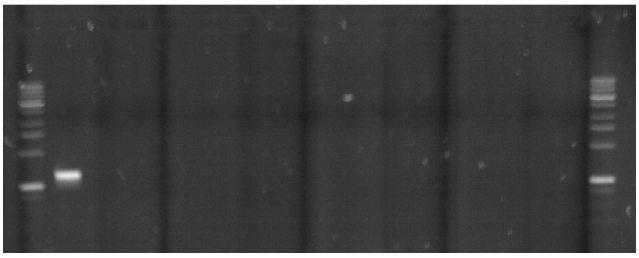

Figure 18 - PCR results of *ermA* (7)

C.2. PCR Results for *ermB* gene

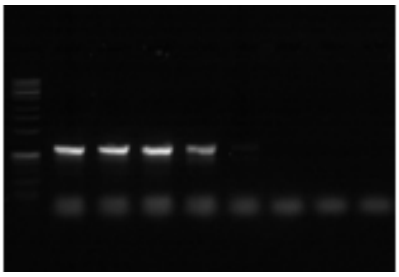

Figure 19 - Gradient PCR results of *ermB*

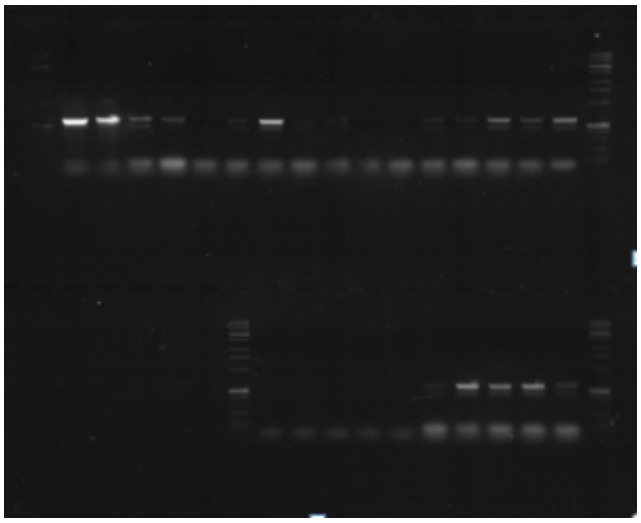

Figure 20 - PCR results of *ermB* (1)

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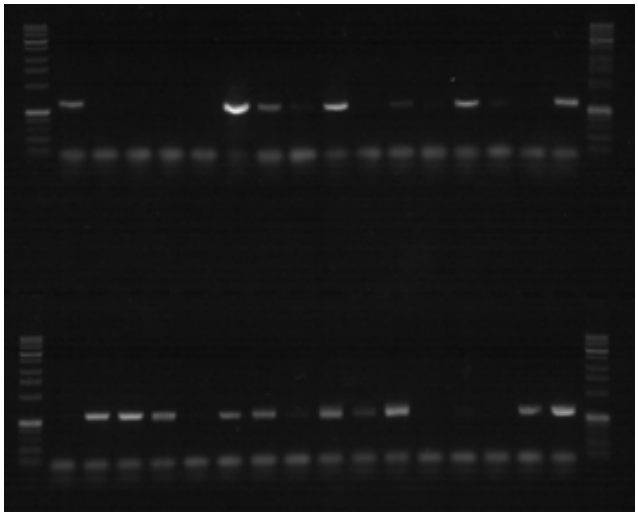

Figure 21 - PCR results of *ermB* (2)

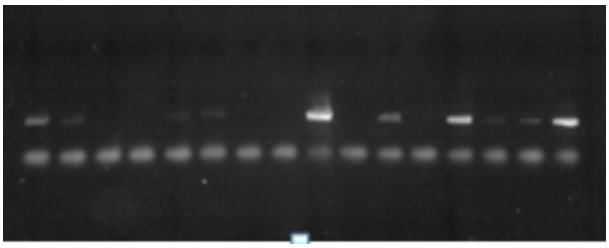

Figure 22 - PCR results of *ermB* (3)

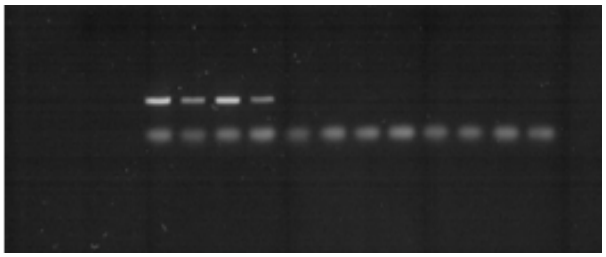

Figure 23 - PCR results of *ermB* (4)

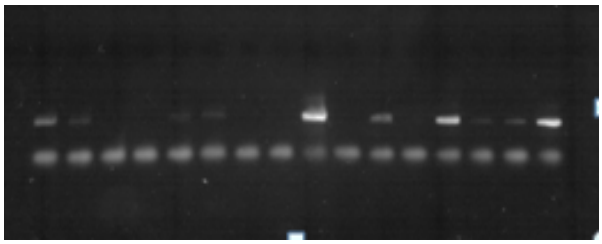

Figure 24 - PCR results of *ermB* (5)

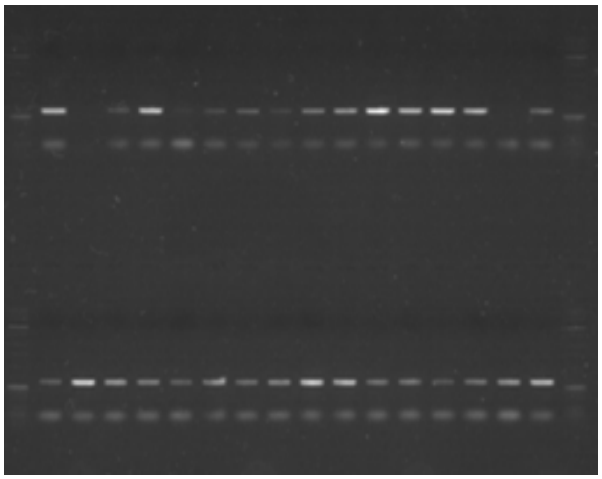

Figure 25 - PCR results of *ermB* (6)

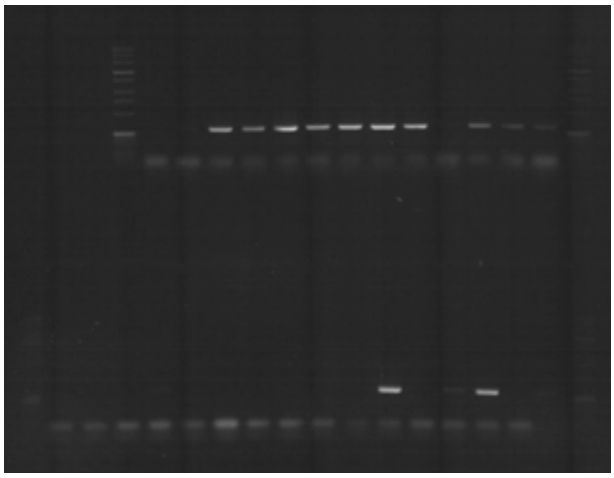

Figure 26 - PCR results of *ermB* (7)

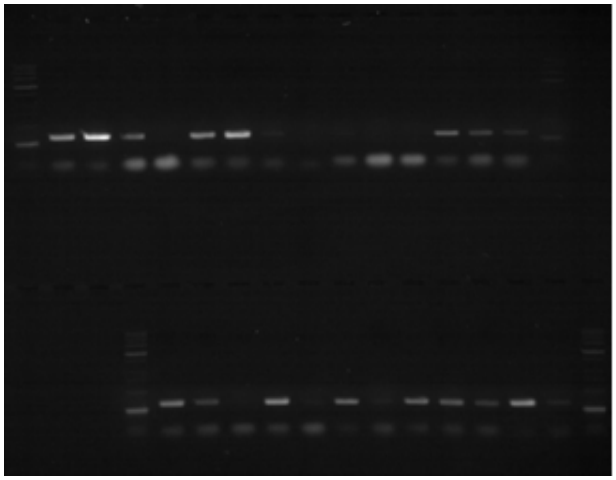

Figure 27 - PCR results of *ermB* (8)

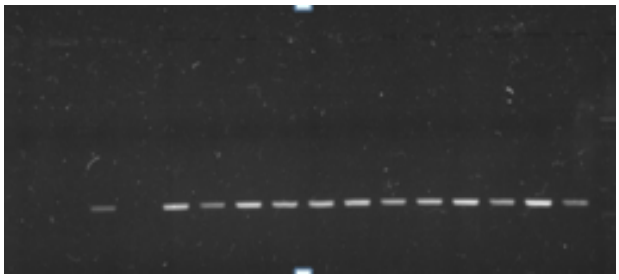

Figure 28 - PCR results of *ermB* (9)

C.3. PCR Results for *ermC* gene

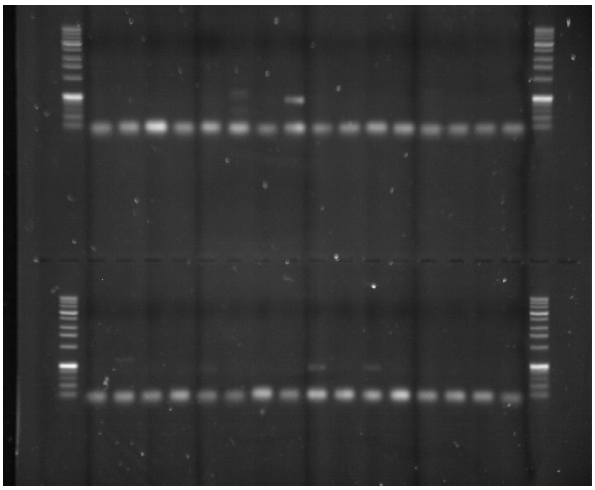

Figure 29 - PCR results of *ermC* (1)

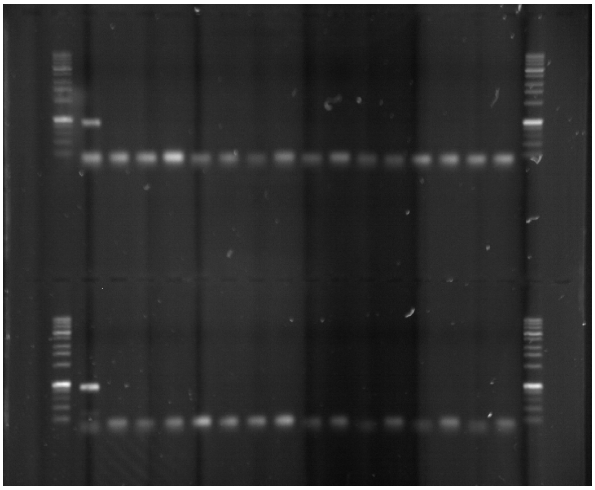

Figure 30 - PCR results of *ermC* (2)

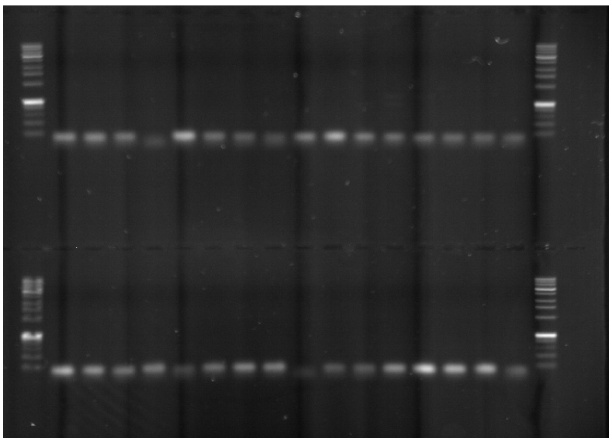

Figure 31 - PCR results of *ermC* (3)

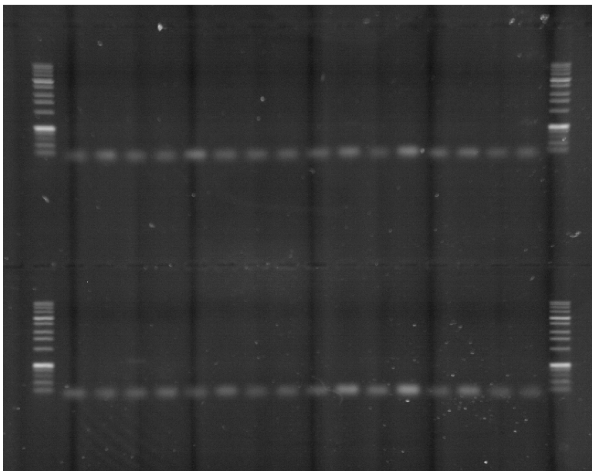

Figure 32 - PCR results of *ermC* (4)

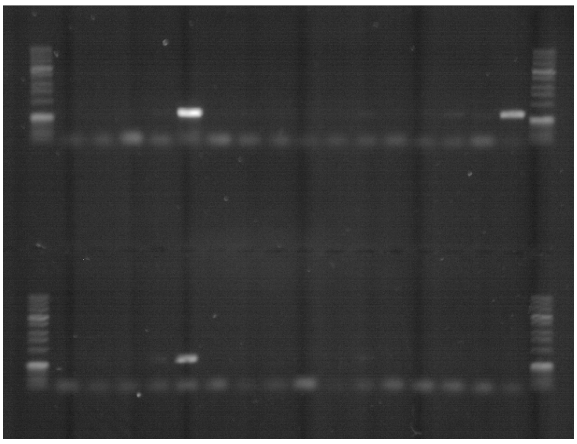

Figure 33 - PCR results of *ermC* (5)

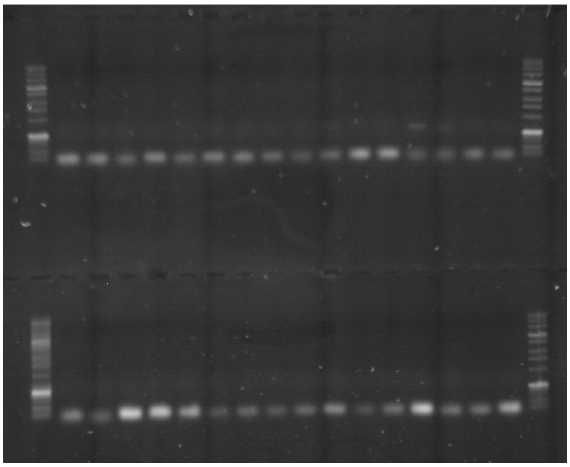

Figure 34 - PCR results of *ermC* (6)

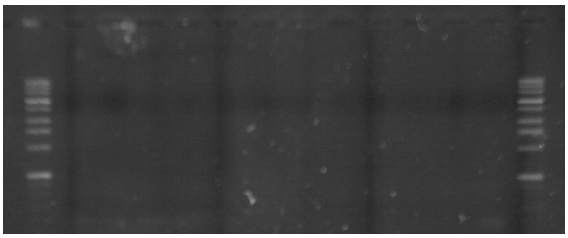

Figure 35 - PCR results of *ermC* (7)

C.4. PCR Results for *ermF* gene

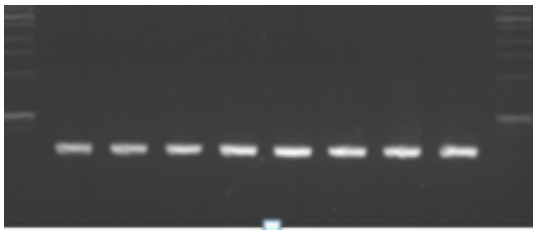

Figure 36 - PCR gradient of *ermF*

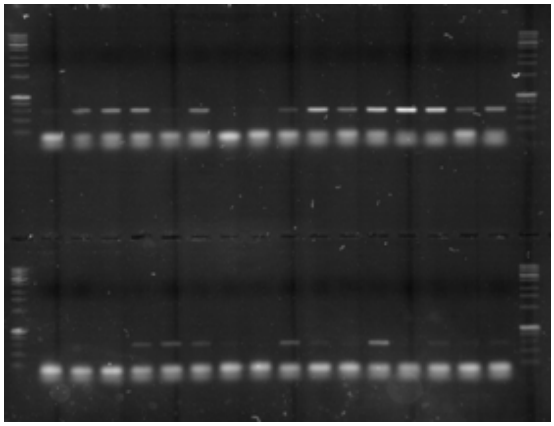

Figure 37 - PCR results of *ermF* (1)

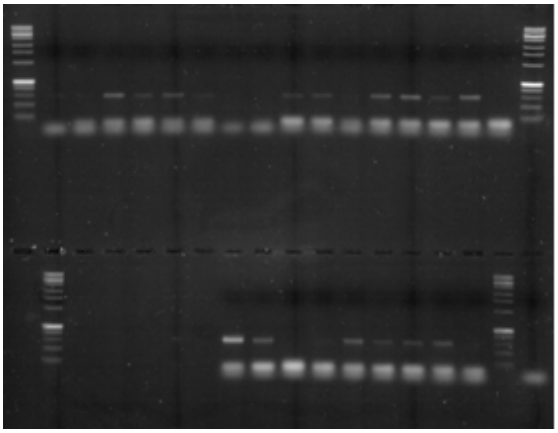

Figure 38 - PCR results of *ermF* (2)

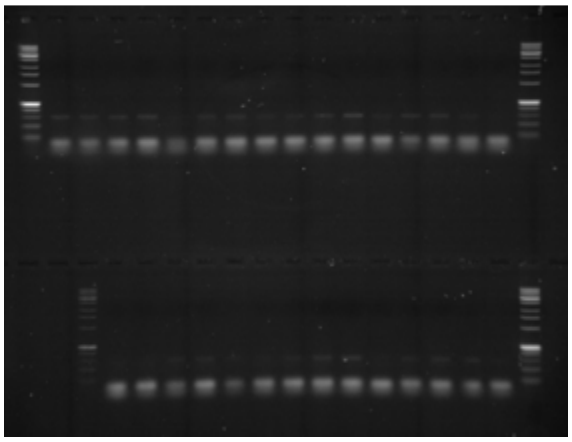

Figure 39 - PCR results of *ermF* (3)

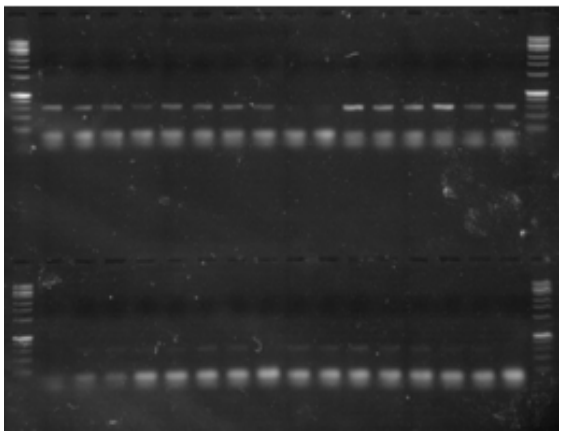

Figure 40 - PCR results of *ermF* (4)

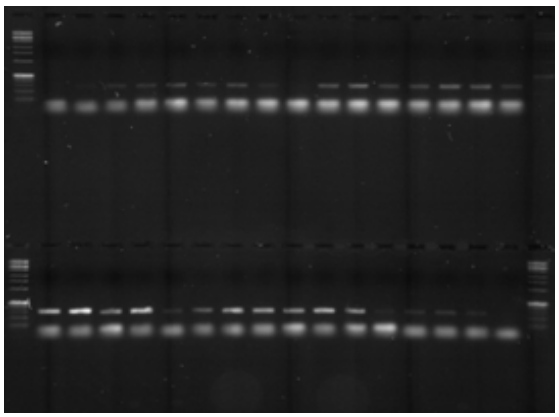

Figure 41 - PCR results of *ermF* (5)

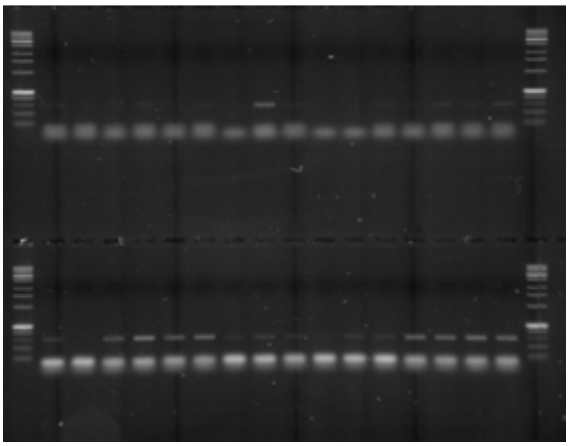

Figure 42 - PCR results of *ermF* (6)

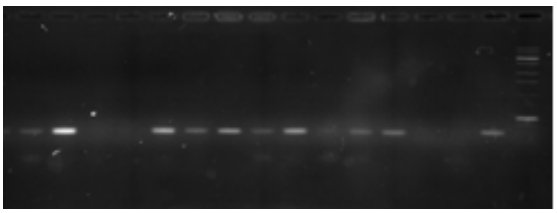

Figure 43 - PCR results of *ermF* (7)

C.5. PCR results for *ermT* gene

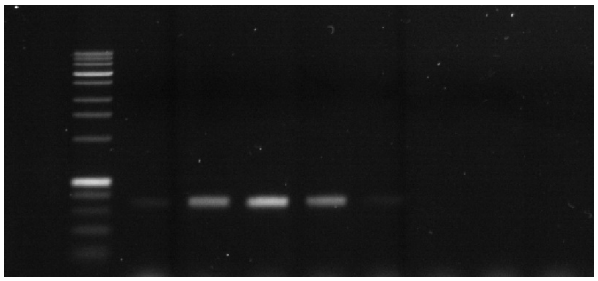

Figure 44 - Gradient PCR of *ermT* gene

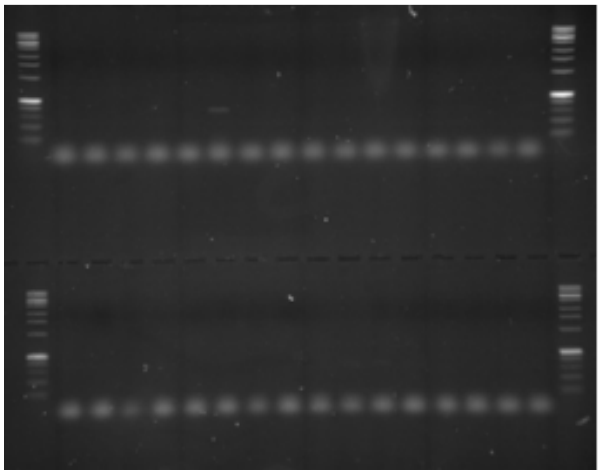

Figure 45 – PCR results of *ermT* (1)

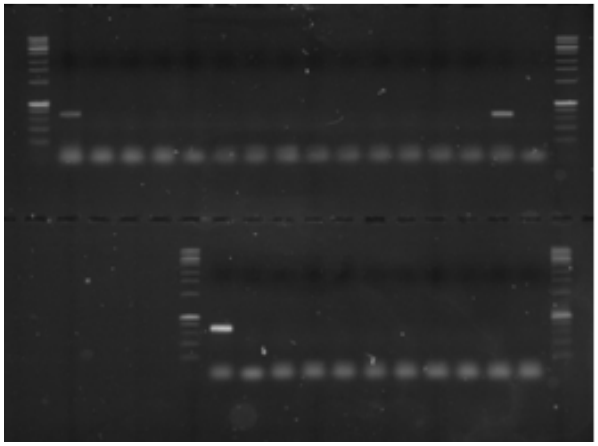

Figure 46 - PCR results of *ermT* (2)

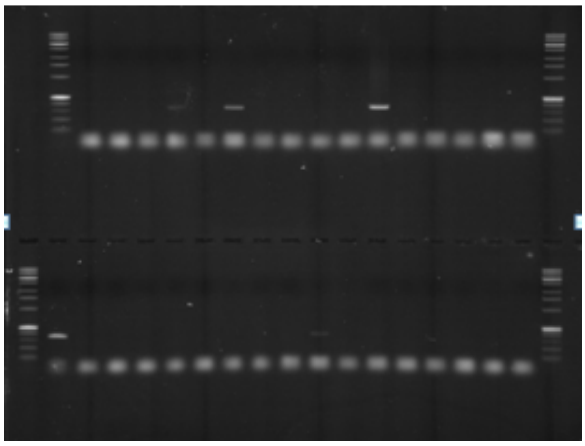

Figure 47 – PCR results of *ermT* (3)

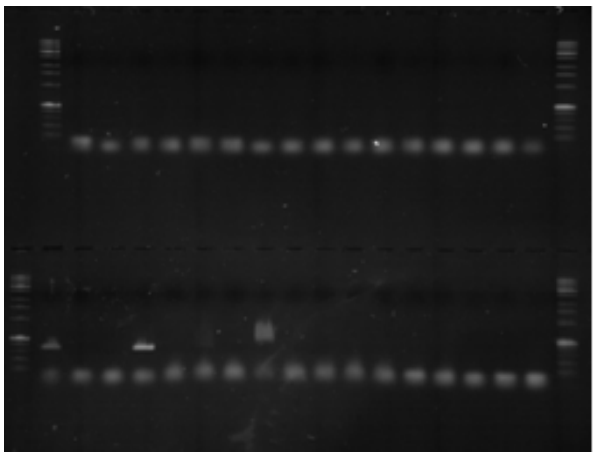

Figure 48 – PCR results of *ermT* (4)

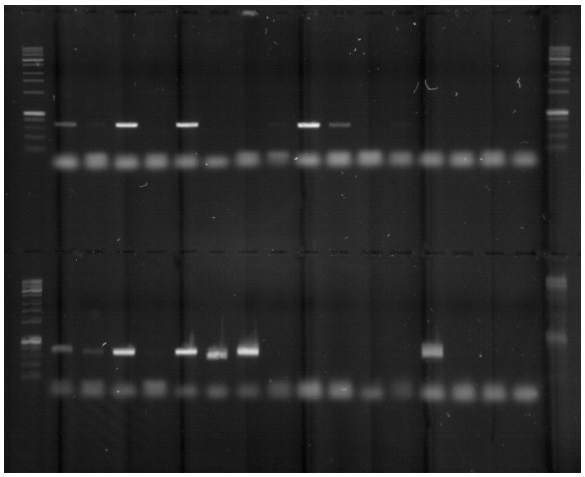

Figure 49 – PCR results of *ermT* (5)

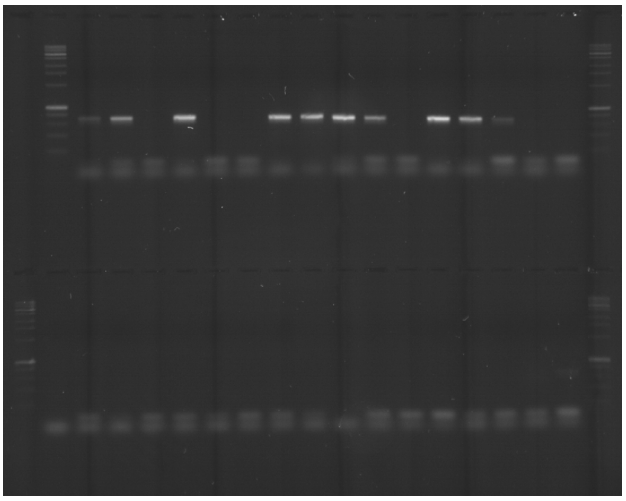

Figure 50 – PCR results of *ermT* (6)

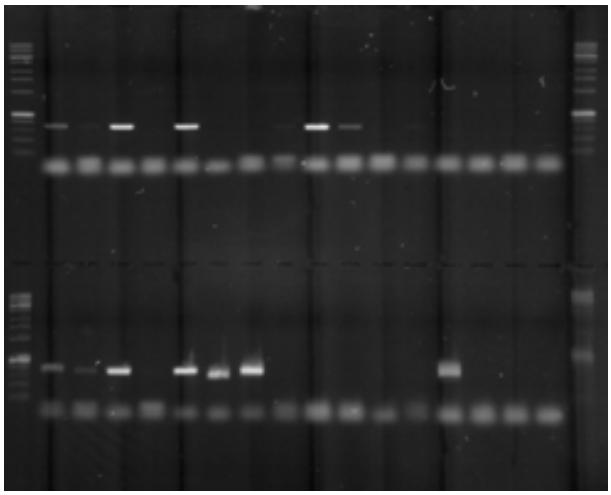

Figure 51 – PCR results of *ermT* (7)

C.6. PCR results for *msrA* gene

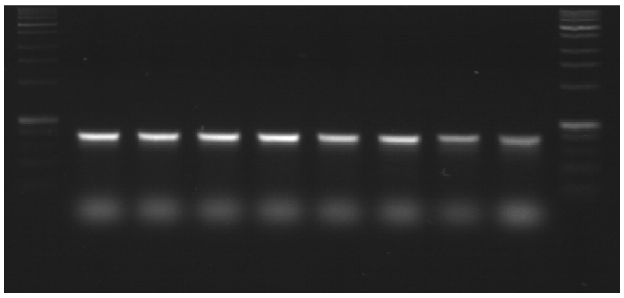

Figure 52 - Gradient PCR of *msrA* gene

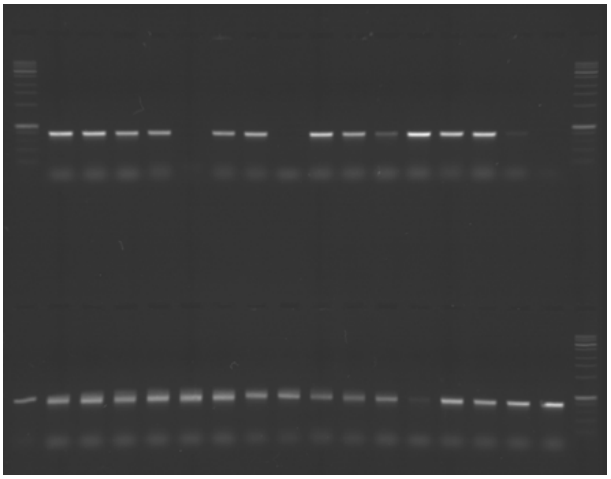

Figure 53 - PCR result of *msrA* (1)

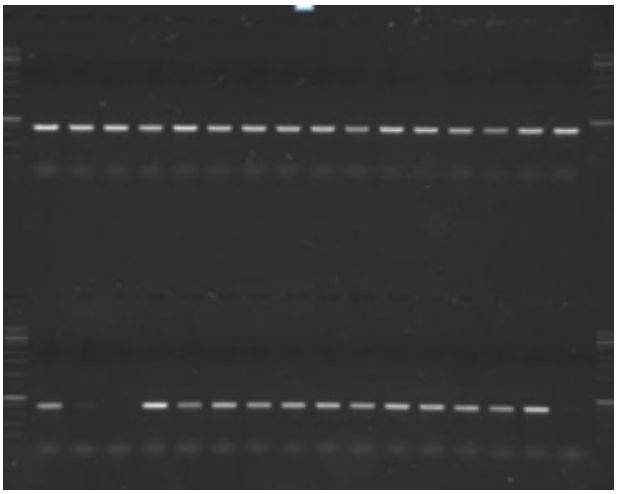

Figure 54 - PCR result of *msrA* (2)

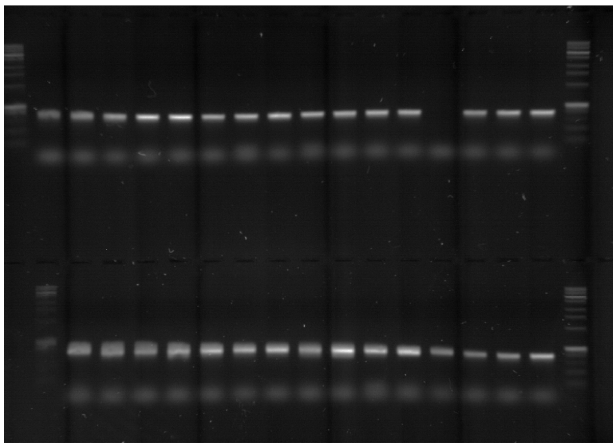

Figure 55 - PCR result of *msrA* (3)

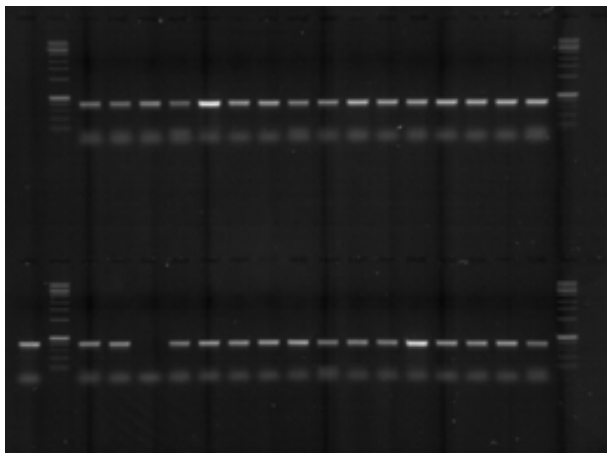

Figure 56 - PCR result of *msrA* (4)

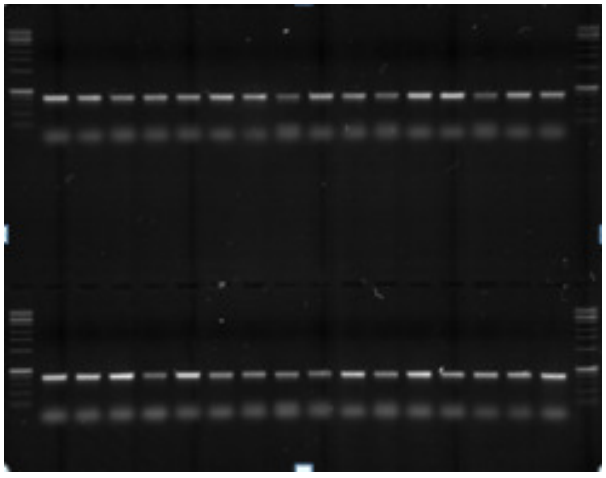

Figure 57 - PCR result of *msrA* (5)

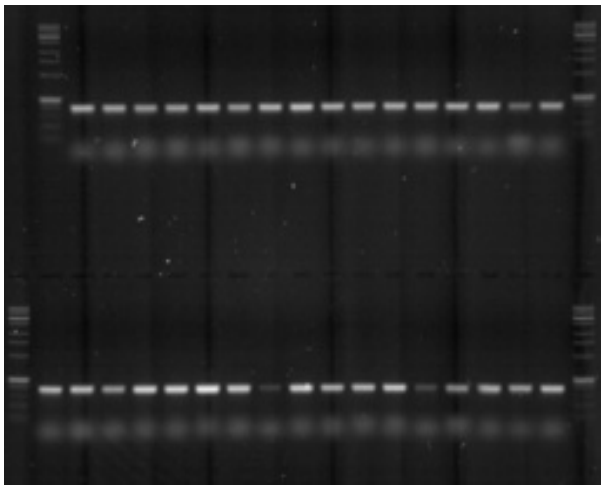

Figure 58 - PCR result of *msrA* (6)

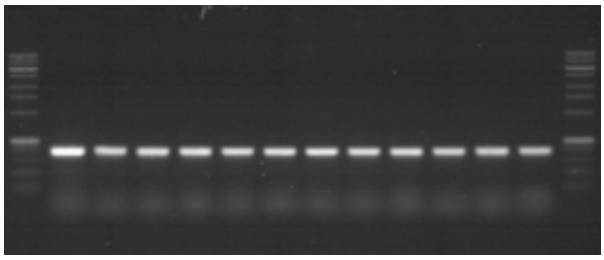

Figure 59 - PCR result of *msrA* (7)

APPENDIX D. STATISTICAL ANALYSIS

Table 9 - R source Code for analysis differences between total and resistant enterococci concentration and loadings

```
# Read the data
dat <- as.matrix(read.csv(file="r_data_spring.csv"))
#we aren't going to work with the difference
#we will work with the log of the ratio
new_response <- log(dat[,1]/dat[,2])

#to check the assumptions of a one sample test
#the plot should resemble a straight line and it does
print(qqnorm(new_response))

#assuming y_(Bockelmann et al.) =approx. c*y_(Aarestrup and Carstensen)
this is an
#estimate of log(c)
lchat <- mean(new_response)
print(lchat)

#an estimate of c would be
chat <- exp(lchat)
print(chat)

selchat <- sqrt((var(new_response))/(length(new_response)))
print(selchat)

test_stat <- lchat/selchat
print(test_stat)

#this is the pvalue of the test c = 1 versus c !=1
pvalue <- 2*(1-pt(q=test_stat, df=(length(new_response)-1)))
print(pvalue)
```

The assumption is valid since the plot resembles a straight line so we can use t-test to check the hypothesis.

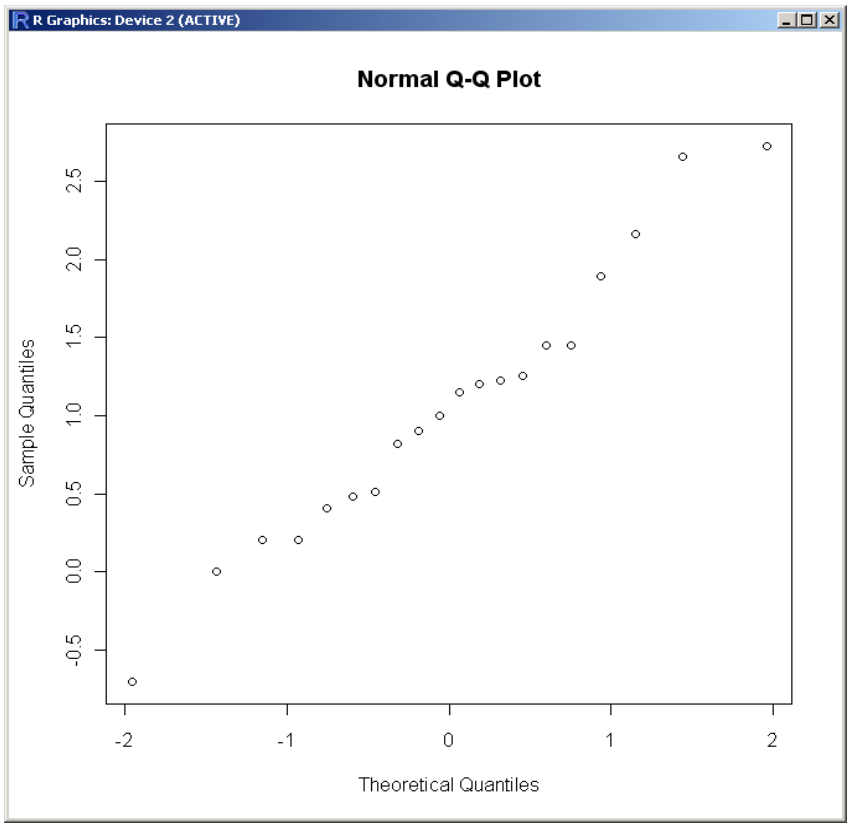

Figure 60 - Q-Q plot for log of ratio between resistant and total enterococci concentrations in Spring

Table 10 - Output screen for bacterial concentrations in Spring

```
> dat <- as.matrix(read.csv(file="r_data_spring.csv"))
> new_response <- log(dat[,1]/dat[,2])
> print(qqnorm(new_response))
$x
 [1] -0.45376219 -1.15034938 -0.31863936 -0.59776013 -1.43953147 -0.06270678
 [7]  0.18911843  0.06270678 -0.18911843  0.45376219  0.59776013 -0.75541503
[13]  1.15034938 -0.93458929 -1.95996398  1.95996398  1.43953147  0.93458929
[19]  0.75541503  0.31863936
$y
 [1]  0.5128236  0.2015794  0.8170550  0.4815439  0.0000000  0.9985288
 [7]  1.2039728  1.1525479  0.9038682  1.2535667  1.4483797  0.4042159
[13]  2.1633433  0.2015794 -0.7081851  2.7324994  2.6592600  1.8976199
[19]  1.4500102  1.2223655
> lchat <- mean(new_response)
> print(lchat)
[1] 1.049829
>
> chat <- exp(lchat)
> print(chat)
[1] 2.857162
>
> selchat <- sqrt((var(new_response))/(length(new_response)))
> print(selchat)
[1] 0.1956296
>
> test_stat <- lchat/selchat
> print(test_stat)
[1] 5.366411
> pvalue <- 2*(1-pt(q=test_stat, df=(length(new_response)-1)))
>
> print(pvalue)
[1] 3.530370e-05
```

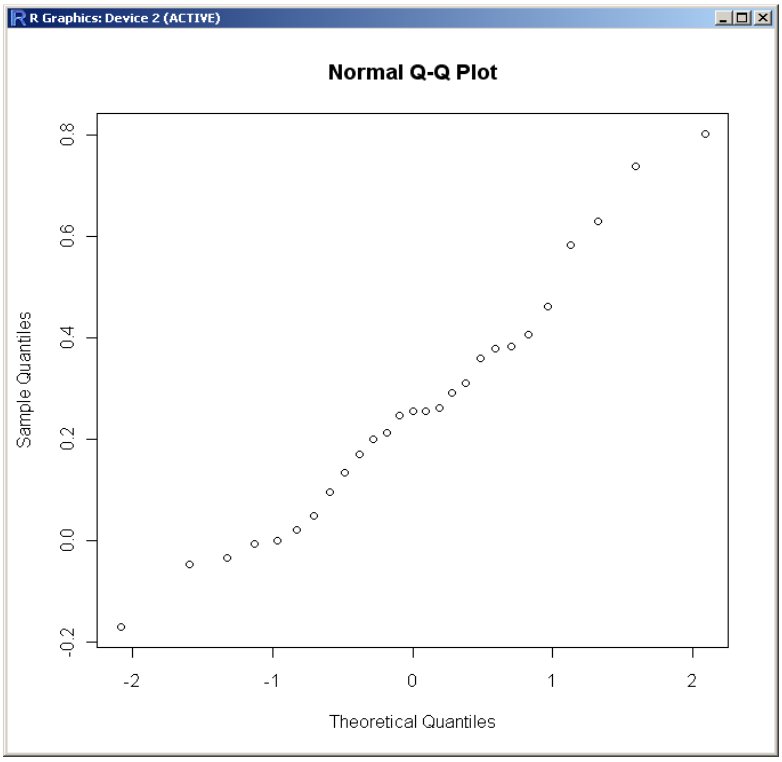

Figure 61 - Q-Q plot for log of ratio between resistant and total enterococci concentrations in Fall

Table 11 - Output screen for bacterial concentrations in Fall

```
> dat <- as.matrix(read.csv(file="r_data_fall.csv"))
> > new_response <- log(dat[,1]/dat[,2])
> > print(qqnorm(new_response))
$x
 [1] -1.32495769 -0.70392179  0.28221615 -0.82846465  1.12814365  0.58945580
 [7] -0.09297185  0.38032564 -0.18675612 -1.12814365 -0.58945580 -1.59321882
[13] -0.48224821 -0.38032564  0.82846465  0.00000000  0.09297185  0.70392179
[19]  0.18675612  1.59321882  0.48224821 -2.08535557  1.32495769  0.96742157
[25]  2.08535557 -0.28221615 -0.96742157
$y
 [1] -0.033901552  0.048790164  0.292987125  0.021202208  0.582967753
 [6]  0.379489622  0.247767585  0.310900363  0.213869915 -0.004987542
[11]  0.097061713 -0.046197591  0.133531393  0.170211488  0.406090304
[16]  0.255073831  0.255839323  0.383169103  0.262364264  0.738018935
[21]  0.361013346 -0.170221150  0.629521485  0.462035460  0.803495238
[26]  0.200670695  0.000000000
>
> #assuming y_(Bockelmann et al.) =approx. c*y_(Aarestrup and Carstensen)
this is an
> #estimate of log(c)
> lchat <- mean(new_response)
> print(lchat)
[1] 0.2592875
> chat <- exp(lchat)
> print(chat)
[1] 1.296006
> selchat <- sqrt((var(new_response))/(length(new_response)))
> print(selchat)
[1] 0.04670556
> test_stat <- lchat/selchat
> print(test_stat)
[1] 5.551535
> > pvalue <- 2*(1-pt(q=test_stat, df=(length(new_response)-1)))
> > print(pvalue)
[1] 7.888963e-06
```

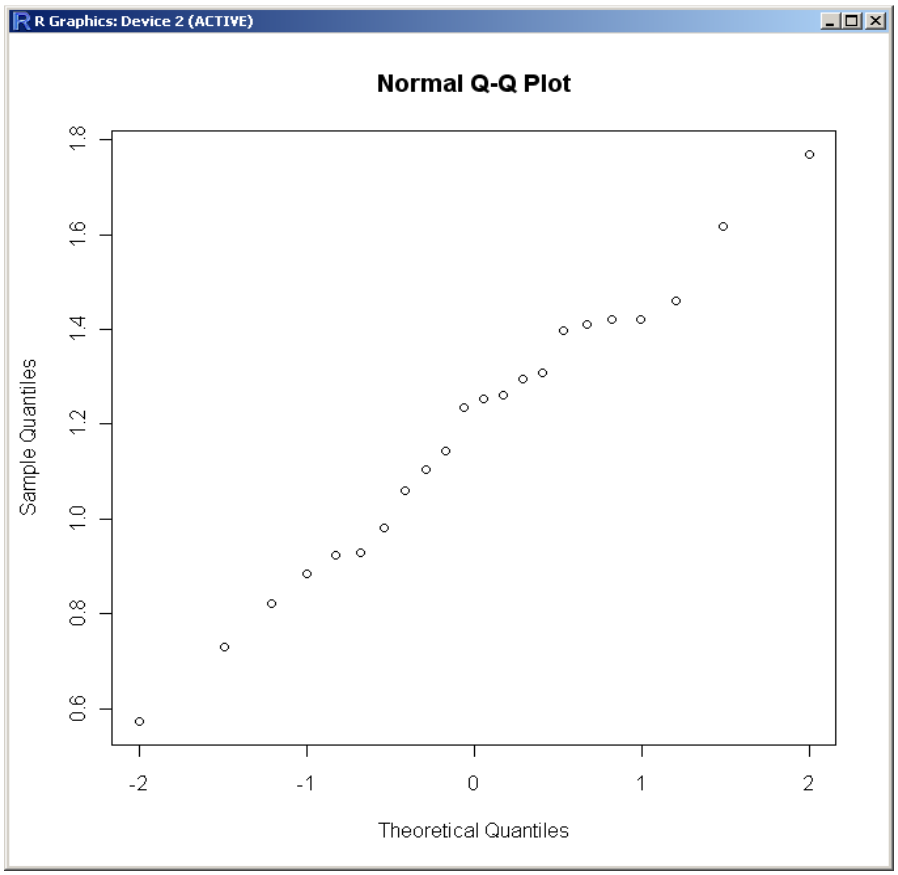

Figure 62 - Q-Q plot for log of ratio between resistant and total enterococci loadings in Spring

Table 12 - Output screen for bacterial loadings in Spring

```
> dat <- as.matrix(read.csv(file="spring_loadings.csv"))
>
> new_response <- log(dat[,1]/dat[,2])
>
> print(qqnorm(new_response))
$x
 [1]  2.00042357  1.48947004 -0.05699967 -0.40998332 -1.20741405 -2.00042357
 [7] -1.48947004 -0.99820117 -0.67448975 -0.82549449 -0.53751911 -0.17174709
[13] -0.28880936  0.28880936  0.17174709  0.05699967  0.40998332  0.53751911
[19]  0.82549449  0.99820117  0.67448975  1.20741405

$y
 [1] 1.7703191 1.6181589 1.2356685 1.0604397 0.8223636 0.5724051 0.7313134
 [8] 0.8858066 0.9283666 0.9248100 0.9806251 1.1430178 1.1049185 1.2957203
[15] 1.2601011 1.2529506 1.3075440 1.3985672 1.4203546 1.4212552 1.4116786
[22] 1.4602107

>
> #assuming y_(Bockelmann et al.) =approx. c*y_(Aarestrup and Carstensen)
this is an
> #estimate of log(c)
> lchat <- mean(new_response)
> print(lchat)
[1] 1.182118
>
> chat <- exp(lchat)
> print(chat)
[1] 3.261274
>
> selchat <- sqrt((var(new_response))/(length(new_response)))
> print(selchat)
[1] 0.06336918
>
> test_stat <- lchat/selchat
> print(test_stat)
[1] 18.65446
>
> pvalue <- 2*(1-pt(q=test_stat, df=(length(new_response)-1)))
>
> print(pvalue)
[1] 1.509903e-14
```

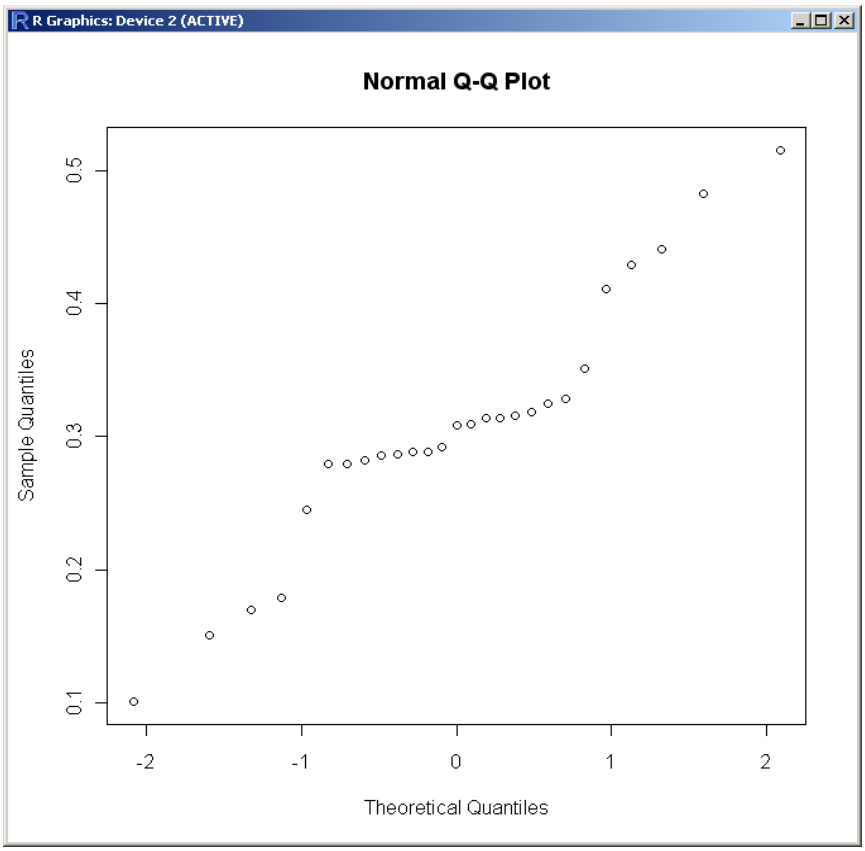

Figure 63 - Q-Q plot for log of ratio between resistant and total enterococci loadings in Fall

Table 13- Output screen for bacterial loadings in Fall

```
> dat <- as.matrix(read.csv(file="fall_loadings.csv"))
>
> new_response <- log(dat[,1]/dat[,2])
>
> print(qqnorm(new_response))
$x
 [1] -1.12814365 -1.59321882 -1.32495769 -2.08535557  2.08535557  1.59321882
 [7]  1.32495769  1.12814365  0.96742157  0.82846465  0.58945580 -0.09297185
[13] -0.70392179 -0.96742157 -0.18675612 -0.58945580 -0.82846465 -0.38032564
[19] -0.48224821  0.28221615  0.48224821 -0.28221615  0.09297185  0.38032564
[25]  0.70392179  0.18675612  0.00000000

$y
 [1] 0.1788670 0.1509835 0.1697005 0.1005908 0.5154894 0.4821591 0.4404281
 [8] 0.4285154 0.4111428 0.3508027 0.3242660 0.2916731 0.2797776 0.2447145
[15] 0.2887922 0.2824456 0.2791926 0.2866077 0.2854832 0.3139201 0.3182918
[22] 0.2885393 0.3097526 0.3160962 0.3285918 0.3138535 0.3086793

>
> #estimate of log(c)
> lchat <- mean(new_response)
> print(lchat)
[1] 0.3070132
>
> #an estimate of c would be
> chat <- exp(lchat)
> print(chat)
[1] 1.359359
>
> selchat <- sqrt((var(new_response))/(length(new_response)))
> print(selchat)
[1] 0.01812271
>
> test_stat <- lchat/selchat
> print(test_stat)
[1] 16.94080
>
> pvalue <- 2*(1-pt(q=test_stat, df=(length(new_response)-1)))
>
> print(pvalue)
[1] 1.554312e-15
>
```

Table 14- Summary of p-value in statistical analysis

	pvalue
Spring concentration	3.530370e-05
Fall concentration	7.888963e-06
Spring load	1.509903e-14
Fall loading	1.554312e-15