FACTORS THAT AFFECT HORIZONTAL GENE TRANSFER IN ENTERIC BACTERIA

by

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B.A., University of Kansas, 2001

M.S., Pittsburg State University, 2004

AN ABSTRACT OF A DISSERTATION

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Department of Diagnostic Medicine/Pathobiology

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Abstract

Antimicrobial resistance (AMR) has arisen as one of the most important public health concerns in the last 60 years. AMR results from pathogenic strains of bacteria adapting to antimicrobial-containing environments through mutations or through horizontal gene transfer (HGT) of genetic material containing resistance genes. Conjugation machinery offers an efficient method for acquisition of AMR and virulence genes, which may be responsible for propelling the evolution of pathogenic bacteria. This dissertation explores the factors, specifically catecholamines and antimicrobials that influence the conjugation frequencies of enteric bacteria including *Salmonella*, *E. coli* and *Enterococcus*. We found that the catecholamine norepinephrine (NE) at physiological concentrations enhanced conjugation efficiencies of a conjugative plasmid from a clinical strain of *Salmonella* Typhimurium to an *E. coli* recipient *in vitro*. Additional experiments determined the influence of the antimicrobial concentrations above, equal to and below the minimum inhibitory concentration (MIC) under *in vitro* conditions on conjugation efficiencies using an *Enterococcus* to *Enterococcus* mating pair in addition to the *Salmonella* to *E. coli* mating pair. Conjugation occurred in all concentrations, but efficiencies of transfer were consistently low in 0 MIC and 1 MIC, with increased activity both above and below 1 MIC. These data were fit to a previously described mathematical model and the rate constant E that relates the rate of gene transfer to drug concentration was determined. The data showed highly similar patterns of conjugation efficiencies when compared to the rate constant E. A final study we measured conjugation frequencies when donor *Salmonella* Typhimurium and the *E. coli* recipient were exposed to both variable concentrations of oxytetracycline and NE. Conjugation was increased pre- and post- MIC, but conjugation frequencies were not enhanced further by the combination of the oxytetracycline and the NE. This dissertation defines the role of outside factors in conjugative gene transfer, and may provide future insight into better control of AMR.

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Dedication

This dissertation is dedicated to my family and friends, with special thanks to my wife Erinn.

Thank you for your love and support throughout this process.

Preface

"*What does not kill me, makes me stronger*." – Friedrich Nietzsche, Twilight of the Idols, 1889.

Chapter 1 - Factors that Effect Horizontal Gene Transfer in Enteric Bacteria, a Literature Review

Abstract

Antimicrobial resistance (AMR) has arisen as one of the most important public health concerns in the last 60 years. AMR results in pathogenic strains of bacteria adapting to antimicrobial-containing environments through mutations or through horizontal gene transfer (HGT) of genetic material containing resistance genes. There are three main methods of HGT, including transformation, transduction and conjugation. In this thesis, conjugation has been shown to be enhanced by the presence of catecholamines as well as antimicrobials. This has been shown in a mating pair of *Enterococcus* to *Enterococcus* (Chapter 3) and *Salmonella* to *E. coli* (Chapters 2-4). Research has shown that HGT may be controlled through a variety of mechanisms, including the damage repair SOS system as well as the pheromone system of *Enterococcus*. Future research will continue to define the role of these factors in HGT.

Antimicrobial Resistance

Bacterial infection has been the leading cause of death historically, allowing for the most minor injury to lead to a patient's eventual demise. All this changed in the last 60 years with the discovery and widespread usage of antimicrobials, one of the most important discoveries in the last century. Since the first report of AMR among *Staphylococcus* spp. as early as the 1940s, the problem of AMR has increased into a serious public health concern leading to economic, social and environmental crisis (122). Bacteria are able to acquire resistance via various genetic phenomena, and multi drug resistance is considered a global consequence of antimicrobial use in

both human and veterinary medicine. Unfortunately, with improper use of these drugs, antimicrobial resistance has developed in bacteria such as vancomycin resistant *Enterococcus* (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA). Rise of AMR among pathogenic bacteria is one of the most telling signs that we have failed to take the threat of infectious diseases seriously, and there are many unanswered questions related to the prevalence, amplification and dissemination of phenotypic resistance and genetic resistance determinants created by medical use of antimicrobials (89, 98, 100).

While recipients of AMR genes survive in environments that contain antibiotics (17, 33, 36, 57, 61, 98-100, 107), recipients of virulence genes are better equipped for invasion and spread (59, 60, 82, 83, 91). The World Health Organization has referred to AMR as the next pandemic (121), and the Centers for Disease Control and Prevention has characterized AMR as one of the world's most pressing public health problems (16). AMR can increase the duration of infection, duration of follow-up care, treatment outcome, and associated costs. The economic impact of AMR has been estimated to extend healthcare costs to over $7 billion annually in the U.S. (4, 58) and over €7 billion annually in the EU alone (40).

Horizontal Gene Transfer

Bacteria display extraordinary variation in their genotype and phenotypes, considering they are single-celled organisms with haploid genomes. The modification, inactivation, or differential regulation of the genes has contributed to the genetic diversification of bacteria on an evolutionary timescale; however, the significant diversity is difficult to account for by mutations alone (85, 88). Bacteria are asexual organisms that reproduce by binary fission resulting in the genetic replication of one cell into two daughter cells. This type of reproduction produces genetically identical organisms that now have equal susceptibilities to environmental pressures,

such as antimicrobial. Over time, systems including transformation, transduction and conjugation have evolved over time to fill the need to diversify the genome to allow for faster adaptation to environmental changes, thereby reducing exclusive dependence of bacteria on random beneficial genomic mutations (97, 106).

Transformation

Transformation increases genetic variation through a competent bacterium uptaking DNA from its immediate environment and incorporating it into the host genetic material to complement its cellular functions allowing for beneficial traits to be passed without evolution (5). Initial indications of this occurrence were first noticed by Dr. Fredrick Griffith in 1928. In these experiments he noted that a strain of *Streptococcus pneumoniae* had two different phenotypes with differing effects on mice (51). A rough strain was found to be nonvirulent, while a smooth strain would cause death in the mouse. Interestingly, if the heat-killed smooth strain bacteria were mixed with the rough strain, a bacterial phenotype was produced that was again able to kill the mouse. The benign bacteria had been "transformed" into a virulent strain by some unknown cause, which we now know to be DNA.

A limitation to transformation is that not all bacteria in a population will simultaneously become competent to uptake DNA, be able to take in high enough amounts of the "correct" gene needed for survival, or the "correct" gene would be in the immediate environment of bacteria (71). Additionally, with the relatively short shelf life of DNA in the environment (0.017 hrs to 28 hours; depending on the matrix) there is even less of a chance for this horizontal transfer method to benefit the recipient cells for their survival (69, 71, 112).

Transduction

Transduction involves DNA transferred from one cell to another through the use of bacteriophages (80). This type of HGT was shown to be originally responsible for the movement of shiga-toxin producing genes (*stx1* and *stx2*) from *Shigella* species into the now highly virulent *E. coli* O157:H7 (91). Once the shiga-toxin producing genes are in the recipient *E. coli*, they can then be spread to other strains of *E. coli* to make them pathogenic (82, 83). A limitation to HGT by transduction is that it is completely phage-dependent, and, similar to transformation, relies on the right gene being present and taken up into the right phage at the right time. Additionally, both the donor and recipient strains must be sensitive to the same bacteriophage (71).

Conjugation

The most significant of horizontal transfer methods bacteria use is conjugation (85). This transfer has been shown to be important to the survival and evolution of many bacterial species (106). Conjugation works by a host cell physically adhering to a recipient cell, and horizontally transferring genetic elements packaged as plasmids or transposons into the cytoplasm for the passage into the recipient cell (85). These transposons and plasmids allow for high efficiency transfer of antimicrobial and virulence genes from single resistant donor bacterium to many recipients which can in turn donate the resistance genes to more recipients (106). Horizontal transfer by conjugation is not exclusive to bacteria of the same species. This is exemplified by the occurance of Vancomycin Resistant *Enterococcus* (VRE) and vancomycin resistant *S. aureus* in ecosystems where they coexist (17, 33, 36, 57, 61, 98-100, 107). Conjugative horizontal transfer has been shown to be not exclusive between bacteria. Studies have shown transfer of genetic elements by conjugation between bacteria to yeast (54), bacteria to plant cells (115), and

recently bacteria to mammalian cells (120). Conjugative transfer between bacteria, especially that of antimicrobial resistance genes, has a considerable impact on human and animal health (112). Many experimental studies have accomplished conjugative transfer using shuttle plasmids including incN, incP, incQ, and incW groups, and by the conjugative transposon Tn916 and Tn925 (7, 20, 31, 66, 69, 108, 109). Of significance, was a study where the plasmid pAT191 which encodes resistance to kanamycin was transferred from gram-positive *E. faecalis* to recipient strain of *E. coli* in the gastrointestinal environments of laboratory mice (31, 37). In another study transposon Tn916 which encodes tetracycline resistance was transferred from a gram-positive *E. faecalis* to a variety of gram-negative bacteria including *E. coli*, from which the Tn916 was reverse transferred to a Tn916-negative strain of *E. faecalis* (12). Recent studies have identified conjugative plasmid-carrying *Salmonella* strains that contain many different AMR genes that are capable conjugative HGT to recipient strains of *E. coli* (15, 47). The plasmid most studied in this thesis is contained within *Salmonella* Typhimurim strain 5678 (Chapters 2-4).

Plasmid Transfer (*tra*) Genes

Conjugative transfer of this $bla_{\text{CMY-2}}$ carrying plasmid involves a complex activation sequence of approximately 30 different transfer (*tra*) genes encoding proteins for direct cell-to-cell mating. Some examples of important proteins include TraI, which encodes a relaxase-helicase and is the central catalytic component of the multiprotein relaxasome complex responsible for beginning the conjugative DNA transfer; TraH, which stabilizes the relaxasome structure; TraJ, which recruits the relaxasome complex to the *oriT* site; TraY, which imparts single-stranded DNA character on the *oriT* site; TraR, which is a LuxR-type quorum-sensing transcription factor; TraG, which is essential for pili assembly and mating pair stabilization; and

TraM, which mediates interaction of relaxase to oriT by stimulating relaxed DNA formation (49).

Transposon Integrase Genes

By comparison, transfer of a conjugative transposon between *Enterococcus* strains (Chapter 3) is rather simple. Transposons are known as "jumping genes" for their ability to remove themselves from their current location and deposit themselves at another site. Transposons are typically flanked by two genes including an integrase (*int*) and an excisase (*xis*) gene. It is the role of excisase to cut the transposon from the genome or plasmid of the host and it is the role of the integrase gene to encode an enzyme that re-integrates the now-free transposon back into a desired location (64, 101). Unlike plasmids containing transfer genes, however, transposons are unable to form a pilus or any type of aggregation factors through which to pass to the recipient cell, so they are reliant on the host cell's machinery to provide the necessary conjugal instigation.

Stress Hormones

In Chapter 2 of this thesis, we explored the role of stress hormones on HGT of a conjugative plasmid. Physiological and psychological stresses of the host have been slow to play an important role in incidence, duration, severity and outcome of host diseases, especially those caused by infectious agents (21, 72-74, 76, 77). Catecholamines are a large group of amine hormones, derived from tyrosine and include epinephrine (adrenaline), norepinephrine (NE; noradrenaline) and dopamine. They are synthesized in the L-DOPA pathway. Adrenaline and NE, are sympathetic neuroendocrine mediators of "fight or flight" (acute stress) response of the host. NE-containing sympathetic synapses are distributed throughout the body, including the

enteric nervous system (ENS), and interestingly, more than half of the NE in the body is synthesized and utilized within the ENS (29, 45). In the gastrointestinal tract the physiological concentration of NE has been shown to be as high as 50 μM (3).

The human gut, especially the colon, harbors a dense, mixed population of bacteria (10^{11} - 10^{12}/g of contents), and the microbial ecology of the gut is dependent on health and disease states of the host. The gut flora evolved specific detection systems to sense host mediators of stress and use such mediators as environmental cues to alter their growth and virulence. The first experimental evidence that the catecholamines increased bacterial growth was gained using a serum-based (iron-depleted) medium (76), and this growth-promoting effect was determined to be due to enhanced iron acquisition and utilization via a catecholate-specific iron transport system with involvement of enterobactin and enterochelin pathways (11, 14, 44, 102). Subsequent studies have demonstrated that catecholamines can influence production of virulence factors, such as toxins and adhesins, biofilm formation, and quorum sensing even in iron-replete conditions (74, 75, 77, 104). For example, *E. coli* O157 responds to catecholamines by increased expression of shiga-toxin (116), exalted chemotaxis, and adherence to eukaryotic cells (6, 21), enhanced attachment and effacement (A/E) lesions (96), attachment to murine cecal mucosa (21), and increased flagella expression and motility (25). In *Salmonella,* catecholamines have been implicated to enhance motility and colonization in the GI tract of pigs (10). This concept provided a non immunological explanation for increased incidence and severity of infectious diseases among stressed individuals. However, the effects of catecholamines on HGT between bacteria in general, especially those in the GI tract, are currently unknown.

Antibiotic Pressure

Past research has shown that the improper use of antimicrobials may be a contributing factor to HGT and AMR. Past studies have shown the enhancement of various antimicrobials to HGT. A common trend in bacteria is that below, or "pre-MIC" levels of antimicrobials cause an increase in mutation rates as well as increases in the efficiencies of HGT of the susceptible recipient bacteria (9, 24, 56). In mating experiments with *Enterococcus*, donor strains containing a conjugative transposon were incubated in pre-MICs of tetracycline have been shown to increase the conjugative HGT frequencies of a transposon up to 119-fold when the donors were subsequently added to recipient *B. thuringiensis* bacterial cultures (103, 108).

Tetracycline and Oxytetracycline

The antibiotics that were used in Chapters 3 and 4 of this thesis were tetracycline and oxytetracycline. The tetracyclines are broad spectrum antibiotics that are commonly produced by *Streptomyces aureofaciens* (34). They were first discovered in 1948 by Dr. Benjamin Minge Duggar (39). Through binding to the 30S ribosomal subunit of prokaryotes they are able to inhibit protein production, leading to bacteriostatic effects (50, 53, 110).

Oxytetracyclines were the second group of tetracyclines to be discovered. They were first identified in 1950 as being produced by the bacteria *Streptomyces rimosus* (43), and are used against a broad range of bacteria as a bacteriostatic antimicrobial through binding to the 30S ribosomal subunit of prokaryotes (50, 53, 110). Oxytetracycline has been used in livestock feed as a prophylactic against infection and as a growth promoter (23, 41).

The effectiveness of tetracyclines has declined in recent years due to the rise of tetracycline resistance among bacteria primarily from the development of drug efflux pumps (50, 68, 123). These pumps are encoded by genes including *tetA* (27, 28). Additional protection is gained through the *tetM* gene operates by protecting the ribosome from the tetracycline (23, 28, 95).

SOS Cellular Response to DNA Damage

DNA can be damaged by a variety of environmental stresses (79). Two well known stresses are with UV light and two different classes of antibiotics (9, 79). The antibiotics include f*luoroquinolones* (such as ciprofloxacin and ceftiofur) and dihydrofolate reductase inhibitors (such as Trimethoprim) (9). Fluoroquinolones act on the DNA topoisomerase to inhibit it's function, which leads to DNA damage (38), and dihydrofolate reductase inhibitors inhibit the cell's uptake of folic acid from the host environment, leading to the inability to replicate DNA (13). These agents can cause single stranded or double strand breakages of cellular DNA, leading to the activation of the cell's SOS system. The SOS system serves as a major defense against environmental damage to cells, and DNA repair machinery which is present in all bacteria (79). In response to DNA damage, activation of SOS genes allows replication to bypass DNA damage and continue replication which can minimize cell death, mutations, replication errors, persistence of DNA damage and genomic instability (79).

The SOS system is regulated and controlled by the proteins LexA and RecA. RecA functions in DNA repair as an ATP-dependent protein that binds tightly to damaged ssDNA, and drives the movement of the three-stranded intermediate in one direction by forming a nucleoprotein filament in which it "spins out" a newly recombined DNA strand (32, 84).

Promoter fusions to *luxCDABE* of *recA* in *E. coli* showed dose-dependent responses to a variety of sub-lethal stresses including antibiotic, and UV light (117).

RecA has been also been shown to interact with the protein LexA (46). LexA is a dimeric protein repressor of SOS response, and in *E. coli*, RecA-dependent cleavage of LexA correlates to increases in SOS system activation (46). The SOS system repairs DNA with the activation of three main pathways, the Nucleotide Excision Repair (NER), RecFOR, and the RecBCD (79). The NER pathway is relatively non-specific and is first activated in response to DNA damage by activating genes *uvrA*, *uvrB*, and *uvrC* (70, 113). The RecFOR pathway performs recombinational single strand gap repairs and is mediated by RecD, RecF, RecO, and RecR proteins and aids in the loading of RecA onto damaged DNA strains (55). Finally, the RecBCD Complex DNA repair of dsDNA breaks by using the Chi sequence 5'-GCTGGTGG-3' that occur. When DNA is damaged, RecBCD attaches to the damaged strand and destroys all ssDNA until reaches this sequence (22, 65, 67). The RecBCD then switches strands to give 3' protrusion, and RecA is then loaded onto 3' protrusion (22, 65, 67). Two studies in *E. coli* have shown that the LexA SOS inhibitor has control over approximately 40 different genes (30, 42, 79). While the majority of these are damage repair genes (not listed), there are several of unknown function (Table 1) (30, 42, 79). Additional studies were conducted in *Enterococcus* where cellular damage by antimicrobials including erythromycin and vancomycin was monitored by microarray and 2D gels, respectively (1, 119). A series of genes were shown to upregulate in these studies as well in response to the cellular damage (Table 1).

SOS and HGT

Induction of the SOS system has been linked to HGT in several different studies. It was observed in early experiments that UV exposure to some bacterial cells allowed for more than

just inhibition of growth, but also contributed to the lytic activation of phages (94, 105, 111). Later studies in *E. coli* began to show that the genes that activate bacteriophage production were shown to be RecA dependent (81). Additionally, in a microarray screen of *E. coli* genes controlled by LexA that were activated under UV stress showed the upregulation of prophage genes (Table 1) (30). In *E. coli*, *stx2* phage genes were shown to activate after quinolone exposure and phages were found to be produced because of SOS induction, leading to spread of *stx2* gene through phage transduction and increases in toxin production (62, 63, 81, 118). This effect has been observed in an *in vitro* mouse model as well (124). Additional studies with *Vibrio cholera*, identified a conjugative transposon called STX that contained multiple antimicrobial resistance genes was induced to horizontally transfer with the activation of the SOS system (8, 9). STX encodes SetR which is a repressor that inhibits the transcription of phage related integrase genes that allow for HGT (8, 9). However, when the SOS response to DNA damage was activated, the effects of SetR were inhibited and transcription and the subsequent transfer of STX were allowed to take place (8, 9). A similar phage gene mediated movement of genes is present in *S. aureus* (60, 78, 86, 87, 111). Various pathogenic islands (PI) including SaPI1-SaPI4, SaPbov1, SaPIbov2 and SaPIn1-SaPIn3 have been shown to encode genes that allow for prophage production and HGT by transduction (60, 78, 86, 87, 111).

Conversely to utilizing the activation SOS system for induction of integrase genes, some conjugative plasmids have actually been shown to have unique machinery which has been shown to inhibit SOS function (48, 90). During conjugation, plasmids enter the recipient cell as single stranded DNA, which is an activator of the SOS response (48, 90). The actions of the SOS can be potentially damaging to the single stranded plasmid DNA, a protein called PsiB protein (present in the bla_{CMY-2} containing plasmid used in Chapters 2-4 (47)) is utilized (48, 90). PsiB

translation activates in a dose-dependent response to increasing levels of RecA protein and binds to free RecA protein to inhibit the activation of SOS by the recipient cell (48, 90). The PsiB has been shown to inhibit the cleavage of the LexA protein as well to further suppress the SOS response (90).

Salmonella

Throughout this thesis we used two types of bacteria as donor strains for HGT mating experiments. These were *Salmonella enterica* and *Enterococcus faecalis*. Gastrointestinal tracts of both humans and animals are a major habitat of *Salmonella enterica*. In 2005, over 36,000 clinical cases of Salmonellosis were reported to the CDC (19). Of these, 15,000 resulted in hospitalization and 400 were fatal (52). In 2007, the number of cases reported in a 10 U.S. state survey was 6,790 and the incidence per 100,000 population was 14.92 (18). Salmonellosis can also cause severe enteritis, decreasing weight gain and reproductive performance in livestock and thereby has a significant economic impact (2). Recent studies have identified plasmid carrying *Salmonella* strains that contain many different AMR genes that are capable of conjugative HGT to recipient strains of *E. coli* (15, 47).

Enterococcus

Enterococcus faecalis is a gram positive facultative anerobe that is commonplace in the gut flora. It has been shown in multiple studies capable of both donating and receiving genetic material horizontally, making it a potential reservoir for antimicrobial resistance in the gut (114). This genetic material can include plasmids, and transposons that have been known to carry AMR genes (89). Many strains of *E. faecalis* have been shown to be multidrug resistant (MDR) and of particular interest has been the recent emergence of vancomycin resistant strains. These strains

have been associated with *nosocomial* infections (114) and in many cases the *Enterococcus* has been shown capable of transferring this vancomycin resistance to other virulent bacteria including *Staphlococcus aureus* (*S. aureus*), giving rise to the dangerous meticillin resistant *S. aureus* (MRSA) (17, 33, 36, 57, 61, 98-100, 107).

Pheromone System and Plasmid Transfer

Potential *Enterococcus* recipient cells have a unique system of attracting *Enterococcus* donor cells for HGT of genetic material. This system is known as the "pheromone" system and it involves the secretion of peptides by a potential recipient cell that are detected by donor cells carrying the conjugative plasmids (26, 35). Pheromones begin as signal peptides of normal lipoproteins that are translated from chromosomal genes (26, 35). These signal peptides that become active pheromones are then cut from the lipoproteins during excretion from the cell. Five well studied pheromones are known as cAD1, cPD1, cCF10, cAM373 and cOB1, and they exclusively attract plasmids pAD1, pPD1, pCF10, pAM373, and pOB1 (respectively) (26, 35). The activation of pAD1 by cAD1 has been well studied and involves the actions of TraA, TraC, and TraE1 (26). TraA normally inhibits the promoter by binding to the nucleic acid sequence TTATTTTTATTT (92, 93) which controls the expression of genes *iad* (inhibitor molecule) *traD*, *traE1*, *sea1*, and *asa1* (aggregation genes) (26). When the pheromone cAD1 binds to the plasmid-encoded surface protein TraC, it is then chaperoned into the cell by the oligopeptide permease system. The cAD1 then goes on to suppress the inhibitory actions of TraA (26, 35). When TraA is suppressed by cAD1, transcription of *traE1* upregulates and protein TraE1 is able to promote the transcription of the aggregation genes and allow for conjugal binding of the donor with the recipient cell and plasmid transfer (26). Interestingly, once pAD1 has entered the recipient cell, the plasmid gene *iad* is then activated to produce the inhibitor iAD1 which binds

to the surface TraC to prevent autoinduction of the cell (26, 35). Finally, another surface protein, TraB moves to suppress production of cAD1 entirely (26, 35).

Conclusions

HGT through conjugation is a relatively straight-forward process where two bacteria (donor and recipient) physically join and pass genetic material. The influence of outside factors is, however, multifaceted. Intriguing past research involving the SOS and pheromone systems, as well as the current research contained within this thesis showing the influence of catecholamines and antimicrobials, demonstrate that we have only begun to understand these most "simple" forms of life. Future research will continue to define the role of these factors in HGT, and in the current AMR crisis.

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Chapter 2 - Catecholamines Increase Conjugative Gene Transfer between Enteric Bacteria

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Abstract

The ability of pathogenic bacteria to sense and respond to periods of host stress is critical to their lifestyle. Adrenaline and norepinephrine are catecholamines that mediate acute host stress in vertebrates and invertebrates. Catecholamines are also used as environmental cues to enhance growth, motility and virulence of bacterial pathogens via specific binding receptors. Incidence of multidrug resistant and highly virulent bacterial pathogens is on the rise, and majority of the genes for antimicrobial resistance (AMR) and virulence are carried on horizontally transferable genetic elements. Conjugation machinery offers an efficient method for acquisition of AMR and virulence genes, which may be responsible for propelling the evolution of pathogenic bacteria. Here we show that norepinephrine (NE) at physiological concentrations enhances horizontal gene transfer (HGT) efficiencies of a conjugative plasmid from a clinical strain of *Salmonella* Typhimurium to an *E. coli* recipient *in vitro*. Expressions of plasmid-encoded transfer (*tra*) genes necessary for conjugation were also significantly upregulated in the presence of NE. Phentolamine, an α-adrenergic receptor antagonist, negated the effects of NE on conjugation more strongly than propranolol, a β-adrenergic receptor antagonist. This study for the first time provides evidence that innate mediators of acute host stress may influence evolution and adaptation of bacterial pathogens.

Introduction

Bacteria display extraordinary variation in their genotypes and phenotypes, considering they are single-celled organisms with haploid genomes. The modification, inactivation, or differential regulation of the genes has contributed to the genetic diversification of bacteria on an evolutionary timescale; however, the significant diversity is difficult to account for by mutations alone [1]. Horizontal gene transfer (HGT) as a major mechanism for bacterial diversity was first proposed based on the observation that virulence determinants could be transferred between pneumococci in infected mice [2], a phenomenon that was later demonstrated to be mediated by the uptake of genetic material, called transformation. Subsequent identification of gene transfer mediated by plasmids, transposons and bacteriophages provided explanation to the current picture of gene flux and the importance of mobile genetic elements in bacterial genetic diversity [3,4]. Compositional *in silico* analyses have revealed that considerable proportions of bacterial genomes consist of horizontally acquired genes [5] and supports the eco-evo principle that organisms coexisting in an ecosystem constantly evolve to adapt to each other, leading to bacterial innovation [1]. HGT provides major milestones in microbial evolution, allowing bacteria to completely bypass adaptation through the process of random mutation [1,6]. This is accomplished through the efficient movement of genetic elements including plasmids, transposons, and bacteriophages [3,4]. However, the most efficient method of HGT is conjugation, which is mediated by the physical adherence of donor and recipient cells and the subsequent transfer of genetic elements into the recipient cell [6,7]. This allows for high efficiency transfer of antimicrobial resistance (AMR) and virulence genes from a donor bacterium to many recipients, and the spread of these genes among bacteria has considerable impact on human and animal health [8].

The recent rise in the incidence of illnesses caused by highly virulent and multidrug resistant strains of bacterial pathogens is of major concern to human and animal health. While recipients of AMR genes survive in environments that contain antibiotics [9-17], recipients of virulence genes are better equipped for invasion and spread [18-22]. The World Health Organization has referred to AMR as the next pandemic [23], and the Centers for Disease Control and Prevention has characterized AMR as one of the world's most pressing public health problems [24]. AMR can increase the duration of infection, duration of follow-up care, treatment outcome, and associated costs. The economic impact of AMR has been estimated to extend healthcare costs to over $7 billion annually in the U.S. [25,26] and over €7 billion annually in the EU alone [27].

Catecholamines are a large group of amine hormones, derived from tyrosine and include epinephrine (adrenaline), norepinephrine (NE; noradrenaline) and dopamine. Catecholamines, especially adrenaline and NE, are sympathetic neuroendocrine mediators of "fight or flight" (acute stress) response of the host. NE-containing sympathetic synapses are distributed throughout the body, including the enteric nervous system (ENS) where more than half of the NE in the body is synthesized and utilized [28,29]. In the gastrointestinal tract the physiological concentration of NE has been shown to be as high as 50 μM [30]. The human gut, especially the colon, harbors a dense, mixed population of bacteria (10^{11} -10^{12}/g of contents), and the microbial ecology of the gut is dependent on health and disease states of the host [31]. The gut flora have evolved specific detection systems to sense host mediators of stress and use such mediators as environmental cues to alter their growth and virulence. The first experimental evidence that the catecholamines increased bacterial growth was gained using a serum-based (iron-depleted) medium [32], and this growth-promoting effect was determined to be due to enhanced iron

acquisition and utilization via a catecholate-specific iron transport system with involvement of enterobactin and enterochelin pathways [33-36]. Subsequent studies have demonstrated that catecholamines can influence production of virulence factors, such as toxins and adhesins, biofilm formation, and quorum sensing even in iron-replete conditions [37-40]. For example, *E. coli* O157 responds to catecholamines by increased expression of shiga-toxin [41], exalted chemotaxis, and adherence to eukaryotic cells [42,43], enhanced attachment and effacement (A/E) lesions [44], attachment to murine cecal mucosa [43], and increased flagella expression and motility [45]. In *Salmonella,* catecholamines have been implicated to enhance motility and colonization in the GI tract of pigs [46]. This concept may provide a non immunological explanation for increased incidence and severity of infectious diseases among stressed individuals. However, the effects of catecholamines on HGT between bacteria in general, especially those in the GI tract, are currently unknown.

The primary objective of this study was to evaluate the role of NE on the intergeneric transfer of conjugative plasmids from *Salmonella* to *E. coli in vitro*. In this study, we used a previously described [47] mating pair of bacteria consisting of a donor strain of *Salmonella* Typhimurium that carries a (>100kb) conjugative plasmid encoding multidrug resistance and a recipient *E. coli*. When we measured the overall ratios of transfer efficiencies with and without exposure to NE, we observed a significant increase in conjugation with NE treatment. Significant upregulation of plasmid transfer (*tra*) genes was observed in the presence of NE. Enhanced conjugative transfer and *tra* gene expression were inhibited by α and β adrenergic receptor antagonists.

Materials and Methods

Bacterial strains and culture media

Salmonella Typhimurium strain 5678 [47] was used as donor strains in the majority of our experiments. This strain carries a type A plasmid that is approximately 100 kb in size and is transferable by conjugation to recipients including *E. coli* C600N (a spontaneous nalidixic acid resistant mutant of strain C600 [47]; kindly provided by Dr. Paul Fey at University of Nebraska Medical Center, Omaha, Nebraska), *E. coli* MG1655N, and a bioluminescent *Citrobacter rodentium* strain ICC180 [60,61] (Table 1). This plasmid contains a bla_{CMY-2} gene that encodes resistance to a large spectrum of β-lactams including ampicillin, ceftriaxone and cefoxitin, and also contains resistance markers for chloramphenicol, streptomycin, sulfisoxazole, trimethoprim-sulfamethaxozole and tetracycline (Table 1). A *Salmonella* Newport 5561 strain that carries a similar sized type C plasmid and AMR profile as that of strain 5678 was also used as donor in some experiments. Based on sequence analysis, these plasmids are closely related to the well-studied plasmid pNF1358, and the organization of the transfer genes is similar to that of IncI plasmid R64 [62] (Genbank DQ017661.1).

Motility study

Motility of *Salmonella* 5678 and *E. coli* C600N was determined by stabbing 0.3 OD cultures (described below) into standard LB plates overlayed with 0.35% LB agar containing 0, 5, 50, 100, or 2000 μM of NE. All plates were incubated overnight at 37ºC before being read. Due to slight irregularities in the motility patterns, we measured motility in cm^2 in an attempt to attain a more accurate data reading. Additional studies were conducted with stabs into standard MIO agar (Becton-Dickinson, Franklin Lakes, NJ).

Pulse Field Gel Electrophoresis

PFGE was conducted with *Salmonella* 5678, *E. coli* C600N and selected transconjugants using standard methodologies [63]. The DNA embedded in agarose was digested with *XbaI* and electrophoresed on a CHEF DR-III instrument (Bio-Rad, Richmond, California) using the following conditions: initial switching time, 2.2 s; final switching time, 63.8 s; total time, 19 h. *Salmonella enterica* serotype Braenderup H9812 (ATCC# BAA-664) was used as the standard.

Southern Blot

DNA separated on the PFGE gel was transferred to nitrocellulose or nylon membranes as described previously [52]. A DIG labeled $bla_{\text{CMY-2}}$ probe (Roche, Indianapolis, Indiana) was created using previously described primers [47,51], and hybridization was detected using NBT/BCIP (Roche).

Determination of plasmid copy number per bacteria

Total DNA from *Salmonella* grown at 0 and 5 μM of NE at the described time points was prepared by boiling the bacteria in Tris-EDTA buffer. Chromosomally-encoded 16S rRNA gene (EUB [64] primers) and *tufA1* [65] gene (which encodes an elongation factor for synthesis of amino acid chains) were used as housekeeping genes (Table 2). Both EUB and *tufA* as housekeeping genes gave highly similar results, but only results from EUB are shown in this study. The plasmid-encoded $bla_{\text{CMY-2}}$ (β-lactamase), and *tnpA* (transposase) were used as genes of interest. The number of copies of plasmid per bacterial cell (determined by ΔΔCt method [66]) was not different when bacteria were grown at 0 or 5 μM of NE; data not shown).

Liquid mating experiments

Liquid mating experiments were conducted as previously described with some modifications [67]. The initial inocula (*Salmonella* strain 5678 and *E. coli* C600N) were grown individually for 18 h at 37°C under aerobic conditions with shaking (at 100 rpm). Overnight inocula were diluted 1:10 in fresh prewarmed LB broth free of catecholamines for approximately 2 h to attain an OD_{600} of 0.3. Cultures were mixed at the frequently used ratio of 1:5 (donor to recipient) to increase the potential mating frequencies by giving the donors more chances to donate the plasmid, and grown in static cultures at 37°C. Mixed cultures were grown in 0, 5, 50, 100, or 2000 μM of NE as previously described [32,34,35,46,68] and reflect the approximate concentrations of NE in the host GI tract under stressful conditions, which ranges from 2-50 μM [69]. Samples were collected at 0, 2, 4, 6, 8, 12, and 24 h post-mixing and were plated on HE-agar containing selective antibiotics (50 μg/ml ampicillin for donor, 12 μg/ml nalidixic acid for recipient, and 50 μg/ml ampicillin and 12 μg/ml nalidixic acid for transconjugants) [67]. Selected transconjugants were replica-plated on to ceftriaxone, cefoxitin, chloramphenicol, streptomycin, sulfisoxazole and tetracycline to ensure all the resistance markers encoded on the plasmid were transferred. These transconjugants were checked for the presence of the bla_{CMY-2} gene by PCR as previously described [50]. Conjugation efficiencies were determined by dividing transconjugant CFUs/mL by CFUs/mL of donor bacteria, as described previously [70].

Catecholamine response and antagonism assays

The adrenergic antagonists phentolamine (with equal affinities to α1 and α2 adrenergic receptors) at 200 μM concentration [57], or propranolol (with equal affinities to β1 and β2) at 500 μM concentration [56] was added to the mating mixtures at the same time as NE (0 or 5 μM) and samples were collected at 0, 2, 4, 6, and 24 h post-mixing, and the conjugation frequency

was determined as described previously [70]. All experiments were repeated from 3 to 5 times as independent biological replicates.

Gene expression studies

Salmonella was grown at 0 or 5 μM concentrations of NE with or without selected adrenergic antagonists, and collected at pre-determined time points. RNA was extracted from each bacterial sample using the Trizol according to the manufacturer's instructions (Invitrogen, Carlsbad, California), and treated twice with the Turbo DNase kit (Ambion, Austin, Texas). The resulting product was tested for DNA contamination and samples with no threshold fluorescence up to cycle 30 were considered to be DNA free. A 16S ribosomal RNA target (EUB[64]) and mRNA from *tufA1* gene [45] were used as house-keeping genes to normalize total RNA yields. qRT-PCR was performed using SuperScript III Platinum SYBR green One-Step qRT-PCR kit (Invitrogen, Carlsbad, California) on a RealPlex PCR machine (Eppendorf, Hauppauge, New York). Expression profiles of plasmid- and chromosomally-encoded genes were calculated as fold-changes using ΔΔCt method [66]. Cycling conditions for qRT-PCR included RT step for 30 min at 50°C, followed by a denaturation step at 95°C for 3 min, and 40 cycles of denaturation for 30 s at 95°C, primer annealing for 30 s at 55°C, and extension for 30 s at 72°C. On all samples, a melt curve analysis was performed in all reactions to confirm amplification of correct product size. All experiments were repeated from 3 to 5 times as independent biological replicates.

Data Analysis

Mean differences in the conjugation frequency and gene expression levels at various concentrations of NE were assessed for wild-type and mutant strains by paired t-tests performed

using statistical functions included in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California). The independent variables were NE concentrations, hours post-mixing, and the types and levels of adrenergic antagonists added. Data is presented as means ± standard error of the means (SEM), and differences were considered statistically significant when the probability of a type I error was <0.05. Further analysis was performed using a mixed effects model, with a repeated measure over hours analysis in a one-way ANOVA, and "unstructured" as the type of variance component. A Bonferroni test was used for post-hoc analysis.

Results

Effect of NE on Bacterial Growth in LB Media

Identical to previous reports [32,48,49], NE enhanced the growth of *Salmonella* and *E. coli* in iron depleted SAPI medium. However, growth of *Salmonella* and *E. coli* was not significantly different in the presence of NE when these bacterium were cultured in Luria-Bertani broth (LB), which was used throughout the study (Figure 1). LB is iron-replete (total iron concentration of 0.951 mg/L; Agilent 7500cx ICP-MS; Kansas State University-Veterinary Diagnostic Laboratory). Adding additional levels of ferric chloride with NE had no effect on growth in LB (data not shown), suggesting the effect of NE was not due to catecholamine/transferrin interaction [36].

NE increased motility of Salmonella

Previous studies have reported an increase in the motility of *Salmonella* in the presence of NE in DMEM [46]. Since LB was used throughout the present study we measured motility in LB agar. We observed similar effects with the donor *Salmonella* strain 5678 with increasing concentrations of NE in 0.35% LB agar. The greatest increase was observed at 50 μM of NE

(Figure 2). Our recipient *E. coli* strain inherently lacked ability to be motile in LB agar or MIO motility medium.

NE enhanced the ratio of horizontal gene transfer from Salmonella to E. coli

Previous experiments [32,48,49] showed NE effects in SAPI medium, and while in our initial experiments we did see enhancement of conjugation in serum-SAPI, we could not rule out if it was due to real enhancement by the NE alone or if it was due to growth enhancement of the transconjugants through the NE-transferrin interactions. LB broth was therefore used in all subsequent mating experiments. Mating in broth was also important in maintaining uniform concentrations of catecholamines, and for ease in the collection of representative samples at predetermined time points to evaluate conjugative transfer and gene expression trends. Luria-Bertani broth (LB) was used in our studies as it is a nutrient-rich medium that supports growth of both *Salmonella* and *E. coli*. LB alone did not contain any detectable levels of catecholamines (ELISA; IBL International, Hamburg, Germany); however, catecholamines added to LB during growth and mating studies remained relatively stable with only a 10% to 22% reduction in their concentrations after 24 h incubation at 37^{o}C under aerobic conditions (data not shown).

Initial experiments involved incubation of mixed cultures of the donor *Salmonella* strain 5678 and the recipient *E. coli* C600N in LB containing 0, 5, 50, 100, or 2000 μM of NE. A significant increase in the conjugation frequency was shown at 5 μM NE concentration between 2 to 6 h of mixed incubation (Figure 3). Therefore, further studies were conducted at 5 μM levels of NE. Successful transfer of plasmid was confirmed by subjecting randomly selected transconjugants to PCR analysis for $bla_{\text{CMY-2}}$ gene [50], and pulse field gel electrophoresis (PFGE) [47,51,52] followed by Southern blotting and hybridization to a previously described

bla$_{CMY-2}$ probe [47,53]. Additionally, replica-plating in Hektoen enteric agar plates for plasmid encoded antimicrobial resistance phenotypes was also performed (data not shown). Filter mating between randomly selected C600N transconjugants as donors and two *Salmonella* Typhimurium strains (LT2 [700720] and 14028) or *Citrobacter rodentium* ICC 180 as recipients (Table 1), followed by PCR analysis for *bla*$_{CMY-2}$ in the *Salmonella* and *C. rodentium* transconjugants confirmed successful transfer of the conjugative plasmid. Replica plating of randomly selected *C. rodentium* and *Salmonella* transconjugants on Hektoen enteric agar containing various antibiotics demonstrated that the plasmid encoded AMR genes were also functional in these recipients (data not shown).

Expression of plasmid encoded genes increased in the presence of NE

There was a significant up-regulation (fold-changes) of *tra* genes G, I, J, R, and Y during the first 6 h of the experiment (Figure 4), which correlated with the time-points when highest efficiencies of conjugation was observed (Figure 3). Up-regulation of chromosomally encoded *invA* (invasin) and *luxS* genes was modest (less than 2.5-fold; data not shown). In previous studies, the quorum sensing genes in *E. coli* (*qseB/C* and *qseE/F*) that belong to two-component signaling pathways have been implicated in interactions with catecholamines [40,54-56]. Levels of expression of orthologues of *qseB/C* and *qseE/F* genes in *Salmonella* 5678 (*preA/B* and *yfhk/A*, respectively) were evaluated in the presence or absence of NE. Although there was a considerable increase in the expression of *yfhK* (mean fold change of 35.2), statistical analyses revealed that they were not significant (p value 0.08; Figure 4).

Catecholamine receptor antagonists inhibited NE-induced conjugation

In previous studies [43,56,57], both α- and β-adrenergic antagonists have been shown to inhibit catecholamine-induced growth and virulence in bacteria. Phentolamine at 500 μM concentration negated the increase in conjugation frequencies observed when exposed to NE and lowered it to levels not significantly different from that of controls (Figure 5). Treatments with phentolamine alone had no significant effects on conjugation frequencies as compared to the controls (Figure 5).

Treatments with propranolol had delayed inhibitory effects on NE enhanced conjugation frequencies. Such inhibitory effects were not observed until the 4 h time-point, but continued through rest of the experimental period (Figure 5). At the 2 h time-point, conjugation frequencies with NE + propranolol treatment was not significantly different from that of treatment with NE alone; but NE treatment with or without propranolol had significantly higher conjugation frequencies as compared to NE-free controls. Treatment with propranolol alone did not significantly influence conjugative transfer (Figure 5).

Effects of adrenergic antagonists on plasmid gene expression

RNA was extracted from *Salmonella* that was treated with 5 μM NE, NE+phentolamine, phentolamine alone, NE+propranolol, or propranolol alone at all time-points when mating mixtures were plated to determine conjugation frequencies. The expression of *traI, traJ, traR and traY* genes that had shown significant increases in the presence of NE was negated to levels not different from that of NE-free medium in the presence of antagonist (Figure 6). Interestingly, the effect of NE –enhanced *traI* and *traJ* gene expression was not totally negated when the β antagonist was added.

Discussion

In this study we have presented evidence that conjugative transfer of plasmids from clinical isolate of *Salmonella enterica* serovar Typhimurium (strain 5678) to an *E. coli* recipient strain C600N was enhanced significantly in the presence of NE. The greatest effects of NE on conjugation were observed at the physiologically relevant concentration of 5 μM (during acute host-stress), and between 2 to 6 h post-exposure. Conjugative transfer of this plasmid involves complex activation sequence of approximately 30 different transfer (*tra*) genes for direct cell-to-cell mating, and we monitored expression of 7 *tra* genes G, H, I, J, M, R, and Y by qRT-PCR analysis. Significant increases in *tra* gene expression seen at 2, 4 and 6 h of NE treatment correlated with the highest levels of conjugation efficiencies. The *traI* gene, which encodes a relaxase-helicase and is the central catalytic component of the multiprotein relaxasome complex responsible for initiating conjugative DNA transfer, increased 10-fold following exposure to NE. Other transfer genes that increased in expression include those that encode TraH, which stabilizes the relaxasome structure; TraJ, which recruits the relaxasome complex to the *oriT* site; TraY, which imparts single-stranded DNA character on the *oriT* site; TraR, which is a LuxR-type quorum-sensing transcription factor; TraG, which is essential for pili assembly and mating pair stabilization; and TraM, which mediates interaction of relaxase to oriT by stimulating relaxed DNA formation [58].

Considerable controversy exists in understanding if α or β receptors are involved in the bacterial response to catecholamines. Sperandio *et al.*, have demonstrated that both α and β adrenergic antagonists, phentolamine and propranolol, respectively, caused decreases in expression of virulence genes [56]. However, Freestone *et al.*, 2007, suggest that growth and virulence were influenced only through α receptors (phentolamine exposure), as this group did

not observe any inhibitory effects when propranolol was used [57]. In the present study we demonstrate involvement of both α and β receptors, with the stronger inhibition being present with the α blocker. The β blocker had a delayed and incomplete inhibition of NE- enhanced effects on conjugative transfer. Further studies are necessary to understand the true role of the β adrenergic receptor in bacterial conjugation.

Physiological and psychological stresses of the host play an important role in incidence, duration, severity and outcome of host diseases, especially those caused by infectious agents. The significance of the present study is multifold. Previous studies have demonstrated that intra- and inter-species communication among bacteria, such as those mediated by pheromones in *Salmonella* , may influence conjugative transfer of genetic material. We have used AMR-carrying plasmids as a model in the present study as they have been implicated in the rise of many multidrug resistant bacterial strains, and evaluation of gain of resistance is easier than evaluating gain of other phenotypes such as virulence. Since conjugative plasmids with similar backbones carry virulence determinants among pathogenic bacteria, it is fair to infer that such plasmids may also be influenced by host stress. Our studies for the first time implicate the involvement of the host hormonal mediators in evolution and adaptation of bacterial pathogens in microenvironments where they are in close contact with the host. Many studies have well established the influence of acute stress in animals, as experienced during transportation, resulting in increased incidence of infectious diseases [59]. Results from this study suggest that host stress may also influence the development and rise of bacterial pathogens that are highly virulent and multidrug resistant, and the results showcase the importance of stress management to prevent illnesses caused by bacterial infections.

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Figures and Tables

Figure 2.1 Average bacterial counts at 0 to 24 h of incubation (x-axis) of donor and recipient, (on left y-axis) and transconjugants (on right y-axis) cultured in LB with 0 or 5 µM NE.

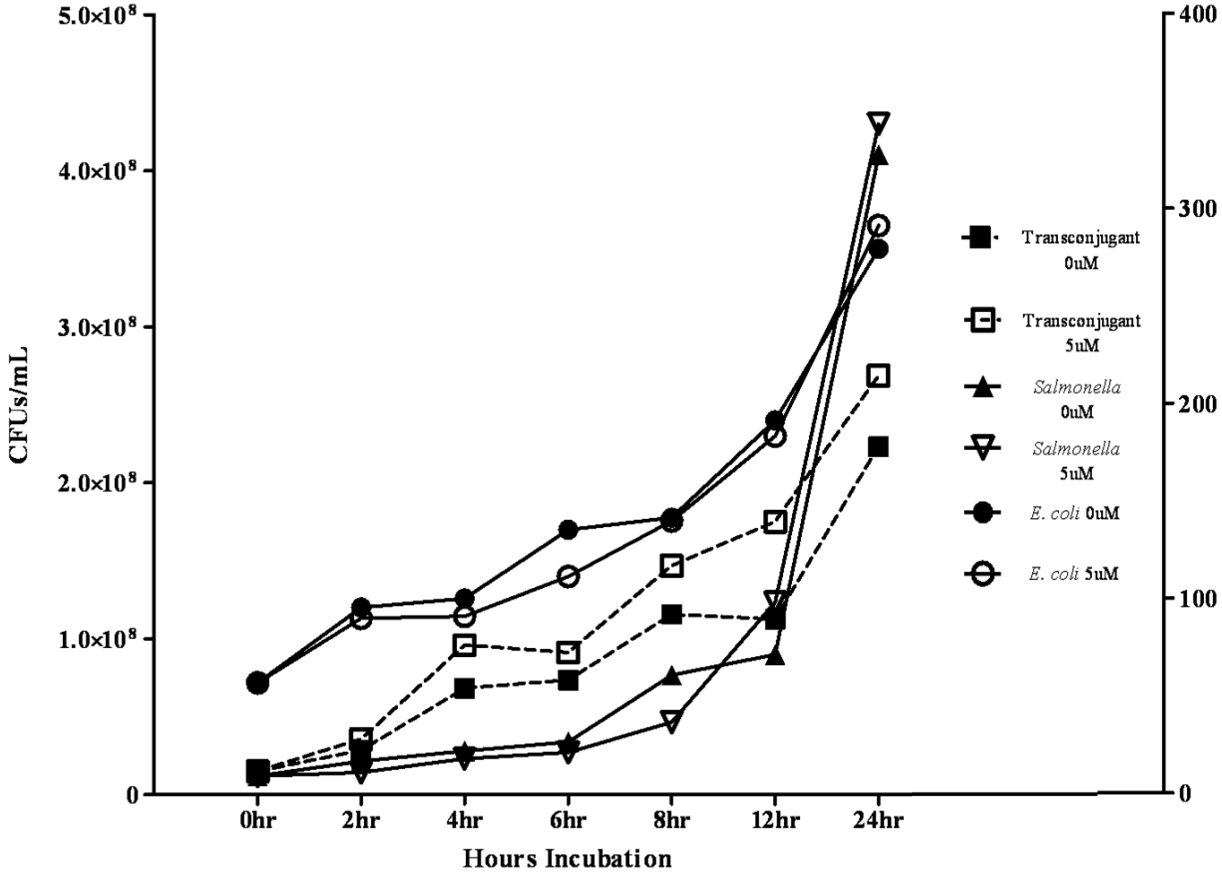

Figure 2.2 Motility of *Salmonella* 5678 in 0.35% agar with increasing concentrations of NE. Motility was measured in cm^2. Error bars represent standard error of the means.

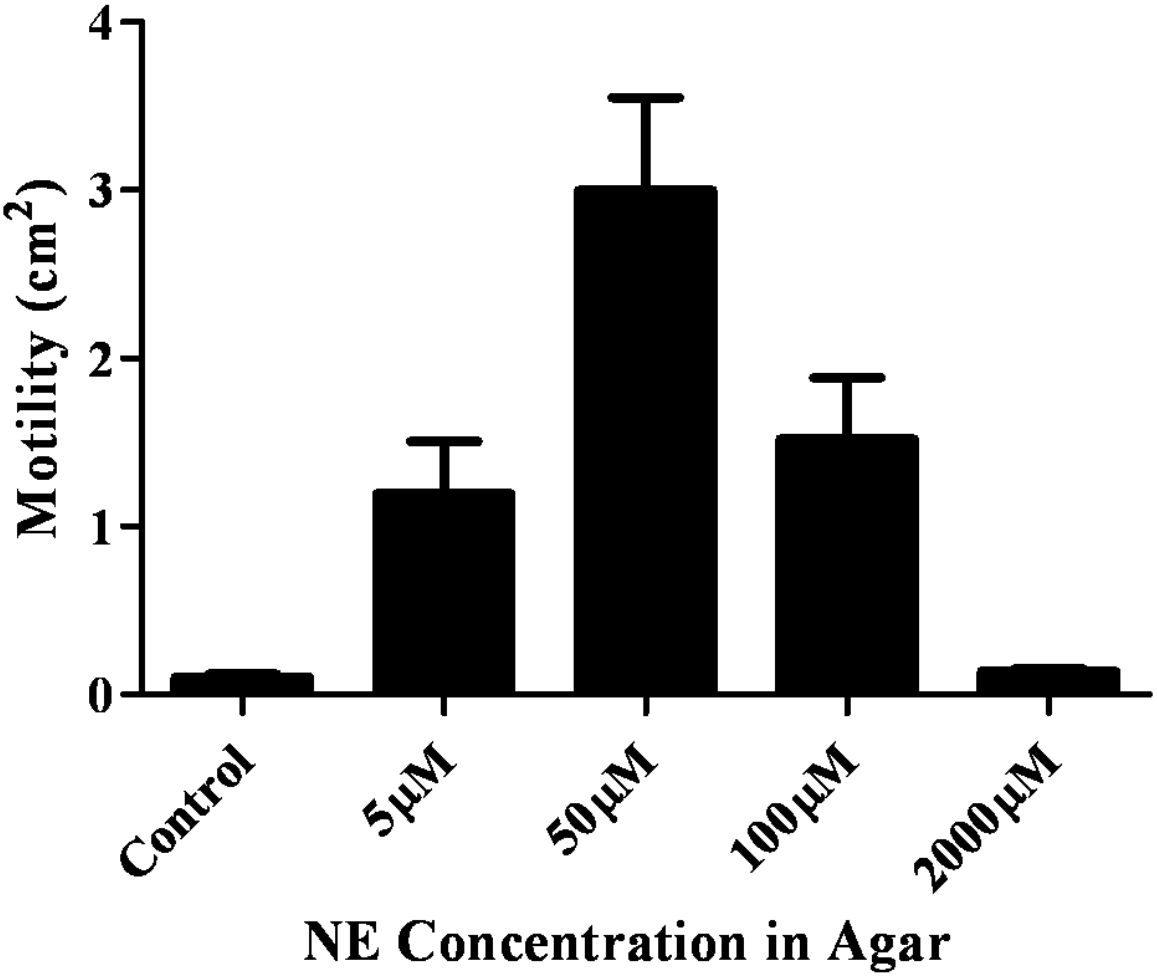

Figure 2.3 Conjugation frequencies increased when *Salmonella* 5678 and *E. coli* C600N were co-cultured in the presence of 5 µM NE compared to no-NE control. Significant (p value ≤ 0.05) increases (indicated by an *) were seen at 2, 4, and 6 h. Error bars represent standard error of the means.

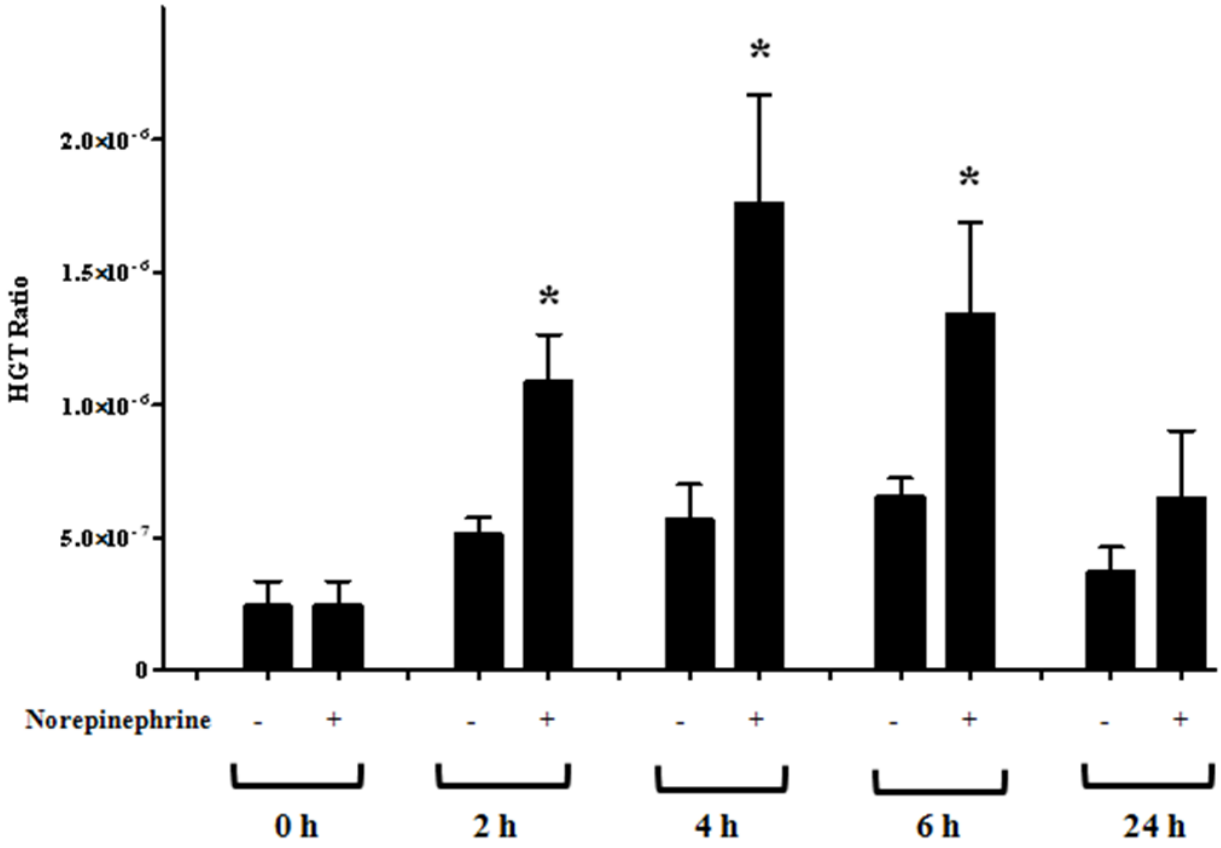

Figure 2.4 Activation (expressed as fold change) of *tra* and quorum-sensing genes in *Salmonella* 5678 between 2-6 h post-NE treatment compared to no-NE control. Significant increases (p value ≤ 0.05; indicated by an *) were observed in *traG*, *traI*, *traJ*, *traR*, and *traY*. Error bars represent standard error of the means.

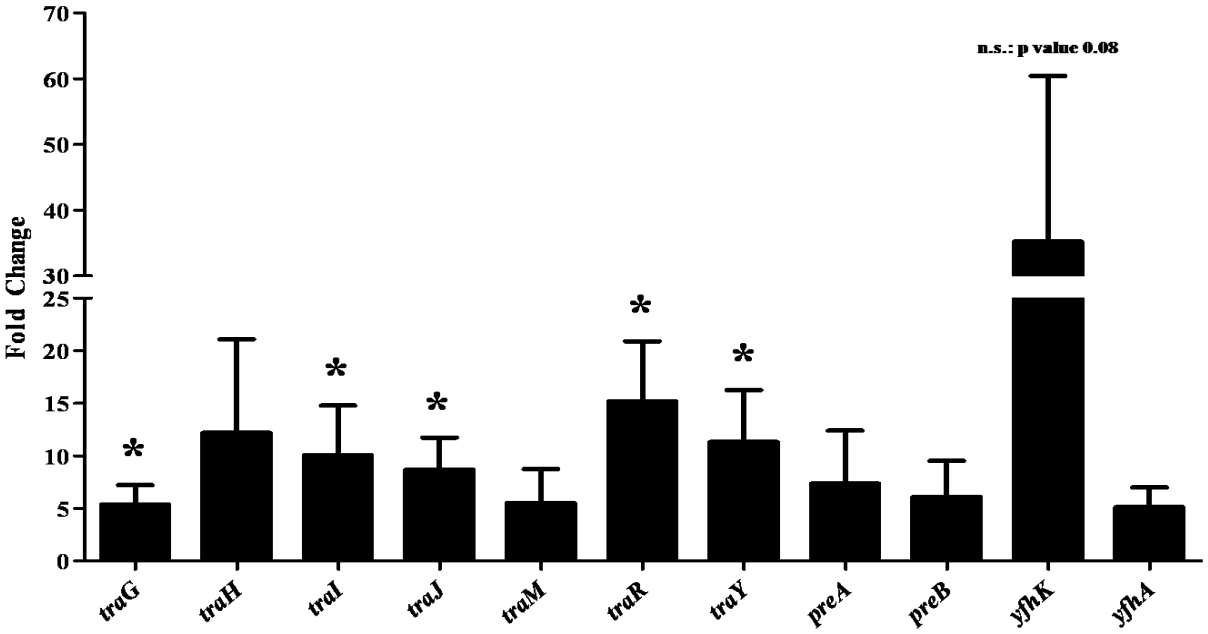

Figure 2.5 Conjugation frequencies between *Salmonella* 5678 and *E. coli* C600N in the presence of NE with or without phentolamine (alpha adrenergic blocker) or propranolol (beta adrenergic blocker). All treatments were compared to untreated controls at their respective hours and significant increases (p value ≤ 0.05) were indicated by an *. No significant difference (indicated by NS) in conjugation efficiency was observed with 5 µM NE and 5 µM NE + propranolol at 2 h. Error bars represent standard error of the means.

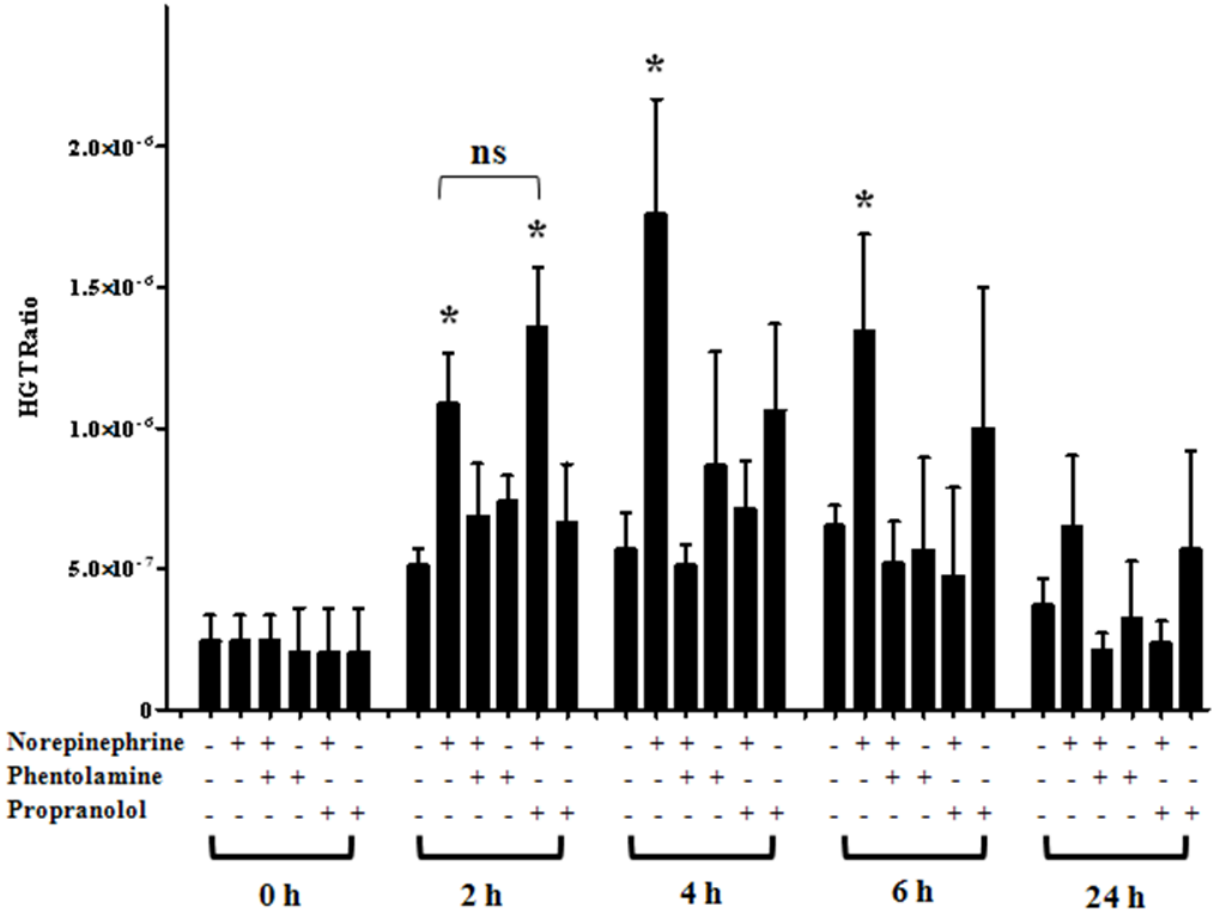

Figure 2.6 Average *tra* gene expression (fold change) between 2-6 h in *Salmonella* 5678 treated with 5 µM NE with or without phentolamine (α adrenergic blocker) or propranolol (β adrenergic blocker). Figure 6a shows results for *traM*, 6b for *traY*, 6c for *traI*, and 6d for *traR*. All treatments were compared to untreated controls, and significant (p value ≤ 0.05) increases are indicated by an *. Error bars represent standard error of the means.

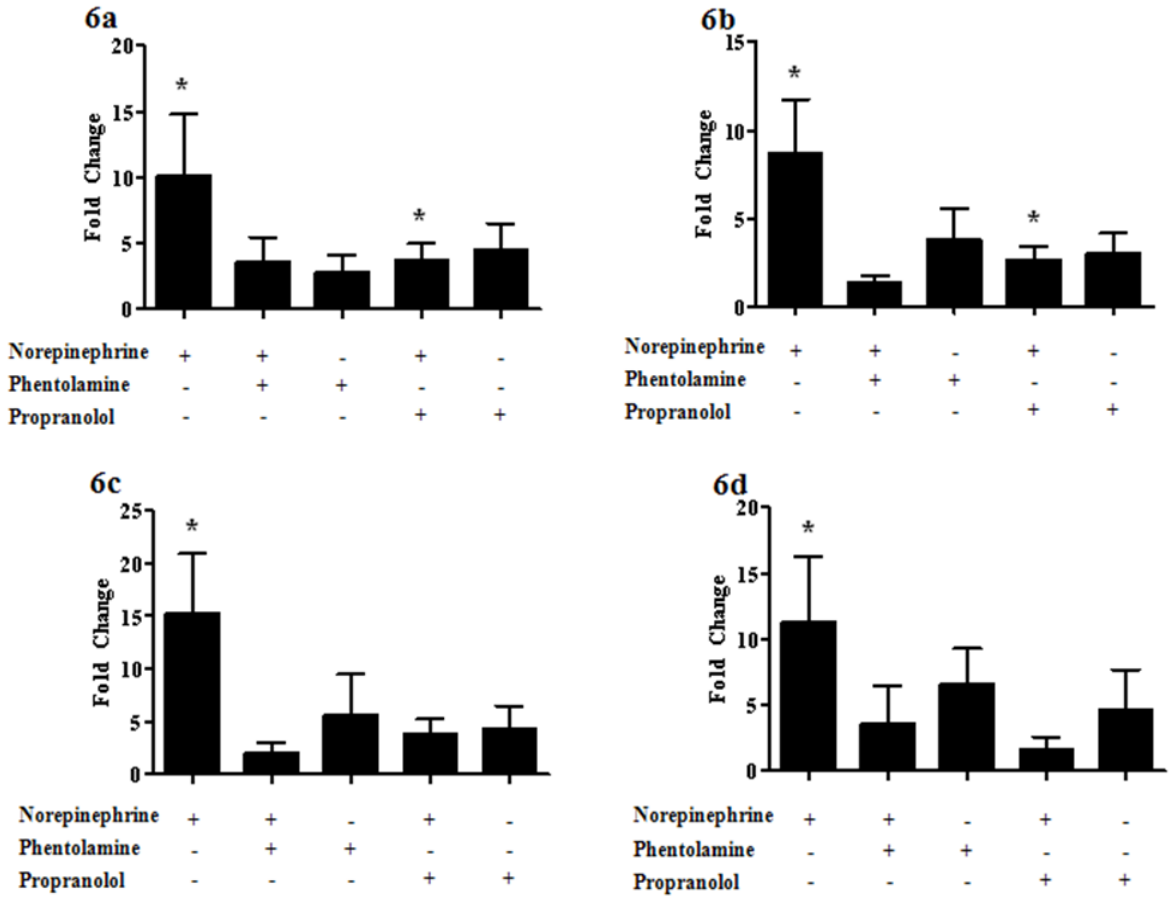

Table 2.1 List of isolates used in this study.

Table 1: List of isolates used in this study.

Strain	Species	AMR Phenotype	Plasmid Type	Reference
5678	*Salmonella enterica* Typhimurium	ACSSuTCroFx	A	[47]
5561	*Salmonella enterica* Newport	ACSSuTCroFx	C	[47]
C600N	*E. coli*	Nal	N/A	[47]
MG1655N	*E. coli*	Nal	N/A	[71]
ICC 180	*Citrobacter rodentium luxCDABE*	Nal, K	N/A	[60,61]
14028	*S. enterica* Typhimurium	UKN	N/A	(ATCC# 14028)
LT2	*S. enterica* Typhimurium	UKN	N/A	(ATCC# 700720)

Abbreviations: A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfisoxazole; Sxt, trimethoprim-sulfamethoxazole; T, tetracycline; K, kanamycin; Cro, ceftriaxone; Fx, cefoxitin, N, nalidixic acid

Table 2.2 Complete list of primers used in this study.

Table 2: Complete list of primers used in this study.

Primer	5' to 3' Sequence	Product Size	Reference
Antimicrobial Resistance			
tnpA F	CATCAAGAAGGTGCGTCAAA	87 bp	Present Study
tnpA R	TAATTCGTCGCAAAATGCAA		
bla_{CMY-2} F	GACAGCCTCTTTCTCCACA	101 bp	[50]
bla_{CMY-2} R	GAATAGCCTGCTCCTGCATC		(Shortened)
Virulence			
invA F	TGCCTACAAGCATGAAATGG	437 bp	[72]
invA R	AAACTGGACCACGGTGACAA		
Quorum Sensing			
luxF F	TTGCAAAAACGATGAACAC	444 bp	[73]
luxF R	AAGACTAAATATGCAGTT C		
luxS F	GTCGACGCCGCTGATACCGAACCG	178 bp	Present Study
luxS R	GTCGACGCGGTGCGCACTAAGTACAA		
preA Sal F	AAAGCGGGCCTGAGTAAAAT	175 bp	Present Study
preA Sal R	CCGGTTCCTGTTTACCCTTT		
preB Sal F	CGACAATGGCTATCTGAAGG	86 bp	[46]
preB Sal R	CGGTAATCCCACTCCTGAC		
yfhK RTF	CGCGCCATGATCTTCGA	61 bp	[54]
yfhK RTR	CCCTTCACCGCCCCTTT		
yfhA RT F	CGCCCCGCCATTCTC	58 bp	[54]
yfhA RT R	CGTAAGCTGCTGCAAATTACCA		
Transfer Genes			
traG F	CTGTCCATAACGACGGGTTC	164 bp	Present Study
traG R	TCGGATAAAAGCGGAATCAC		
traH F	GGACGTGAAGGTTGACTGGT	109 bp	Present Study
traH R	GACTGGGAAGGTGATGCAAT		
traI F	TTGTCTTCCTTCCTGCCATC	163 bp	Present Study
traI R	TGAACGCTTTGTCAGCAATC		
traJ F	GCTTTACGACCACCGTCATT	98 bp	Present Study
traJ R	CCTGTCATCAGGGATTCGAT		
traM F	AATATTCGCGCTCCACATTC	126 bp	Present Study
traM R	AACAGCGGGCAAATAATGTC		
traR F	TCGACATTGCGAACCATATC	103 bp	Present Study
traR R	GCCGGAGCAAACTGACTAAG		
traY F	TGCGACGAAACTCAGTATGC	153 bp	Present Study
traY R	GGAAGCATGTTCTGGGTGTT		
Positive Controls			
EUB F	TGGAGCATGTGGTTTAATTCGA	161 bp	[64]
EUB R	TGCGGGACTTAACCCAACA		
tufA1 F	TGATGACGAAGAGCTGCTGGAACT	146 bp	[65]
tufA1 R	CTTTCAGACCAGAACCACGAACGA		

Chapter 3 - Bimodal Distribution Pattern of Conjugative Gene Transfer Ratios pre- and post-MIC

Abstract

The widespread use of antimicrobials in medicine as well as in food production has resulted in pathogenic bacteria becoming resistant to the antimicrobials used to treat them. Antimicrobial resistance (AMR) can result from horizontal gene transfer (HGT) through plasmids and transposons. The exact influence of the antimicrobial concentration on HGT through direct cell-to-cell conjugation is, however, not clearly defined. The objective of this study was to address this deficiency by quantitatively characterizing the efficiencies of conjugation in two mating pairs of enteric bacteria during exposure to concentrations above, equal to and below the minimum inhibitory concentration (MIC) under *in vitro* conditions. The first mating pair consisted of a donor *E. faecalis* INY1010 which transferred a conjugative transposon Tn925 to a recipient *E. faecalis* OG1RF, and the second pair involved the transfer of a 100kb conjugative plasmid from *Salmonella* Typhimurium 5678 to a recipient *E. coli* C600N. Broth mating were performed in the presence of increasing concentrations of tetracycline or oxytetracycline, respectively, and compared to antimicrobial-free medium. Conjugation occurred in all concentrations, but efficiencies of transfer were consistently low in 0 MIC and 1 MIC, with increased activity both above and below 1 MIC. Expression of plasmid encoded transfer (*tra*) genes was significantly upregulated in response to the lowest and highest MICs when *Salmonella* and *E. coli* were co-cultured, but were only upregulated in the highest concentrations when *Salmonella* was cultured alone. A previously described mathematical model was fit to these data and the rate constant E that relates the rate of gene transfer to drug concentration was determined. The *in vitro* data showed highly similar patterns of conjugation

efficiencies when compared to the rate constant E. This study provides important insight in defining the role of antimicrobial concentration on conjugation efficiencies and may provide future insight into better control of AMR.

Introduction

Bacteria are asexual organisms that reproduce by binary fission, resulting in the genetic replication of one cell into two daughter cells. This type of reproduction produces genetically identical organisms that have equal susceptibilities to environmental pressures, be it antimicrobial or otherwise (29). Over time, systems utilizing transformation, transduction and conjugation have evolved to diversify the genome allowing for faster adaptation to environmental changes, thereby reducing exclusive dependence of bacteria on random beneficial genomic mutations (34, 41). As a consequence, horizontal transmission of resistance elements is considered the predominant mode for the dissemination of bacterial resistance (6) and is accomplished through the efficient movement of genetic elements including plasmids, transposons, and bacteriophages (40, 41).

The most efficient method of HGT is through conjugation. Conjugation is mediated by the physical adherence of donor and recipient cells and the subsequent transfer of genetic elements into the recipient cell (2, 29). These genetic elements allow for high efficiency transfer of antimicrobial and virulence genes from single resistant donor bacterium to many recipients which can in turn donate the resistance genes to more recipients (41). Horizontal transfer by conjugation is not exclusive to bacteria of the same species. This is exemplified by the occurrence of vancomycin resistant *Enterococcus* (VRE) and vancomycin resistant *Staplococcus aureus* (VRSA) in ecosystems where they coexist (11, 12, 20, 24, 35-37, 42). Conjugative HGT has been shown to be not exclusive between bacteria either. Several studies have shown

successful transfer of genetic elements by conjugation between bacteria to yeast (18), bacteria to plant cells (45), and recently bacteria to mammalian cells (46). This transfer is important to the survival and evolution of many bacterial species (41), and has allowed for high efficiency transfer of AMR and virulence genes between bacteria which has had a considerable impact on human and animal health (44).

Gastrointestinal tracts of both humans and animals are a major habitat of *Salmonella*. In 2005, over 36,000 clinical cases of Salmonellosis were reported to the CDC (8). Of these, 15,000 resulted in hospitalization and 400 were fatal (17). In 2007, the number of cases reported in a 10 U.S. state survey was 6,790 and the incidence per 100,000 population was 14.92 (7). Salmonellosis can also cause severe enteritis, decreasing weight gain and reproductive performance in livestock and thereby has a significant economic impact (1). Recent studies have identified plasmid carrying *Salmonella* strains that contain many different AMR genes that are capable of conjugative HGT to recipient strains of *E. coli* (5, 15).

The objective of this study was to determine the conjugative transfer efficiencies of genetic elements of two pathogenic bacterial mating pairs under *in vitro* conditions following antimicrobial exposure, and to fit these date ot a mathematical model to aid in future AMR research (14).

Materials and methods

Bacterial strains

Enterococcus faecalis INY1010 is a clinical isolate that was used as a donor in the first set of mating experiments (21). This strain carries a conjugative transposon Tn925 that highly similar to the well-characterized Tn916 (21, 22) that provides resistance to tetracycline (4). The

recipient was *Enterococcus faecalis* OG1RF which contained chromosomal mutations that provided resistance to rifampicin and fusidic acid (31) (Table 1).

Salmonella Typhimurium strain 5678 (15) was used as the donor strain in the second set of mating experiments. This strain carries a type A plasmid that is approximately 100 kb in size, and is transferable by conjugation to the recipient *E. coli* C600N (a spontaneous nalidixic acid resistant mutant of strain C600 (15); kindly provided by Dr. Paul Fey at University of Nebraska Medical Center, Omaha, Nebraska). This plasmid contains a $bla_{\text{CMY-2}}$ gene that encodes resistance to a large spectrum of β-lactams including ampicillin, ceftriaxone and cefoxitin. This plasmid also contains resistance markers for chloramphenicol, streptomycin, sulfisoxazole, trimethoprim-sulfamethaxozole and tetracycline (Table 1). Based on sequence analysis, this plasmid was closely related to the well-studied plasmid pNF1358, and the organization of the transfer genes was similar to that of IncI plasmid R64 (23) (Genbank DQ017661.1).

MIC determination

The minimum inhibitory concentration of tetracycline or oxytetracycline for donor and recipient strains was determined using a slight modification of micro-broth dilution method recommended by CLSI (30). Briefly, 10 µL of a 0.5 McFarland bacterial suspension was pipetted into 11 mL of Luria-Bertani (LB) broth. Aliquots of the bacterial suspension (100 µL) was added to the wells of a 96-well plate containing 100µL of increasing concentrations of tetracycline or oxytetracycline (Sigma, St. Louis, MO) in duplicates, for donor and recipient strains. The 96-well plates was placed in an incubated (37^{o}C) spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA) set to read absorbance at 600 nm with readings taken hourly. The MIC was the lowest concentration at which optical density readings was reduced by

60% (25). The MIC of tetracycline for the *E. faecalis* INY1010 donor and *E. faecalis* OG1RF recipient strains were 125 µg and 2 µg/ml, respectively. The MICs of *Salmonella* 5678 donor and *E. coli* C600N recipient strains for oxytetracycline were determined to be 60,000 and 62.5 ng/ml, respectively.

Pulse Field Gel Electrophoresis

PFGE was conducted with *Salmonella* 5678, *E. coli* C600N, and selected transconjugants using standard methodologies (33). The DNA embedded in agarose was digested with *XbaI* and electrophoresed on a CHEF DR-III instrument (Bio-Rad, Richmond, CA) using the following conditions: initial switching time, 2.2 s; final switching time, 63.8 s; total time, 19 h. *Salmonella enterica* serotype Braenderup H9812 (ATCC# BAA-664) was used as the standard (data not shown).

Southern Blot

DNA from the PFGE gel was transferred to nitrocellulose or nylon membranes as described previously (38). A DIG labeled $bla_{\text{CMY-2}}$ probe (Roche, Indianapolis, IN) was created using previously described primers (5, 15), and hybridization was detected using NBT/BCIP (Roche) (data not shown).

Liquid mating experiments

Broth cultures were used in all mating experiments to maintain uniform concentrations of antimicrobials, and for ease in the collection of representative samples at predetermined time points to evaluate HGT and gene expression trends. All *Enterococcus* matings were performed

in static Brain Heart Infusion (BHI) broth and all *Salmonella* to *E. coli* matings were conducted in static Luria-Bertani (LB) broth. *E. faecalis* INY1010 and *E. faecalis* OG1RF were inoculated into separate tubes and grown overnight (12-18 h) at 37^{o}C with shaking. Inocula were mixed at a 1:1 ratio for a total volume of 100 ml and incubated in 0x, 0.5x, 1x, 2x, 4x, 8x and 16x MIC of tetracycline. Samples were collected at 1, 2, 4, 6, and 8 days post-exposure and plated on Tryptic Soy Agar (TSA) plates containing selective antibiotics (30 µg/mL tetracycline for donor, 50 µg/mL rifampicin for recipient, and 30 µg/mL tetracycline and 50 µg/mL rifampicin for transconjugants). Selected transconjugants were checked for presence of transposon by *tetM* PCR amplification (data not shown).

Liquid mating experiments between *Salmonella* and *E. coli* were conducted as previously described with some modifications (26, 32). The initial inocula (*Salmonella* strain 5678 and *E. coli* C600N) were grown individually for 18 h at 37°C under aerobic conditions with shaking (at 100 rpm). Overnight inocula were diluted 1:10 in fresh prewarmed LB broth free of antimicrobials for approximately 2hrs to attain an OD 600 of 0.3. Cultures were mixed at a ratio of 1:5 (donor to recipient) oxytetracycline was added to the mixed cultures at 0, 0.25, 0.5, 1, 1.5, 2, 4, 8, 16, 32, and 64x recipient MIC levels, and incubated at 37^{o}C.under aerobic conditions. Samples were collected at 0, 2, 4, 6, 8, 10, 12, and 24hrs post-mixing and were plated on HE-agar containing selective antibiotics (50µg/ml ampicillin for donor, 12 µg/ml nalidixic acid for recipient, and 50µg/ml ampicillin and 12µg/ml nalidixic acid for transconjugants) (26). Selected transconjugants were replica-plated on to ceftriaxone, cefoxitin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline to ensure all the resistance markers encoded on the plasmid were transferred. These transconjugants were checked for the presence of the $bla_{\text{CMY-2}}$ gene by PCR as previously described (50). HGT ratios were determined by dividing transconjugant CFUs/mL

by CFUs/mL of donor bacteria, as described previously (22). All antimicrobials were purchased through Sigma-Aldridge, St. Louis, MO, and all media was purchased through Fisher Scientific, St. Louis, MO.

Gene expression studies

RNA was extracted from *Salmonella* and *E. coli* together, or *Salmonella* alone grown at 0 through 64X MIC of oxytetracycline using the Trizol (Invitrogen, Carlsbad, CA). The RNA was treated with DNase treatment kit (Invitrogen, Carlsbad, CA) to remove residual DNA to acquire samples with no threshold fluorescence before cycle 30 in a SYBR Green qPCR reaction. A 16S ribosomal RNA target, EUB (49), was used as a house-keeping gene to normalize total RNA yields. Quantitative RT-PCR was performed on listed primer sets (Table 2) using SuperScript III Platinum SYBR green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) on a RealPlex PCR machine (Eppendorf, Hauppauge, NY). Expression profiles of plasmid and chromosomally encoded genes (calculated as fold-changes using $\Delta\Delta C_t$ method) are summarized in Figure 2. A melt curve analysis was performed following all PCR to confirm a single amplicon of adequate size.

Mathematical Modeling of Mating Pair Data

The number of bacteria (CFU/mL) from each time point and condition were organized by donor, recipient and transconjugant for each concentration and time point. These files were uploaded to a Susceptible-Infected-Recovered (SIR) mathematical model (14) designed in acslX modeling platform (AEgis Technologies, Huntsville, AL). Variables including drug concentration, carrying capacity, and starting CFUs/mL were inputted into the model. Outputs

from each run including the E value (rate constant that relates the rate of gene transfer to the drug concentration) and standard deviations (how well the *in vitro* data fit to the model) were considered for each run, and the E values and predicted transconjugant CFUs/mL were compared by observed value by linear regression analysis (Figure 6;7).

Data Analysis

Mean differences in the ratio of conjugative frequencies and gene expression levels at various concentrations of antibiotics were assessed by paired t-tests performed using statistical functions included in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). The independent variables were antimicrobial concentrations, hours post-mixing, and the types and levels of adrenergic antagonists added. Data was presented as means ± SEM, and differences were considered statistically significant when the probability of a type I error was <0.05. Further analysis was performed using a mixed effects model, with a repeated measure over hours analysis in a one-way ANOVA, and "unstructured" as the type of variance component. A Bonferroni test was used for post-hoc analysis.

Results

Inhibition of recipient in the presence of antimicrobial

In the *Enterococcus* mating pair experiments there was a dose-dependent decrease in the CFUs/mL of the OG1RF recipient population as the tetracycline concentrations increased (Figure 1a). This differed from the donor INY1010 population which increased in response to antibiotic pressure (Figure 1b). In the *Salmonella* to *E. coli* mating pair, the recipient *E. coli* population also responded in a dose-dependent decrease in CFUs/mL as oxytetracycline

concentrations were increased (Figure 1c). Contrary to the *E. faecalis* INY1010 donor population, the *Salmonella* population remained relatively consistent in response to increasing concentrations of oxytetracycline (Figure 1d).

Bimodal pattern present when conjugative transfer ratios were measured across increasing MICs

Resistance gene transfer occurred in both the *Enterococcus* (Figure 2) and the *Salmonella* to *E. coli* (Figure 3) mating pairs. Resistance gene transfer occurred in the populations exposed to all the concentrations of antimicrobials, but the efficiency of transfer varied between antibiotic concentrations. In both of these pairs, the resistance gene transfer was consistently low in populations exposed to 0x MIC and 1x MIC. Exposure to sub-inhibitory (0.25x – 0.5x MIC) and supra-inhibitory (1.5x – 64x MIC) concentration of antimicrobials had increased numbers of transconjugants and higher efficiencies of conjugation, producing a bimodal distribution pattern (Figure 2, 3). The experiments were repeated at least 10 times. Successful transfer of plasmid was confirmed by subjecting randomly selected transconjugants to PCR analysis of $bla_{\text{CMY-2}}$ gene; pulse field gel electrophoresis (PFGE) (5, 15, 38) followed by Southern blotting and hybridization to a $bla_{\text{CMY-2}}$ gene region probe; as well as by replica-plating in Hektoen enteric agar plates for plasmid-encoded antimicrobial resistance phenotypes (data not shown). Filter mating between randomly selected C600N transconjugants as donors and *Citrobacter rodentium* ICC180 or two *Salmonella* Typhimurium strains (LT2 700720 and 14028) as recipients (Table 1), followed by PCR analysis for $bla_{\text{CMY-2}}$ in the *C. rodentium* and *Salmonella* transconjugants, and plating in appropriate antimicrobial-containing plates confirmed successful transfer of the conjugative plasmid (data not shown).

Transfer gene expression when exposed to antimicrobials

Two plasmid transfer (*tra*) genes present on the *Salmonella* plasmid were selected for analysis based on their ability to upregulate in the presence of catecholamines, as shown in a previous study (32). There was a significant up-regulation (fold-changes) of plasmid-encoded *tra* genes I and Y in the presence of antimicrobials (Figure 4), which correlated with the sub- and supra-MIC concentrations of oxytetracycline and time-points when highest levels of HGT occurred (Figure 4). Interestingly, when *Salmonella* alone was incubated with the increasing concentration of oxytetracycline, the significant upregulation of genes only correlated with the post-MIC concentrations. Additional *tra* genes J and R were tested and the trend was the same (Figure 5). There was found to be a dose-dependent correlation (as determined by r^2 values) between fold changes and antimicrobial concentrations both pre and post the MIC when *Salmonella* was co-cultured with *E. coli* and correlation above the MIC when *Salmonella* was cultured alone.

Use of a mathematical model for analysis

The data acquired during the *Salmonella* to *E. coli* mating experiments were fitted to a mathematical model (14) and the conjugation efficiencies in increasing concentrations of oxytetracycline were determined. The same bimodal pattern observed in Figure 3 was again found when E values (rate constant that relates the rate of gene transfer to the drug concentration) were compared by linear regression (deviation was significantly above 0, p value >0.001) to observed conjugation ratios (Figure 6). Predicted vs. observed CFUs/mL of transconjugants at all concentrations were also analyzed by linear regression, and all showed

significant (p value < 0.001) deviation from zero (Figure 7). Additional analysis of donors and recipient populations by linear regression analysis also demonstrated significant (p value <0.001) deviations from zero at all concentrations (data not shown).

Discussion

Perhaps most interesting observation in this study was that there were bimodal effects on conjugative transfer efficiency ratios across the increasing concentrations of antimicrobials surrounding the 1 MIC level (Figures 2; 3). A common trend in bacteria is that pre-MIC levels of antimicrobials cause an increase in mutation rates as well as increases in the efficiencies of HGT of the susceptible recipient bacteria (3, 10, 19). The pre-MIC side of the bimodal curve was expected and was believed to be reflective of this. For the conjugation increases post-MIC we hypothesized that the increase in conjugation was another pre-MIC effect, this time for the resistant donor bacteria. This is supported in the literature with two past studies in *Enterococcus*. In these experiments, pre-incubation in sub-MIC concentrations of tetracycline of a donor *E. faecalis* population containing transposons Tn916 and Tn925 were shown to enhance conjugation efficiencies up to 119-fold when the donors they were added to the recipient *B. thuringiensis* bacterial cultures (39, 43).

Additional support for this hypothesis was gained through the *tra* gene expression data. Conjugative transfer of the Type A plasmid involves complex activation sequence of approximately 30 different transfer (*tra*) genes for direct cell-to-cell mating (16). We monitored expression of two *tra* genes I and Y by qRT-PCR analysis. RNA was collected from a mixed culture of *Salmonella* and *E. coli* and the *traI* gene (encoding a relaxase-helicase and is the central catalytic component of the multiprotein relaxasome complex responsible for beginning the conjugative DNA transfer) showed significant upregulation in both the pre- and post- MIC

concentrations of oxytetracycline (Figure 4a). The same bimodal pattern was found for *traY*, which imparts single-stranded DNA character on the *oriT* site (Figure 4b).

To support the hypothesis that there was a separate pre-MIC effect of the oxytetracycline on the *Salmonella* donor population, *Salmonella* was incubated alone and gene expression of *traI* and *traY* were monitored. The gene expression data indicated a high amount of post-MIC *traI* and *traY* upregulation when *Salmonella* was incubated alone with increasing amount of oxytetracycline (Figure 5a;b). Additional transfer genes were quantified including *traJ* (Figure 5c), (recruits the relaxasome complex to the *oriT* site) and *traR* (a LuxR-type quorum-sensing transcription factor; Figure 5d) which both showed the same pattern of increased gene expression in the higher concentrations of oxytetracycline. Based on the gene expression study data, it appears that the increase in the HGT in both the pre- and post- MICs may be due to pre-MIC effects of both the recipient and the donor.

In order to determine if the bimodal pattern of AMR acquisition was unique for tetracycline treatment, an additional study was performed using the *Salmonella* to *E. coli* mating pair in the presence of 0, 0.25, 0.5, 1, 2, 4, 8, and 16X MIC of ceftiofur (MIC of ceftiofur for *Salmonella* donor and *E. coli* C600N was 10,000 and 100 ng/ml, respectively). Preliminary results showed a similar bimodal pattern of AMR acquisition was demonstrated with ceftiofur treatment as well (data not shown).

Dynamics of any system changes as the number of variables are increased. In the present study we considered the each separate bacterial population's growth and death, horizontal transfer of genetic element between them, the individual time points of the experiment, and the influence of increasing concentrations of antimicrobials. When the susceptible population of *E. faecalis* OG1RF and *E. coli* C600N were cultured alone, their growth was visibly inhibited in

increasing levels of antimicrobials with the smallest inhibitory concentration being the MIC. When the recipients were co-cultured with the donor strains (*E. faecalis* INY1010 or *Salmonella* 5678, respectively), inhibition is observed (Figure 1a, 1c), however, recipients are still positively identified at the highest MIC concentrations (16x for *E. faecalis* OG1RF and 64x for *E. coli* C600N) perhaps due to some protective effects by the donor strains. Some of these potential protective effects may be explained by a recent study by Lee *et al*, 2010 (25). In this study, protective effects from indole were instrumental in increasing the MIC of a susceptible *E. coli* population when they were exposed to antimicrobials.

There was an interesting growth dynamic with the donor population as well. In *Enterococcus,* the growth of the donor population increases in response to the increase of antimicrobials, or perhaps due to the decrease in the recipient population while the *Salmonella* population remains relatively stable in most antimicrobial concentrations, only showing inhibition at 64x MIC.

The many factors (with varying degrees of knowledge of their true effects) that contributed to the results in this study are, in a sense, only a superficial glimpse at the bigger picture. Undoubtedly if we were to change key factors (donor to recipient ratio, carrying capacity, efficiency of transfer, etc.) the dynamics of the entire system would change as well. In future studies it may be useful to run experiment of this nature, but the amount of time and resources required might be limited. In order to save resources in future work, we utilized the data acquired in the *Salmonella* to *E. coli* mating experiments to set up parameters in a previously developed SIR computer model (14). Mathematical models can be defined as conceptual models that use mathematical language to represent a particular context. As technology and resources become more readily available and user-friendly, mathematical models

are being used more frequently to answer complex questions in the biomedical sciences. The quantitative nature of mathematical models offers numerous advantages over other conceptual models. The use of mathematical language ensures precision in the description of hypotheses and assumptions. Also, it facilitates the logical manipulation of statements that can readily be updated as our knowledge of the subject increases and evolves. Finally, a mathematical model provides quantitative conclusions that can be compared with measurements taken from real life. They are particularly useful in cases where a specific numerical outcome is needed such as a dosage regimen for drug administration (27, 28). This model incorporated the SIR format in defining the interactions between the donor and recipient strains with the subsequent development of transconjugant bacteria (14). Using these data, the model simulated the same bimodal pattern as demonstrated in the *in-vitro* studies with the conjugation frequency increases (measured as E, or transfer efficiency values) pre- and post-MIC (Figure 6). With this model we now have the capability of predicting conjugation efficiencies at a variety of conditions including carrying capacity, starting concentrations of antimicrobials, starting populations of donors, recipients and transconjugants, hours incubated, and effectiveness of antimicrobials used.

In this study we provide evidence that the concentration of antimicrobials influences the efficiencies of conjugation in an *Enterococcus* INY1010 to *Enterococcus* OG1RF and *Salmonella* to *E. coli* mating pair. This study for the first time reports significant activity in supra-MIC concentration and through gene expression analysis attempts to interpret this occurrence. The mathematical modeling systems developed here for analysis add a valuable tool for measuring conjugation frequencies in real-time. These results demonstrate the complexity of the interactions between donor and recipient bacteria as environmental factors are altered. Future investigation will further define additional factors affecting conjugative HGT.

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Figures and Tables

Figure 3.1 Growth curves (CFUs/mL) of donor and recipient populations throughout the experiments at increasing concentrations of antimicrobials. Growth of *E. faecalis* mating pair OG1RF (1a) and INY1010 (1b) shows population change over 8 days. Growth of *E. coli* C600N (1c) and *Salmonella* 5678 (1d) mating pair shows growth over 24 hours.

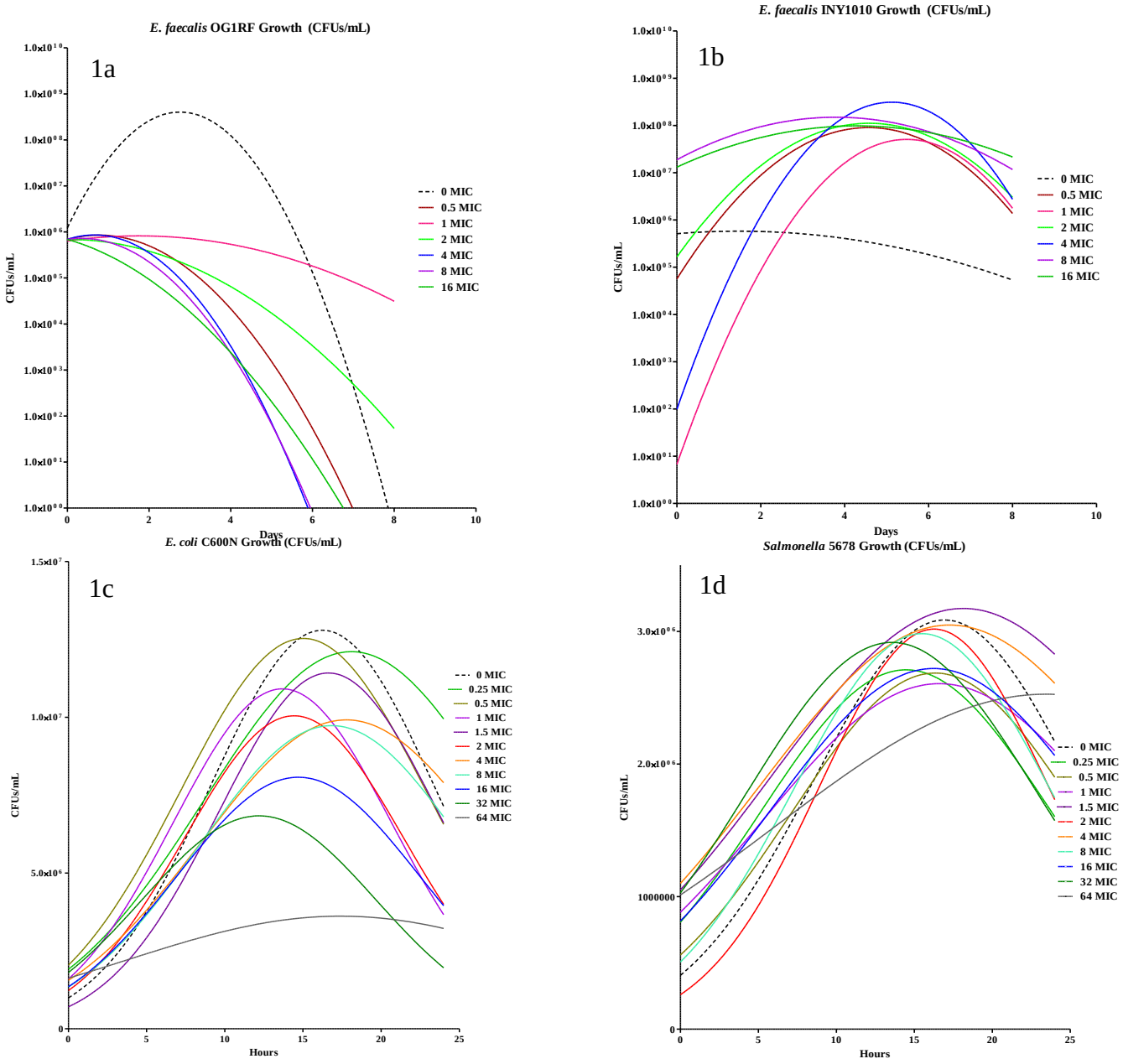

Figure 3.2 Conjugation efficiencies (measured as log ratios) of conjugative Tn925 between *E. faecalis* strains INY1010 and OG1RF at increasing concentrations of tetracycline. Each bar represents al HGT reads over the course of the 8 day experiment. An * indicates an increase significant at p value <0.05, and ** indicates significant increase at p value <0.01 compared to 0 MIC.

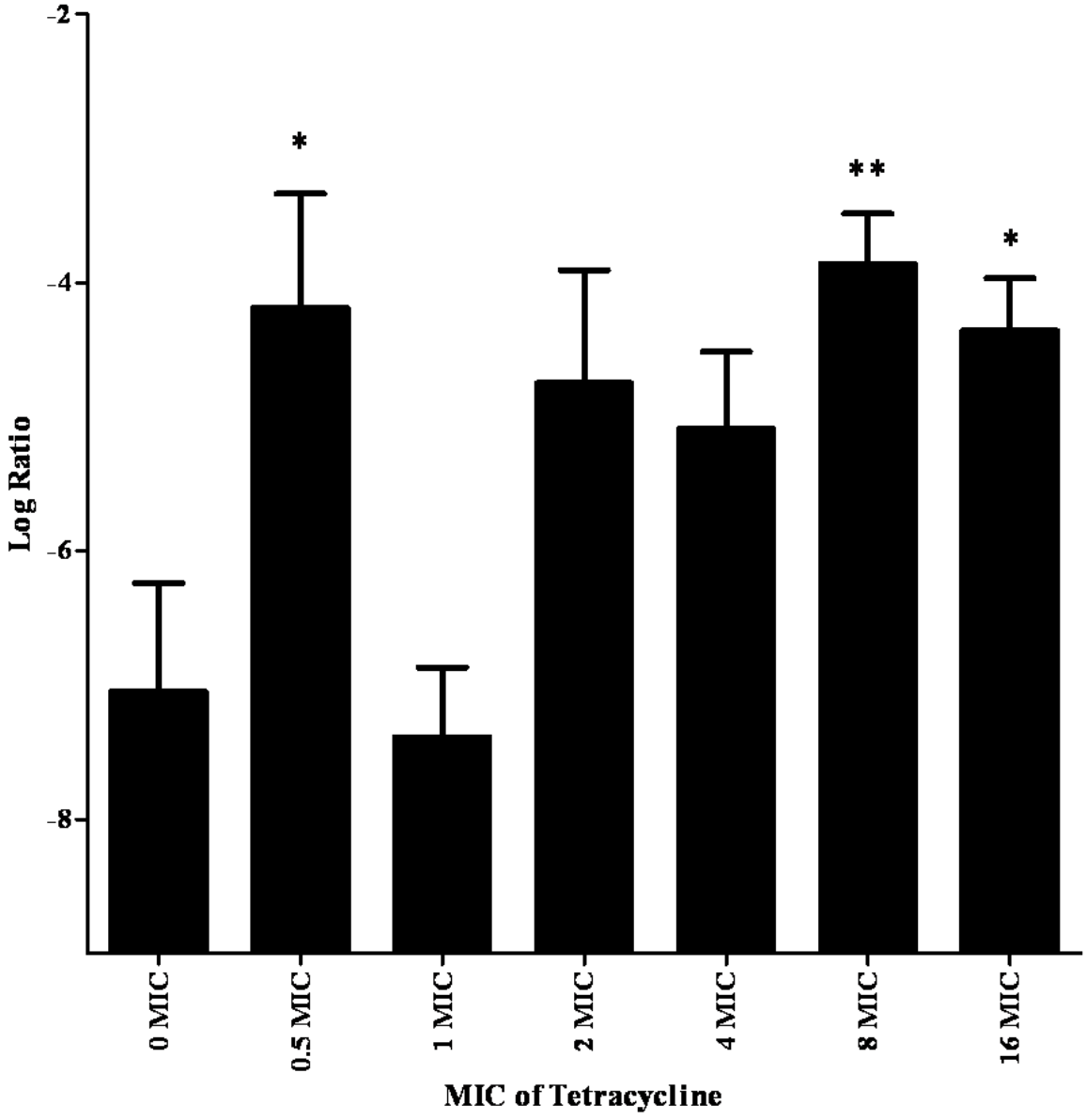

Figure 3.3 Conjugation efficiencies (measured as log ratios) of type A conjugative plasmid from *Salmonella* to *E. coli* at increasing concentrations of oxytetracycline. An * indicates an increase significant at p value <0.05, ** indicates significant increase at p value <0.01, and * indicates a significant increase at p value <0.001 compared to 0 MIC.**

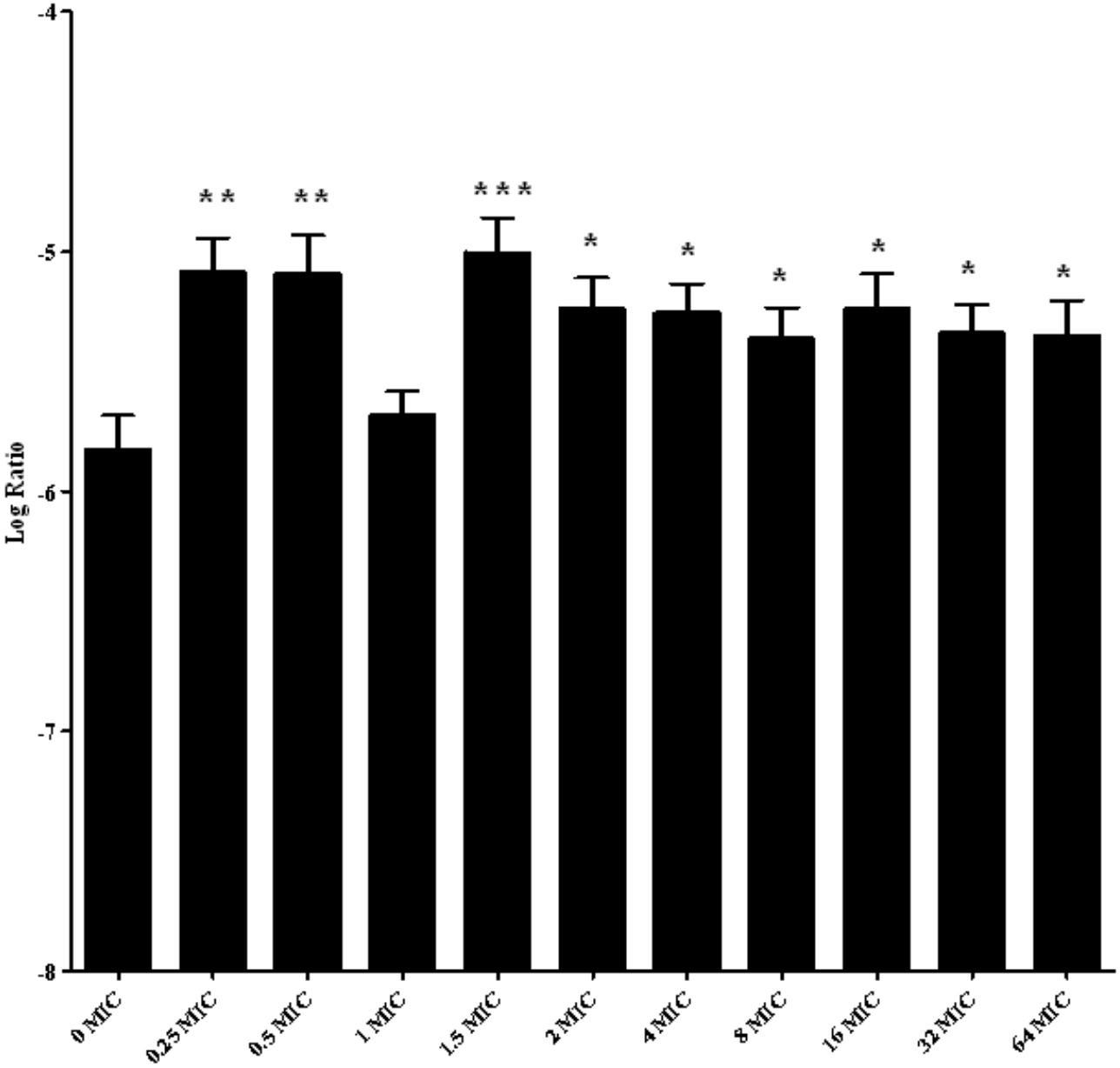

Figure 3.4 Gene expression studies for plasmid *tra* genes *traI* (3a) and *traY* (3b) in *Salmonella* co-cultured with *E. coli* in increasing concentrations of oxytetracycline. An * indicates an increase significant at p value <0.05, ** indicates significant increase at p value <0.01, and * indicates a significant increase at p value <0.001 compared to 0 MIC.**

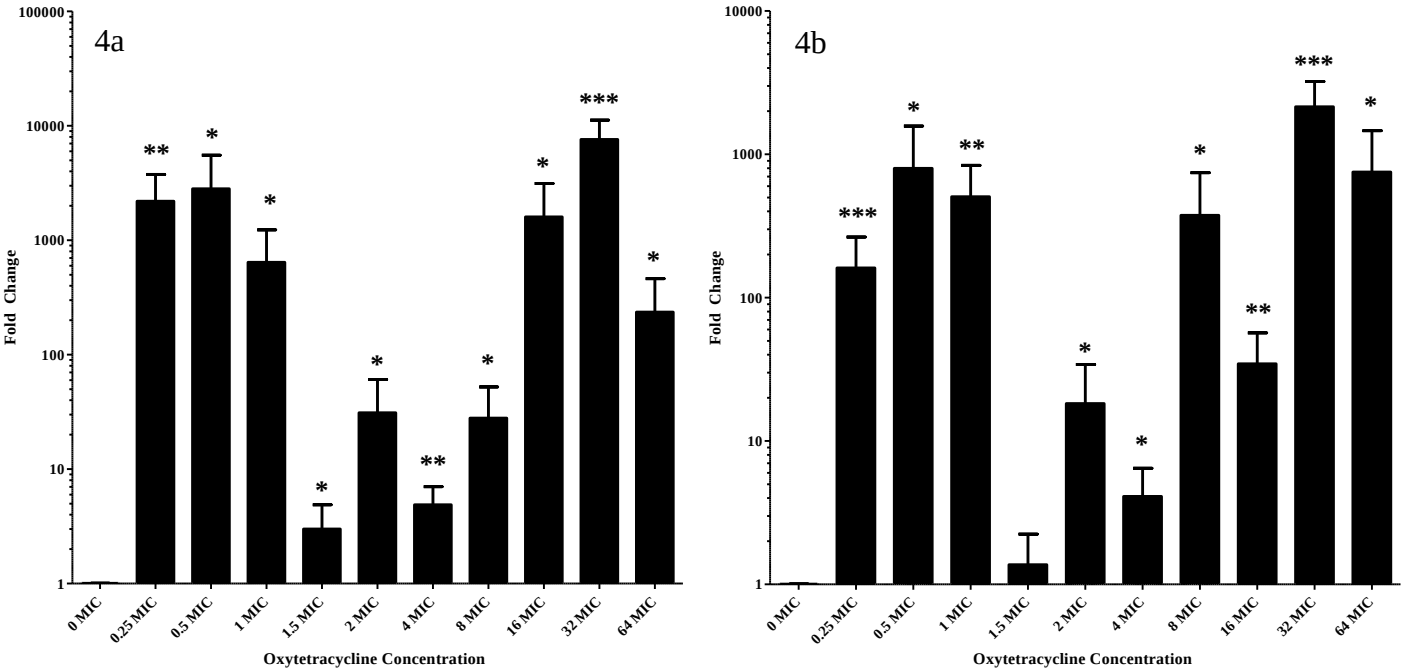

Figure 3.5 Gene expression studies for plasmid transfer (*tra*) genes *traI* (5a), *tray* (5b), *traJ* (5c), and *traR* (5d) in *Salmonella* exposed to increasing concentrations of oxytetracycline. An * indicates an increase significant at p value <0.05, ** indicates significant increase at p value <0.01, and * indicates a significant increase at p value <0.001 compared to 0 MIC.**

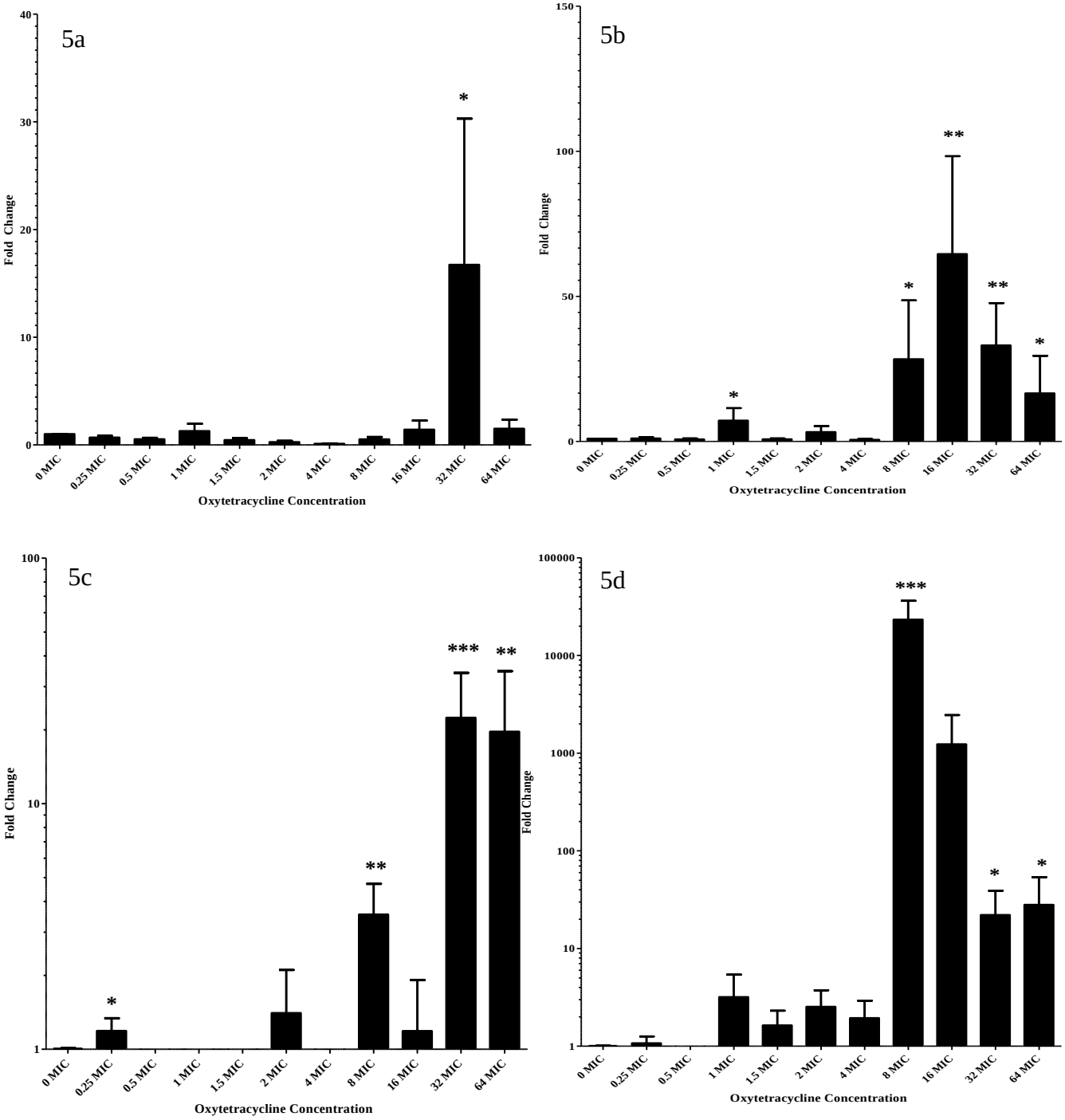

Figure 3.6 Linear regression analysis of observed vs. predicted HGT efficiencies. The bimodal pattern is present with 0 and 1 MIC at lower points on the line as compared to all other values. Significant (p value < 0.001) deviation from zero was found.

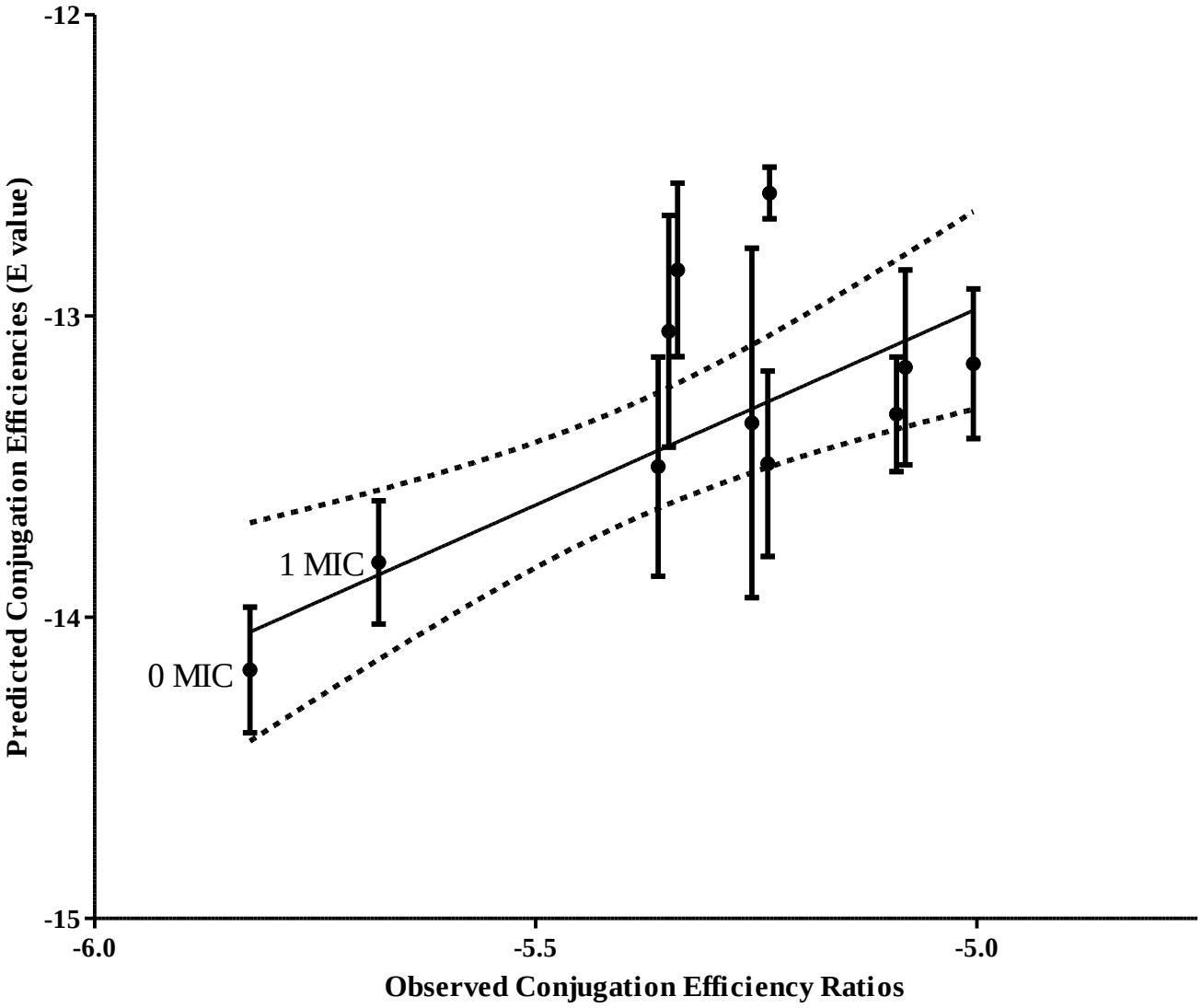

Figure 3.7 Linear regression analysis (7a-k) of observed vs. predicted transconjugants. (7a) is 0MIC, (7b) 0.25 MIC, (7c) 0.5 MIC, (7d) 1 MIC, (7e) 1.5 MIC, (7f) 2 MIC, (7g) 4 MIC, (7h) 8 MIC, (7i) 16 MIC, (7j) 32 MIC, and (7k) 64 MIC. Significant (p value < 0.001) deviation from zero was found.

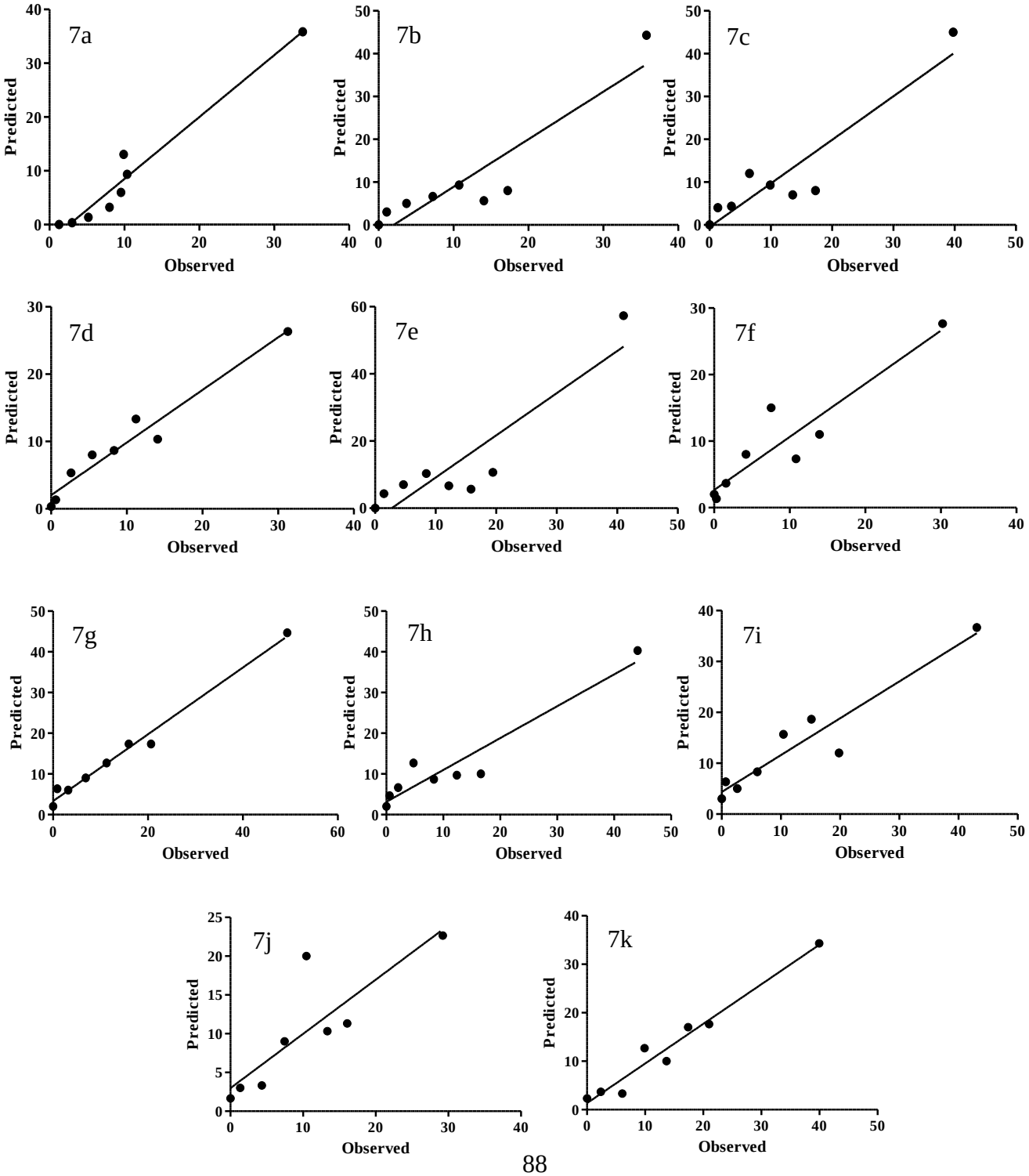

Table 3.1 List of isolates used in this study.

Strain	Species	AMR Phenotype	Mobile Element	Reference
INY1010	*Enterococcus faecalis*	T, S, Sp	Tn925	(9, 21)
OG1RF	*Enterococcus faecalis*	Rif, Fus	N/A	(13)
5678	*Salmonella enterica* Typhimurium	ACSSuTCroFx	Type A Plasmid	(15)
C600N	*E. coli*	Nal	N/A	(15)
ICC 180	*Citrobacter rodentium luxCDABE*	Nal, K	N/A	(46, 47)
ATCC 14028	*S. enterica* Typhimurium	UKN	N/A	(ATCC)
ATCC 700720	*S. enterica* Typhimurium	UKN	N/A	(ATCC)

Abbreviations: A, ampicillin; C, chloramphenicol; Cro, ceftriaxone; Fus, fusidic acid; Fx, cefoxitin; K, kanamycin; N, nalidixic acid; S, streptomycin; Sp, Spectinomycin; Su, sulfisoxazole; T, tetracycline; Rif, rifampicin

Table 3.2 Complete list of primers used in this study.

Primer	5' to 3' Sequence	Product Size	Reference
Antimicrobial Resistance			
bla$_{CMY-2}$ F	GACAGCCTCTTTCTCCACA	101 bp	(49)
bla$_{CMY-2}$ R	GAATAGCCTGCTCCTGCATC		(Shortened)
Transfer Genes			
traI F	TTGTCTTCCTTCCTGCCATC	163 bp	(This study)
traI R	TGAACGCTTTGTCAGCAATC		
traJ F	GCTTTACGACCACCGTCATT	98 bp	(This study)
traJ R	CCTGTCATCAGGGATTCGAT		
traR F	TCGACATTGCGAACCATATC	103 bp	(This study)
traR R	GCCGGAGCAAACTGACTAAG		
traY F	TGCGACGAAACTCAGTATGC	153 bp	(This study)
traY R	GGAAGCATGTTCTGGGTGTT		
Positive Controls			
EUB F	TGGAGCATGTGGTTTAATTCGA	161 bp	(48)
EUB R	TGCGGGACTTAACCCAACA		

Chapter 4 - The Effects of Stress Hormones and Antibiotics on Conjugative Gene Transfer

Abstract

The widespread use of antimicrobials in medicine as well as in food production has resulted in pathogenic bacteria becoming resistant to the antimicrobials used to treat them. Antimicrobial resistance (AMR) can result from conjugative horizontal gene transfer (HGT) through plasmids and transposons. Adrenaline and norepinephrine are catecholamines that mediate acute host stress in vertebrates and invertebrates. Past research has shown that catecholamines can also be used as environmental cues for pathogenic bacteria to enhance growth, motility, virulence and increase conjugation frequencies between enteric bacteria. The exact influence of the antimicrobials and catecholamines together on conjugation is, however, not clearly defined. The objective of this study was to address this deficiency by quantitatively characterizing the efficiencies of conjugation in a mating pair of *Salmonella* Typhimurium and *E. coli* C600N during exposure to concentrations above, equal to and below the minimum inhibitory concentration (MIC) of oxytetracycline under *in vitro* conditions with or without norepinephrine (NE). Conjugative HGT occurred in all concentrations of oxytetracycline, but efficiencies of transfer were consistently low at 0 MIC and 1 MIC, with increased activities both pre- and post- MIC. While the addition of NE enhanced conjugation frequencies at all oxytetracycline concentrations, the effects were not cumulative. Expression of plasmid encoded transfer (*tra*) genes was significantly upregulated in response to the highest concentrations when *Salmonella* was cultured alone, and NE exposure enhanced the fold changes further. This study for the first time provides insights into the effects of oxytetracycline and NE on conjugation, and

may provide future insight into better control of AMR in animals and humans whose bacterial infections are treated with antimicrobials while experiencing stress.

Introduction

Random mutation has allowed bacteria to adapt to a variety of environmental niches (38, 48); however, HGT greatly accelerates bacterial evolution (7, 47, 48). HGT is accomplished through the efficient movement of genetic elements including plasmids, transposons, and bacteriophages (47, 48). Conjugative transfer is the most efficient (33). Conjugation works by a host cell physically adhering to a recipient cell, and horizontally transferring genetic elements packaged as plasmids or transposons into the cytoplasm for the passage into the recipient cell (33). These transposons and plasmids allow for high efficiency transfer of antimicrobial and virulence genes from single resistant donor bacterium to many recipients which can in turn donate the resistance genes to more recipients (48).

In a previous study, a significant increase in conjugation was reported in response to exposure to catecholamines (35). Catecholamines are a large group of amine hormones, derived from tyrosine, and include epinephrine (adrenaline), norepinephrine (NE; noradrenaline) and dopamine. Catecholamines, especially adrenaline and NE, are sympathetic neuroendocrine mediators of "fight or flight" (acute stress) response of the host. NE-containing sympathetic synapses are distributed throughout the body, with more than half located in the gastrointestinal (GI) tract in the enteric nervous system (12, 16). In the gastrointestinal tract the physiological concentration of NE has been shown to be as high as 50μM (1).

Subsequent studies have demonstrated that catecholamines can influence production of virulence factors, such as toxins and adhesins, biofilm formation, and quorum sensing even in iron-replete conditions (26-32, 35, 45, 46). For example, *E. coli* O157 responds to

catecholamines by increased expression of shiga-toxin (51), exalted chemotaxis, and adherence to eukaryotic cells (2, 8), enhanced attachment and effacement (A/E) lesions (37), attachment to murine cecal mucosa (8), and increased flagella expression and motility (11). In *Salmonella,* catecholamines have been implicated to enhance motility and colonization in the GI tract of pigs (4).

The development and widespread use of antimicrobials in the last 60 years has resulted in many drug resistant strains of bacteria. Less than inhibitory concentrations (pre-MIC) of antimicrobials are thought to play a role in increasing mutation rates as well as increases in the efficiencies of HGT of the susceptible recipient bacteria (3, 10, 19). In two studies, in *Enterococcus,* pre-incubation with pre-MIC concentrations of tetracycline of a donor *E. faecalis* population was shown to enhance transposon HGT up to 119-fold when the donors were added to recipient *B. thuringiensis* bacterial cultures (44, 50).

The objective of this study was to determine the effects on the conjugative transfer of a plasmid from *Salmonella* Typhimurium to *E. coli* C600N under *in vitro* conditions following exposure to oxytetracycline and/or NE.

Materials and methods

Bacterial strains

Salmonella Typhimurium strain 5678 (17) was used as the donor strain in all mating experiments. This strain carries a type A plasmid that is approximately 100 kb in size, and is transferable by conjugation to the recipient *E. coli* C600N (a spontaneous nalidixic acid resistant mutant of strain C600 (17); kindly provided by Dr. Paul Fey at University of Nebraska Medical Center, Omaha, Nebraska). This plasmid contains a $bla_{\text{CMY-2}}$ gene that encodes resistance to a

large spectrum of β-lactams including ampicillin, ceftriaxone and cefoxitin; as well as resistance markers for chloramphenicol, streptomycin, sulfisoxazole, trimethoprim-sulfamethaxozole and tetracycline (Table 1). Based on sequence analysis, this plasmid is closely related to the well-studied plasmid pNF1358, and the organization of the transfer genes is similar to that of IncI plasmid R64 (22) (Genbank DQ017661.1).

MIC determination

The MIC of oxytetracycline for donor and recipient strains was determined using a slight modification of micro-broth dilution method recommended by CLSI (34). Briefly, ten microliters of a 0.5 McFarland bacterial suspension was pipetted into 11 mL of Luria-Bertani (LB) broth. Aliquots of the bacterial suspension (100 μL) was added to the wells of a 96-well plate containing 100μL of increasing concentrations of oxytetracycline (Sigma, St. Louis, MO) in duplicates, for donor and recipient strains. The 96-well plate was placed in an incubated (37^{o}C) spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA) set to read absorbance at 600 nm with readings taken hourly. The MIC was the lowest concentration at which optical density readings was reduced by 60% (24). The MIC of *Salmonella* 5678 donor and *E. coli* C600N recipient strains for oxytetracycline was determined to be 60,000 and 62.5 ng/ml, respectively.

Pulse Field Gel Electrophoresis

PFGE was conducted with *Salmonella* 5678, *E. coli* C600N, and selected transconjugants using standard methodologies (36). The DNA embedded in agarose was digested with *XbaI* and electrophoresed on a CHEF DR-III instrument (Bio-Rad, Richmond, CA) using the following

conditions: initial switching time, 2.2 s; final switching time, 63.8 s; total time, 19 h. *Salmonella enterica* serotype Braenderup H9812 (ATCC# BAA-664) was used as the standard (data not shown).

Southern Blot

DNA from the PFGE gel was tranfered to nitrocellulose or nylon membranes as described previously (42). A DIG labeled $bla_{\text{CMY-2}}$ probe (Roche, Indianapolis, IN) was created using previously described primers (6, 17), and hybridization was detected using NBT/BCIP (Roche) (data not shown).

Liquid mating experiments

Broth cultures were used in all mating experiments to maintain uniform concentrations of antimicrobials and catecholamines, and for ease in the collection of representative samples at predetermined time points to evaluate conjugation frequencies and gene expression trends. All *Salmonella* to *E. coli* matings were conducted in static Luria-Bertani (LB) broth.

Liquid mating experiments between *Salmonella* and *E. coli* were conducted as previously described with some modifications (25). The initial inocula (*Salmonella* strain 5678 and *E. coli* C600N) were grown individually for 18 h at 37°C under aerobic conditions with shaking (at 100 rpm). Overnight inocula were diluted 1:10 in fresh prewarmed LB broth free of antimicrobials for approximately 2hrs to attain an OD 600 of 0.3. Cultures were mixed at a ratio of 1:5 (donor to recipient) oxytetracycline was added to the mixed cultures at 0, 0.25, 0.5, 1, 1.5, 2, 4, 8, 16, 32, and 64x MIC levels with and without 5μM of NE, and incubated at 37^{o}C under aerobic conditions. Samples were collected at 0, 2, 4, 6, 8, 10, 12, and 24hrs post-mixing and were

plated on HE-agar containing selective antibiotics (50µg/ml ampicillin for donor; 12 µg/ml nalidixic acid for recipient; and 50µg/ml ampicillin and 12µg/ml nalidixic acid for transconjugants) (25). Selected transconjugants were replica-plated on to ceftriaxone, cefoxitin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline to ensure all the resistance markers encoded on the plasmid were transferred. These transconjugants were checked for the presence of the $bla_{\text{CMY-2}}$ gene by PCR as previously described (55). HGT ratios were determined by dividing CFUs/mL of transconjugant by CFUs/mL of donor bacteria, as described previously (21).

Gene expression studies

RNA was extracted from *Salmonella* and *E. coli* together, or *Salmonella* alone grown at 0 through 64X MIC of oxytetracycline using the Trizol (Invitrogen, Carlsbad, CA) and was treated with DNase treatment kit (Invitrogen, Carlsbad, CA) to remove residual DNA to acquire samples with no threshold fluorescence before cycle 30. A 16S ribosomal RNA target EUB (54) was used as a house-keeping gene to normalize total RNA yields. Quantitative RT-PCR was performed on listed primer sets (Table 2) using SuperScript III Platinum SYBR green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) on a RealPlex PCR machine (Eppendorf, Hauppauge, NY). Expression profiles of plasmid and chromosomally encoded genes (calculated as fold-changes using $\Delta\Delta C_t$ method) are summarized in Figure 2. A melt curve analysis was performed following all PCRs to confirm a single amplicon of adequate size.

Data Analysis

Mean differences in the ratio of HGT and gene expression levels at various concentrations of NE were assessed for wild-type and mutant strains by paired t-tests performed using statistical functions included in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). The independent variables were oxytetracycline concentrations (with or without NE), hours post-mixing, and the types and levels of adrenergic antagonists added. Data is presented as means ± SEM, and differences were considered statistically significant when the probability of a type I error was <0.05. Further analysis was performed using a mixed effects model, with a repeated measure over hours analysis in a one-way ANOVA, and "unstructured" as the type of variance component. A Bonferroni test was used for post-hoc analysis.

Results

Effects of NE and oxytetracycline on donor and recipient growth

There was a dose-dependent decrease in the CFUs/mL of the drug-susceptible *E. coli* population as the concentration of oxytetracycline increased (Figure 1a). The addition of 5μM of NE to the antimicrobials did not significantly enhance or suppress the effects of the oxytetracycline. The drug-resistant *Salmonella* donor growth did not show any significant inhibition by the oxytetracycline or any enhancement with the addition of the 5μM NE (Figure 1b).

NE and oxytetracycline enhance HGT, but effects are not compiled

Transfer of the conjugative plasmid occurred in all concentrations of oxytetracycline tested, both with and without NE. As was shown in a previous study (unpublished data), a bimodal pattern emerged with the highest transfer efficiencies being observed below (0.25x – 0.5x MIC) and above (1.5x – 64x MIC) 1 MIC (Figure 2a). The addition of 5μM NE to each of

the concentration of oxytetracycline only increased HGT efficiencies significantly at 0 MIC. A similar bimodal pattern was seen in transconjugant CFUs/mL with increases in population again below and above 1 MIC (Figure 2b). The number of transconjugants was greater at 0 MIC with 5μM NE (Figure 2b). Successful transfer of plasmid was confirmed by subjecting randomly selected transconjugants to PCR analysis for $bla_{\text{CMY-2}}$ gene (55), and pulse field gel electrophoresis (PFGE) (6, 17, 42) followed by Southern blotting and hybridization to a previously described $bla_{\text{CMY-2}}$ probe (5, 17). Additionally, replica-plating in Hectoen enteric agar plates for plasmid encoded antimicrobial resistance phenotypes was also performed (data not shown). Filter mating between randomly selected C600N transconjugants as donors and two *Salmonella* Typhimurium strains (LT2 700720 and 14028) or *Citrobacter rodentium* ICC 180 as recipients (Table 1), followed by PCR analysis for $bla_{\text{CMY-2}}$ in the *Salmonella* and *C. rodentium* transconjugants confirmed successful transfer of the conjugative plasmid. Replica plating of randomly selected *C. rodentium* and *Salmonella* transconjugants on Hectoen enteric agar containing various antibiotics demonstrated that the plasmid-encoded AMR genes were also functional in these recipients (data not shown).

Transfer gene expression

The plasmid transfer (*tra*) genes present on the *Salmonella* 5678 type A plasmid were selected for gene expression analysis to upregulate in the presence of NE and oxytetracycline. *Salmonella* incubated alone with oxytetracycline upregulated *tra* gene expression when exposed to concentrations of oxytetracycline above the MIC of *E. coli* (8x – 64x MIC) (Figures 3a;3b). Interestingly, the addition of 5μM NE to increasing concentrations of oxytetracycline resulted in

the upregulation of the *tra* genes significantly above the fold changes caused by oxytetracycline alone in both *traI* and *traY* (Figure 3a;3b).

Discussion

In this study we have presented evidence that the conjugative transfer of a plasmid from a clinical strain of *Salmonella* to a recipient *E. coli* was significantly affected by the addition of oxytetracycline and/or 5 μM NE. Past studies showed significant increases in HGT when NE or oxytetracycline was added to the *Salmonella* to *E. coli* mating pair (unpublished data). It was, however, unknown if the combination of NE and oxytetracycline would cause additive effects to conjugation efficiencies. In the present study, we report that this particular mating pair had a limit to the enhancement of conjugal transfer efficiencies that was reached by the addition of NE or oxytetracycline, and this limit was not exceeded with the addition of the combination of the two (Figure 2a).

A divergent result was found in the gene expression portion of this study. Conjugative transfer of the type A plasmid involves a complex activation sequence of approximately 30 different transfer (*tra*) genes for direct cell-to-cell mating (18). We monitored expression of two *tra* genes I and Y by qRT-PCR analysis based on their abilities to respond to NE and oxytetracycline in past studies (Chapters 2; 3). The *traI* (encoding a relaxase-helicase and is the central catalytic component of the multiprotein relaxasome complex responsible for beginning the conjugative DNA transfer) and the *traY* (which imparts single-strand DNA character to the *oriT* site) genes were monitored. While maximizing limits were reached when HGT efficiencies were measured, gene expression studies showed a cumulative increase in *tra* gene expression when NE was added to increasing concentrations of oxytetracycline. This may suggest that there

is a bottleneck-type effect between conjugative signals and actual conjugation at a critical, but unknown control point.

This study is relevant to human and animal health as stress and antimicrobial use often occurs in the same environment. In wards including the ER, OR and ICU, stress levels are extremely high for patients with life-threatening afflictions, and to prevent infection, a wide variety of antimicrobials are used to inhibit bacterial infection. In food animal health, the feedlot setting where small pens with multiple animals create stress, and antibiotics which serve as prophylactics and growth enhancers are mixed into the feed in less than inhibitory doses (9, 15). Perhaps due to this deadly combination we have witnessed the rise of vancomycin resistant *Enterococcus* (VRE) and vancomycin resistant *Staplococcus aureus* (VRSA) (13, 14, 20, 23, 39-41, 49).

This study for the first time attempts to determine the effects of catecholamines and antimicrobials on HGT. While the combination of oxytetracycline and NE did not have a cumulative effect on HGT efficiencies, there was an intriguing enhancement of *tra* gene upregulation responsible for the transfer of the plasmid. These results demonstrate complexity of the interactions between bacteria undergoing conjugative HGT as environmental factors are altered. In the case of the present study, it was not sufficient to monitor bacterial actions on Petri dishes. Only through gene expression analysis were we able to begin to define the complete picture. Future investigation will further enhance our knowledge of how multiple elements contribute to efficiencies of conjugative HGT.

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Figures and Tables

Figure 4.1 Growth averages (CFUs/mL) of mixed donor and recipient populations throughout the experiments with increasing concentrations of antimicrobials (x-axis) with and without 5µM of NE. Average growth of *Salmonella* 5678 (1a) and *E. coli* C600N (1b) are shown.

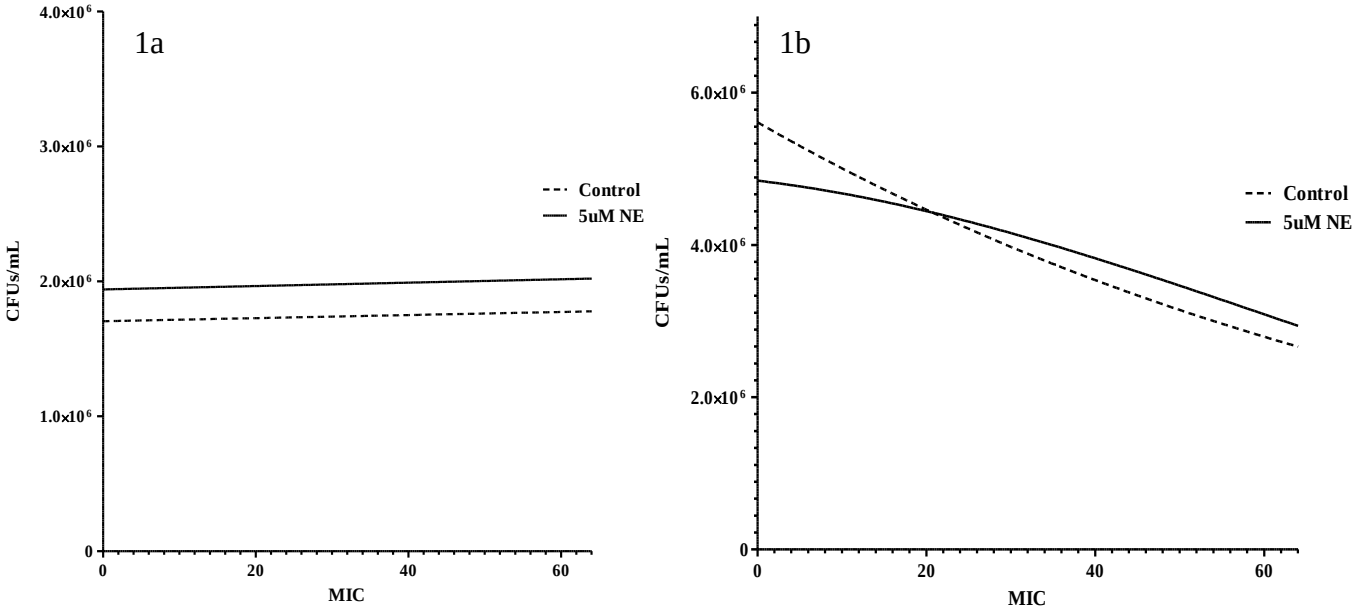

Figure 4.2 Conjugative transfer efficiencies (measured as log ratios) of type A conjugative plasmid from *Salmonella* to *E. coli* at increasing concentrations of oxytetracycline either with NE (black bar) or without NE (white bar) (2a). Transconjugant numbers (CFUs/mL) in the presence of increasing concentration of oxytetracycline either with (black bar) or without NE (white bar) (2b). An * indicates an increase significant at p value <0.05, ** indicates significant increase at p value <0.01, and * indicates a significant increase at p value <0.001 compared to 0 MIC.**

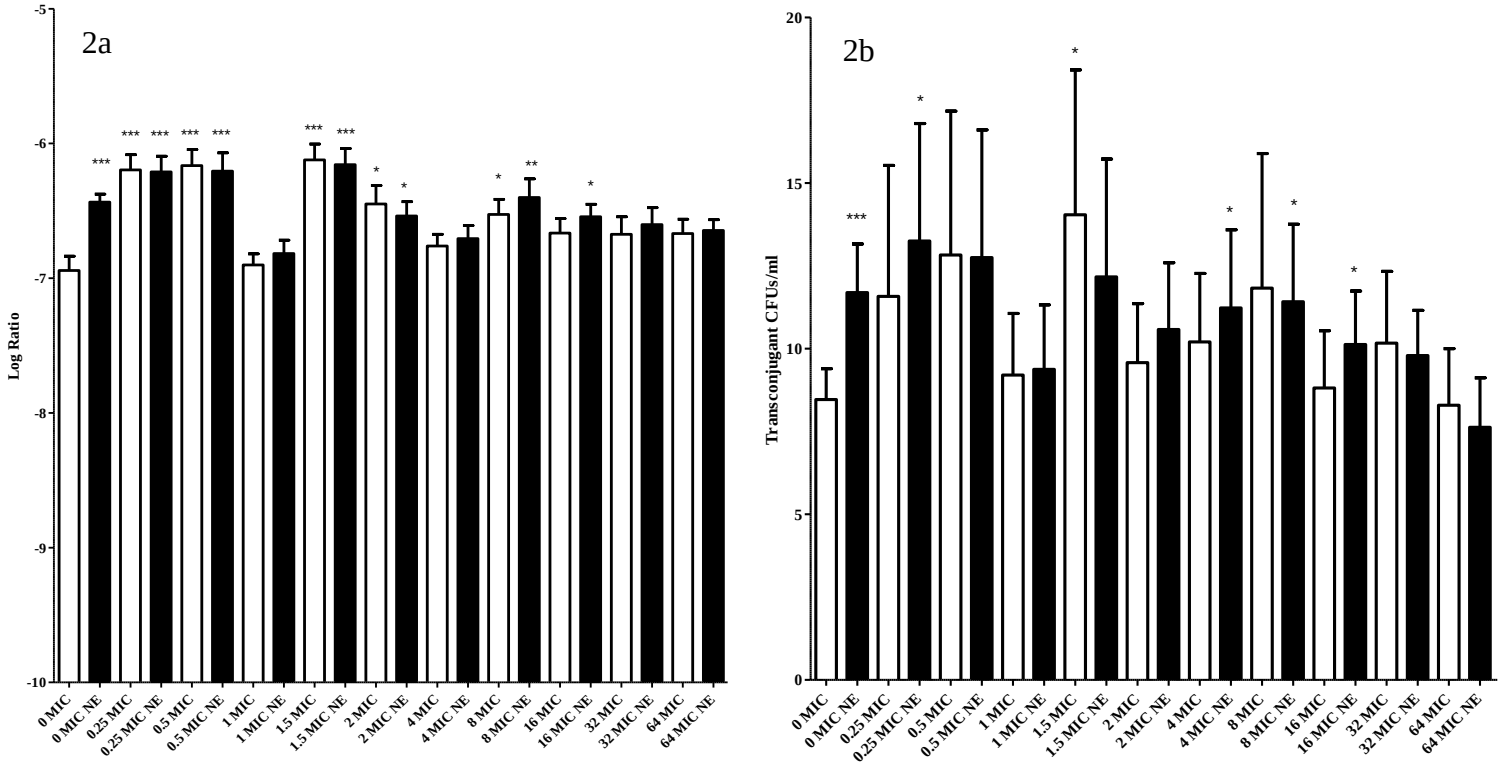

Figure 4.3 Gene expression studies for plasmid transfer (*tra*) genes *traI* (3a), and *traY* (3b) in *Salmonella* exposed to increasing concentrations of oxytetracycline with NE (black bar) or without NE (white bar). An * indicates an increase significant at p value <0.05, ** indicates significant increase at p value <0.01, and * indicates a significant increase at p value <0.001 compared to 0 MIC.**

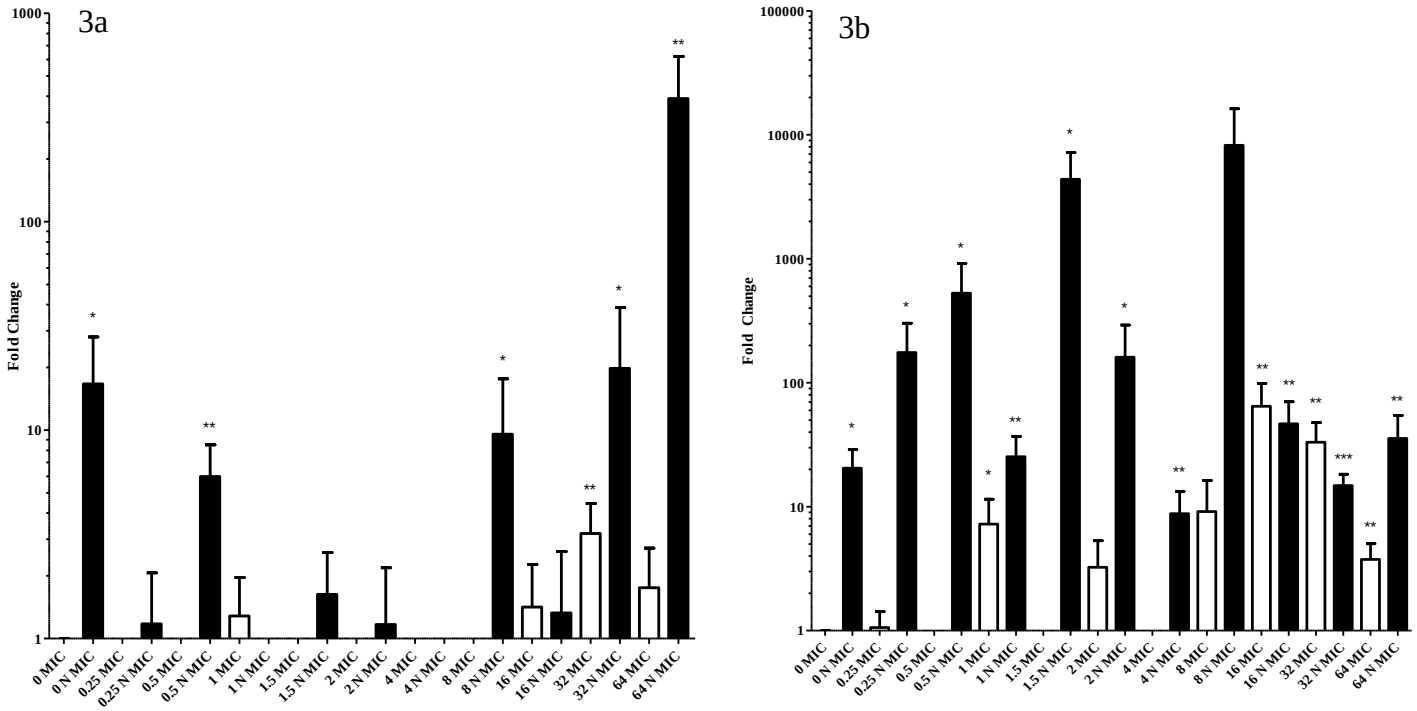

Table 4.1 List of isolates used in this study.

Strain	Species	AMR Phenotype	Plasmid Type	Reference
5678	*Salmonella enterica* Typhimurium	ACSSuTCroFx	A	(17)
5561	*Salmonella enterica* Newport	ACSSuTCroFx	C	(17)
C600N	*E. coli*	Nal	N/A	(17)
MG1655N	*E. coli*	Nal	N/A	(38)
ICC 180	*Citrobacter rodentium luxCDABE*	Nal, K	N/A	(46, 47)
14028	*S. enterica* Typhimurium	UKN	N/A	(ATCC# 14028)
LT2	*S. enterica* Typhimurium	UKN	N/A	(ATCC# 700720)

Abbreviations: A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfisoxazole; T, tetracycline; K, kanamycin; Cro, ceftriaxone; Fx, cefoxitin, N, nalidixic acid

Table 4.2 Complete list of primers used in this study.

Primer	5' to 3' Sequence	Product Size	Reference
Antimicrobial Resistance			
bla$_{CMY-2}$ F	GACAGCCTCTTTCTCCACA	101 bp	(49)
bla$_{CMY-2}$ R	GAATAGCCTGCTCCTGCATC		(Modified)
Transfer Genes			
traI F	TTGTCTTCCTTCCTGCCATC	163 bp	(This study)
traI R	TGAACGCTTTGTCAGCAATC		
traY F	TGCGACGAAACTCAGTATGC	153 bp	(This study)
traY R	GGAAGCATGTTCTGGGTGTT		
Positive Controls			
EUB F	TGGAGCATGTGGTTTAATTCGA	161 bp	(48)
EUB R	TGCGGGACTTAACCCAACA		

Appendix A - A Co-Printed Oligomer to Enhance Reliability of Spotted Microarrays

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Abstract

Successful printing and hybridization is essential for efficient and reliable data acquisition in a spotted microarray experiments. In this study we demonstrated that printing a 25mer (printed 25mer) with a standard 70mer probe in each spot followed by the use of a fluorescently labeled 25mer complement in the hybridization mixture ensures monitoring overall printing quality of the chip. This system can also be used as a control to evaluate adequate hybridization, washing, and alignment of spots to position the tracking grids during scanning. A print correction value incorporated in data analysis enhances consistency and reliability of results.

Introduction

Spotted microarrays are reliable, efficient, and relatively inexpensive tool used in diagnosis of infectious diseases in laboratories (Chittur, 2004, Ojha and Kostrzynska, 2008). The customizable feature of spotted array makes it a very important technique in molecular analysis, especially in the field of pathogenic microbiology where preprinted arrays (such as Affymetrix) are unavailable or can be very expensive. The importance of uniformity in printing of oligonucleotide on glass slides is an essential step to attaining usable and accurate data. In

order to assess quality of spotted array printing, fluorescently labeled oligomers (3-6mers) (SpotQC, IDTDNA, Coralville, IA), and SYBR Green II, which binds to ssDNA, have been used (Battaglia et al., 2000, Gupta et al., 2007, Tran et al., 2002). While these strategies do allow for visualization and assessment of the overall printing, sacrificing a chip for this type of analysis can be expensive, and the chip used may not be representative of the entire series that was printed. Additionally, poor printing of even a limited number of oligos on the untested slides could potentially lead to important genes being false negatives.

Some methods have been developed to address this problem, including the addition of a fluorescein label to each printed 70mer probe (Hessner et al., 2003), or by co-printing a labeled 70mer probe with each standard 70mer (Hessner et al., 2004). While they are beneficial for print checking on every chip, they do not address the important aspect of its use as control to normalize variations associated with hybridization.

Hybridization controls are important for use in microarray experiments, but the currently available systems, such as external RNA controls, ArrayControl™ (Ambion, Austin, TX) or ToxArray™ involve multiple probes and complex algorithms, and are expensive to set up and perform (van Bakel and Holstege, 2004, Yauk et al., 2006). In another study, a 40mer control was printed alongside the standard 40mer probe. A complement for the co-printed 40mer was used to evaluate hybridization efficiency (Zhao et al., 2006). The effects of such probe-complement system on the binding of fluorescently labeled sample DNA to the standard 40mer or its ability to correct for variations due to printing was not described. In this study, our goal was to identify an inexpensive hybridization system that would serve as a reliable printing and hybridization controls that can be used on every chip without affecting the efficiency of hybridization of the probes. We describe the use of a unique 25mer oligonucleotide (printed

25mer) that is printed in the same spot as the standard 70mer. The hybridization mixture contains fluorescently labeled sample DNA along with the fluorescently labeled 25mer complement (25mer complement) that allows for easy and consistent visualization of spots containing the printed 25mer. This system is subtle enough to prevent inhibition of binding of fluorescently labeled sample DNA to printed oligos and is specific and flexible enough to be used at a wide range of hybridization temperatures. Additionally, it has allowed for development of an analysis method called Print Correction Value for normalization in a diagnostic microarray.

Materials and Methods

2.1 Development of 25mer probe

A 25 oligomer sequence was chosen by first generating an arbitrary sequence of 25 nucleotides that at the time of its selection had no matching sequences on a BLAST search. If any matches were identified, the entire sequence was reshuffled until an appropriate sequence was determined. Possible candidate oligos were checked for secondary structures and self-dimers using the OligoAnalyzer 3.0 software (IDT, Integrated DNA Technologies, IA). Single nucleotides were changed and analyzed until the sequence 5'-GGATGCTAGATCGTGTGCTCTCGCC-3' was found. A complementary sequence 5'-GGCGAGAGCACACGATCTAGCATCC-3' was then synthesized (IDT) with either a 5' Cy3 or Cy5 dye attachment.

Modifications to 25mer sequence was carried out by incorporating degenerate nucleotides (A,T,G,or C represented by N; IDT) in different positions in the printed 25mer sequence. Overall intensities were monitored for each of these sequence modifications, and compared to the control 25mer.

2.2 Microarray chip printing

25mer primers with sequence modifications (Table 1) were created and printed in replicates of ten on UltraGap slides (Corning Inc., Corning, NY). Two fields containing the oligos, at 35 μM concentrations were printed using a Genetix QArray2 System slide printer (Genetix, New Milton, UK). The fluorescent hybridization signals from the array were visualized using a GenePix 4000B slide reader (Molecular Devices, Sunnyvale, CA) and matched to the GenePix Array List (GAL) file previously created by the microarray slide printer. The 70mer primers; *Salmonella*-specific gene *invA* (Chiu and Ou, 1996) and the positive control EUB targeting the 16S ribosomal RNA gene for all eubacteria (Yang et al., 2002) were printed alone or in combination with each of the modified 25mer primers.

2.3 DNA labeling

Genomic DNA from *Salmonella enterica* DT104 was directly labeled with the BioPrime Plus Array CGH Genomic Labeling System (Invitrogen, Carlsbad, CA), with slight modifications. After random primer incorporation, 1.5μL of 1mM Cy3 or Cy5-dCTP (Amersham, Piscataway, NJ) was spiked in to increase overall fluorescence signal. Dye incorporation and amplification was checked by the microarray feature on a Nanodrop ND-1000 spectrophotometer (Nanodrop-Thermo Fisher Scientific, Wilmington, DE).

2.4 Hybridization

After labeling, the genomic DNA was mixed with a 2X Hybridization Solution (GeniSphere, Hatfield, PA) and 1μL of 1nM stock of either Cy3 or Cy5-labeled 25mer was added to the mixture. The mixture (total volume of 30μL) was heated to 80ºC for 5 minutes and then added onto the prepared chips. The functional temperature range of the 25mer complement was determined by overnight hybridization to printed slides in water baths or incubators at

temperatures 42ºC, 47ºC, 53ºC, 59ºC, and 65ºC. This test was performed three times, and colors of Cy dyes switched each time.

2.5 Sample scoring

To determine if the signals were positive, a previously described method by Frye et. al was employed (Frye et al., 2006). The average of the median fluorescence signal for each spot was divided by average of the median fluorescence emitted by all spots on the chip to acquire a ratio. This value was called the relative signal intensity, and a threshold of 2.0 or above was considered positive.

2.6 Statistical analyses

Unpaired t-tests were performed using statistical functions included in Microsoft Excel (Microsoft Corp., Seattle, WA) and GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). A p-value of < 0.05 was considered significant.

Results

3.1 Changes in affinity with increasing temperature

At low temperatures, the control 25mer produced the highest relative signal intensity, but as temperatures increased, there was a net decrease in relative signal intensities (Figure 1). The exceptions to this trend were oligos 11 and 5 which had NNN tag on the 3' end and a single N on the 5' end, respectively (Table 1). As the hybridization temperature increased (42°C to 65°C), the relative intensities of these oligos began to increase, probably due to a relaxation of secondary structures on the chips (Table 2), as determined by using AutoDimer v1 software (http://www.cstl.nist.gov/div831/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm) (Vallone and Butler, 2004).

The 25mer complement was able to bind to the printed 25mers up to hybridization temperatures of 59ºC. Even at a hybridization temperature of 65ºC there was still fluorescent signal present; however, lower temperatures ranging between 42ºC and 53ºC were optimal. At no temperature did the 25mer complement bind to spots that had only 70mers (Figure 1).

3.2 Changes in affinity with increasing modifications

Affinity of binding was determined by different sequence modifications of the 25mer complement. As the number of modifications to the printed 25mer increased (Table 1), there was an overall reduction in their relative signal intensities (Figure 1). This was exemplified in modified probes 13, 14, 15, 16, 17, and 19 (in which Ns were added into the middle of the printed 25mer), where there were rapid reduction in relative signal intensities as the number of Ns increased (Table 1 and Table 2).

3.3 Co-detection assay

Co-detection assays using 25mer complement and fluorescently labeled *Salmonella enterica* DNA were performed to determine the effects of the 25mer on the hybridization of the bacterial DNA. The labeled 25mer complement was able to hybridize to the printed 25mer, and the labeled genomic DNA was able to hybridize to *Salmonella*-specific probes. The most optimal temperature range for these reactions was between 42ºC and 47 ºC, where no statistically significant differences in fluorescence signal were observed between the 25mer + 70mer and the 70mer alone. As temperature increased, there was statistically significant reduction in relative signal intensities of the 25mer + 70mer spots compared to the 70mer spots alone. Interestingly for *invA*, printing 25mer + 70mer actually had better relative signal intensities compared to 70mer at 47 ºC (Figure 1).

Oligo probes numbers 18 and 20 which had fluorescence intensities similar to that of the unmodified 25mer in the affinity assays (Table 2), were shown to inhibit the overall hybridization functions of the 70mer (data not shown), when co-printed with 70mer. Thus, the original, unmodified printed 25mer was shown to be the best for use in co-detection assays. In other words a combination of a printed 25-mer and its exact complement had optimal hybridization kinetics for use in this control system.

The optimal temperature for use of the printed 25mer was determined to range from 42ºC to 47ºC. However, it should be noted that we performed labeling of the genomic DNA only using BioPrime Plus Array CGH Genomic Labeling System, and use of another kit or alternate (SDS-based) hybridization buffers would possibly yield increased functionality at higher hybridization temperatures.

3.4 Print correction value

In order to correct for possible printing variations (over-printing or under-printing) with spotted array, this 25mer probe-complement system can be used to normalize the signals from the 70mer by a Print Correction Value (PCV). The PCV can be defined as the ratio of the mean of the median 25mer signals of a particular spot, and the mean of median 25mer signal of all spots on the chip. The 70mer value (numerator) will then be divided by this PCV (denominator) on that spot to adjust printing variation (Figure 2).

PCV = 25mer value / mean or median net 25mer values

Corrected value of 70mer = 70mer value / PCV

This method is based on the assumption that printing variation is normally distributed and should be tested for each study using frequency distribution charts. If the assumption were found

to be not true, subpopulations and/or outliers should be identified and these values should be accounted for by using a non-parametric summary statistic (e.g., median) as an estimate of the PCV. This PCV method has been used in microarrays in our laboratory and has provided us with additional confidence in the data acquired in our experiments (data not shown).

3.5 BLAST results

When the 25mer was originally designed (fall 2006), there were no similarity to any sequence deposited in GenBank. However, our 25mer sequence has recently shown some limited homologies to a few recently submitted sequences on a blastn search using the megablast option for highly similar sequences. Depending on the host system (bacterial, *Drosophila*, mouse, human, etc.), it would be relatively simple to identify a new 25mer that has no sequence homology to any genes that they will likely encounter in that system, for its use as a printing and hybridization control.

Discussion

4.1 Use in diagnostic arrays

Our 25mer system is ideal for incorporation in the newly developing field of diagnostic microarray (Chandler et al., 2006, Dankbar et al., 2007, Frye, et al., 2006, Kostic et al., 2007, Li et al., 2006, Perreten et al., 2005, Strommenger et al., 2007, Tembe et al., 2007, Tomioka et al., 2005, Volokhov et al., 2002, Yu et al., 2004) as it is useful in nearly all steps of a DNA microarray experiments (Figure 3). This system will eliminate false negative readings due to lack of printing of the oligos, ensuring that a negative in this test is a true negative. The 25mer is not only useful for determination of printing and its variations; it is also useful for checking overall hybridization results of an array. Any type of technical mishaps during hybridization

steps including air bubbles, shifted cover slips, unequal distribution of hybridization mix, improper buffer concentrations, improper hybridization temperatures etc., can be easily visualized and accommodated when analysis of the data is performed.

This system will allow the user to detect and optimize slide washing procedures. Often times, when new microarray protocols are being developed, determining the appropriate washing conditions (stringency and duration) can be challenging, time consuming and expensive; especially if chips are lost in the work-up process. Being able to visualize the quality of each printed spot after washing provides us greater knowledge on the molecular kinetics of hybridization that occurs between printed probes and labeled targets, and will allow for optimization of the washing temperatures.

In routine scanning of spotted array slides, fluorescent spots representing the positive controls are essential to align tracking files. The use of the 25mer in printed spots makes this alignment of the tracking files simple and easy as each and every printed spot is visualized and detected by the software, allowing for less time spent positioning the tracking grids. Finally, incorporation of the 25mer allows for increased confidence in the data acquired by allowing for easy identification of problem spots due to hybridization problems, and by use of a print control calculation to account for any printing issues. This control system can be adapted easily in a three-color microarray by using complementary probes that have different fluorescence emission wavelength compared to that of the samples.

The only steps which it will not be of benefit would be during the initial design and synthesis of the 70mer oligos. Caution will have to be taken in these stages to ensure that the 25mer used will not cross-react with any of the 70mers designed or with any of the labeled sample DNA (see above). The 25mer system will not eliminate false prints (switched oligos),

cross reactions, or lack of hybridization due to incorrect probe design.

4.2 Conclusions

We have presented in this study a 25mer control system that is easy and inexpensive to use, and provides consistent results in spotted microarray. The ability to check every microarray chip for printing and hybridization efficiency allows for greater confidence and enhances reliability in the obtained results.

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Figures and Tables

Figure A.1 Box plot of representative trial (T3) of co-detection experiment. The p-values ofeach 70mer with and without 25mer are listed above each box plot. For *invA* 47°C (marked with *) the addition of the printed 25mer caused a significant increase in 70mer fluorescence signal, though this was not generally typical at this temperature.

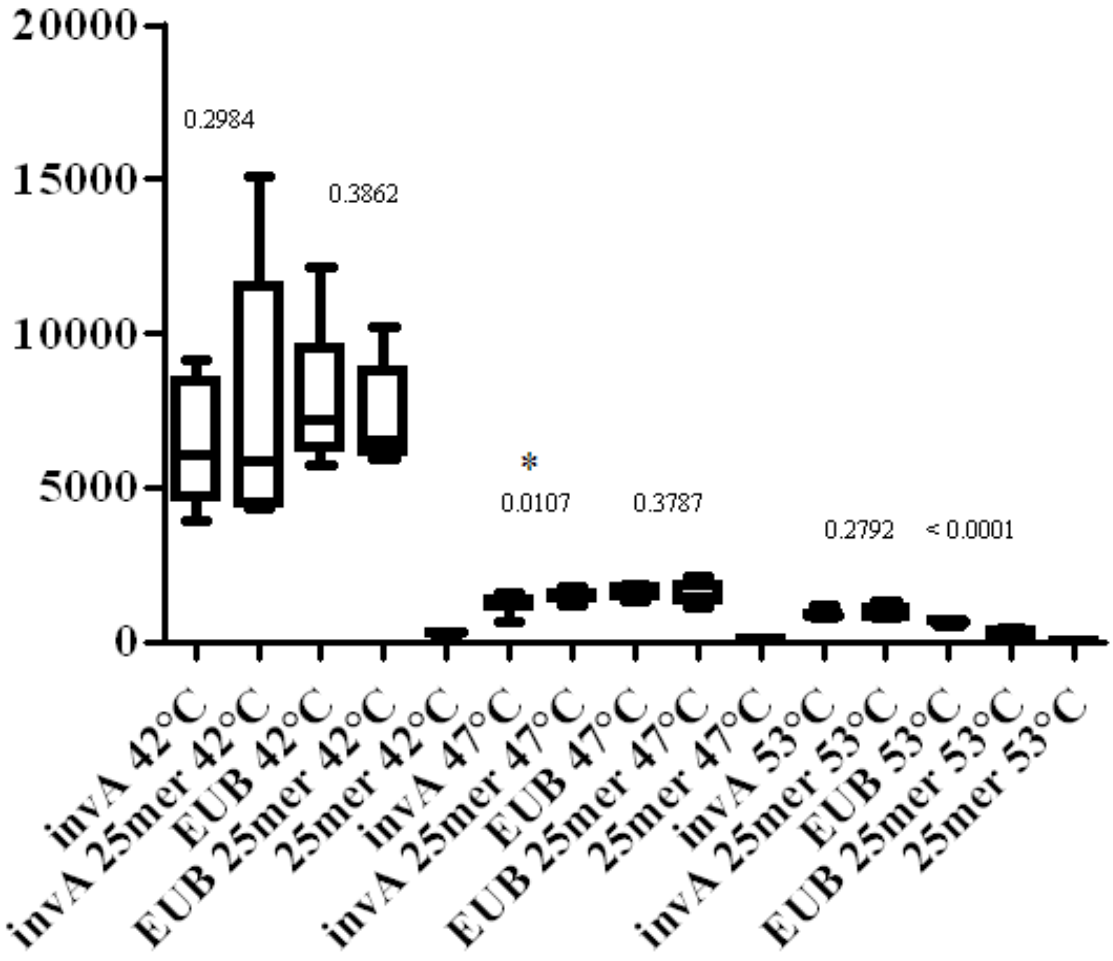

Figure A.2 Appearance of 25mer on microarray chip as detected by GenePix 4000B two color scanner. Figures A.2a and A.2d show labeled DNA bound to 70mer oligos (Cy 5 and Cy3 respectively). A.2b and A.2e show two-color ratio scan of labeled oligos. Figures A.2c and 296 nd A.2f show hybridization of labeled 25mer (Cy 3 and Cy 5 respectively) to its printed 25mer target. Variations in printing that is present in A.2c and A.2f can be corrected by the Print Correction Value option that the 25mer offers.

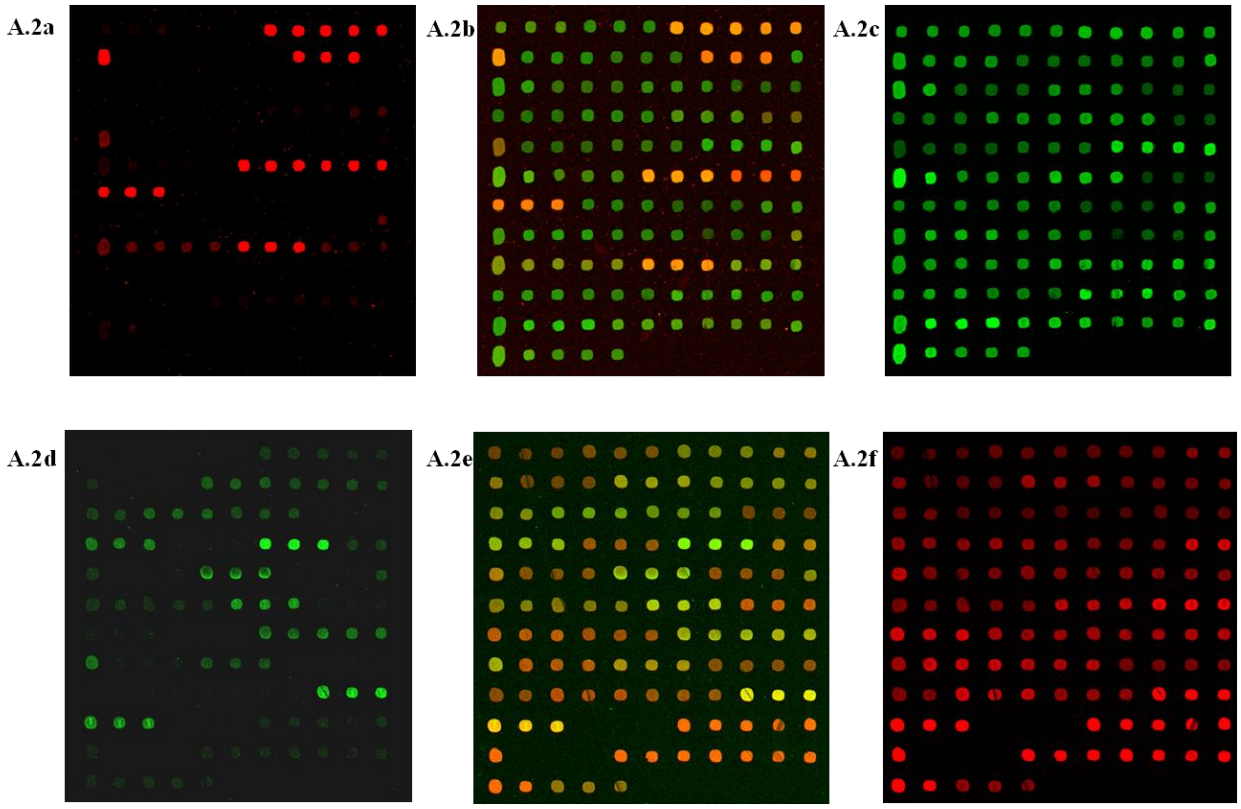

Figure A.3 Flow chart of microarray work flow showing which steps are benefited by the addition of the 25mer printing and hybridization system.

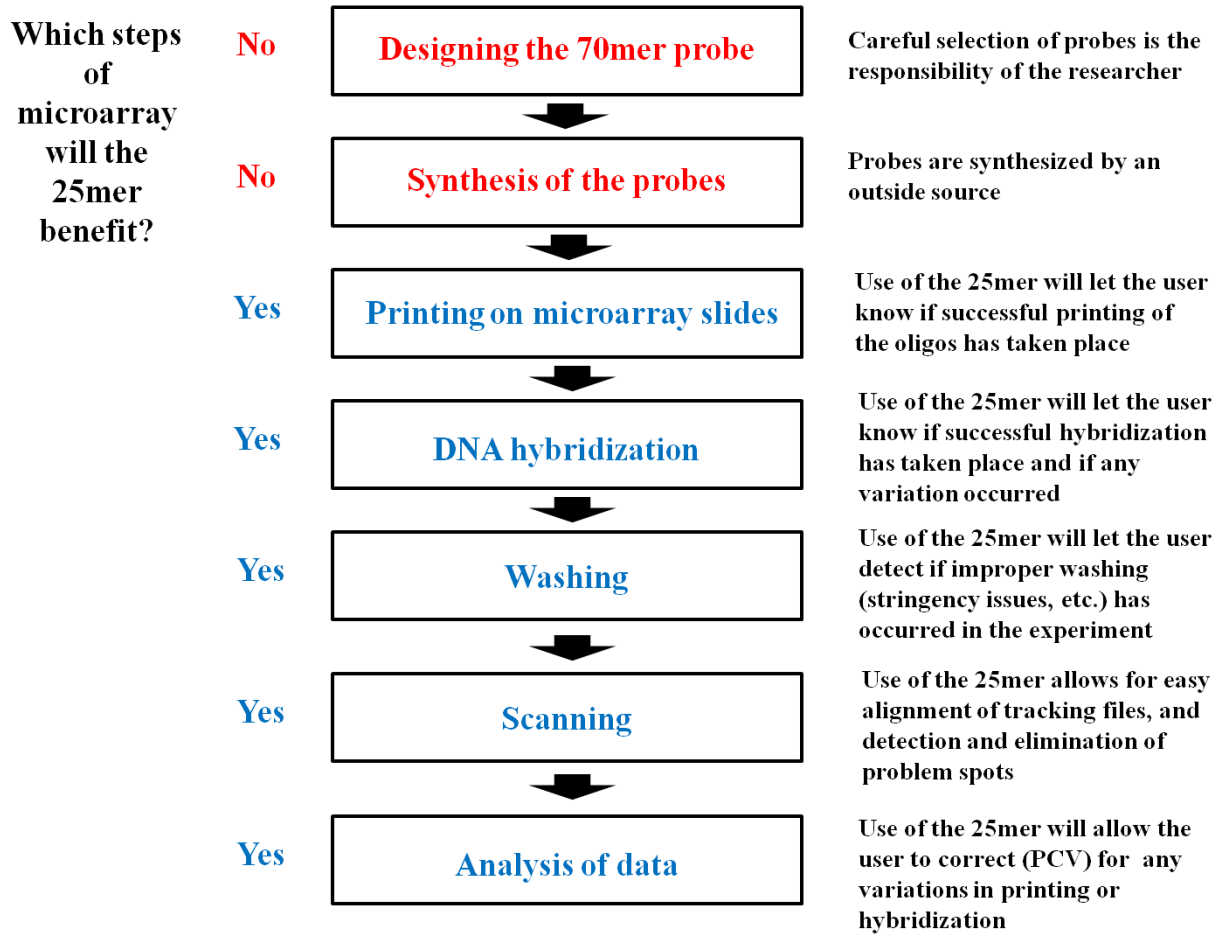

Table A.1 Complete list of all oligos with modifications used in this study. Column marked rank indicates the oligos producing the most fluorescent signal at 42°C.

Probe Name	Probe Sequence	Rank
Printed 25mer	GGATG CTAGA TCGTG TGCTC TCGCC	1
oligo 1	NGATG NTAGA TCGTG TGCTC TCGCC	6
oligo 2	NGATG NTAGA NCGTG TGCTC TCGCC	17
oligo 3	NGATG NTAGA NCGTG NGCTC TCGCC	19
oligo 4	NGATG NTAGA NCGTG NGCTC NCGCC	22
oligo 5	NGATG CTAGA TCGTG TGCTC TCGCC	2
oligo 6	GGATG NTAGA TCGTG TGCTC TCGCC	13
oligo 7	GGATG CTAGA NCGTG TGCTC TCGCC	11
oligo 8	GGATG CTAGA TCGTG NGCTC TCGCC	12
oligo 9	GGATG CTAGA TCGTG TGCTC NCGCC	5
oligo 10	GGATG CTAGA TCGTG TGCTC TCGCN	14
oligo 11	GGATG CTAGA TCGTG TGCTC TCGCCNNN	9
oligo 12	NNNGGATG CTAGA TCGTG TGCTC TCGCC	7
oligo 13	GGATG CTAGA TCGNNNTG TGCTC TCGCC	4
oligo 14	GGATG CTAGA TCNTG TGCTC TCGCC	16
oligo 15	GGATG CTAGA TCNNG TGCTC TCGCC	15
oligo 16	GGATG CTAGA TNNNG TGCTC TCGCC	20
oligo 17	GGATG CTAGA TNNNN TGCTC TCGCC	10
oligo 18	NNNNN CTAGA TCGTG TGCTC TCGCC	3
oligo 19	GGATG CTAGA NNNNN TGCTC TCGCC	21
oligo 20	GGATG CTAGA TCGTG TGCTC NNNNN	8
oligo 21	NNNNN CTAGA NNNNN TGCTC TCGCC	23
oligo 22	GGATG CTAGA NNNNN TGCTC NNNNN	24
oligo 23	NNNNN CTAGA NNNNN TGCTC NNNNN	26
oligo 24	NNNNN NNNNN NNNNN NNNNN NNNNN	25
oligo 26	NNNNN CTAGA TCGTG TGCTC NNNNN	18

Table A.2 Results of each probe and each trial (T1, T2, T3) by temperature. Black rectangles indicate positives and white rectangles indicate negatives. No hybridization occurred for T1 at 65°C, so data was not analyzed.

Appendix B - Diagnostic Microarray for Human and Animal Bacterial Diseases and Their Virulence and Antimicrobial Resistance Genes

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Abstract

Rapid diagnosis and treatment of disease is often based on the identification and characterization of causative agents derived from phenotypic characteristics. Current methods can be laborious and time-consuming, often requiring many skilled personnel and a large amount of lab space. The objective of our study was to develop a spotted microarray for rapid identification and characterization of bacterial pathogens and their antimicrobial resistance genes. Our spotted microarray consists of 489 70mer probes that detect 40 bacterial pathogens of medical, veterinary and zoonotic importance (including 15 NIAID Category A, B and C pathogens); associated genes that encode resistance for antimicrobial and metal resistance; and DNA elements that are important for horizontal gene transfer among bacteria. High specificity and reliability of the microarray was achieved for bacterial pathogens of animal and human importance by validating MDR pathogenic bacteria as pure cultures or by following their inoculation in complex and highly organic sample matrices, such as soil and manure.

Introduction

Laboratory diagnoses of bacterial diseases continue to be predominantly cultivation-based methods, including isolation, identification and antimicrobial susceptibility determinations. Molecular methods, such as PCR, have enhanced detection abilities and have replaced traditional methodologies for many infections (Versalovic & Lupski 2002; Sabat *et al.* 2006). However, the limitations of PCR include expense and human hours involved in developing and optimizing each assay, especially those that are specialized, such as multiplex-PCR, Real Time PCR, etc. Although high throughput PCR amplification and fluorescence-based detection systems have improved the number of target genes detectable (Brenan *et al.* 2005; Lucero & Spurgeon 2007), the number of genes detected in parallel is considerably less compared to microarray methodologies.

DNA microarrays, which work on nucleic acid hybridization principles, are widely used and have multiplex capability to detect hundreds of thousands of genes in a single experiment (Bryant *et al.* 2004). Spotted DNA microarray platforms are cost-effective, flexible and easy to use in any laboratory with basic facilities and equipment (Chittur 2004).

Most species of pathogenic bacteria have strains that vary in their disease-causing abilities (virulence). Such variations have been associated with virulence factors that provide a selective advantage to bacteria's parasitic lifestyle compared to less virulent strains belonging to the same species. Therefore, an approach that focuses on detecting genes that encode these virulence factors is essential to identify and characterize bacterial pathogens. Many of these genes are carried on horizontally transferable genetic elements, such as pathogenicity islands, lysogenic phages, plasmids, etc (Galan & Curtiss 1989; Chiu & Ou 1996; Paton & Paton 1998; Plunkett *et al.* 1999). Screening bacterial strains for such genetic elements can provide

understanding on the mode of acquisition of the virulence factors by that particular strain and can establish a clonal relationship between isolates.

Antimicrobial resistance is considered to be the cause of the next pandemic, and with the horizontal transfer of multidrug resistant (MDR) plasmids and transposons being a factor, potentially deadly strains are on the rise (Phillips *et al.* 2004). The significance of MDR bacteria is exemplified by vancomycin-resistant *Enterococcus* (VRE), which can not only withstand treatments with the most powerful antimicrobials but also has shown the ability to horizontally transfer the resistance genes to other species of bacteria (CDC 2002; Ray *et al.* 2003; NNIS 2004).

Intraspecies and interspecies horizontal transfer of genetic elements packaged as plasmids or transposons is an integral part of survival and evolution for many bacterial species and is often accomplished through bacterial conjugation (Thomas & Nielsen 2005). Horizontal transfer of virulence and antimicrobial resistance genes between bacteria has had a considerable impact on human and animal health and on medical practices (van den Eede *et al.* 2004) as demonstrated by the occurrence of VRE and methicillin resistant *Staphylococcus aureus* in ecosystems where they cohabitate with other pathogens (Hiramatsu 1998; Rice 2001; CDC 2002; Khan *et al.* 2002; DeLisle & Perl 2003; Ray *et al.* 2003; Rosenberg *et al.* 2004; Rice 2006; Tiwari & Sen 2006). Another significant group of genes that is horizontally transferred is the group that encodes metal resistance. Since these genes are frequently present in close proximity to the antimicrobial resistance (AMR) genes, selection for metal resistance may select for AMR genes and vice versa (Hasman & Aarestrup 2002; Hasman 2005; Hasman *et al.* 2006).

The objective of this study was to develop a spotted microarray that is capable of identifying pathogenic bacterial species based on their virulence factors and characterizing them

based on the presence of antimicrobial resistance genes, DNA segments essential for horizontal transfer of genetic material and genes for metal resistance. The objective included validation of the array using MDR pathogenic bacteria as pure cultures and following their inoculation into complex and highly organic sample matrices like soil and manure.

Materials and Methods

2.1. 70mer oligo development and selection

Conserved regions (300 to 400 bp) of the genes that encode virulence factors, antimicrobial resistance and metal resistance were identified by reviewing current literature available at PubMed (www.ncbi.nlm.nih.gov). One or more oligonucleotide probes (approximately 70 bp; 70mers) were identified for each gene using Picky 2.0 (size: 65-75mer with 40-60% GC content; Iowa State University, Ames, IA) and Oligowiz 2.1.0 (size: 70mer, with default settings; CBS, Lyngby, Denmark); and a consensus probes were selected for printing. The specificity of the selected sequences was confirmed by a blastn search (www.ncbi.nlm.nih.gov) in the GenBank database. Positive hybridization controls, based on 16S rRNA sequences (EUB (Yang *et al.* 2002), Frye 1, 2, and 3 [personal communication, Dr. Jonathan Frye]) 23S rRNA, and the beta subunit of RNA polymerase (*rpoB* (Aliyu *et al.* 2004)) were also synthesized and printed. Negative controls including H_2O only, hybridization buffer only and a 25 bp DNA probe without homology to any listing in GenBank were also printed (Peterson *et al.* 2009). Probes were synthesized by Invitrogen (Invitrogen Co., Carlsbad, CA) and Operon Biotechnologies (Operon, Huntsville, AL). A total of 113 virulence genes from 43 pathogenic bacteria, 227 AMR genes conferring resistance to 30 antimicrobials, 99 genes that encode resistance from 20 metals, 31 horizontally transferable elements and 7 positive control

oligos were chosen for this study. The entire list of the genes and their 70mer probe sequences is provided in Table 1 (supplemental).

2. 2. Microarray oligomer printing

A complete array containing 489 70mer oligos was printed at a concentration of 35µM in replicates of 3, 10 or 16. Two identical fields were printed on each Ultra Gap slide (Corning, Lowell, MA) using a Genetix QArray2 System slide printer (Genetix, Hampshire, UK) crosslinked in a UV Stratalinker 2400 (Stratagene, La Jolla, CA) at 600µJ, and stored in the dark at room temperature until used.

2.3. Bacterial cultures

DNA was isolated from the following bacterial strains: *Enterococcus faecalis* V583 PMV158GFP (Dr. Lynn Hancock, Kansas State University, *gfp, asc10, asa1*, *ermBCT*, and *vanB* positive), *Enterococcus faecium* R2-Tx5034 (Dr. Ludek Zurek, Kansas State University, *esp*, *ermBCT*, and *vanA* positive), *Escherichia coli* O157:H7 (ATCC 43894; *eae*, *stx1*, and *stx2* positive), *E. coli* O157 Neo 5-13-005 (clinical isolate with a plasmid containing *aphA1* gene that encodes for neomycin resistance), *Salmonella* Typhimurium DT104 (ATCC 700408; *invA* and *spvC* positive, *sopE* negative), *Salmonella* Munster (T. G Nagaraja, Kansas State University, *invA* and *sopE* positive) and *Fusobacterium necrophorum* subspecies *necrophorum* strain A25 (T. G Nagaraja, *lktA*, and *HAEM* positive).

2.4. DNA isolation

Pure cultures of *Salmonella* Typhimurium DT104, *Enterococcus faecalis* V583 PMV158GFP, and *E. coli* O157:H7 were grown overnight at 37^{o}C in 5 ml of TSB culture media, and *F. necrophorum* A25 were grown overnight at 37^{o}C in 5 ml of pre-reduced anaerobically sterilized brain-heart infusion broth (PRAS-BHI). DNA was extracted using a

DNeasy Blood & Tissue kit (Qiagen, Valencia, CA).

2.5 DNA extraction from environmental samples

DNA from bacteria-spiked manure and soil samples was isolated using a FastDNA Spin kit for Soil (MP Biomedicals, Solon, OH) and further purified using Geneclean Turbo kit (MP Biomedicals). This stepwise procedure was selected for DNA isolation from soil because it provided consistently higher yields and quality of DNA as shown by Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE) than all other kits used in our studies, including PowerSoil DNA kit (Mo Bio, Carlsbad, CA), UltraClean Fecal DNA kit (Mo Bio), Qiagen RNA/DNA Mini Kit (Qiagen, Valencia, CA), and QIAamp DNA Stool Mini kit (Qiagen, Valencia, CA).

A protocol that combined the FastDNA Spin kit for soil (MP Biomedicals) followed by purification using Geneclean Turbo kit (MP Biomedicals) was effective in extracting adequate amounts of DNA from flies (personal communication, Dr. Ludek Zurek), soil and cattle manure as well as water and fecal samples from dogs and cats (Narayanan, *et al*., unpublished data). The labeling and hybridization protocols are optimized in such a way that the same protocol could be used on all samples (pure cultures or complex matrices). Therefore, no modifications were necessary to perform microarray analysis of bacteria from soil and cattle manure, as well as from water and fecal samples from dogs and cats.

2.6. Environmental samples

Manure samples were surface-grabbed from feedlot cattle pens and mixed to represent many animals in individual pens. The soil samples at depths of 15.2 cm were collected from a corn field near the feedlot. *Salmonella* Typhimurium DT104 was added to manure and soil at concentrations of 10^{10}, 10^{8}, 10^{6}, 10^{4}, and 0 (i.e. unspiked) CFUs/g. Total DNA from these

samples was isolated, fluorescent labeled and used for hybridizing microarray chips (see below). A second series of spiking experiments was conducted using *Enterococcus faecalis* strain V583 PMV158GFP at concentrations of 10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6}, 10^{5}, and 10^{4} CFUs/gram in manure and soil samples.

2.7. Bacterial isolation from environmental samples

The viability of the bacteria spiked into manure and soil samples was assessed by suspending 250 mg of each environmental sample in 2.5 ml of PBS. serial dilutions of this suspension were plated on Brilliant Green agar (BD, Franklin Lakes, NJ) containing 10µg/ml tetracycline and 10µg/ml ampicillin (both antibiotics from Sigma, MO) was used for *Salmonella* Typhimurium DT104. *Salmonella* isolates from plates were confirmed using a *Salmonella* Poly O antigen sera agglutination test (Oxoid Inc., Ontario, Canada).

M-*Enterococcus* agar (BD, Franklin Lakes, NJ) with 10 µg/ml vancomycin (Sigma, St. Louis, MO) was used as a selective medium for *Enterococcus faecalis* V583 PMV158GFP. The identity of the strain was confirmed by their green fluorescence under UV light when grown on TSA plates.

2.8. Enrichment of environmental samples

Single colonies of *E. coli O157* (ATCC 43890, 4055397, 4064932, or 4027993) were grown on TSA plates overnight at 37°C, and was inoculated in 10mL of TSB and incubated overnight in a shaking airbath (37°C, 150 rpm). 1ml of the overnight cultures were mixed with 10ml of fresh TSB and incubated for an additional 6 hours to allow the bacteria to attain log phase growth. The bacterial suspensions were centrifuged at 5000 x g for 10 minutes, and the pellets were resuspended in 1ml TSB. Serial dilutions were made in TSB and 200µl of the diluted bacterial suspensions were mixed with 1g of autoclaved cattle feces. The final

concentrations of *E. coli*, as determined by plating, were 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFUs/g. Unspiked manure was again used as a negative control. Enrichment of fecal samples was carried out in Gram Negative (GN) (Greenquist *et al.* 2005; Jacob *et al.* 2008a) which contained Cefixime (0.5µg/ml) (Dynal, Lake Success, NY), Cefsulodin (10µg/ml) (Sigma, St. Louis, MO), and Vancomycin (8µg/ml) (Sigma, St. Louis, MO). After incubation (37°C, 150 rpm), DNA was extracted from 250µl of the solution using the FastDNA Spin kit for soil (MP Biomedicals) followed by purification using Geneclean Turbo kit (MP Biomedicals) as described above. Presence of the bacteria was determined by a positive signal of a microarray probe or a PCR specific for the O antigen-specific polymerase of *E. coli* O157 (O157 *wzy*) (Table 1; Table 1 Supplemental). The unspiked manure sample failed to show the presence of this gene even after enrichment.

2.9. DNA labeling and hybridization

DNA from all sources was labeled directly using the BioPrime Plus Array CGH Genomic Labeling System (Invitrogen Co., Carlsbad, CA) per the manufacturer's protocol with slight modifications. After random primer incorporation, an additional 1.5 µl of 1 mM Cy3 or 1 mM Cy5-dCTP (Amersham, Piscataway, NJ) was added to the labeling mixture to improve dye incorporation and amplification. For each sample, the overall labeling efficiency was determined with the Microarray Feature on Nanodrop ND-1000 spectrophotometer, and only samples that had a labeling efficiency of 1 pmol/µl were used for hybridization.

The microarray chips were prehybridized in blocking solution (0.1% BSA, 5X SSC, 1% SDS) at 42^{o}C for 1 hour with shaking, and then spun at 2,200 x g in a minicentrifuge (Fisher Scientific) to dry. The labeled DNA was mixed with an equal volume 2X hybridization solution (2% SDS, 30X SSC, 50% Formamide) or 2X Hybridization Solution #7 (Genisphere Inc.**,**

Hatfield, PA) and the mixture was added onto the chips. The labeled DNA mixture was hybridized overnight at 42^{o}C, and the chips were then washed for 10 min in each of the following wash buffers: 10X SSC + 0.2% sarkosyl, 10X SSC, and 0.2X SSC. Lastly, the slides were dipped quickly in water, spun dry at 2,200 x g and visualized on a GenePix 4000B slide reader (Molecular Devices, Sunnyvale, CA). The spotted probes that printed irregularly provided no signals, had inconsistent hybridization, or exhibited nonspecific binding were excluded from further consideration.

2.10. Spiked Primer DNA Labeling

In an attempt to increase the detection limit of our microarray, specific primers were added to random primers used in fluorescent labeling reactions (Figure 3). The primers were designed in two ways: either as "inside" primers, which were reverse complements of the last 18-25 nucleotides in the 3' end of the 70mer, or as "outside" primers (18-25 bp), which were reverse complements of a region that is 5 nucleotides downstream to the 70mer probe (Figure 3). The gene targets chosen for this experiment included *invA*, *intl1* (*Salmonella* specific), *sopE* (a *Salmonella* marker, but not present in the strain used), *asc10* (*Enterococcus* specific, negative control for *Salmonella* experiments) and EUB (positive control for all eubacteria). In total, 3 nM of the specific primers were spiked into the labeling mix both with and without random hexamers.

2.11. Data analyses

The fluorescent hybridization signals from the array were visualized using a GenePix 4000B slide reader (Molecular Devices, Sunnyvale, CA) and matched to the GenePix Array List (GAL) file previously created by the microarray slide printer. A GenePix Report (GPR) file was generated measuring the overall intensities. The fluorescence signal from each probe set was

averaged and used for further analyses. Data analysis was performed using the TIGR MultiExperiment Viewer (TMEV) program (TIGR, Rockville, MD) with one-color setting. Additional data analyses were performed using a previously developed relative pathogen signal (RPS) ratio method (Tomioka *et al.* 2005). Briefly, the averaged signal intensities for each probe were divided by signal intensities of positive control spots to gain an RPS ratio, and the values over 0.25 were considered positive and values below 0.25 were considered negative. Since EUB and Frye 3 produced the most consistent results they were considered for further analysis. The detection limit of a test is defined as is the lowest concentration of an analyte that can be detected in a given matrix (Morrison *et al.* 1979; Ripp 1996). The lowest concentration of *E. coli* O157 (measured in CFUs/g) that still gave an RPS ratio above the threshold in manure was considered to be the detection limit of the microarray.

Results

3.1. Specificity of microarray analysis

Total DNA from pure cultures of strains of *Salmonella* Typhimurium DT104, *F. necrophorum* A25, *Enterococcus faecalis* V583 PMV158GFP, and *E. coli* O157:H7 were used to evaluate the specificity of the chip. The chip was capable of detecting antimicrobial resistance genes, as well as distinguishing bacterial species based on species-specific virulence genes (Figure 1).

Microarray detected virulence genes *eae*, *stx1* and *stx2* from *E. coli* O157:H7 DNA; *lktA* and HAEM (hemagglutinin) from *F. necrophorum* DNA; *asa1* and *asc10* from *Enterococcus* DNA , and *invA* from *Salmonella* DNA. *Salmonella* DNA also hybridized with known markers for DT104; *tetG*, *tetR*, *sul1*, *flo*, and *aadA2*, and *Enterococcus* DNA was further identified by the presence of *ermB, ermBCT* and *vanB* genes. Minimal non-specific hybridizations infrequently

occurred, as was shown by total DNA from *F. necrophorum* hybridizing with the *hemA* probe for *C. parvum* (Figure 1).

Specificity of the platform was determined to be high for the array as it was capable of differentiating two clonally related species of *Enterococcus* (Figure 2). DNA from *E. faecium* R2-Tx5034 hybridized with probes for *esp*, *msrC*, *pbp5*, and *vanA* and was compared to *E. faecalis* V583 PMV158GFP used in the first study. Both *Salmonella* were positive for *ermB* and *ermBCT*, but the gene for *vanB* and *gfp* was only detected in *E. faecalis* V583 PMV158GFP (Lakticova *et al.* 2006). PCR was again performed for confirmation on selected genes (Table 1) (data not shown). Each of these genes corresponded with known genetic markers for the strain and was confirmed by PCR analysis or as reported in previous literature (Table 1) (data not shown).

Our microarray analysis confirmed a previous report that *msrC* was present only in *E. faecium* resistant to methicillin (Portillo *et al.* 2000) and *pbp5* was present in *E. faecium* resistant to penicillin (Fontana *et al.* 1983). When *Salmonella* Typhimurium DT104 and *Salmonella* Muenster were compared, both strains were positive for *invA*. However, the virulence gene *sopE* was present only in *Salmonella* Muenster. These results were also confirmed using PCR assays (data not shown). When *E. coli* O157:H7 ATCC 43894 was compared against *E. coli* O157 Neo 5-13-005 (a clinical isolate that has a plasmid with neomycin resistance), genes *stx1* and *stx2* were detected only in ATCC 43894, whereas *aphA1* for neomycin resistance was present only in *E. coli* O157 Neo 5-13-005 (data not shown).

3.2. Microarray of environmental samples

In order to determine the detection limit of the microarray platform, *Enterococcus faecalis* V583 PMV158GFP and *Salmonella enterica* DT104 were serially diluted and mixed

into manure and soil to obtain concentrations from 10^{10} to 10^{1} CFU/g. As expected, the positive control EUB probe for 16s rRNA was detectable at all dilutions, confirming adequate labeling and hybridization. The highest detection limit for species-specific genes, virulence factors or antimicrobial resistance genes was 10^{9} CFUs/gram for cattle manure or soil. To improve the detection limit of this array, a variety of methods was evaluated with mixed results. Indirect labeling and a Templiphi amplification kit (Amersham Biosciences, Piscataway, NJ) previously reported to be effective for increasing the detection limit (Wu *et al.* 2006), did not significantly improve the overall detection limit of the array. Additional experiments were conducted using specific primers that bound inside and outside of the 70mer sequence spiked along with the random hexamers in the hybridization mix to enhance specific labeling of the DNA. The inside primers mixed in with random hexamers consistently provided improvements to the detection limit (10^{8} CFU/g of *Salmonella* in cattle manure) as compared to random hexamer alone (no detectable signal) or random hexamers mixed in with outside primers (10^{9} CFUs/g, data not shown) (Figure 3; Figure 4; Table 2). The efficiency of labeling was poor when the inside and outside primers were used without random primers in the labeling mixture (data not shown). It is interesting to note that the 18-25 nucleotides in the 3' end of the 70mer did not impact the binding of the sample DNA. The detection limit was greatly improved to from 10^{9} to 10^{3} following enrichment in GN broth (Figure 4; Table 2).

Discussion

The spotted microarray developed in this study contained 489 oligomers, including a total of 113 virulence genes, 227 AMR genes, 99 metal resistance genes, 31 transferable elements and 7 positive control oligos. This encompasses a total of 43 bacterial species and resistance for 30 different antimicrobial agents. This microarray will add to the growing list of

microarrays used to aid in the diagnosis and characterization of disease-causing bacteria (Volokhov *et al.* 2002; Yu *et al.* 2004; Perreten *et al.* 2005; Tomioka *et al.* 2005; Chandler *et al.* 2006; Frye *et al.* 2006; Li *et al.* 2006; Dankbar *et al.* 2007; Kostic *et al.* 2007; Strommenger *et al.* 2007; Tembe *et al.* 2007).

The ability to rapidly and specifically identify and characterize bacterial pathogens is important to human and animal health. In our array, we designed oligomer probes to identify both veterinary and zoonotic human bacterial pathogens, which include many categories of select agents belonging to Category A, B and C as listed by NIAID, (http://www3.niaid.nih.gov/topics/emerging/list.htm) by targeting genes that encode known or putative virulence factors. This approach that focuses on detecting genes that encode these virulence factors is essential to identify and characterize virulent bacterial pathogens.

High specificity of our microarray was confirmed by the ability to differentiate between aerobic and anaerobic bacteria, and between gram-negative and gram-positive bacteria, including *Salmonella* Typhimurium DT104, *F. necrophorum* A25 *Enterococcus faecalis* V583 PMV158GFP, and *E. coli* O157:H7. It could also differentiate between two strains of *Enterococcus* (*E. faecium* R2-Tx5034 and *E. faecalis* V583 PMV158GFP), two strains of *E. coli* (O157 ATCC 43894, and O157 Neo 5-13-005) and two strains of *Salmonella enterica* (Typhimurium DT104 and Muenster). The specificity of our system proved to be very high, as probes corresponding to species-specific virulence genes distinguished the four species of bacteria and even between strains within species, some of which differ by as little as one gene (Figures 1 and 2). Probes corresponding to AMR, metal resistance and horizontally transferable genetic elements further characterized the bacterial strains and corresponded to known genetic markers of each of the tested bacteria.

Based on the ability of this microarray to accurately characterize bacterial strains, this technology could be standardized for use in routine diagnosis in human or animal hospitals. This "diagnostic lab on a chip" approach can be optimized to achieve a fool proof, versatile, high throughput, low-cost system for use in field setting, and could provide an important primary step in the diagnosis of disease as it is able to identify many virulence genes in a single test. With the rapid results that diagnostic microarrays such as this can provide, it may allow for more specific antimicrobial treatments to begin earlier and would aid greatly in source tracking as well as fingerprinting of any strain of bacteria. Spotted microarrays have an extra advantage of being easily customizable by allowing printing of probes to detect additional genes without having to refabricate the entire chip.

Rapid identification of causative agents of natural and maliciously introduced infectious disease outbreaks is pivotal in immediate treatment and control strategies. In such a situation, it is crucial to identify all pathogens involved in the outbreak, their total virulence profile and their overall antimicrobial susceptibilities. Since future bioterrorism threats could involve a mixture of agents, new or uncommon variants of known agents, or a single highly transmissible agent with multiple virulence and AMR genes (Phillips *et al.* 2004; Tomioka *et al.* 2005), we designed probes to detect genes encoding virulence factors. This gene-centered rather than pathogen-centered approach was the basic criterion for selection of probes to detect Group I (emerging), Group II (re-emerging), and Group III (agents with bioterrorism potential belonging to categories A-C) bacterial pathogens, antimicrobial resistance genes, metal resistance genes and horizontally transferable elements.

Many studies that use traditional bacteriology and PCR assays are laborious, time-consuming and are focused on phenotype characterization that limit the number of species,

subspecies or serotypes of the pathogen that can be identified (Ziebell *et al.* 2002; Inglis *et al.* 2006; Kim *et al.* 2006; Bolton *et al.* 2007; Kerouanton *et al.* 2007; Jacob *et al.* 2008a; Jacob *et al.* 2008b). With a spotted microarray, the genetic analysis of many bacterial species in various types of samples can be performed in one rapid test allowing for a global view of all species existent in a microcosm (Porwollik *et al.* 2003; Yu *et al.* 2004; Wu *et al.* 2005; Frye *et al.* 2006; Ballmer *et al.* 2007; Han *et al.* 2007; Malorny *et al.* 2007; Strommenger *et al.* 2007). Although microarrays in the past were designed to determine relative gene expression profiles, recent arrays like the one in the present study have focused on using this powerful technology for identification and characterization of pathogens (Chandler *et al.* 2006; Ballmer *et al.* 2007; Bonhomme *et al.* 2007; Ojha & Kostrzynska 2008). For example, this technology has been used in high throughput detection of methicillin-resistant *Staphylococcus* in hospitals (Strommenger *et al.* 2007) and in the identification of antimicrobial resistance genes (Perreten *et al.* 2005; Frye *et al.* 2006). Additional studies have included arrays that detect bioterrorism agents in blood samples (Tomioka *et al.* 2005) and those that detect pathogenic strains of *Shigella* and *E. coli* in fecal samples (Li *et al.* 2006).

Detection limits in complex matrices such as manure and soil are complicated by the presence of other competing DNA, inhibitors of labeling reactions (tannins, silica, etc.) and low yields of DNA (Gentry *et al.* 2006; Schadt CW 2006). Previous attempts to improve detection limits including using the Templiphi amplification kit (Amersham Biosciences, Piscataway, NJ) yielded marginal improvements (Wu *et al.* 2001; Wu *et al.* 2005; Wu *et al.* 2006). The detection limit of our microarray was marginally improved by the inside primer technique to 10^8 CFUs/gram, which is consistent with detection limits reported in previous studies (Schadt CW 2006). However, this technique may not be applicable for large arrays because of increased

probability of producing heterodimers, as well as increased cost. When the fecal samples were enriched with the GN broth, the detection limit improved to 10^3 CFUs/g. At this detection level, the array will be able to characterize most enteric bacteria, as studies have shown that infectious doses of enteric bacteria, such as *Salmonella*, is 10^3 CFUs/gram (Blaser & Newman 1982).

Conclusion

We have developed a microarray chip that is capable of identifying and characterizing bacterial pathogens that can cause human, animal and zoonotic diseases including 15 NIAID Category A, B and C bacterial pathogens. This array was developed and validated for four different bacterial species that carry resistance for multiple AMR, metal resistance and horizontally transferable elements. Although the detection limit of the current array limits its use in the presence of large amounts of contaminating DNA, it can be used with confidence in pure and enriched cultures.

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Tables and Figures

Figure B.1 Microarray gene profile of total DNA from four different pathogenic species of bacteria: A, DNA from *Salmonella* Typhimurium DT104 (ATCC 700408); B, *E. coli* O157:H7 (ATTC 43894); C, *Enterococcus faecalis* V583 PMV158GFP; and D, *Fusobacterium necrophorum* subspecies *necrophorum* strain A25.

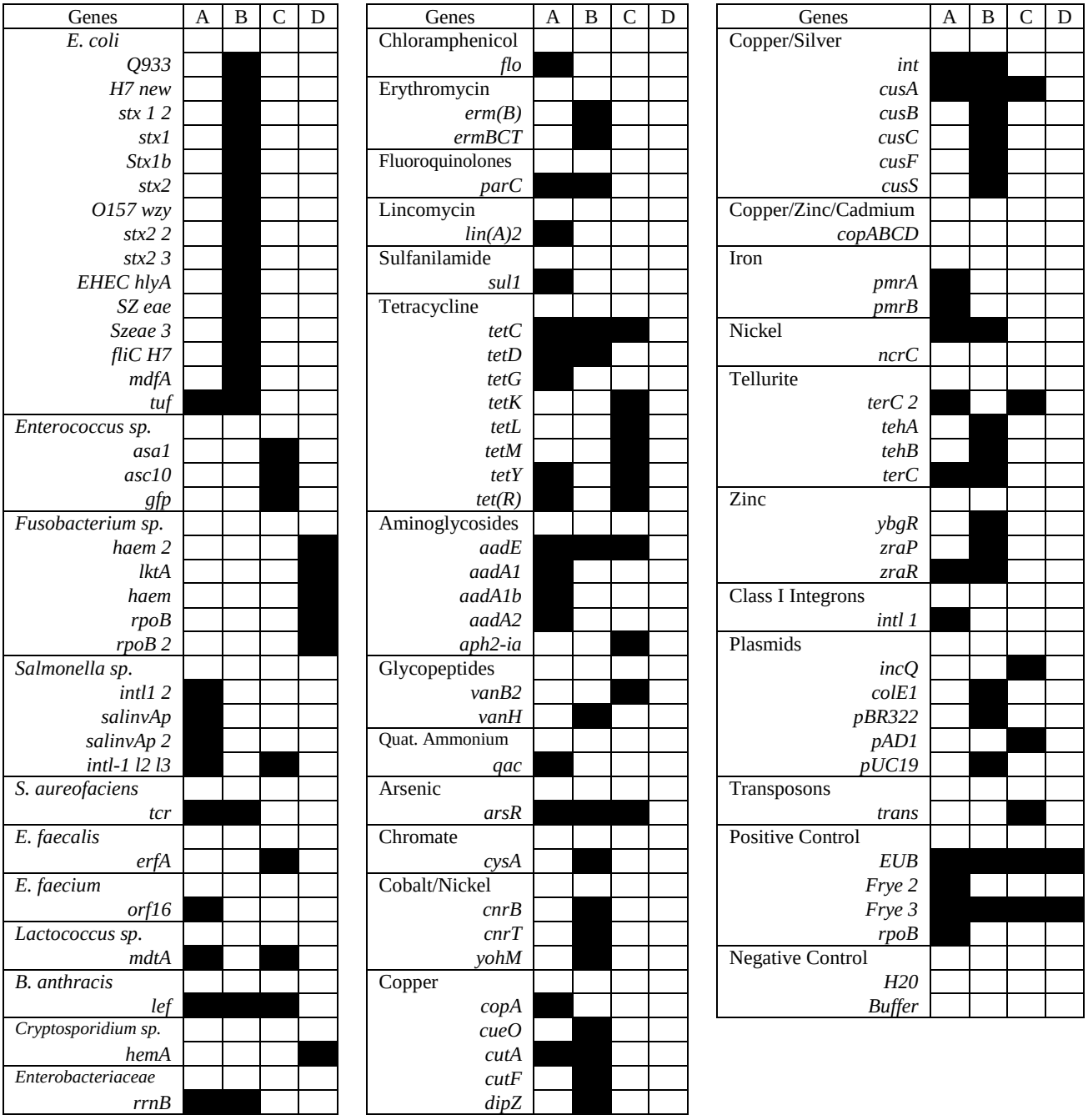

Figure B.2 Microarray gene profile of total DNA from two enterococcal strains: A, DNA from *Enterococcus faecalis* V583 PMV158GFP; and B, *Enterococcus faecium* R2-Tx5034.

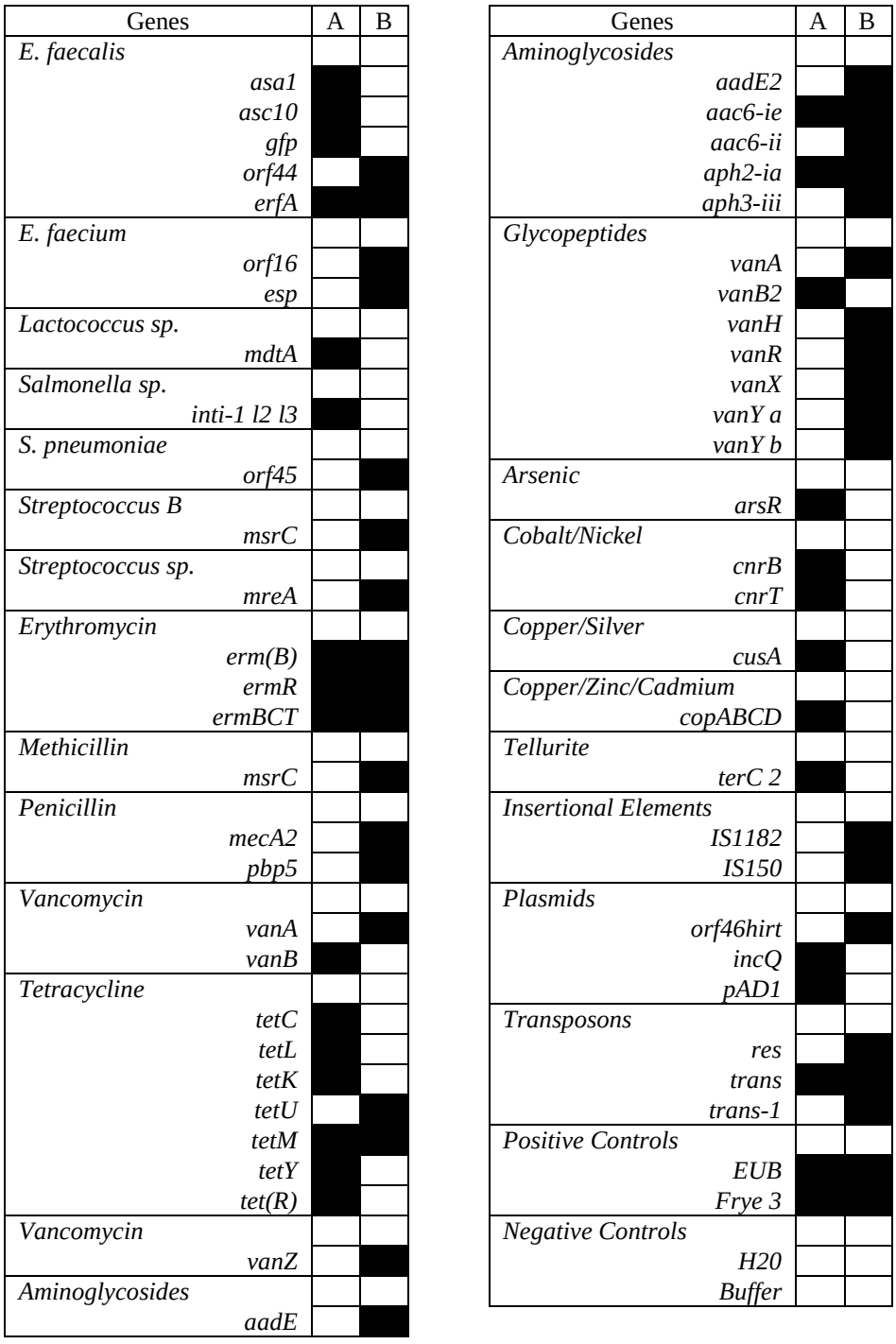

Figure B.3 Schematic alignment of inside and outside primers used in this study. A >>> symbol indicate the position of the original probe in relation to the primers.

Gene	Sequence
	>>
invA	5 ' ACCACCTATCTGGTTGATTTCCTGATCGCACTGAATATCGTACTGGCGATATTGGTGTTTATGGGGTCGTTCTACATTGACAGAATCCTCAGTTTT 3 ' ||||||||||||||||||||||||| |||||||||||||||||||| 3 ' CCGCTATAACCACAAATACCCCAGC GTAACTGTCTTAGGAGTCAA 5 '
	>>>
sopE	5 ' ATTGGCCCCTGCGCCGCTTGGCGTAAAAACGTCATTTTTGGTTGTTCCGGGCAGACCTGCATTTTCCGCTGCTTCGCCAATTTCCTGAAGAAAAGGC G CT 3 ' ||||||||||||||||||||||||| |||||||||||||||||||||| 3 ' AGGCCCGTCTGGACGTAAAAGGCGA TTAAAGGACTTCTTTTCCG C GA 5 '
	>>
intI 1	5 ' AAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAGTTTACGAACCG A 3 ' ||||||||||||||||||||||||| ||||||||||||||||||| 3 ' AAGACCTGGTCAACGCACTCGCGTA TGAACGTAATGTCAAATGC 5 '
	>>
asc10	5 ' ACGCAACCAGGATCAACGACAGTGCACCCCGATAACCCCGATCCGCAGTTAGGTAGTGCAACGCCTAATACGGCAGTATCTGAAGAATCTGCAGTAC A AA 3 ' ||||||||||||||||||||||||| |||||||||||||||||||| 3 ' ATCCATCACGTTGCGGATTATGCCG GACTTCTTAGACGTCATG T T 5 '
EUB	5 ' >> CCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCTCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCTACCCGAAA 3 ' |||||||||||||||||||||| |||||||||||||||||| 3 ' CGAGCGTGTTCGCCACCTCGTA AATTAAGCTACGATGGGC 5 '

Figure B.4 The detection limit (CFUs/g) following the inside primer technique and enrichment protocol.

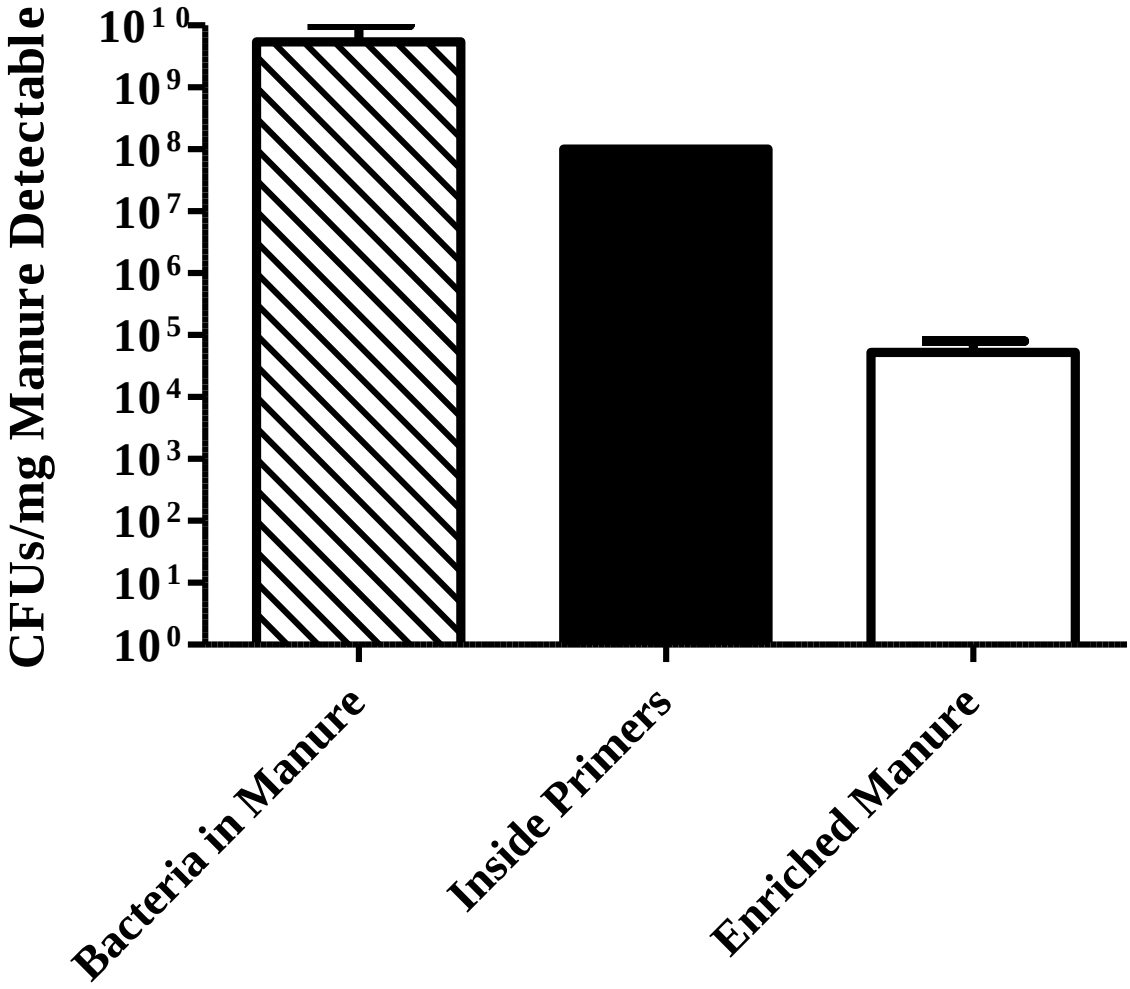

Table B.1 Complete list of primers used for PCR confirmation of 70mer oligos.

Gene	Forward Primer 5' to 3'	Reverse Primer 5' to 3'	Product Size	Reference
asa1	GCACGCTATTACGAACTATGA	TAAGAAAGAACATCACCACGA	375	(Vankerckhoven *et al.*, 2004)
asc10	GGTAGTGCAACGCCTAATAC	TTCAGCTCCTACTGTTGGTT	171	This study
ermB	GAATCCTTCTTCAACAATCA	ACTGAACATTCGTGTCACTT	175	(Jacob *et al.*, 2008a)
ermBCT	GAAATTGGAACAGGTAAAGG	TTTACTTTTGGTTTAGGATG	404	(Jost *et al.*, 2004)
esp	AGATTTCATCTTTGATTCTTGG	AATTGATTCTTTAGCATCTGG	510	(Vankerckhoven *et al.*, 2004)
EUB	TGGAGCATGTGGTTTAATTCGA	TGCGGGACTTAACCCAACA	161	(Yang *et al.*, 2002)
gfp	CAATGCTTTTCAAGATACCC	GTGTCCAAGAATGTTTCCAT	213	This study
haem	CATTGGGTTGGATAACGACTCCTAC	CAATTCTTTGTCTAAGATGGAAGCGG	310	(Tadepalli *et al*., 2007)
Intl 1	GTTCGGTCAAGGTTCTG	GCCAACTTTCAGCACATG	923	(Zhang *et al.*, 2004)
invA	TGCCTACAAGCATGAAATGG	AAACTGGACCACGGTGACAA	500	(Guerra *et al.*, 2000)
lktA	AAATGGTGAAAGAATGACAA	TGCATAATTTCCTACTCCTG	194	(Tadepalli *et al.*, 2007)
O157*rfb*	CGGACATCCATGTGATATGG	TTGCCTATGTACAGCTAATCC	259	(Paton & Paton, 1998a)
rpoB	TACGTATGCCTCACGGATCA	CTCTCGAAACAACCCCTTTG	183	(Narongwanichgarn *et al.*, 2003)
stx1	ACACTGGATGATCTCAGTGG	CTGAATCCCCCTCCATTATG	614	(Gannon *et al.*, 1992)
stx2	CCATGACAACGGACAGCAGTT	CCTGTCAACTGAGCAGCACTTTG	779	(Gannon *et al.*, 1992)
tufA1	TGATGACGAAGAGCTGCTGGAACT	CTTTCAGACCAGAACCACGAACGA	146	(de Sablet *et al.*, 2008)
vanA	CATGAATAGAATAAAAGTTGCAATA	CCCCTTTAACGCTAATACGATCAA	1030	(Kariyama *et al.*, 2000)
vanB	GTGACAAACCGGAGGCGAGGA	CCGCCATCCTCCTGCAAAAAA	433	(Kariyama *et al.*, 2000)

Table B.2 Results from sensitivity improvement experiments. Inside primers and outside primers with and without random hexamers results are not noted as they did not produce measureable levels of fluorescence. Values are listed as CFUs/g.

		Manure		Spiked Primers in Manure	Soil
Bacteria	**Genes**	**Unenriched**	**Enriched**	**Inside Primers with Hexamers**	**Unenriched**
S. enterica Typhimurium DT104	*invA*	10^{10}	-	10^{8}	10^{10}
	EUB	Unspiked	-	Unspiked	10^{4}
E. faecalis V583 PMV158 GPF	*asa1*	10^{9}	-	-	-
	asc10	10^{9}	-	-	-
	GFP	10^{9}	-	-	-
	vanB	10^{10}	-	-	-
	EUB	Unspiked	-	-	-
E. coli O157 48390	O157 *wzy*	10^{10}	10^{3}	-	-
	EUB	Unspiked	Unspiked	-	-
E. coli O157 4055397	O157 *wzy*	10^{10}	10^{4}	-	-
	EUB	Unspiked	Unspiked	-	-
E. coli O157 4064932	O157 *wzy*	10^{10}	10^{5}	-	-
	EUB	Unspiked	Unspiked	-	-
E. coli O157 4027993	O157 *wzy*	10^{10}	10^{5}	-	-
	EUB	Unspiked	Unspiked	-	-

Table B.3 Complete list of 70mer oligos designed using Picky 2.0 and Oligowiz 2.0 printed and used in this study. Oligos with a * were derived from 26-33mer probes origionally created by Perreten et al., 2005. Oligos with a ** were previously published by Frye et al., 2006.

	Virulence Genes		NCBI accession no
A. baumannii			
	aar3	CATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAG	AY038837
B. anthracis			
	cya	ACTATTTGCTATATCCTCCTCACAGGCTATAGAAGTAAATGCTATGAATGAACATTACACTGAGAGTGA	DQ889680
	lef	AGTGGTCCCGTCTTTATCCCCCTTGTACAGGGGGCGGGCGGTCATGGTGAT	M29081
	pX01	AGGGGTTGATGATTCATATTTAAAAAACGCATATAAGCAAATACTTAATTGGTCAAGCGATGGAGTTTCT	AE017336
	pX02	AGCCTTGATAGTGCGAGAAGACATATGAAAAACATAAAAATTGTAAGAATATTGAAACATGATGAGGCAA	AE017335
B. pseudomallei			
	BpeAB-oprB	TCCTTGCGCATCGCGGGGCTCGTGCGCAGCATGTCGAAGCAGCCGTCGACGAGTTTTTCCGCATGCCGTT	CP000572
Brucella sp.			
	brucella FB4U	AGCGAGGAAAGCCGCATGTTCCTGGTCAAGCAGGGGCATCATTCGATGATTTGCCGCCTGGAGCT	CP000709
	VirB	AGCCCGACTCGACCTGCACGATTGCTGCCATAGTCTGAGGTGCAACAGTCGGTGCGCATTGTTGCGAGG	AF141604
Campylobacter sp.			
	racR	CGGCTAGAGGTGACTTAAGCGATAAAGTTGTGGGGCTTCAAATCGGTGCTGATGATTATTTACCAAAGCCT	AF053960
	VirB11	AGTCTCAGGTGGAACAGGAAGTGGAAAAACTAGCTTTTTAAATTCTTTAATGGGTGAAATTGATCCAAAT	CP000550
C. difficile			
	ToxA	CCAATACAAGCCCTGTAGAAAAAAATTTACATTTTGTATGGATAGGTGGAGAAGTCAGTGATATTGCTCT	X51797
	ToxB	ATGGAGAGTCATTCAACTTATATGAACAAGAGTTGGTAGAAAGGTGGAATTTAGCTGCTGCTTCTGACA	X53138
C. glutamicum			
	ImrB	ACCTCTCCGGCAGCTGCGAAGCTGCCTAGGGAGGTCGTTGTTGTTCTTTCGATCCTCGTGGTTTCCGCGA	AF237667
C. botulinum			
	bont/A	ACTGATCTTGGAAGAATGTTGTTAACATCAATAGTAAGGGGAATACCATTTTGGGGTGGAAGTACAATAG	CP000727
	botulinum nt	AGCTTTGGACATGAAGTTTTGAATCTTACGCGAAATGGTTATGGCTCTACTC	CP000727
C. perfringens			
	catDP	AGCAGATTATGAAAGTGATACGCAACGGTATGGAAACAATCATAGAATGGAAGGAAAGCCAAATGCTCCGGAAAACAT	U15027
	cpe	AGGAGATGGTTGGATATTAGGGGAACCCTCAGTAGTTTCAAGTCAAATTCTTAATCCTAATGAAACAGGTACCT	CP000312
	cpa	ACAAGCTACATTCTATCTTGGAGAGGCTATGCACTATTTTGGAGATATAGATACTCCATATCATCCTGCTAATGT	EU839838
	cpb	AAAGCGAATATGCTGAATCATCTACAATAGAATATGTCCAACCTGATTTTTCTACTATACAGACAGATCAT	EU839838
	cpb2	ACACCATCATTTAGAACTCAAGTTTGTACATGGGATGATGAACTAGCACAAGCAATTGGGGGAGTTTATCCACA	EU085384
	ext	ATCTTGTAAAAAGTTTAGCAATCGCATCAGCGGTGATATCCATCTATTCAATAGTTAATATTGTTTCACCAACT	AY858558
Cryptosporidium sp.			
	DNAJ	AGCGAAGATGACCTTTTTGATTTGTTTATGAAGGAGGTTAATGAAGCAAACAGTAAAAGCACTAACCAAGGA	XM_625506
	hemA	ATTCTTTCACTCCTACAAATTCTGGTTCTTTGGAACTTTTCCAATTTGAAAGAAGAGATAGTCCTGT	U18120

E. coli

ereA3	ATGCCATCGTCTGTGTTACAAGCGCCGGCAAGGACAGCCTGGTTGCCC	AY183453
EHEC hlyA	ATAAGACGGATGTTGGTAAACTAACAATTGATGCAACAGGAGCATCAAAACCTGGTGAGTATATAGTTC	X94129
fliC H7	TCAAAACGTGATGCGTTAGCTGCCACCCTTCATGCTGATGTGGGTAAATCTGTTAATGGTTCTTACACCA	AM228905
flicC H11	TTAATATCGATGGCGCGCAGAAAGCAACTGGCAGTGACCTGATTTCTAAATTTAAAGCGACAGGTACT	AY973413
H11 new	TGGGAATGGTGTATATTCTGCAGAAATTGATGGTAAGTCAGTGACATTTACTGTGACAGATGCTGACAAA	AY337465
H7 new	ATGCTGCATTCGATAAATTAGGGAATGGCGATAAAGTCACAGTTGGCGGCGTAGATTATACTTACAACGC	AF228488
invE	AGCATTTTTTCATCTATGGAGCTCTCACATCAGAGCTCCACAAGAATATTATTCTTTTATCCAA	AF386526
ipaH	ACCGCCTTTCCGATACCGTCTCTGCCAGCAATACCTCCGGATTCCGTGAACAGGTCGCTGCATGGCTGGA	DQ132807
LT	AGCGGCGCAACATTTCAGGTCGAAGTCCCGGGCAGTCAACATATAGACTCCCAAAAAAAAGCCAT	CP000795
O157 wzy	GTCAAAGGATAACCGTAATCCTAAAATAAAAAGAATAATAGGGTATTTTTTATTGGTAGGGGTTGTATGC	AY647261
Q21	ATGCCTCGTTGTTTATGATTTACCCGATTCTGGATAGTGCGTTTAAAAACCGGAAACGTGTAGAGAAAAT	AJ605767
Q933	AGCGTGAATTGCCGGGAGGGAGAACCTCTGTATTTTATCAGCGAAAAAATAGTTTACGATCGTAAAAATC	AF548457
ST Sta	CCGTTTAACTAATCTCAAATATCCGTGAAACAACATGACGGGAGGTAACATGAAAAAGCTAATGTTGGCAA	AJ555214
Stb	AGCCAAGGAAAGTTGTAAAAAAGGTTTTTTAGGGGTTAGAGATGGTACTGCTGGAGCATGCTTTGGCGC	AY028790
stx 1 2	ACCTTTACAGTTAAAGTGGGAGATAAAGAATTATTTACTAACAGATGGAATCTTCAGTCTCTTCTTCTCA	AY135685
stx1	ACGCAGTCTGTGGCAAGAGCGATGTTACGGTTTGTTACTGTGACAGCTGAAGCTTTACGTTTTCGGCA	EF685162
Stx1	GATCTCAGTGGGCGTTCTTATGTAATGACTGCTGAAGATGTTGATCTTACATTGAACTGGGGAAGGTTGA	EF079675
STX1	AGTGGAACCTCACTGACGCAGTCTGTGGCAAGAGCGATGTTACGGTTTGTTACTGTGACAGCTGAAGCT	EF685162
STX-1 FAM IBQ-1	CACTGACGCAGTCTGTGGCAAGAGCGATGTTACGGTTTGTTACTGTGACAGCTGAAGCTTTACGTTTTCGGCA	EF685162
stx2	ACGCCGGGAGACGTGGACCTCACTCTGAACTGGGGGCGAATCAGCAATGTGCTTCCGGAGTATCGGGGAG	AF525041
StX2	TCAGATTTTACACATATATCAGTGCCCGGTGTGACAACGGTTTCCATGACAACGGACAGCAGTTATACCA	EF079674
stx2 2	AGTACCTGTGAATCAGGCTCCGGATTTGCTGAAGTGCAGTTTAATAATGACTGAGGCATAACCTGATTC	AM230664
stx2 3	CTCTTGAACATATATCTCAGGGGACCACATCGGTGTCTGTTATTAACCACACCCCACCGGGCAGTTATTT	AB168111
stx2 3	CTCTTGAACATATATCTCAGGGGACCACATCGGTGTCTGTTATTAACCACACCCCACCGGGCAGTTATTT	EF079674
STX-2 FAM IBQ-1	ACGCCGGGAGACGTGGACCTCACTCTGAACTGGGGGCGAATCAGCAATGTGCTTCCGGAGTATCGGGGA	EU086525
Stx2e	TGCTCAGCTGACAGGGATGACTGTAACAATCATATCTAATACCTGCAGTTCAGGCTCAGGCTTTGCCCAGG	DQ449665
SZ eae	ACCAGCGCCGGGATCCCATCGTTTCGTCTAAATATATCCATAATCATTTTATTTAGAGGGAGGGAGGGGGGA	BA000007
SZ eae 2	AACCTAATAACACAAAACCCTCTTCCTGGGGTTAATGTTAATACTCCAAATGTCTATGCGGTTTGTGTAGAA	EF079676
Szeae 3	TGAGCAGTATTATGGTGATAATGTTGCTTTGTTTAATTCTGATAAGCTGCAGTCGAATCCTGGTGCGGC	EF079676
Tuf	ACTTCCCGGGCGACGACACTCCGATCGTTCGTGGTTCTGCTCTGAAAGCGCTGGAAGGCGACGCAGAGT	CP000800
Tuf 2	GTTCGAATCTGAAGTGTACATTCTGTCCAAAGATGAAGGCGGCCGTCATACTCCGTTCTTCAAAGGCTAC	X57091

E. maxima

ww2	CAATTAATGGATTTGAAGCTCTTTAGAAGGGTGGATGTCCTCTTGCATGAAGAGGTTTGTGAGCGCGCGC	AY779455

Enterbacteriaceae

rrnB	AGCGGGTGAAAAGCCCGCTCGCCGGAAGACCAAGGGTTCCTGTCCAACGTTAATCGGGGCAGGGTGAGT	V00331

Enterobacter sp

nrpA	GATAATCATCAGGCTGTACTTATTTGGACATTTCAACTGCTTGAAAGGGAACCAGCCTTAAATGAATTAG	DQ264843
nrpB	AATACGCTGACAAGTATGTATTATGACCGTGCTATACGTATCGAATTGCCAGTCGCTGATAAGTTATGTA	DQ264843

Enterococcus sp.

Asa1	GCATGAATGATGTCTTGGACACGACCCATGATCGTTTCACAGGAAAATGGCACGCTATTACGAACTATGA	X62656

Asa1 2	CGCTCCTCTTTTATATATAGAGAGGCTACCCAGCAATGATAACTAGGTAGCCACAGACATAAAATT	X17214
Asa1 Hirt	GGGGCATGAATGATGTCTTGGACACGACCCATGATCGTTTCACAGGAAAATGGCACGCTATTACGAACTATGACCT	X62656
cyt	ATTGTTGCATCAGCAGGAAATGAGTCGCGTGATATAAGCACTGGTAATGAAAAACATATACCAGGAGGAC	AF454824
asc10	ACGCAACCAGGATCAACGACAGTGCACCCCGATAACCCCGATCCGCAGTTAGGTAGTGCAACGCCTAATACGGC	AY855841
esp	ATGTTGACACAACAGTTAAGGGGAAAGTATTCATCCACGAGTTAGCGGGAACAGGTCACAAAGCGCAACT	AF034779
esp 2	AAAAGATACAGTAAAAGTTCCAGTAGAAGTAACAGACAATCGCTCTGACGCTGATAAATATGAGCCAACA	AF034779
gelE	TCAGAGTAACTTCTTCACCAACTGGTGACCCCGTATCATTGGTTTATAAAGTGAACGCTACAGATGGAACAAT	D85393
GFP	TGTTCAATGCTTTTCAAGATACCCAGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGT	AF286456
GFP 2	TGGATGAACTATACAAATAAGCTTAATTAGCTGAGCTTGGACTCCTGTTGATAGATCCAGTAATGACCTC	DQ493885
oppD	GCGCAACATCCGTATACAAAAGTCCTTCTAAATGCTATTCCTATTGCAGATCCACAACTAACTAGGTAACG	CP000803
RD5	GAAGAGCAAGAGCAATATGAAACTGTAAAAACTCAAGCGGAGAAAGAAGCAGAGCAAAAATACCAACCTC	CP000608
RD7	ATGCAATTGCTCAAGGTTCATCGGAGAGAAGTGTGACATTTGCTGTAAAAGCAGAGGATGAAGTTCGAGGT	CP000608

F. tularensis

Fusobacterium sp.

HAEM	ATGGAATGTTTATTCTGGTTCATTGGGTTGGATAACGACTCCTACTTTGGATCCAGGAACGGGAAAAGT	AF529887
HAEM 2	AAAGAATCCAAGCAACCGCTTCCATCTTAGACAAAGAATTGAAATATCTCAAGAAAGAATGGGAT	AF529887
lktA	ACAGCAAAACGTTATAGTTCTGTTGCAATTGGAAATGCCGCAGTCGGAGTGGCTGCAAAAGGAGCTGGA	DQ672338
rpoB 2	GGGGAAAAAGCGAGAGATGTTCGAGACAGTTCTCTACGTATGCCTCACGGATCAAAAGGAACGGTTGTGGA	AF527637

Listeria sp.

actA	ACCGCCAACTGCATTACGATTAACCCCGACATAATATTTGCAGCGACAGATAGCGAAGATTCCAGTCTGA	EF661572
iap	AGCAACTATCGCGGCTACAGCTGGGATTGCGGTAACAGCATTTGCTGCGCCAACAATCGCATCCGCAAGC	AY072791
inlA	ACATCAGTCCCCTAGCAGGTTTAACCGCACTCACTAACTTAGAGCTTAATGAAAATCAGCTGGAAGATATTAGC	AB276427
inlB	ACGGGGCGAAAGTACAAGCGGAGACTATCACCGTGCCAACGCCAATCAAGCAAATTTTTCCAGATGATGC	DQ302480
hlyA1	ACCACGGAGATGCAGTGACAAATGTGCCGCCAAGAAAAGGTTATAAAGATGGAAATGAATATATCGTTGT	DQ844159
inlD	AACAAAACAGCATTACGTATCTTAGTAACTCTGGCTGTAGTAATGGCAATTAGCTTTTGGGTAGGGACGA	DQ347810

M. elsdenii

phy	ACGCCGGGCGAAATCGACGCCTTCCTGGCTTTCGTCCGTACCTTGCCAGCTGACGCCTGGCTGCACGACC	DQ257441

N. meningitidis

terC 2	CCCGATTTCCGTTTCACTGTCGGTCGTGTTCGGCGCGTTGGGTGCATCGATACTGACCTCGTTAATTTAT	AL157959

P. aeruginosa

mexC/D	TCCAGATCGATCCGGCACCGCTGAAGGCTGCGGTGTCGCGCGCCGAGGGTGAGCTGGCGCGGAACCGCGC	U57969
nfxB	ACCTCACCCACCGCGAGCTGCTGGTATTCCTGGTATTCCAGTACCGCCCGGACTTCCTCGACCCGCACGG	AY180395

Rickettsia sp.

tsa686	ACAACTGGCCTGCCATTTGGTGGTACATTAGCTGCTGGTATGACAATTGCTCCAGGTTTTAGAGCAGAGC	U80635

Salmonella sp.

fliC	GTCGCTGATATTGCCACTGGCGCGACGGATGTTAATGCTGCTACCTTACAATCAAGCAAAAATGT	AY864776
IntI-1 L2 L3	AAACCTTGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAGGTTGCCGGGT	EU006711
Intl1	AAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCAT	EU052800
SalInvAP	ACCTACCTATCTGGTTGATTTCCTGATCGCACTGAATATCGTACTGGCGATATTGGTGTTTATGGGGTCG	DQ644633
SalInvAP 2	TTATTGATTGCACATAAAGATCTTGTCCTCCTTACGTCTGTCGATGTCCGTCGATTTATTAAGAAA	U43273
Salmonella InvA	ACAGGATACCTATAGTGCTGCTTTCTCTACTTAACAGTGCTCGTTTACGACCTGAATTACTGATTCTGGT	AE008832

sopE	AAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCAT	AY168875
sopE 2	GGTCTTTACTCGCACTACCTCTAATATCTATATCATTGAGCGTTTGAAGCATAAAATCTTTAACG	AY167930

S. aureofaciens

tcr	ACGGCGAACAGTTCGGTGGTGATGTCACTCAAGGTCTCGGCCGCGGGGGAGAGATCGGTCATGGGGGCAG	D38215

S. fradiae

tcl	ACGGCCGAGAACAGGGGCTTTCGCATGCGTACATCACCTTCCTCCCAGCTTTCCCTGCACGGTGTCACCA	M57437

S. rimosus

otrB	ATGCCGTTCTACGGCAAGCTGTCCGACATCTACGGGCGCAAGCCCATGTACCTGATCTCCATCGTGGTGT	AF061335

S. suis

mrp	TGGGGCAGCAAGCGTTTTGCTTGGTGTGTCGTTAGTTTTAGGTGCTGGTGCACAGGTTGTTAAGGCTGATGA	CP000408

S. aureus

can	ACAGTACCATTAACTGTTAAAGGTGAACAGGTGGGTCAAGCAGTTATTACACCAGACGGTGCAACAAT	M81736
FnBPA	CGGAACAAAACAATACTACAGTAGAGGAAAGTGGGAGTTCAGCTACTGAAAGTAAAGCAAGCGAAACACA	X62992
vga	ACGTGGTGAAGATGTCTCGGGTACAATTGAAGGACGGGTATTGTGGA	M90056

S. agalactiae

bca	ATGGGGATGTTTCTCAGTTGCAGAGTACAGGAAGGGCTAGTCTTACCTATAATATATTTGGTGAAGATGG	M97256
bac	AGTAGCTAGTGTAGCGGTACGTAGTTTGTTCATGGGAAGCGTTGCTCATGCAAGTGAGCTTGTAAAGGACGA	X58470
cyl	AGCCAACGAAGCCACTGTCTCTAACTATAAAGAATCATATGGTGTTGTTTTGCGTAATGCTGATGGAGAAAGGCT	CP000114
sec	ACACCCAACGTTTTAGCAGAGAGTCAACCAGACCCTATGCCAGATGATTTGCACAAATCAAGTGAGTTTA	AP009324

S. equi

fnz	AGGTGCAACATTATTGTTTGGTTTAGGTCATAATGAGGCCAAAGCCGAGGAGAATTCAGTACAAGACGT	Y17116
SeM	TGCCGGTGCAGCATCAGTATTAGTTGCAACAAGTGTGTTGGGAGGGACAACTGTAAAAGCGAACTCTGAGGT	AF012927

S. pneumoniae

Hyal1	AGTCATTGAGGCTAAGGATGGGGCTATCACTATCTCAAGCCCTGAGAAATTAAGGGCAGCGGTTCACCGTATGGT	CP000410
Nana	AGCTCAAGAAGGGGCAAGTGAGCAACCTCTGGCAAATGAAACTCAACTTTCGGGGGAGAGCTCAACCCTAACT	CP000410
ply	CCGACTTCTTATCTAGCCAGACGGCGACGCAATGTCTCAGAAGAATTGTACGAGGAAATTTTGGATCACT	X52474
orf45	ACCGGTACGCTTATATAGAAGATATCGCCGTATGTAAGGATTTCAGGGGGCAAGGCATAGGCAGCGCGCT	AM490850

S. pyogenes

scp	CTGGTCAAGGGTATAGAAACCGTACTGATTGCGGTTATAGTCAGGTTTTTCTTTGTGTAACATAGGAGAACCT	CP000261

Streptococcus G

Protein G	AGGAAGCAACCCATTCTTCACAGCAGCTGCGCTTGCAGTAATGGCTGGGGCGGGTGCTTTGGCGGTCGCT	X06173

S. zooepidemicus

zag	GCGGACATTACAGGAGCAGCCTTGTTGGAGGCTAAAGAAGCTGCTATCAATGAACTAAAGCAGTATGGCA	U25852

V. cholerae

eltor	AGGCTAGGTGGGCCAGGCGACGGTTGTCCTGGTTCAAGTGCGTAGGCTTGAGAGTTAGGTAAATCCGGCT	AE003852

Y. pestis

yfeA	ATGTCCCGTCTGCCGTCGTGACCGCAGGTATTACACCCCTGCCTATCCGAGAAGGCCCCTATAGCGGCAT	YPU50597

Species Specific AMR

C. difficile

catD	ATGTATCTATGATACCGTGGTCAACCTTCGATGGCTTTAATCTGAATTTGCAGAAAGGATATGAT	X15100
cme	TGGCTCATCACTTGTACAAATGGCAATGATATGGTATGTCACACTTCAGACATCATCAGGTGTATGGGTA	AY362981

Corynebacterium sp.

cmr	TCGTCCCATTCCGCCAACAAGGCCGCGTATTTGGACTAGCCATGGCAGTGGAAATGGCAGCCAACCCGCT	CGU43535
lmrB	ACCTCTCCGGCAGCTGCGAAGCTGCCTAGGGAGGTCGTTGTTGTTCTTTCGATCCTCGTGGTTTCCGCGA	AF237667

E. coli

mdfA	ACTTGCTGTTAGCGCGTCTGACCTCGCGCCGCACCGTACGTTCGCTGATTATTATGGGCGGCTGGCCGAT	Y08743

E. faecalis

aad9	CAGCAAGAAATGGTACCGTGGAATCATCCTCCCAAACAAGAATTTATTTATGGAGAATGGTTACAAGAGCT	M69221
erfA	TGTGTGTTTGGTTTTATTTTGATTGAATTAGGCTTGCCGACCATTTTAGCACGAATGATTGACAAAGGAA	AE016830
erfB	ACGGCTAATTTCTGTCGTCGGTAATCCAGTCAAATAAGGCAACGCTGCATTAAAGGCAACGGT	AE016830
orf44	CCGAGGATTTGTGGGAGAAACTTTTGTCCACCTACCGGATGGATTCCTATGAAAACATATGGGAAGCAT	X92945

E. faecium

emtA	AGGTGGTTCATTCACGGCTTTTTTGTGTTCTTAAAACGGCGTTCAAAAAAGTGGAGGCGTAAGTATGACT	AF403298
inuB *	CATCCAACTGGTTGTTTGACGTAGCTCCGTACTTGATGCTTTATAAAAATGAGTACGGAACAGAGGTAGT	AJ238249
orf16	ATGCCATCAATTGTTCAGGGCGGTATCCGGTGAGGTGGCGGAAAGGCTTCATTATGCCTATCCGGAGTAT	AM490850
pbp5	AGCACGGCAAAAATCGAACAGGCGCTTATATTGCCGGCGCAGTGATCTTAATAGCAGCTGCGGGTGGCGG	X92687

Lactococcus sp.

mdtA	TGGCGGCACGATTGTCCACGCGGGTTGGGTTGAAGTTCTATTTGCTGCTGCGATGCTGATTGGCTCTTTTGCTGT	X92946
mdtA 2 *	ACCGCTCAGATGCCAACAGTCCAATCTATTATGCCCACAATGGTACCAGAAGACGAAATTACTCGAGTCAACGG	X92946

M. elsdenii

tetOW	ATGGGGCCATCTTGGTGATCTCCGCGAAAGATGGCGTGCAGGCCCAGACCCGTATTCTGTTCCATGCCCT	AY485126

S. aureus

inuA *	ACTCATTGGTTAGATGGAGGCTGGGGCGTAGATGTATTAACTGGAAAACAACAAAGAGAACACAGAGA	J03947
NorA	AGGACCAGGGATTGGTGGATTTATGGCAGAAGTTTCACATCGTATGCCATTTTACTTTGCAGGAGCAT	D90119

Strep. B

flo	ACTTGGGTTTAAATGAAGCACTTGAGCGTTCTTGTAATGTTTTGAGTGGTGGGGAAAGAACGAAATTATCG	M81802
msr(SA)	AGTGAAAAACAAAGATTGTCCCAAGCCAGTAAAGCTAAACGAAATCAAGCGCAACAAATGGCACAAGCATC	AB013298
msrC	AGGGTTTGCTCAGGAAACTCTGACGAAACCGTTGTGTACGTTAAGTGGGGGAGAAGCGACTCGTTTGACG	AY004350
msrD mel	CCGTAGCATTGGAACAGCTTTTCACACCCCGGCTCTCAATGCGGTTACGCCACTTTTAGTACCAGAAGAACAGCT	AF227521
orf5	AGCTGCAGAATACGAACAATTTATTGCGGAACGTGCTCGATTGGAAAGGGCTGCGGAGGAAAAGCGAAAACAGGCT	AF227520
vgb	AGGGCGAATAACTCCTCTGGGGGAAATTACCGAATTCAAAATTCCAACGCCAAACGCTCGACCTCATGCA	M20129

Streptococcus sp.

mreA	ACCTGTGGCATGGTAGTACATGGAGATGCTAGAGGACGAACTATAGGGTTCCCAACTGCTAATCTAGCT	U92073

Antimicrobial Resistace

Bleomycin

ble **	ATGGATTCGCAGTTCTAATGTGTAATGAGGTTCGGATTCATCTATGGGAGGCAAGTGATGAAGGCTGGCG	D86934

Chloramphenicol

cat **	CGACATGAAGAGTTCAGGACCGCATTAGATGAAAACGGACAGGTAGGCGTTTTTTCAGAAATGCTGCCTT	M35190
cat	ACCAGTTGCTCTGCAACTTCATCATTCTGTATGTGATGGTTACCATGCTTCACTATTTAT	X92945
cat4 **	CCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGAAATTCCGTATGGCAATGAAAGACGGTGA	AJ401050
cat-86 *	AGCAAAAGCAGCAACCTATTTCCGAAACCTCATATGCCAGAAAACATGTTCAATATTTCAAGTCTACCGTGG	K00544
catB *	AGATTGAAATGGCTAGGTCACGGAAAAAAGCCTTCTAAAATAGAATTACGAAAATTTTTAGGAGGCCCGA	M93113

*catDP1 **	AGCAGATTATGAAAGTGATACGCAACGGTATGGAAACAATCATAGAATGGAAGGAAAGCCAAATGCTCCGGAAAACAT	U15027
cat-DPS	ATGTATCTATGATACCGTGGTCAACCTTCGATGGCTTTAATCTGAATTTGCAGAAAGGATATGAT	X15100
*cat-LM **	ATGAAATGTTTCCTAAAAAACCAATACCTGAAAACACAGTTCCTATTTCGATGATTCCTTGGATTGATTT	X68412
*catP ***	TGGCAATTCAAGTTCATCACGCAGTATGTGACGGATTTCACATTTGCCGTTTTGTAAACGAATTGCAGGA	L02937
*catP **	GTGTTTAGAACAGGAATTAATAGTGAGAATAAATTAGGCTATTGGGATAAGTTAAATCCTTTGTATACAG	M64281
*catQ **	ACTGCAAATATAGAGATAACTGGTTTACTGCGTGAAATTAAACTTAAGGGCCTGAAACTGTACCCTACGCT	m55620
*catS **	ACAGAGACATTTTCCAACCTTTGGACACCATACATACCAGATTTTGAAGCATTTTCTATGGCGTATGCGA	X74948
*cat-TC **	AGGGTGATAAACTCAAATACAGCTTTTAGAACTGGTTACAATAGCGACGGAGAGTTAGGTTATTGGG	U75299
*cfr **	ATGGGAATGGGTGAAGCTCTAGCCAACCGTCAAGTATTTGATGCTCTTGATTCGTTTACGGATCCTAAT	AJ249217
*flo ***	GATATTCATTACTTTGGCTATACTGGCGATGCTCGCACTCCTAAATGCGGGTTTCAGGTGGCACGAAACC	AF252885

Erythromycin

*ere(A) ***	CGCAATTGGCCGAAATTATCCAGCTCATCGATCACCTCATGAAACCGCACGTTGATATGTTGACTCACTT	A15069
*ere(A2) ***	CCGGTGCTCATGAACTTGAGCGATTTTCGGATACCCTGACCTTTTCTTTGTATGGCTCAGTGCTGATTTG	AF512546
*ere(B) ***	GCAGGGCGATATGGGTGCAAAAGACAAATACATGGCAGATTCTGTGCTGTGGCATTTAAAAAACCCACAA	A15097
*erm(A) ***	AAGTGGGTAAACCGTGAATATCGTGTTCTTTTCACTAAAAACCAATTCCGACAGGCTTTGAAGCATGCAA	D86934
*erm(B) ***	ACAAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATT	AJ243541
*ermC ***	TTTGAAATCGGCTCAGGAAAAGGCCATTTTACCCTTGAATTAGTAAAGAGGTGTAATTTCGTAACTGCCA	NC001386
*erm(F) ***	GATTTGAAACTTGTCTATGAGGTAGGTCCTGAAAGTTTCTTGCCACCGCCAACTGTCAAATCAGCCCTGT	U30830
*erm(G) ***	TTTGAAATAGGTGCAGGGAAAGGTCATTTTACTGCTGAATTGGTAAAGAGATGTAATTTTGTTACGGCGA	M15332
*erm(TR) ***	AGAGGGGATTTGCTAAAAGGTTGCAAAATACCCAACGAGCTTTAGGTTTGCTGTTAATGGTGGAAATGGA	AF002716
*ermA **	TGGGTAAACCGTGAATATCGTGTTCTTTTCACTAAAAACCAATTCCGACAGGCTTTGAAGCATGCAAATGTCAC	X03216
*ermB **	ACCGTTTACGAAATTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACG	Y00116
ErmBCT	ACCGATACCGTTTACGAAATTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAAC	EF525477
ermC	ATCGGCTCAGGAAAAGGCCATTTTACCCTTGAATTAGTAAAGAGGTGTAATTTCGTAACTGCCAT	V01278
*ermC **	ATCGGCTCAGGAAAAGGCCATTTTACCCTTGAATTAGTAAAGAGGTGTAATTTCGTAACTGCCAT	J01755
ermC	CGTCTAATAGCCGGTTAAGTAATAGCCGGTTAAGTGGTCAAACTTTGGGAAAATCTCAACCCGCATTAAG	X82664
*ermD **	TGCGTTTCGGGGACTTGCCGAATACGCGCTAAAGGAGCCGAATATCCCTCTCTGTGTTGCTTTACGCGGA	M29832
*ermF **	ATGTTCAAGTTGTCGGTTGTGATTTTAGGAATTTTGCAGTTCCGAAATTTCCTTTCAAAGTGGTGT	M14730
*ermG **	CCTAGGTATTATTTCCATCCAAAACCTAAAGTGGATAGCGCATTAATTGTATTAAAAAGAAAGCCAGC	M15332
*ermQ **	CACCAACTGATATGTGGCTAGTTATGGAGAAAGGTTCCGCAAAAAGATTTATGGGAATACCTAGAGAGAG	L22689
*ermT **	AGATTGGTTCAGGGAAAGGTCATTTCTCGTTTGAATTAGCTAAAAGGTGTAATTATGTAACCGCCAT	M64090
*ermX **	ACCTCCTCGGCGGCGGTCGAAGTGGTCCATGATGATTTCCTTAACTTCCGGTTACCCGCCACTCCCTGCGT	M36726
*ermY **	ATTGGTTCAGGAAAAGGGCATTTCACACTAGAACTGGTTCAAAAATGTAATTATGTAACAGTTATCGAGA	AB014481

Fluroquinilones

parC	TGTCGCAGATGGGCTGGGTACGCAGCGCTAAAGGCCATGATATCGACGCGCCGGGCCTGAATTATAA	M58408

Lincomycin

car(A)	ACTTCACTCACCCAGATCAGGAGGACTCCTGCGTGTCGACAGCGCAACTAGCTCTGCATGACATCACCAAGC	M80346
lin(A)	ACCCTTAACAATCCCAAAACTTGTCGAATGGTCGGCTTAATAGCTCACGCTATGCCGACATTCGTCTGCA	M14039
lmr(A)	ACCGCGGATTACCGGTTCCCTATTCCCTATTCCCAGGTAATGCACCGGATATCGAGGGCCGTGGCGGCACGT	X59926
lsaB orf3	CCTCATGGAAGTTGAACTTTGACCGACAGCAGGGACATGAACAAGCAACAAATGAACGCTTGCAGAAGGA	AJ579365

Methicillin

ccrA	AGAAAAGTTGGCACAAGGCATCATTGATGCAGAAACGTTCAGAGAACAAACGCAATCATTACGTC	DQ483067
ccrB	GTGAAAAACGTCGTAAAGGGCTGAATGATAAACCAGTGATAGCTGAAGGTAAGCATTCCCCCATT	DQ483075

Oleandomycin

ole(B)	TGGGCCGCTCCCTTTTGGCCACACTGCCTCTTTTTCTCCGAATGGCGGATCACCCATGCAGAACGCACACCGT	L36601
oleC	ACTGCTCGCCATGATGGGCATGAACATGGCCCAGGGCGTGGGAACCGGCTTCAACCAGGACTTCAACTCCGGT	L06249

PCN-binding prot 2

pbp4	ACCGGTACGCTTATATAGAAGATATCGCCGTATGTAAGGATTTCAGGGGGCAAGGCATAGGCAGCGCGCT	AM490850
*mecA **	AATTGGCAAATCCGGTACTGCAGAACTCAAAATGAAACAAGGAGAAACTGGCAGACAAATTGGGTGGTTT	AB096217
*mecA ***	AATTGGCAAATCCGGTACTGCAGAACTCAAAATGAAACAAGGAGAAACTGGCAGACAAATTGGGTGGTTT	AY271717
mecA-2	AGCTCCAACATGAAGATGGCTATCGTGTCACAATCGTTGACGATAATAGCAATACAATCGCACATACA	AB266532
*penA ***	ACTTATCCGACGTTGGATGGCGAGAACGTCTTATAGTGACTCTGTCAACTGCCACACTCAACCTATCTCG	L02928

Pristinamycin

vgaB	TGGAGCAAGCTATAAAGCTAAAAGAGAATAAGGCGCAAGGAATGATTAAGCCCCCTTCAAAAACAATGGGAACAT	U82085

Rifampin

*arr-3 ***	ATAATTACAAGCAGGTGCAAGGACCGTTCTATCATGGAACCAAAGCCAATTTGGCGATTGGTGACTTGCT	AY038837

Spiramycin

srm(B)	ACCACCTCACCCCGGTGCTGGTGGAGGAGTTGGAGCAGGCACTCGCGGACTACCGCGGCGCCGTCGTGGT	X63451

Streptogramin A

satA	CGGTGCCATAGAAATAGAAATGCAACTTGTTCCAGGCTTTACTCCTCGTGCTTGCGGAACATTCCGAAGCCAGCA	L12033
satG	ACGTGTGGTTTGGGCAAAATGTGACCGTCCTACCAGGCGTAAAAATAGGTGACGGTGCCATTATCGGAGCA	AF139725
vat	ATGCCTGGGGTGAAAATTGGGGACGGGGCAATCATTGCTGCAGAAGCTGTTGTCACAAAGAATGTTGCT	L07778
vatB	TGTGTGGATTGGTCAGAATGTTACTGTTATGCCAGGAATTCAAATAGGAGATGGAGCAATTGTTGCTGCG	U19459
*vatB 2 **	TGGTCAGAATGTTACTGTTATGCCAGGAATTCAAATAGGAGATGGAGCAATTGTTGCTGCGAATTCAGT	U19459
*vatC **	ACACGGAAATTGGGAACGATGTTTGGATTGGACGAGATGTGACAATTATGCCCGGTGTAAAAATAGGAAACGGGGC	AF015628

Streptogramins

*sat(G) vat(E-8) ***	ACTGACTGATTTGCCGTTGAAAGGTGATACTGTAGTCGGAAATGACGTGTGGTTTGGGCAAAATGTGACC	AY043213
vga(A)	CGGGTACAATTGAAGGACGGGTATTGTGGAAAGCAAAAAGTTTTAGTATTCGCGGAGGAGACAAGATGGC	M90056

Streptothricin

*sat4 **	TTGGAACCGGTACGCTTATATAGAAGATATCGCCGTATGTAAGGATTTCAGGGGGCAAGGCATAGGCAGC	AF516335

Sulfanilamide

*sul1 ***	CTACCTGAACGATATCCAAGGATTTCCTGACCCTGCGCTCTATCCCGATATTGCTGAGGCGGACTGCAGG	AY458224
*sulI1 ***	GAATAAATCGCTCATCATTTTCGGCATCGTCAACATAACCTCGGACAGTTTCTCCGATGGAGGCCGGTAT	NC005324

Tetracycline

otrA	AGGCGACCACGAATCCGGCAGGAAAGGTCCGCATCACGACGCTAAGGAATCCGAACCCCCTCCA	X53401
otrC	ACATCGCCATGTCGGTCGGCTCCGGCGTCAACGACGACTTCAACAAGGGGGTGATGGACCGCTTCCGCACCAT	AY509111
tcr	ACGGCGAACAGTTCGGTGGTGATGTCACTCAAGGTCTCGGCCGCGGGGGAGAGATCGGTCATGGGGGCAG	D38215
tet30	CTGCCGGAAAGCCGAAAGGCCGGTCCGGGCAAGTTTGCGTTCAAGGAACTTAACCCGTTGGCGCCATTGGT	AF090987
tet31	CCTTGCAGGGCTAGGGTTAATGCATATTATCTTTCAGGCTTTTGTCGCAGGATATATCGCATCTCGCTGGA	AJ250203
tet32	AGGTTTCCCTGTCGCCAAAGATAACCATGACCGATATTTCTGATTTGGACAAATGGGATATGATTATTTCCGGA	AJ295238
tet33	ATTGGGCAGGCTCCAGGTGCGACCTGGGTGCTGTTTACTGAACACCGCCTCGACTGGAGTCCCGTCGAAGT	AJ420072
tet34	ACGACGAAGATTGCGATACCAACGCTGACCTGAGTTTTCACGCATTTCATCGAGCAACGCACGATTGGTTGGCA	AB061440

tet35	AGCTAACTACGCGTTCTGGCAGGATGCCATTTTAGACTTCGACAGCCACGACAGAATCGCACAAACGA	AF353562
tet36	CAGCAGAGGTCAGTTCCTACACACAAGGCTTGGGCGTTTTTGTTACTCGATTTTCGGGTTATCGACCTACCA	AJ514254
tet37	AGACTGATCCAGGCTTGGTTATCTGCTACGGAACGATGTATGGGAACACAGAGGATCGCACACCGTCGAT	AF540889
tet38	ACGACAGTAAGTTGGCAAGCGACATTAGCCGGTTTAGTAATTGGTATTGGCGCTGTAGTATACGCT	AY825285
tet39	ATGGCTGCTGCTCCTTCTCTATTGTGGCTATATATTGGTCGTATTTTTGCGGGAATTACAGGTGCCAACATGGCTGT	AY743590
tetA	TGCCGGCCGCGCTTTGGGTCATTTTCGGCGAGGATCGCTTTCACTGGGACGCGACCACGATCGGCATTT	AF534183
tet(A) **	CAGCCTGACCTCGATCGTCGGACCCCTCCTCTTCACGGCGATCTATGCGGCTTCTATAACAACGTGGAAC	AJ634602
tetAP *	ATTGTATGGGGATTAGGGTCTACTTTTATCAGTGGCTCGCTTGAAGCTTGGATTGCAGAAGAAGAGAAGA	L20800
tetB	TGCCGATACCACCTCAGCTTCTCAACGCGTGAAGTGGTTCGGTTGGTTAGGGGCAAGTTTTGGGCTTGGT	AB084246
tetBP	ATGGAGCAATACTAGTTATATCAGGAGTAGAGGGGATTCAGTCACAAACAAGAATATTATTTGACACA	L20800
tetC *	TGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGT	AY171578
tetD	ACTCGGGGCGATGCATGCACTGTTTCAGGCGGTGGTTGCCGGGGCGCTGGCAAAACGGCTGAGTGAGAAAACCA	L06798
tetE	ATGCCTGTCTTGCCGGCGTTATTACGGGAGTTTGTTGGAAAGGCTAATGTTGCAGAGAACTACGGTGT	Y19116
tetG	ATTGCCGGGCCAGCACTTGGTGGCATGCTCGGTGGTATTTCTGCTCATGCTCCGTTTATCGCCGCTGCCCT	AF133139
tetH	GGCGCATCATTGCGGGGATCACAGGCGCAACAGGTGCCGTATGTGCATCAGCGATGAGTGATGTGACTCCCGC	Y15510
tetJ	ACGGGACTTGGCTTCTACAGGGTGGTTTCAATCTATTCTTGCTGAAGCCATGGCCATCACGGGAAAAGCAT	AF038993
tetK	ACTACTCCTGGAATTACAAACTGGGTAAACACTGCATATATGTTAACTTTTTCGATAGGAACAGCAGTA	S67449
tetK 2 *	ATTTTTGGTAGGTTAGTACAAGGAGTAGGATCTGCTGCATTCCCTTCACTGATTATGGTGGTTGTAGCTAGA	M16217
tetL *	ACCACCTGCGAGTACAAACTGGGTGAACACAGCCTTTATGTTAACCTTTTCCATTGGAACAGCT	M11036
tetM	ATCCCCTCCCTCTGCTGCAAACGACTGTTGAACCGAGCAAACCTCAACAAAGGGAAATGTTACTTGATGCACT	X04388
tetM 2	CCGCACCCTCTACTACAAACAACTGTTGAACCGAGTAAACCTGAACAGAGAGAAATGTTGCTTGATGCCC	AP009324
tetO	AGGCACAACAAGGACAGATACAATGAATTTGGAGCGTCAAAGGGGAATCACTATCCAGACAGCAGTGACA	M18896
tet(O) **	GAAAAGCAGAATATACCATCCACATAGAAGTCCCGCCAAATCCTTTCTGGGCTTCTGTCGGGTTGTCCAT	M18896
tetO 2	AGCGTCAAAGGGGAATCACTATCCAGACAGCAGTGACATCTTTTCAGTGGGAGGATGTAAAAGTCAACAT	M18896
tetQ	CCGCAAAGGAAGGCATACAAGCGCAAACAAAGTTGCTGTTCAATACTTTACAAAAACTGCAAATCCCGAC	Z21523
tet(R) **	ATGTTTATCAGTGATAAAGTGTCAAGCATGACAAAGTTGCAGCCGAATACAGTGATCCGTGCCGCCCTGG	AJ634602
tetS *	CGGTATCTTAGCACATGTTGATGCAGGAAAAACTACTTTGACAGAAAGCTTACTATACAGTAGCGGAGCAAT	L09756
tetT *	ACCTGGGCACATGGATTTCATAGCCGAAGTTGAGCGAACTCTGAAAGTGTTAGATGGAGCTAT	L42544
tetU *	ACGTGGCAAAGCAACGGATTGGCATGCGATGGTTCAGGAAAGCTTAGATAGTTTTGCAAGCCCGCAT	U01917
tetV	AGTTCTACCCGGTGGTGGTGTCGACGCTGATCTACATCGTGACCACGATGGCCTCGGGCAACCCCATGCT	AF030344
tetW 2	ACCGGGGAGCGTCGAAAAAGGGACAACGAGGACGGACACCATGTTTTTGGAGCGGCAGCGTGGGAT	DQ294299
tetW *	TGCCCATGTAGACGCTGGAAAGACGACCTTGACGGAGAGCCTGCTATATGCCAGCGGAGCCAT	AJ222769
tetX	CTTGCCCTAAAAGATTGATTGGAATTTATTCAGGACTGCCTATCACTTAGATAGAAGTCCAAGGTCTGCT	M37699
tetY	ACCGGCACTCTTTCAAAGCGACTGGGTGACCGCGGTGTGTTGCTGCTTGGAATGGGCGCTGATATGTGCGGGT	AF070999
tetZ *	AGCAAACCGCAGAGGTCGGCAATGCGAGCGTTCCTGAACGCGACCGGGAATTCGATTTCGGCCTGACAGCACT	AF121000

Trimethoprim

dfr1 **	AGCCGGAAGGTGATGTTTACTTTCCTGAAATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGC	AJ400733
dfrA1 **	TTATCTCTCCTCCCGTCGTAACAGCAAAGCTGCATACCGGTTTCTGGGTAAAATCCTCAACAACGTGAAG	AJ628353
dhfrI **	CAGTTTTGATTATGGGTAGAAAAACTTTTGCCTCACTGCCTAAAGTGCTGCCCGGACGACTTCATGTGGT	X57730

Tylosin

tlcC	ACGGCCGAGAACAGGGGCTTTCGCATGCGTACATCACCTTCCTCCCAGCTTTCCCTGCACGGTGTCACCA	M57437

Vancomycin

*VanB **	ACCGGAGGCGAGGACGCTTACCTACCCTGTCTTTGTGAAGCCGGCACGGTCAGGTTCGTCCTTTGGCGT	U00456
*vanC **	AGGCAATTCACCGGAATACACCGTTTCTTTAGCTTCAGCAACTAGCGCAATCGAAGCACTCCAATCATCT	AF162694
vanC2	CACCGTTTCTTTAGCTTCAGCAACTAGCGCAATCGAAGCACTCCAATCATCTCCCTATGACTACGACCTC	L29638
vanD4-5	AGCCCTGCCGGGACTGGGAGAACTATGCGGGATACCCGGCTGTGATTTCTCCGGACAGAAGGATCCATGGC	AF277571
vanZ	ACAAATACTGTTGGAGGCTTTCTTGGACTGAAATTATATGGTTTAAGCAATAAGCATATGAATCA	M97297

Virginiamycin

vgbA	ACAGAGTACCCACTACCGACACCAGATGCAAAAGTCATGTGTTTAACTATATCCTCAGATGGGGAAGT	M20129
vgbB	AGGTGGCTTTACAGAATATCCATTGCCACAGCCGGATTCTGGTCCTTACGGAATAACGGAAGGTCTAAATGGCG	AF015628

Miscellaneous AMR

Acetyltransferase

vatA	ACCATCGGATGGATGGATCAACATATCCTTTTCATCTATTCAGGATGGGTTGGGAGAAGTATATGCCTTCCT	L07778
vatD	CGGGGATGGTGCAATAGTAGCTGCTAATTCTGTTGTTGTAAAAGATATAGCGCCATACATGTTAGCTGGAG	L12033
vatE	TCGGAAATGACGTGTGGTTTGGGCAAAATGTGACCGTCCTACCAGGCGTAAAAATAGGTGACGGTGCCAT	AF139725

Aminoglycosides

aadB	TACTTTTACTATGCCGATGAAGTACCACCAGTGGACTGGCCTACAAAGCACATAGAGTCCTACAGGCTCG	AY204504
aac(3)-Id	ATTAAAAAACTCAAGGCTATAGGCGCAGCGCGTGGAGCTTATGTGATTTACGTCCAAGCTGATAAAGGCG	AY458224
aac(3)-III	CCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGG	X13542
*aac(6)-Ib**	AAGTCGCCTGGAAAACGGCATCAGAATACGATTCAAACGGCATTCTCGATTGCTTTGCTATCGAAGGAAA	AY103455
AAC6-Ie	CCAAAAACTGATGAGATAGTCTATGGTATGGATCAATTTATAGGAGAGCCAAATTATTGGAGTAAAGGAATTGG	M18086
AAC6-Ii	ACAGCTCGGCAGAAGAAGTAGAAGAAATGATGAATCCAGAACGAATCGCGGTAGCAGCGGTAGACCAAGAT	L12710
AAC6-Im	AGCGAGTTTCCTTTCGCCCGATGAATGAGGATGATTTAGTTCTAATGTTAAAATGGCTGACAGATGACCG	AF337947
aacC1	CCGCAGTGGCTCTCTATACAAAGTTGGGCATACGGGAAGAAGTGATGCACTTTGATATCGACCCAAGTAC	U04610
aadA1	TCATATCGTTTTAACCCTGGCGCGTATCTGGTACACCCTTTCTACCGGGAGATTTACCTCTAAGGATGCG	NC003198
aadA1b	AGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGC	AJ62853
*aadA2**	AAAACGCCTACCTGCCCAGTATCAGCCCGTCTTACTTGAAGCTAAGCAAGCTTATCTGGGACAAAAAGAA	AY263741
aadA7	GATCTCTTCAGCTCAGTCCCAGAAAGCGATCTATTCAAGGCACTGGCCGATACTCTGAAGCTATGGAACT	AY458224
aadE	AGCCACAGCCGCCAGAGAACATGATGGATATTCCGGGGATGGCGGATGAAATGCGACGCCCAATGATGCT	CP000408
aadE 2	GAAGCATTATTTCTATGCCATCAATTGTTCAGGGCGGTATCCGGTGAGGTGGCGGAAAGGCTTCATTATG	AF516335
aadK	AGTGGCTCGAAATCTTTGGGAAGCGCATTATGATGCAAAAACCAGAAGATATGGAGCTTTTTCCTCCCGA	M26879
ANT4-Ia	GGGGATGATGTTAAGGCTATTGGTGTTTATGGCTCTCTTGGTCGTCAGACTGATGGGCCCTATTCGGA	PB0110CG
ANT6-Ia	AGCGCAAGGGAGTATGATGATTGCTGCAATGAATTTTGGAATGTAACACCTTATGTTATTAAAGGATTGTGCCGT	AF516335
ANT9-Ia	AGGAGTGAAGTTGTCCCTTGGCAATATCCTCCAAAAAGAGAATTTATATACGGTGAGTGGCTCAGGGGT	X02588
aph	TTCTTGAGCTTCTCGGGCAGACGGAACTAACCGTCAACAAAATCGGATATTCCGGAGATCACGTCTATCA	X03364
APH2-Ia	ACTATGTCAGAAGAAGAACAAAATTTGTTAAAACGAGATATTGCCAGTTTTTTAAGACAAATGCACGGT	M18086
APH2-Ib	AGGATGCCCTTGCATATGATGAAGCGACGTTTTTGAAAGAGTTACATTCCATAGAGATTGATTGTTCTGT	AF207840
APH2-Ic	AGCATACAATCCGTCGAGTCGCTTGGTGAGGGCTTTAGGAATTACGCGATCCTCGTCAATGGAGATTGGGT	U51479
APH2-Id	CCATTCCGGAGGTGGTTTTTACAGGAATGCCATCAGAAACGTACCAAATGTCTTTCGCAGGT	AF016483
aph3''	TTTTTGGTGAATCGCATTCTGACTGGTTGCCTGTCAGAGGCGGAGAATCTGGTGATTTTGTTTTTCGACG	AYO55428
APH3-III	ATGCTATGGCTGGAAGGAAAGCTGCCTGTTCCAAAGGTCGTGCACTTTGAACGGCATGATGGCTGGAGCA	M36771
APH3-Iva	ACCGTCAACAAAATCGGATATTCCGGAGATCACGTCTATCACGTGAAAGAGTACAGGGGCACCCCCGCAT	X03364

aph6	TCATTGCCAGACGGGACTCCTGCAATCGTCAAGGGATTGAAACCTATAGAAGACATTGCTGATGAACTGC	AYO55429
aphA-3	CAGGCTCTTTCACTCCATCGACATATCGGATTGTCCCTATACGAATAGCTTAGACAGCCGCTTAGCCGAA	AF516335
aphAI	TGCTCGAGGCCGCGATTAAATTCCAACCTGGATGCTGATTTATATGGGTATAGATGGGCTCGCGATAATG	U13633

Beta-lactams

bla1	GCGATTGATACTGGTACAAATCAAACAATCGCTTATCGACCTAACGAAAGGTTTGCCTTTGCATCAACT	AF367983
ampC	CACTATTTGAGCTCGGATCTGTAAGTAAAACTTTCACAGGTGTGCTGGGTGCGGTTTCTGTGGCGAAAAA	AJ237702
ampR	CTGGCCGGATTCTATGACAGCCATCCGCATATTGATCTGCATATCTCCACCCATAACAATCATGTGGACC	AJ237702
bla2	AGCAGTTCCTTCGAACGGTTTAATCCTTAATACTTCTAAAGGATTAGTACTTGTCGATTCTTCTTGGGAT	AF367984
blaCMY-2	ATATCGCCAATAACCACCCAGTCACGCAGCAAACGCTGTTTGAGCTAGGATCGGTTAGTAAGACGTTTAA	X91840
blaCTX-M-1	TTGCCGAATTAGAGCGGCAGTCGGGAGGAAGACTGGGTGTGGCATTGATTAACACAGCAGATAATTCGCA	DQ663489
blaCTX-M-12	AGACTGGGTGTGGCATTGATTAACACAGCGGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTCG	AF305837
blaFOX-2	AATGACAAGATGCAAACTTACTATCGGAGCTGGTCACCGGTTTATCCGGCGGGGACCCATCGCCAGTATT	Y10282
blaIMP-2	ATTGGTTTGTGGAGCGCGGCTATAAAATCAAAGGCACTATTTCCTCACATTTCCATAGCGACAGCACAGG	AJ243491
blaKPC-3	CATCCGTTACGGCAAAAATGCGCTGGTTCCGTGGTCACCCATCTCGGAAAAATATCTGACAACAGGCATG	AF395881
blaOXA-2	GCAGGCCACAATCAAGACCAAGATTTGCGATCAGCAATGCGGAATTCTACTGTTTGGGTGTATGAGCTAT	M95287
blaOXA-26	GTTACTCCACAGGTAGGTTGGTTGACTGGTTGGGTGGAGCAAGCTAATGGAAAAAAAATCCCCTTTTCGC	AF201287
blaOXA-27	GGCGAGAAAAGGTCATTTACCGCTTGGGAAAAAGACATGACACTAGGAGAAGCCATGAAGCTTTCTGCAG	AF201828
blaOXA-2b	AGCAATAAAGAGGTGGTAAATAAAAGGCTGGAGATTAACGCAGCCGATTTGGTGGTCTGGAGCCCGATTA	AY303807
blaOXA-61	ATGATGGAAAAACTTGGGCGAGTAACGACTTTTCAAGGGCTATGGAGACTTTCTCTCCCGCTTCCACTTT	AY587956
blaOXA-9	TCCGTGCTCGTCTTTTAAACTTCCATTGGCAATCATGGGGTTTGATAGTGGAATCTTGCAGTCGCCAAAA	M55547
blaOXY-K1	GCACCACCAATGATATTGCGGTTATCTGGCCGGAAGATCACGCTCCGCTGATATTAGTCACCTACTTTAC	AF473577
blaPER-2	TTATGGAAATGGATGGTTGAAACCACCACAGGACCACAGCGGTTAAAAGGCTTGTTACCTGCTGGTACTA	X93314
blaPSE-1	TGTGGAGTGAGCATCAAGCCCCAATTATTGTGAGCATCTATCTAGCTCAAACACAGGCTTCAATGGCAGA	AB126603
blaROB-1	TATTATTGCTGACATTAACGGCTTGTTCGCCCAATTCTGTTCATTCGGTAACGTCTAATCCGCAGCCTGC	AFO22114
blaSHV-37	CTTGAGCAAATTAAACTAAGCGAAAGCCAGCTGTCGGGCCGCGTAGGCATGATAGAAATGGATCTGGCCA	AF317502
blaSME-1	GATGAGCGGTTCCCTTTATGCAGTTCATTTAAAGGTTTTTTGGCGGCTGCTGTTTTAGAGAGGGTGCAAC	Z28968
blaSME-2	TTAGGTTAGATCGCTGGGAACTGGAACTTAACACTGCAATCCCAGGAGATAAACGTGACACTTCAACGCC	AF275256
blaTEM	TTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGAT	AJ634602
blaVIM-2	TTATTGGTCTATTTGACCGCGTCTATCATGGCTATTGCGAGTCCGCTCGCTTTTTCCGTAGATTCTAGCG	AF369871
blaZ	AGTGGTCAAGCAATAACATATGCTTCTAGAAATGATGTTGCTTTTGTTTATCCTAAGGGCCAATCTGAACCTAT	M60253
mecI	TGCAAGTGCGAATAATATAATAGAAGAAATACAAATGCAAAAGGACTGGAGTCCAAAAACCATTCGTACA	D86934

Dihydrofolate reductase

dfrA	ATTGTCGCTCACGATAAACAAAGAGTCATTGGGTACCAAAATCAATTACCTTGGCACTTACCAAATGAT	AF051916
dfrD	AGGTAGAAAGAACCTTCAATCAATCGGAAGGGCTTTACCTGACAGAAGAAATATTATTTTGACTAGAGA	Z50141

Efflux pumps

cmeA	TTTAGGTGTTGTGCTTTTACTCGCTGCTTGCAGCAAAGAAGAAGCACCAAAAATACAAATGCCGCCTCAA	AR466820
cmeB	TTGCCAATGATTTTCGCAACAGGAGCAGGAAGTGCTTCAAGACACTCTTTAGGAACAGGGCTTATTGGTG	AR466820
cmeC	AAAATTTGATGGTAGCGCAAGCGGAAGTCGTGCAAAAACAGCTATAAATGCTCCAAGCAATCGAACTGGG	AR466821
cmeR	ACTCAAATAGAACACCATCACAAAAAGTTTTAGCCAGACAAGAAAAAATCAAAGCAGTGGCCTTAGAGCT	AR466820
mef	ATTTTGGGACCTGCCATTGGTGTGCTAGTGGATCGTCATGATAGGAAGAAGATAATGATTGGTGCCGAT	u70055
mef(A/E)	CGATTTTGGGACCTGCCATTGGTGTGCTAGTGGATCGTCATGATAGGAAGAAGATAATGATTGGTGCCGA	AY319932

msr(A)	CACCACGGAAATCGCTAACGCCACACCGTTTTATTATGCCGAAGATGACCACCAGCAATATCTGCATAAA	NC_002655

Glycopeptides

vanA	AAAACCTTGCGCGGAATGGGAAAACGACAATTGCTATTCAGCTGTACTCTCGCCGGATAAAAAAATGCAC	AF516335
vanB2	AAAAGTCGCAATCATCTTCGGCGGTTGCTCGGAGGAACATGATGTGTCGGTAAAATCCGCAATAGAAATT	AY145441
vanD	TTTACTTCCTACAGCCGTTATCCCCGCATGATGACAGCAGCCGGTTTTACGCTTACTGAAATACTGGATC	AY489045
vanE	TGGTTGTGGTATTTTAGGAAATGAACAATTGGTCGTTGGAGAATGTGACCAAATCAGTCTTGTGGATGGC	AF430807
vanG	AATTGGCAGGAATACCTGTTGTTGGCTGCGATACACTCTCATCAGCTCTTTGTATGGATAAGGACAGGGC	AF253562
vanH	GAATCCAACGCCAAATCCGCGCCTTTCAATCAATGTATCAGTGTGGGACATAAATCAGAGATTTCCGCCT	AF516335
vanR	ATCATGCTTCCCGGCACAAGCGGCCTTACTATCTGTCAAAAAATAAGGGACAAGCACACCTATCCGATTA	AF516335
vanX	TACCGTCCTAATCGTGCTGTAAACTGTTTTATGCAATGGGCTGCACAGCCGGAAAATAACCTGACAAAGG	AF516335
vanY	TTGATGAGCAAAGTGTGCTTTACCAAGAAATGGGGGCTGAGTATGCCTTACCAGCAGGTTATAGTGAGCA	AF516335

Lincosamides

linB	TGAAACATAGTATAACCTCGAACTTTGATTCATCCAACTGGTTGTTTGACGTAGCTCCGTACTTGATGCT	AJ238249

Macrolides

mph(A)	CCGACATGGGCTCAAGCTCCATGGCCCGCTGACTGTCAATGAGCTTGGGCTCGACTATAGGATCGTGATC	AY522923
mphB	AGCCGCAGCGCTTGATCTTGTAGTACACACACCAGAAGAAGCAAGAATGTCAATGAAGCAGCGTATGGATGCAG	D85892
mphBM	ACGTCACAGGTCTCATAGACTGGACTGAAGCAACCCACTCCGACCCATCAATGGACTTTATGGGACACCATCGT	AB013298
mphK	ACTGTACGCACTTGCAGCCCGACATGGGCTCAAGCTCCATGGCCCGCTGACTGTCAATGAGCTTGGGCT	U36578

O-nucleotidyltransferases

lnuA	ACTCATTGGTTAGATGGAGGCTGGGGCGTAGATGTATTAACTGGAAAACAACAAAGAGAACACAGAGA	J03947
lnuB	CATCCAACTGGTTGTTTGACGTAGCTCCGTACTTGATGCTTTATAAAAATGAGTACGGAACAGAGGTAGT	AJ238249

Quat. Ammonium

qac	GCAATAGTTGGCGAAGTAATCGCAACATCCGCATTAAAATCTAGCGAGGGCTTTACTAAGCTTGCCCCTT	AY458224

Metal Resistance

Aluminum

BnALMT1	AAGGACTAAATCGAGGAGTGGCAACATTAGTAGCAGGAGGACTAGCACTTGGAGCTCATCAGCTAGCA	110082270
BnALMT 2	TAGATACGAGTTGCGTACATGGGGTCGGGATGATGCTAGAACATTCATTGGGTGTTCGTATACTTCAGAT	AB194301
ybaX	CAGTAGCCTGACGCGTGACAGCATTCCGGTGCCTGATTATGAACCTGAAGCCGATGGTATCCCGAATACG	U00096

Arsenic

arsB	GGCGTCATGACGGCGTTTCTGTCTTCGATCATGAACAACATGCCCACCGTGCTCATCGGGCTGCTGTCC	71553748
arsC	GGCGCACCGCCGGACATCGTCATCACCGTTTGCGACGCCGCCGCGGGGGAAGCCTGCCCGCTGTATCTCG	CP000438
arsD	TCGAAGTGTTTGACCCATCGCTGTGTTGCAGCACCGGCGTCTGCGGCGTGGATGTTGACCAAGCCTTGGT	DQ057986
arsH	CAGTTGCGCGTGCTGGGTCGCTGGATGCGCATGTTCACCATCCCCAACCAGTCCTCGGTTCCCAAGGCCT	CP000438
arsR	ACCGCCTGAACCCTGCGCTGCCGGCCTGGATCCACGAAGTCCTGCAAGTGACCCTGCGGGCCAACGGCGA	CP000438

Cadmium

cadA	TGTTAAAAAGATTCCAGGCGTTCAGGACGCAAAAGTAAACTTTGGCGCTTCTAAAATTGATGTATATGGA	AB179623
cadD	TGAGATGTTTTATGATTCAAAATGTCGTTACTTCAATAATCCTGTATTCTGGGACAGCCGTAGACTTACT	AL157959
cadD2	TTGGAAAAATATAGCAGATGGTTTGTTGCTGTTGTTTATTTAGGATTGGGGGTATATATCCTGATTGAAA	AL157959
cadD3	GGTGCTGACAATATTGGTGTCTTTGTTCCATATTTTACTACCTTAAATTTAGTGAATTTGATAGTGGCTT	AL157959
cadD4	TTTAGTGAATTTGATAGTGGCTTTACTTACCTTTCTAGTCATGATTTATCTCTTGGTTTTTTCTGCCCAA	AL157959
colR	CGCACCCTGCAGGTGGCTGACCTGAGCTACGACCTCGATACCCTCGAGGTAACCCGCCAGGGTCGTCTGC	AM279159

colS	TGGCGCGACACCATCGAACAGAAAGGCCTCACCCTGTATTTCGATGGCCGCGTGAGCGCTAGCCCTGTGC	AM279160

Chromate

cysA	CATGCGCGGCTGTATAACGGCGACGAGCGTATCGAAACCCGCGATGAGGAACTTGCTCTCGCACAAAGCG	U00096

Cobalt/Nickel

cnrA	GTGGAGCCGGACACGGGGAAGATGGCAGCATACGGAGTTTCCTACGCGGACCTAGCCCGGGCGCTCGAAG	AJ276513
cnrB	TCCCAGTGTCGGAGAAGTCCCCCGCATCTACACAGGCCCCGGAAGCACAGAAGCCACAATCCGCCCCAGT	AJ276513
cnrC	TGGCGTCAGTGCGGGCGTCCGCCGCTATGGCTGGACCAATTCCAGTGGCTATGTGGTTGGGGTCACGGCC	AJ276513
cnrH	CGTCCAGGACACCTTTGTTGCCGCCTGGCACGCCCTGGATGACTTCGATCCCGACAGGCCATTTCGCGCC	AJ276513
cnrT	TCCTGCTTGCGTTTGCGATCTGGCCTGAGGTGCCAGGCTTCGCATTCTGGGCGGCTTTGGCTTTGATGGC	AJ276513
cnrX	GCTGTTCCACTAGATGCCAACGAGCGCGAGATTCTTGAGTTGAAAGAAGATGCCTTTGCGCAGCGTCGGC	AJ276513
cnrY	TGCAGCCTTGGTCGCGTTTGCGGCAATCAATCGCGTGGCGACCATCATGTTGGAAAAGCCTGCCCCGACG	AJ276513
ncrA	AGGCTTCATTCATCGCCCTGACTGTTGTGGCAACATTGTCGCTGGTCACCGCCGTACTTATCTGGCGTCC	DQ517331
ncrB	TTTTATAAACAGCGACCACAGACGCATCAAAACTCAAGCTCTGCACCACAGAGCTTTTACTATCAAAATC	CP000241
yohM	GGATGCCCATGCACGAGCCCATGCCAATGACATTAAACGACGCTTTGATGGTAGAGAGGTCACCAACTGG	U00096

Copper

copA	GCCGCGATCCGAGCCGCACGATAGAGCTGCACCTGACCGGCCACATGGAGAAATTCGCCTGGTCGTTCAA	AY321492
copB	TGGAATCGCACTTACGGCAACACCGCCGACTACGCCCGCGAAGAAGACGAGGATCGCAGTGAGGCGCGCC	AY321492
copC	TGGTCACGCAATTCTCCGGGGCCAAACTGGTAATGACCGAAATGGGTGGCAGGGCCCACTCGCCGATGCC	AY321492
copD	TTTCATCTTGGACCGTCACTGCAACAATCGCTGCGCGACGGCCAGTACCTCATCGCCGCGAACGCGCTGC	AY321492
copP	ATTTGACGCTCCAGCGACACAGGATTTGATCAAAGAAGCTTTATTAGATGCTGGGCAAGAAGTGGTGTGA	AE000511
cueO	GCTAATGGAGAAATATGGCGATCAGGCGATGGCCGGGATGGATCACAGCCAGATGATGGGCCATATGGGG	U00096
cueR	AAACGGCGCACGCTGGAGAAGGTGGCGGAGATCGAACGACACATTGAGGAGCTGCAATCCATGCGCGACC	U00096
cutA	GCTGGAATGCCTGAAGTCTCATCATCCATATCAAACCCCGGAACTTCTGGTTTTACCTGTTACACACGGA	U00096
cutF	ACGGAACATGGGTGATGAATGAGCGTTATCTCGGTGCTCGTGAAGAACCTTCCTCCTTCGCTTCCTACGG	U00096
dipz	TGGGCATTTGGTGCGACGCATACCGCGCAAACTCAGACGCATCTCAACTTTACACAAATCAAAACGGTAG	U00096
int	TGGACGGTGAGTTGCGTGATAAAGGTAGCTCGCTGGTAACCGGGATTGTCGACGCGCGTCTCAATAAGCA	U00096
pcoA	TGAAAATGACGGTCGTGGCTGCAGATGGCCAGTATGTAAACCCGGTTACCGTTGACGAATTCAGGATTGC	AY378100
pcoB	TACTCCAGCCATCCTATGAGGTGAATTTCTACAGTCAGGATGATGAATCGCGGGGTCGCGGCAGGGGACT	AY378100
pcoC	ATTCTCAGGTGCAAAATTAACGATGACGGGTATGAAAGGCATGTCATCACATTCTCCGATGCCGGTCGCG	AY378100
pcoD	GCTGGATTGTCAGGCTCTGTGCCCTGTTTACCACACTCGGTGCTTTGTTCCTTTACACTAATAAGAGAGT	AY378101
pcoE	CACATCGATTTGTTAATAATGCCTCAGCCGTCAGTCATGTGAACTCCTCGACGCATGAAAACTTACCGGA	AY378102
pcoR	TTCTGCTTGAGTTGCTGCTGCAACGCACCGGAGAAGTGTTACCCAGGAGTCTTATCTCGTCCCTGGTCTG	AY378103
pcoS	TGCTCCGACGTGCTTTCAGTAACCTGCTTTCCAATGCAATCAAGTATTCTCCCGATAACACCTGTACAGC	AY378104

Copper/Silver

cusA	CTGGTTTCTCAAATATGAGCTAAAAACCATCCCTGACGTTGCGGAAGTGGCGTCGGTGGGCGGTGTGGTG	CP000468
cusB	CGAGTATCAGTATGCCATTGTGCAGGCCCGCGCCGCCGGGTTTATCGACAAGGTGTATCCGCTTACCGTG	CP000469
cusC	GCATCGCTGCAAATTACTTTGCAACGGGCGCGGGCATTATATCAGCACGGCGCAGTAAGTTATCTGGAAG	U00096
cusF	CTGCAAGTCGCAATGTTCAGTCTGTTTACCGTTATTGGCTTTAATGCCCAGGCTAACGAACATCATCATG	U00096
cusS	GAGATTAGCGCCACCCTTGAACGGGTACTAAATCACCCTGACGAAACGCAAGCCCGACGCTTAATGACGC	U00096

Copper/Zinc/Cadmium

copABCD	GCCGCGATCCGAGCCGCACGATAGAGCTGCACCTGACCGGCCACATGGAGAAATTCGCCTGGTCGTTCAA	AY321492

czcA	TGAGTGGGCGAAATATCCGGGAAAGCGTCCACTGGAGTGTTGATAAAACTATAAGTCCCAAAAAATAAAA	AE000511
czcA 2	GCCGCGATCCGAGCCGCACGATAGAGCTGCACCTGACCGGCCACATGGAGAAATTCGCCTGGTCGTTCAA	AY321492
czcB 1	GTCTTTAGGCTGTGAGCCTTGAATATACATTTCTACCATTTCCCCCACATGGTAATTGCCCTCATCTAAT	AE001439
czcB 2	GCGCGCTTCAATGTGCCTAATCTTAAATTGCTTTATTACCCTAACATGTTCGCTCAAGTAGAAATCTTTC	AE001439
czcC	CTGACCGTCAGCGTCGGCAGCCAGTACAGCCGCGAAGAGCGCGAACGGGTCAACGTAGTGGGTCTGTCGA	CP000058
cznA	TTTTCCATTTCTTCAGGGCTAGAGCCGGGGAGTTTTAAAATGATTTTAACTTGAGTGGGCGAAATATCCG	CP000241

Iron

iroA	CAGACATACGGCATTAAAAAACCAAGTGAAGGAGGGGAATATTTCCTGGCGGAGGGCGAGAGTGAGCTCA	X69214
iroB	GGCTGCGGGATTATCCCCGGCAACGTCGGCCTGTCGAGTAACATGATCAATGCTTTCCTCAACAATCGCT	AY365116
iroC	GGCGCTGATTGTTGCACATCGTCTGACCACCGCGCAACGCTGCGATCTGATTGCCGTTATTGATAAGGGG	AY365116
iroD	TGGTGCTCAGCCACTCTCCTTCAATGTGGTGGACGCCAGAAAGAACCAGTCGACCAAGCTTGTTCAGCGA	AY365116
iroE	CGTGGCTGTCGGGTATCAGACCAACTTCCCTTTCGATCTCAACAGCAGGGCTTACGACTATACGCCAGCA	AY365116
iroN	TGAACTGGAATACCAATGCCACATGGATGATCACTTCGGAGCAAAAAGACACCGGTAATCCTCTGTCGGT	AY365116

Iron/Aluminum

pmrA	AATATAGGCCGCCATCAGGCATGGAGGGATGGACAGGAACTGACCCTGACGCCTAAGGAGTACGCGCTGC	AL627282
pmrB	GATCGCCATTCACAGCTCCACGCTTGAGATTGAGTCCGTCGTCTCGGCGATCAATCAACTGGTTACGCGT	AL627282

Lead

pbrA	GAATTCAACGCGCTGCCCGGTCGGGGTGTGCAAGGCCAAATCAACGGTGCGACCTACCATCTGGGCAACC	AY378100
pbrBC	GATGGCTGTGTTCGCCGATATGGGTGCCAGCTTGCTTGTTGTCGGCAACGGCTTGAGGTTGTTGCGCCGA	AY378100
pbrR	TCGCCAAGCGCTCTGGGTGCGAGGTCGTGACCATCCGCTACTACGAGAAGGAAGGGCTACTGCCAAAGCC	AY378100

Mercury

merA	TCCGAAGGGGACGGCGCAAATCGCTACCGACCCCGGCACTTCGACGGATGCGCTGACTGCGGCCGTGGCC	CP001068
merE	TGCAGGAACAGCACTGGGATCATACTTCACGGAATTTAAAAATGTTATTTTCATCATGATGGGTCTGTTA	AB066362
merP	TGATGTTGCTATCCCTTATGCTAGTGGTTAGTGCATGCAGTAACGAACAAGAAGTCCAAAAAACTGAAGT	AB066362
merR	CTGAGAGAAACGATGGCTCCATATCTAACAATGGCAGAAGGATTGAAGCTGGCTGTCCTAACTTTTGATA	AB066362
merR1	TGCTGATGGATCTTAAAGAAAGATGTCCCGAAAACAAAGATATTTACGAATGCCCCATTATTGAAACACT	AB066362
merR2	TTTATTTAACAAGCCTACTTCAAAAGAGTGTAACATCCAATCTACTAAGTACTTAGATCGAATTATTAAG	AP006716
merT	CACTAATCTTGATTCCCCTTGGACTCACTGGGTTTGCAGGCGCAATCGCATTCTACTCGTTGAAGTATCG	AB066362

Nickel

ncrC	GGATACGCTGGCGCGGCGGGCTCCATACTTCTCAAGCGTACTGATTGCACTTGTCGGCATTTACATGGGC	DQ517331
ncrX	AGGCTCTAGCCTGGCAAATGCTTTAATCAGCATCTCATCAACCATGTCCATAGCAGGCCGTTCTGAAACG	AF322866
ncrY	AGGCAATGTTGTCAGCCAGCAGCAGGACGAGTGGTATACGTTCCGCGACCCAACCGGTACCATCAAAGTC	DQ517331

Nickel/Cobalt/Cadmium

nccB	TTTTATAAACAGCGACCACAGACGCATCAAAACTCAAGCTCTGCACCACAGAGCTTTTACTATCAAAATC	AE000511

Silver

silA	CAACCGGGATCAAAAGCCCGATAGGTATCAAAGTGTCCGGGACTGTTCTGTCCGATATCGACGCGACGGC	AF067954
silB	ATTCAAAGGCTGCGTTCAACCCGCACAATCCAGACCCGTTTTACCATTAAAGCACCTATTGATGGTGTCA	AF067954
silC	TGCCAGAGGATTGTATGCAAGTGGTGCTGTCAGTTACATCGAAGTGCTGGATGCAGAACGTTCCCTCTTC	AF067954
silE	TGTCGGGATCCAGGGGACTGCACCTCGTATGGCCGGTATGGACCAGCATGAACAGGCCATTATTGCTCAT	AY009387
silP	CGACCTGATGATACTGAACAGGGCCCGTCATCTGTCAGAGATCACCATGAAAAATATCCGACAGAATCTG	AF067954
silS	GTTCAGAAGGGCGATCAATAATCTGTTATCCAATGCCCTGCGTTATACCCCGGAGGGACAGGCAATCACC	AY378100

sliR	TCACGGAAAGCCAGTTTAGGGTGGCTGATCTCTCGATGGATCTCGTATCCAGAAAAGTCAGTCGCGCCGG	AY378100

Tellurite

tehA	TGCAGAGCGATAAAGTGCTCAATTTGCCGGCAGGCTACTTTGGTATTGTGTTGGGGACGATAGGGATGGG	CP000468
tehB	AGAGGGGGAATTACGTCGATATTACGAAGGCTGGGAGAGGGTGAAATACAATGAAGACGTCGGCGAGCTG	CP000468
terA	ACCTTTTGCTCAATTAGATGGAGATGACAGAACAGGAAGTAATACACAAGGAGAAAATTTAAGAATAAAT	AM180355
terB	ATATATTTTGTAGCGAGTGTGGAGCAAAGTGTTTAAAAGGAAGCAAGTTTTGCAGTGAATGTGGAACGGA	AM180355
terC	CGCGTTTCATCATTCCTCGGCGCTATCGTGCAGATTATGCTGCTGGATATTATCTTCAGCCTCGACTCGG	CP000468
terD	CTATAGTAGTAGCTGAAATATATAAGCACAATGGAGAATGGAAGTTTAATGCACTAGGTTCTGGCTTTGA	AM180355
terE	TTAGATGTAAGTGTTTTCATGGTTGGAGAATCTCAAAGAGTTGAAAAAGATGAAGATTTTATATTCTACA	AM180355
terY	CCTTTAAGCACGAGTGGTGGCACCCCTTTAGATCAAGCGTTTAGATTGGCTAAGGATCTTATTGAAGATA	AE000511

Transferable Copper

tcrB	TCGAAGGTGGATCTAGTCACCCTATCGCTCAGTCAATTATTAGTTACGCAGAACAGCAAGGGATACGTCC	AY048044
tcrZ	GCCAATACAGTGCAAGAAAAATTTTCAGCTATTGAAGGGGTTGAATCTGTAGAGGTTGATTTAGCGACTA	AY048044

Zinc

ybgR	CGCGTGGCGACTGTTGAAAGATAGTGTGAATGAATTACTTGAAGGTGCACCGGTATCGCTGGATATCGCT	CP000468
zraP	TCGTTAGATGAGTTACGGGTGAAACGAGATATTGCGATGGCTGAAGCGGGTATTCCGCGCGGTACCGGAA	CP000468
zraR	CTGGTGGAAGTCGAAAAAGAGGTGATTCTGGCGGCGCTGGAGAAAACGGGCGGCAACAAAACCGAAGCCG	CP000468
zraS	TGCAAACAGCCGGGAGATCCAGTTACGCTTTACCGCCAACGACACATTACCGGAAATTCAGGCCGATCCG	CP000468

Plasmids and Trasposons

Class I Integrons

intI 1	AAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCAT	DQ787712
intI1 2	CTACTTGCATTACAGTTTACGAACCGAACAGGCTTATGTCAACTGGGTTCGTGCCTTCATCCGTTTCCAC	AY458224
intl 1 3	ATCAGTTGGAAGAATTTGTTCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGT	DQ274503

Insertional Elements

IS1182	CGGGCCGCCAAATACAAAATATGCTGATAGATAGTATTCGGATGCGCTGCTTATCTCAAGAGCAATTCCC	AF516335
IS150	AAAAGGGAACTGAGGGTTCACTGATTCTACATTCAGATCAAGGATGGCAGTATCAGATGCCACAATATCA	AF516335

Plasmids

Alpha 1	CCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAA	AY333433
ColE1	CGCTCTGCTAATCCGGTTACCAGTGGCTGCTGCCAGTGGCGTTAAGGCGTGCCTTACCGGGTTGGACT	V00270
incN oriT	ATGATGTATTTTTGATGTACTTTTGTTGTACTGGCTACCTCAGTACCAGATAATGATGTAATTCTGTTGT	M30197
incP oriT	GGTGCGAATAAGGGACAGTGAAGAAGGAACACCCGCTCGCGGGTGGGCCTACTTCACCTATCCTGCCCGG	X54459
IncQ	ACCCTCAGCCGAAATGCCTGCCGTTGCTAGACATTGCCAGCCAGTGCCCGTCACTCCCGTACTAACTGT	M28829
incQ oriT	TCGTAGGCTATCATGGAGGCACAGCGGCGGCAATCCCGACCCTACTTTGTAGGGGAGGGCGCACTTACCG	M28829
incW oriT	CATCATTGTAGCACCATCATAGCATTATAGTTGCATCATTGCTGCACGATAACCCAATGCGCATAGCGCA	X51505
M13	GTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCT	U46018
mphC	AGTGGAAAGTACATGCAAAAGACCTTATTGCTTACCCAAAACTTACAGGTAAACCCGCAGCCACAATAG	AF167161
Orf14	ACCGATACCGTTTACGAAATTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAG	EF525477
orf46 hirt	CTGATCGAAAAATACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCTGCTAAGGTATAT	AB264038
pAD1	TTTTGAACCAGAGAGGAAGCAATTTATAGCGCCTAAATTAATCAGAGCAGCTACTAAGGGATTTAGGCTA	X62658
pAM alpha 1	GTGCTAAGTTATTGTTTTCGATTTTAAGGTGCTTATTTTGCGTTCTAAGCGTCTCGAAATCTTTCGTAAT	AF503772
pBR322	AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT	J01749

pMMB66EH	TTCAAGCGGTACAACGGCAAGACCCCGGAGAAGGGCGGGGCACAGAAGACCGAAGCGCTCAAGCCCAAGG	X15234
pSLT	GTACATAATGAGACTCAGGAAAGGTCATGGATTGACGTTGGTCACACTTGCGTGTTTATCCCTGCTGGGC	AE006471
pUC19	TCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCG	X02514
qnrS1	CAATCATACATATCGGCACCACAACTTTTCACATAAAGACTTAAGTGATCTCACCTTCACCGCTTGCACA	DQ885572
RP4 IncP	CGAGCTTGCGCCGGCTTCGATTGAAGGCTGGCCTCTCCCTGCGAAGCGTGAGGACAGTCGGCCCAACAGC	X59793

Transposons

res	AATGGCTGGTGTTAACCAATTAGAGCGAGATCTTATTCGGATGAGACAACGTGAAGGGATTGAATTGGCT	AF516335
Tn916	CCAGTGATAAGAGTATTTATCACTGGGATTTTTATGCCCTTTTGGGCTTTTGAATGGAGGAAAATCACAT	EFU09422
Tn925	CACACGTCCGTAAAAGCATGAGCCAAAGAGGCGAAATTTTAATGCACTTAAAAGAACTAGACGCAAAGCG	AY855841
tnpA	GATGATCAAAACGCAGGTTGTCAAACTGACTACGTTCACGCGGCGATTATAGCCGATCAAATGATGAGCA	AJ628353
tnpM	CCAATGGAGGAACACCACCATGAACGCCAATGAACCGAGCACCAGTTGCTGCGTGTGCTGCAAGGAAATC	AJ628353
trans	CGGGACACACAAGCAGCCTATGCTTTTCTTAAGCGGTTAGTGAAGCAGTTTGATGAACCGAAGGTTGTAG	AF516335
trans-1	GAAGGCGGTGCTTCTTCACTTGAGAGCCAAAAAAGGGGCAGAAAAATTAGTATGAATTCCAAGCTAAACA	AF516335

Controls

Positive Control

23S	CGGAACGCTAGTTTCGATGGAGGCGCTGGTGGGATACTACCCCTGCGTTATGGCCACTCTAACCCGCACCAC	AY116904
23S rRNA	ATCAACCTGTTGTCCATCGCCTACGCCTATCGGCCTCGGCTTAGGTCCCGACTAACCCTGGGCGGACGA	AE016830
EUB	' CCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCTCGCACAAGCGGTGGAGCAT	DQ787712
EUB 2	ACGCTGTAAACGATGAAAACTAGATGTTAGTCCAGCTATTAAATCATAATTAATAAACTGATATGATTTA	DQ787712
Frye 1	AGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATA	EU779389
Frye 2	CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGT	EU779389
Frye 3	TGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT	EU779389

Negative Control

buffer		
H2O		

Appendix C - Development of a Microarray and Multiplex Polymerase Chain Reaction Assays for Identification of Serovars and Virulence Genes in *Salmonella enterica* of Human or Animal Origin

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Abstract

Salmonella enterica is an important enteric pathogen consisting of many serovars that can cause severe clinical diseases in animals and humans. Rapid identification of *Salmonella* isolates is especially important for epidemiological monitoring and controlling outbreaks of disease. Although immunological and DNA-based serovar identification methods are available for rapid identification of isolates, they are time-consuming and/or costly. In this study we developed and validated two molecular methods for identification of *Salmonella* serovars. A 70mer oligonucleotide spotted microarray was developed that consisted of probes which detected genes responsible for genetic variation between isolates of *Salmonella* that can be used for serotyping.

A multiplex PCR assay was also developed that are capable of identifying 42 serovars and provided a valuable prediction of the pathogenicity of the isolates by detecting the presence of virulence genes *sseL*, *invA*, and *spvC*. The gene *spvC* was the best predictor of pathogenicity. In a blind study, traditional serological methods correlated 93.3% with the microarray-based method and 100% with the multiplex PCR- based serovar determination.

Introduction

Gastrointestinal tracts of both humans and animals are a major habitat of *Salmonella*. In 2005, over 36,000 clinical cases of salmonellosis were reported to the CDC[10]. Of these, 15,000 resulted in hospitalization and 400 were fatal[22]. *Salmonella enterica* subspecies *enterica*, with the majority of clinical cases belonging to subgroup I, is categorized into serogroups (based on lipopolysaccharide or O antigen) and serotypes (based on the flagellar or H antigens)[40].

In order to identify the individual serovars (based on O and H antibody-antigen tests) of *Salmonella*, testing is routinely performed in state reference laboratories and several veterinary laboratories, which due to high number of samples submitted, may lead to long turnaround times. In an attempt to streamline the process, molecular techniques have been developed such as ribotyping[14], PCR single-strand conformation polymorphism analysis[34], restriction fragment length polymorphism[23], pulsed-field gel electrophoresis[4,25], IS200 fingerprinting[15] automated 5' nuclease PCR[24], and random amplified polymorphic DNA analysis[43] are major improvements in serovar determination, but the success of these assays require high technical skill, are prone to inter-laboratory variation, are time consuming, and are incapable of processing large number of samples. Multiplex PCR assays are efficient platforms that detect many gene targets in a single sample preparation by incorporating multiple primer-pairs. In the past, they were limited to identifying a small number of serovars, and these assays did not attempt to predict the overall

pathogenicity of *Salmonella* isolates[2,6,26,46]. A recent study described multiplex PCR procedure consisting of two multiplex PCR panels for determining 30 clinically relevant serovars of *Salmonella*[27]. The objective of the present study was to increase the number of serovars identified using the multiplex procedure, and to develop an additional multiplex PCR panel called *Salmonella* Typing Virulence (STV) that determines the presence or absence of the genes *spvC*, *invA*, and *sseL*, to allow us to predict the overall pathogenicity, invasiveness and replication ability of the *Salmonella* isolates respectively.

DNA microarrays, which work on nucleic acid hybridization principles are widely used, and have multiplex capability to detect hundreds of thousands of genes in a single experiment[8]. Spotted DNA microarray platforms are cost-effective, flexible, and easy to use in any laboratory with basic facilities and equipment[11]. This technology has been used in high throughput detection of pathogens such as methicillin-resistant *Staphylococcus* in hospitals[44] and in the identification of antimicrobial resistance genes[16,36]. More recently three studies[29,42,50] have focused on molecular serotyping of *Salmonella* using microarray protocols. These microarrays could identify only a limited number of serovars, and required multiple probe sets for each serovar. For example, 414 probes and a software program were necessary to identify 14 different serovars[42]. Since there are presently 1531 *Salmonella enterica* subspecies *enterica* group I serovars[19], such assays can get complicated and expensive. Therefore, the objective of our study was to develop an economical, high throughput and adaptable microarray that is capable of determining a large number of common serovars using a limited number of probes, as well as to compare the results with a modified multiplex PCR assay and established serological assays.

Materials and Methods

Bacterial strains, culture and DNA extraction

Salmonella isolates used in this study were acquired from human clinical sources, or isolated from the feces of feedlot cattle in Midwestern USA, or from various other sources including turkey, swine, horse, and reptiles. Isolates were sent to Kansas State University – Veterinary Diagnostic Laboratories (KSU-VDL) for species and serogroup identification and later to The National Veterinary Services Laboratories (NVSL) for serotyping by traditional antibody -based methods, using the Kauffman-White scheme. Isolates were grown overnight at 37ºC on Tryptic Soy Agar[a], and a single colony was picked and inoculated into 5 ml of Tryptic Soy Broth[a] and grown at 37ºC with shaking. Bacterial genomic DNA was extracted from 500 µl of overnight cultures using DNeasy Tissue Kit[b] according to the manufacturer's instructions. Final DNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer[c] ().

STV multiplex PCR and primer design

A new multiplex panel to detect virulence genes was developed. Primers to amplify *sseL* gene[13,41] were designed using Primer 3 version 4.0 software[d] , based on conserved sequences from GenBank (Accession # AE008802) so that the melting temperature (Tm) and product size was compatible with other reactions in the multiplex panel (Table 3). Potential primer candidates were checked with BLAST searches for nucleotides until an acceptable set was found. The primers PT4 (amplifying sequences of a *S.* Enteritidis phage type 4 strain) and STM 7 (amplifying sequences of a *S.* Typhimurium strain) and *sseL* were combined with primers for *spvC* and *invA*, to create a unique multiplex PCR (STV). All the primers included in this

panel were analyzed using AutoDimer v1 software[e] to ascertain that they do not form homo- or hetero-dimers[47].

Multiplex PCR reaction protocols

The multiplex PCR was modified from Kim *et al.* 2006[27]. The protocol consisted of two multiplex PCR assays each consisting of 5 primer sets. The primer sets correspond to arbitrary regions on the genome of *Salmonella* Typhimurium[33] (STM) and *Salmonella* Typhi[35] (STY) as determined by microarray analyses[27]. The completed multiplex PCR reactions were electrophoresed on agarose gels, and the amplicons were numbered 1 through 5 based on their sizes (1 being the largest amplicon). If the PCR products were detected at a predicted location on a gel it was considered positive for that reaction. For *e.g.*, in the STM multiplex PCR reaction, if products corresponding to STM 1 and STM 5 primer sets amplified, then the amplicon code for STM reaction for that particular isolate will be designated 1, 5. Similar amplicon codes were also generated for the STY multiplex panel. Occasional faint, non-specific bands did appear which was consistent with the previous study[27], however, only the most prominent bands were considered for amplicon coding. The incidences and the prominence of these bands were considerably reduced when the Qiagen Multiplex PCR kit[b] was used.

The STM assay was modified to use the Qiagen Multiplex PCR Kit[b] as per the manufacturer's instructions. Briefly 12.5µl of 2x Buffer was added to 4 µl of Q Solution with 50-100 ng of template DNA for a final volume of 25 µl. The reaction conditions included initial denaturation step at 94ºC for 15 min, followed by 35 cycles of 94ºC for 30 sec, 62ºC for 45sec, a step down to 58ºC for 45 sec, and 72ºC for 1 min, and a final extension of 72ºC for 5 min. All reactions were performed on a Mastercycler Gradient thermocycler[f].

The STY assay was modified to contain 1.25 units Takara Hot Start *Taq*[g], 1.6 X reaction buffer, 0.2 mM dNTPs, and 50-100 ng of template DNA for a final volume of 50 µl. The STV reaction contained 1.25 units *Taq* polymerase[h], 1 X Ex *Taq* reaction buffer[g], 0.2 mM each of dNTPs, and 50-100 ng of template DNA at a final volume of 50 µl. Both STY and STV used the same cycling parameters: 1 cycle of 94ºC for 6 min, followed by 35 cycles of 94ºC for 30 sec, a step up to 62ºC for 15 sec, 58ºC for 15 sec, and 72ºC for 1 min, and a final extension of 72ºC for 5 min.

Sample analysis and scoring of multiplex PCR products

PCR products were separated by electrophoresis in a 3% agarose gel[h] in 1 X TBE (90 mM Tris, 90 mM Borate, 20 mM EDTA [pH 8.0]) for approximately two hours at 5.6 V/cm. The gel was stained using ethidium bromide (10 mg/ml), visualized under UV light[h] and captured by a digital imaging system[i]. PCR amplicon sizes were determined by comparison to molecular weight markers[j] and scored as described above.

Microarray probe design and printing

Initially, three to eight candidate probes for each gene target were selected (a total of 63 probes) and printed in replicates of three or ten. These corresponded to genetic regions determined to be important for serovar differentiation by previous studies and with our multiplex PCR assays discussed above[27,39]. These genetic regions were originally chosen since heterogeneity in the region was reported to be helpful to differentiate between common serovars of *Salmonella*[39]. Based on their sensitivities, intensities of signals, and correlation with multiplex PCR data, 37 probes were selected (Table 4, supplementary data) and were considered

for further studies. The remaining 26 probes were excluded during our validation step (described later) from further consideration because of low intensity/negative hybridization, false positives/increased background, or signal inconsistency (data not shown). Candidate oligos were designed using OligoWiz 2.0[48] and synthesized[k]. On each microarray, the positive hybridization controls EUB (16S region conserved in all Eubacteria)[49], and *rpoB* [1] were included. Negative controls including H_2O, hybridization buffer only and a 25 bp DNA probe without homology to any listing in GenBank were also printed[38]. Two fields containing the 70mer oligos were printed on Ultra Gap slides[l] using a Genetix QArray2 System slide printer[m] at a concentration of 35 μM each in replicates of 3 or 10. After printing, the DNA was cross linked in a UV Stratalinker 2400[n] at 600 mJ and stored in the dark at room temperature until used.

Microarray DNA labeling and hybridization

Extracted genomic DNA was labeled directly using the BioPrime Plus Array CGH Genomic Labeling System[o] as per manufacturer's protocol with slight modifications. After random primer hybridization, an additional 1.5 μl of 1 mM Cy3 or 1 mM Cy5-dCTP[p] was spiked into the labeling mixture to improve dye incorporation and amplification. For all DNA labeling reactions, the efficiency of labeling was determined using the 'Microarray Feature' on Nanodrop ND-1000 spectrophotometer[c], which measures the fluorescent dye incorporation in the sample DNA. The labeled DNA was mixed with an equal volume of 2X Hybridization Solution #7[q] and used for hybridization. The microarray chips were prehybridized in blocking solution (0.1% BSA, 5X SSC, 1% SDS) at 42^{o}C for one hour with shaking, and then spun at 2200 x G to dry. The labeled DNA mixture was hybridized overnight at 42^{o}C, followed by washing for 10 min in each of the buffers: 10X SSC, 0.2% Sarkosyl; 10X SSC; and 0.2X SSC. Lastly, the slides were

quickly dipped in water, spun dry at 2200 x G and visualized on a GenePix 4000B slide reader[r].

Other *in vitro* labeling kits including Ares Alexa Fluor 546 and 647[o], DNA labeling kits (Indirect)[o], and Array 900DNA Labeling Kit for DNA[q] were employed, but the BioPrime Plus Array CGH Genomic (Direct) Labeling System[o] was used in further experiments since it provided a consistently higher efficiency of dye incorporation as evidenced by Nanodrop ND-1000 spectrophotometer[c] and by total spot fluorescence on microarray chip as measured by a GenePix 400B slide reader[r].

Validation of molecular methods

For the multiplex blind study, 111 *Salmonella* culture samples, and 31 bacterial DNA samples were submitted to our laboratory. All samples were processed with STM, STY and STV multiplex PCR and scored as described above. For the microarray blind study, 20 *Salmonella* pure culture samples, and 36 bacterial DNA samples were submitted to our laboratory. All samples were hybridized to serotyping microarray chips and STM, STY and STV scoring was conducted. Serotyping results were compared against results from traditional antibody-based serotyping and statistical analyses (see below) were conducted.

Data analyses

The fluorescent hybridization signals from the array were visualized using a GenePix 4000B slide reader[r] and matched to the GenePix Array List (GAL) file previously created by the microarray slide printer. A GenePix Report (GPR) file was generated measuring the overall intensities. Each target region was represented by 3 or 10 replicate spots. The fluorescence signals from each set were averaged and used for further analyses. Data were analyzed using the

TIGR MultiExperiment Viewer (TMEV) program[s] with one color setting. Data were also analyzed using an relative pathogen signal (RPS) ratio method previously described[45]. Briefly, the averaged signal intensities for each probe were divided by signal intensities of positive control spots to attain an RPS ratio. Mean hybridization signals to spots containing a unique DNA probe (25 bp) that shares no significant homology to sequences in GenBank were subtracted from the RPSs of test spots[38]. A final ratio was determined from this, and values over 0.25 were considered positive, and values below 0.25 were considered negative.

The sensitivity and specificity of multiplex and microarray were calculated using the antibody based testing as the gold standard. Specificity is number of true negatives/(number of true negatives + false positives) and sensitivity is true positives/(number of true positives + false negatives).

Results

Multiplex PCR assay development and validation

Our multiplex PCR panels successfully identified 42 serovars of *Salmonella*. This included Bareilly, Choleraesuis , Kentucky, London, Meleagridis, Minnesota, Muenster, Orion, Reading, Senftenberg, Tennessee, and Uganda (Figure 1, Table 1); as well as the 30 serovars published previously[27] (data not shown).

A third multiplex panel STV containing five sets of PCR primers was created to predict the virulence of a *Salmonella* isolate and to further discriminate some serovars (Figure 2). The amplicons for *sseL* (169 bp) and *invA* (244 bp) were present in all isolates tested. As previously noted, *invA* has been used as a genetic marker for all pathogenic *Salmonella*[12]. The PCR targeting *spvC* gene amplified a 571 bp product only from serovars Choleraesuis , Dublin,

Enteritidis, and Typhimurium (Figure 2). Amplicon codes were generated as described above, for all serovars tested, including the 12 new serovars (Table 1; Table 2).

In the blind study, the multiplex PCR based serovar determination correlated with the traditional antibody-based serotyping results (performed by NVSL) 100% of the time (total 111 isolates) belonging to 23 serovars when their serogroup was known. In the absence of the serogroup data the multiplex PCR assay successfully identified the serovars of 135 out of 142 *Salmonella* isolates, in which case, the overall sensitivity and specificity were both 95.3%.

Microarray serovar identification

A spotted microarray platform was developed containing 70mer probes for STM, STY, and STV genes. Based on the hybridization patterns, unique signatures were developed for tested serovars. This array of 37 probes was successful in determining all serovars of *Salmonella* tested (total of 86 isolates belonging to 40 serovars, ten of these isolates are shown in Figure 3).

The correlation between the signals generated in microarray and bands amplified in multiplex PCR matched 30 out of the 30 isolates tested in our initial validation step (data not shown). In the blind study, we successfully identified serovars of 52 out of 56 total isolates representing 28 different serovars (Table 2). The sensitivity and specificity of the microarray based serotyping assay were determined to be 93.3%. Since many of the isolates used in the blind study were submitted as DNA samples from diagnostic labs from across the country, the serogroup data was unknown.

Discussion

In this study the discriminatory power of a multiplex PCR assay for molecular serovar identification of *Salmonella* was greatly improved from the existing techniques[27] with addition of

a new multiplex PCR panel (STV), that not only incorporated two gene targets STM 7 and PT4 that differentiates certain serovars[27], but also detects the presence of the virulence markers *spvC, invA* and *sseL*. All isolates tested were positive for virulence genes *invA* and *sseL*. Since only strains of *Salmonella* isolated from clinical sources were included in this study, the presence of these genes in less virulent strains, especially those from environmental sources is not known.

The *invA* gene encodes an invasion protein and has been reported to be present in most strains of *Salmonella* isolated from animals and humans[31]. The *sseL* encodes a deubiquitinase enzyme that contributes to intracellular survival and invasion by *Salmonella* in macrophages, and is claimed to be present only in highly virulent strains[13,41]. The virulence gene *spvC* is generally carried in a plasmid, but may also be present in the genome, and has been shown in previous studies to improve the survival in the presence of starvation stress in host tissues[7,12,20,28]. The *spvC* gene encodes a phosphothreonine lyase that has significant similarity to OspF of *Shigella flexneri*, and has an inhibitory effect on signal transduction mediated by ERK and JNK pathways, and NF*k*B activation in eukaryotic cells, thereby inhibiting proinflammatory cytokine responses[32]. Previous studies have reported that *spvC* gene is predominant in four serovars tested including Typhimurium, Choleraesuis, Dublin, and Enteritidis[12,20], with *Salmonella* Typhimurium and Enteritidis being the most common serovars isolated from diarrheal patients[20]. Therefore, the presence of *spvC* gene may predict the ability of a *Salmonella* strain to survive in a host environment, its capacity to infect other hosts, and the possibility of causing an outbreak. The potential of the assay was demonstrated by the testing of 111 blinded isolates with a sensitivity of 100% when serogroup data was available. When calculations were performed without considering the serogroup data (135/142 isolates), the sensitivity and specificity was determined to be 95.3%.

Microarray has been used previously to identify serovars of bacteria such as *E. coli*[3,21], *Salmonella*[9,18,29,30,39,50], and *Bartonella*[5], due to the rapidity of the assay and its ability to provide high throughput and consistently reproducible results. Also the charged coupled device (CCD) detection of fluorescent spots in microarray has been reported to provide high sensitivity[17]. The microarray developed in this study consisted of probes that targeted the genetic regions shown to be important for serovar differentiation by previous studies[39] and our multiplex PCR assays. The data from microarray-based serotyping was compared with analysis of the same isolates using a multiplex PCR, and the sensitivity and specificity was determined to be 93.3% (see materials and methods). A previous multiplex PCR study by Arrach *et al.* noted that some target DNAs may not be amplified due to intra-serovar variation at the primer binding regions which could lead to the compiling of false amplicon codes[2]. In this study, the 95.3% concordance of multiplex PCR and microarray; suggests that such mutations may not significantly affect the effectiveness of multiplex PCR to determine the serovar of a *Salmonella* isolate.

This study describes two rapid molecular methods that can accurately identify the serovars of common clinical isolates of *S. enterica* subsp. *enterica*. The multiplex PCR is straightforward and can currently be applied in any laboratory with access to PCR and gel electrophoresis equipment. The growing number of *Salmonella* genome sequences available for analysis, and comparison by complete genomic hybridization and other methods, will identify future targets to further improve the discrimination of serovars that may share the same amplicon codes. Of the 1531 serovars of *Salmonella enterica* currently known[19], our blind studies only covered a relatively limited number of them. While our studies and those conducted by Kim *et al.* 2006[27] included isolates of some of the most common serovars encountered[20], larger validation studies will be necessary for using these test as a diagnostic tool. Also, higher

throughput for diagnostic samples may be achieved by performing a fluorogenic 5' nuclease PCR assay.

All blind samples (n=142) were processed for multiplex PCR and a subset (n=56) were also tested by microarray analysis (Table 2). The smaller number of samples used in the microarray blind studies were due to the relatively higher costs to perform the assay. Conventional antibody-based serotyping has become increasingly expensive in the recent past due to high costs associated with licensing and transportation of live or frozen cultures via mail; maintenance of freezers to store these cultures; and production of large collection of specific antisera. Expense of the microarray can be reduced by low density arrays with support matrices such as nitrocellulose, by multiple arrays printed on each glass slide and each array hybridized with DNA labeled by different fluorescent markers from different isolates, as well as by labeling targets with colorimetric instead of fluorescent dyes.

While multiplex PCR arrays provide a molecular method to rapidly identify the serovar of a *Salmonella* isolate, microarray has the potential to acquire over one million data points in a single experiment. Also, a microarray probe set can be easily incorporated into any established diagnostic microarray protocol, thereby increasing the overall strength of that platform. For example, we have incorporated the serovar identifying array in an antimicrobial resistance gene array to provide a platform that evaluates resistance profiles of *Salmonella* serovars[37] (Supplemental Figure 1).

We have identified multiplex PCR amplicon codes for 42 serovars, and have determined microarray hybridization profiles for 40 serovars of *Salmonella enterica*. In this study we report a newly developed multiplex PCR reaction (STV) that detects virulence genes (*sseL*, *invA*, and *spvC).* The presence or absence of *spvC* was successful in identifying a subset of serovars that

have been shown previously to cause significant human and animal diseases. We believe that the tests developed in the present study will aid in the understanding the epidemiology and diversity of *Salmonella*.

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Sources and manufactures

a. Difco, Becton Dickson, Sparks, MD.

b. Qiagen, Valencia, CA.

c. Nanodrop, Wilmington, DE.

d. Whitehead Institute, Cambridge, MD; http://frodo.wi.mit.edu/

e. (http://www.cstl.nist.gov/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm)

f. (Eppendorf, Westbury, NY)

g. (Takara Bio Inc, Japan)

h. (Fisher Scientific)

i. (Fotodyne Inc., Hartland, WI)

j. (100bp ladder; Promega, Madison, WI)

k. (Operon, Huntsville, AL)

l. (Corning, Lowell, MA)

m. (Genetix, New Milton, UK)

n. (Stratagene, La Jolla, CA)

o. (Invitrogen Co., Carlsbad, CA)

p. (Amersham, Piscataway, NJ)

q. (Genisphere Inc.**,** Hatfield, PA)

r. (Molecular Devices, Sunnyvale, CA)

s. (TIGR, Rockville, MD)

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Figures and Tables

Figure C.1 STM (A) and STY (B) multiplex PCR products of 12 previously unscreened serovars. If PCR products (five primer-sets for STM and five primer-sets for STY) were present at the predicted location on a gel it was considered positive for those reactions. An amplicon code was designated based on the bands that were present. For example, serovar Bareilly (in panel A; second lane) had PCR products for primer sets STM 2 and STM 5. Therefore, the amplicon code for STM multiplex PCR for Bareilly is 2, 5 (see Table 1 for amplicon codes for all serovar tested in this study).

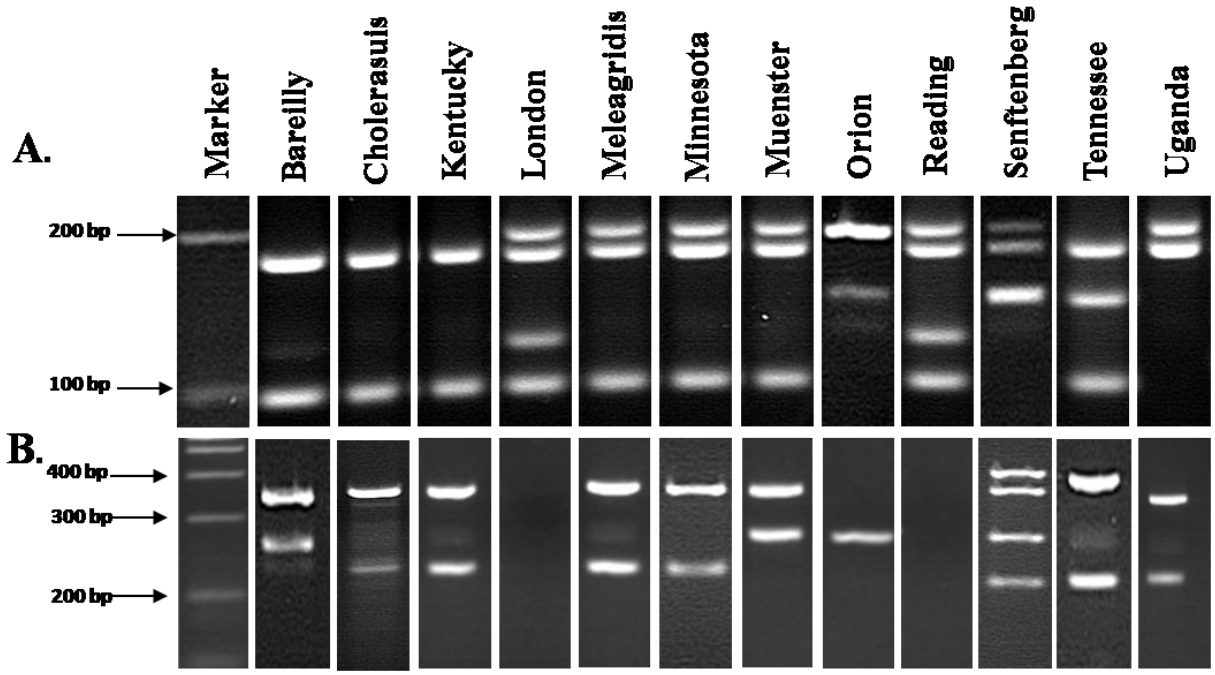

Figure C.2 Multiplex PCR STV products of 29 serovars for which STM and STY were screened previously [27] (A and B). Multiplex PCR STV (*Salmonella* Typing Virulence) products of the 12 new *Salmonella enterica* serovars (C). An amplicon code was designated based on the bands that were present. For example, serovar Agona (in panel A; second lane) had PCR products for primer sets STV 2, STV 4, and STV 5. Therefore, the amplicon code for STV multiplex PCR for Agona is 2, 4, 5 (see Table 1 for amplicon codes for all serovars tested in this study).

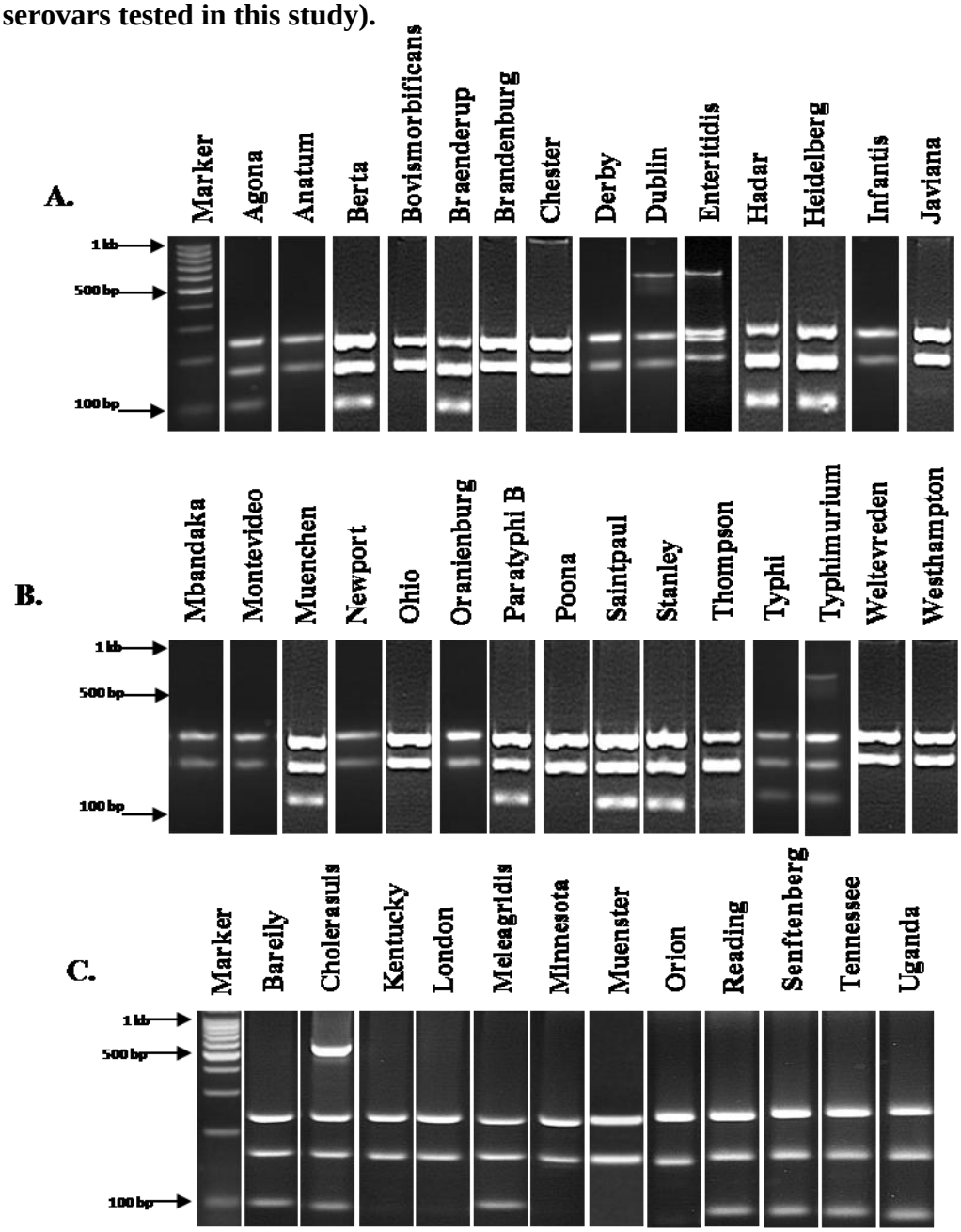

Figure C.3 Results from microarray (A; black is positive and white is negative) and multiplex PCR (B) for the STM 1-5 (STM), STY 1-5 (STY), and STV 1-5 (STV) genes of ten *Salmonella* isolates representing seven serovars.

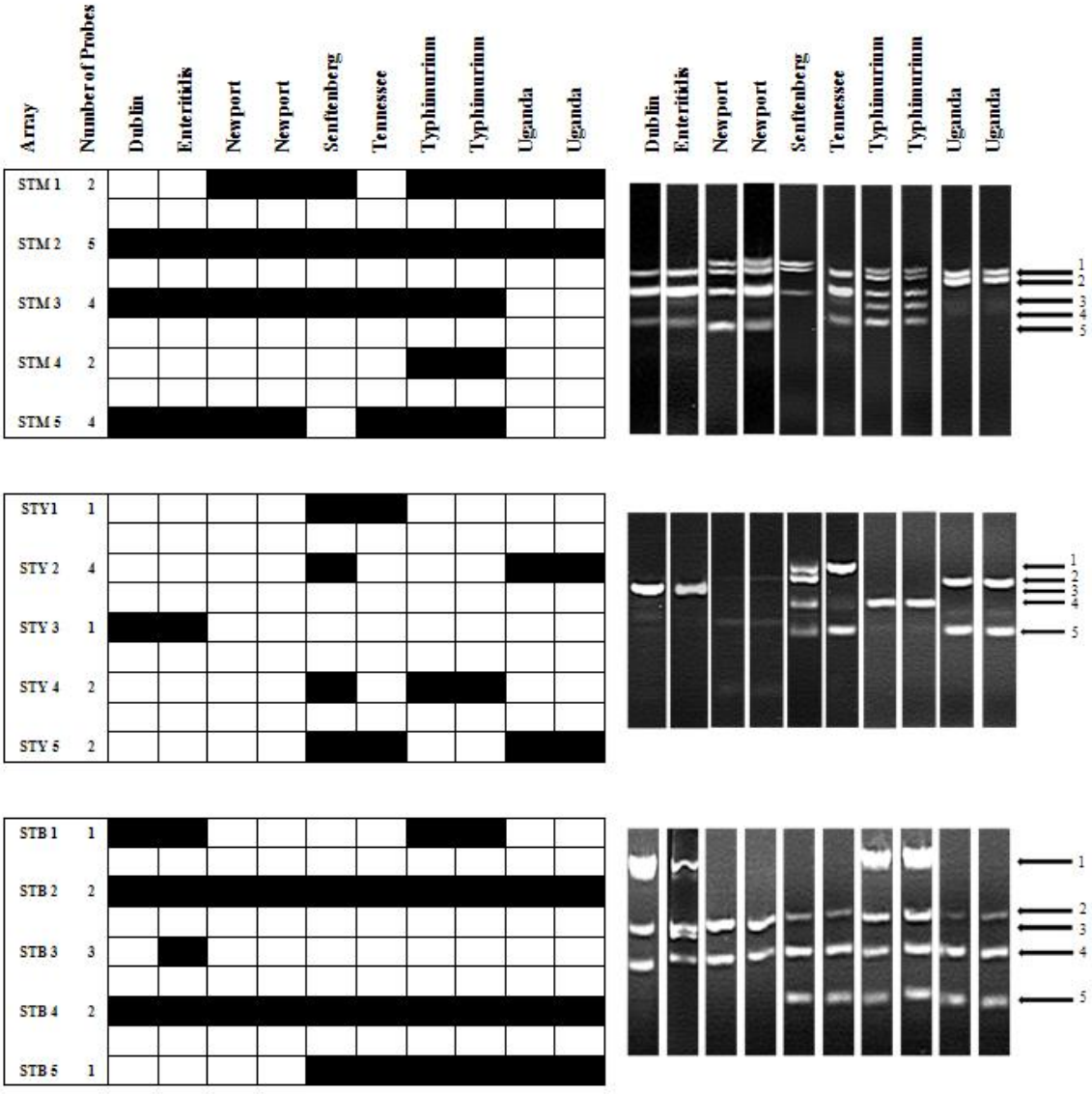

Figure C.4 (Supplementary Data) Microarray slide showing *Salmonella* Typhimurium DNA hybridization to probes for serovar identification. Each 70mer probe was printed randomly in triplicate. Probes for serovar identificiation are listed for STM (blue), STY (green), and STV (orange). Positive hybridizations appear as red signals and negative hybridizations appear as green signals (due to hybridization by the print and hybridization control 25mer probe)[38]. The *Salmonella* serovar determining probe set was incorporated into a microarray chip containing probes for virulence factors, antimicrobial resistance genes, and metal resistance genes[37].

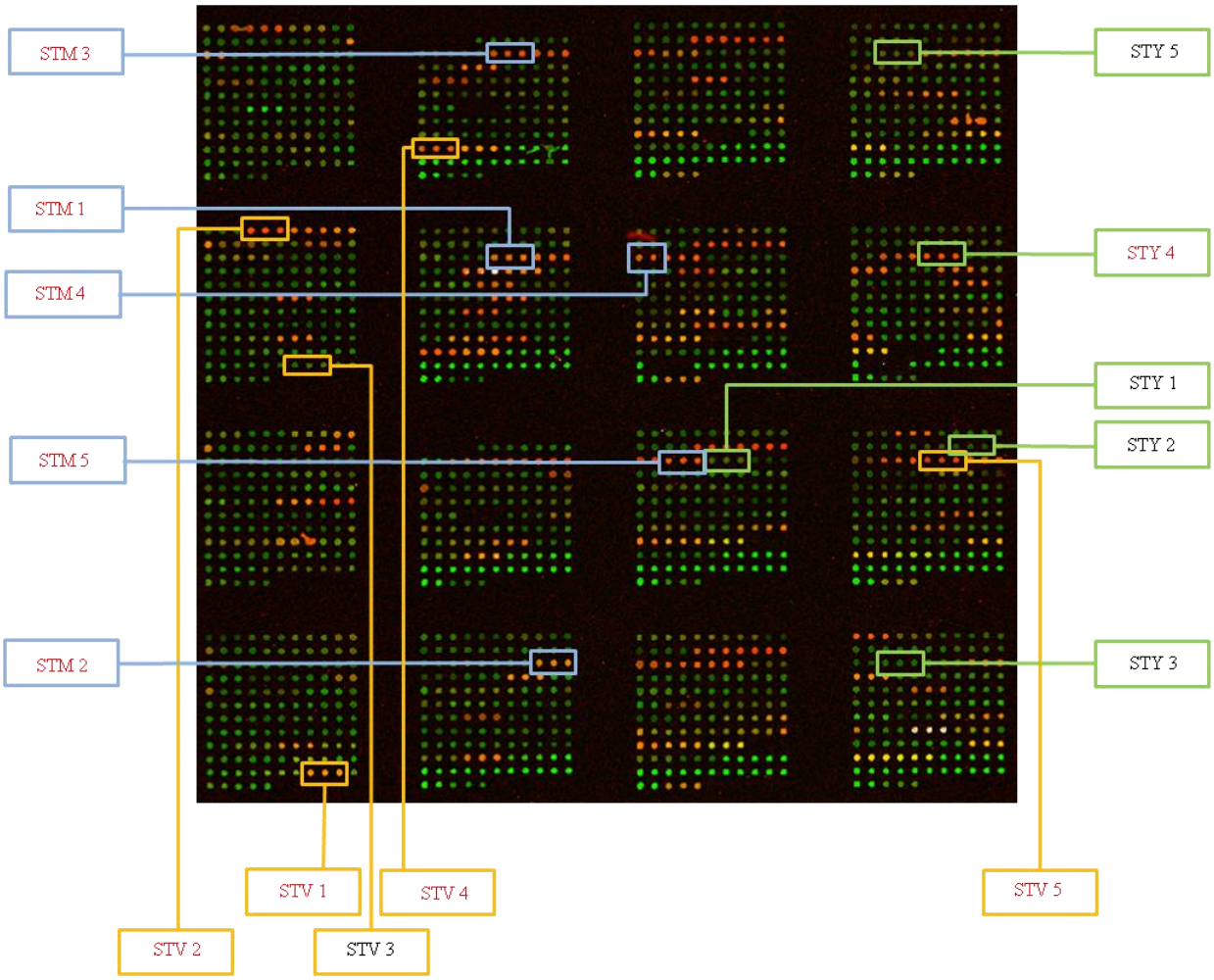

Table C.1 Complete list of all amplicon codes for STM, STY and STV. An * indicates serotypes previously unscreened by STM and STY, and ND indicates that amplicon code was not determined in our study.

Serotype	STM	STY	STV	Serotype	STM	STY	STV
1. Agona	2, 3	5	2, 4, 5	23. Montevideo	5	2, 4	2, 4
2. Anatum	1, 2, 3, 4, 5	0	2, 4	24. Muenster*	1, 2, 5	2, 4	2, 4
3. Bareilly*	2, 5	2, 4	2, 4, 5	25. Muenchen	1, 2, 5	0	2, 4, 5
4. Berta	2, 3, 5	2, 3	2, 4, 5	26. Newport	1, 2, 3, 5	0	2, 4
5. Bovismorbificans	2, 3, 5	0	2, 4	27. Ohio	2, 5	0	2, 4
6. Braenderup	2, 5	0	2, 4, 5	28. Oranienburg	2, 5	2, 4	2, 4
7. Brandenburg	1, 2	2	2, 4	29. Orion*	1, 3	4	2, 4
8. Chester	1, 2	0	2, 4	30. Paratyphi B	1, 2, 4, 5	0	2, 4, 5
9. Choleraesuis*	2, 5	2, 5	1, 2, 4, 5	31a. Poona 1	1, 5	1, 2	ND
10. Derby	1, 2, 3, 5	5	2, 4	31b. Poona 2	1, 2	1, 2	2, 4
11. Dublin	2, 3, 5	3	1, 2, 4	31c. Poona 3	1, 5	2	ND
12. Enteritidis	2, 3, 5	3	1, 2, 3, 4	32. Reading*	1, 2, 4, 5	0	2, 4, 5
13. Hadar	3, 5	0	2, 4, 5	33. Saintpaul	1, 2, 3, 4, 5	0	2, 4, 5
14. Heidelberg	1, 2, 4, 5	2	2, 4, 5	34. Senftenberg*	1, 2, 3	1, 2, 4, 5	2, 4, 5
15. Infantis	2	2	2, 4	35. Stanley	1, 2, 5	2	2, 4, 5
16a. Java 1	1, 2, 4, 5	0	ND	36. Tennessee*	2, 3, 5	1, 5	2, 4, 5
16b. Java 2	2, 4	0	ND	37. Thompson	2, 3, 5	5	2, 4
17. Javiana	1, 2	2, 5	2, 4	38. Typhi	1	1, 2, 3, 5	2, 4, 5
18. Kentucky*	2, 5	2, 5	2, 4	39. Typhimurium	1, 2, 3, 4, 5	4	1, 2, 4, 5
19. London*	1, 2, 4, 5	0	2, 4	40. Uganda*	1, 2	2, 5	2, 4, 5
20. Mbandaka	2, 3, 5	2, 5	2, 4	41. Weltevreden	1, 2, 4, 5	1, 4, 5	2, 4
21. Meleagridis*	1, 2, 5	2, 5	2, 4, 5	42. Westhampton	1, 2, 3	1, 2, 4, 5	2, 4
22. Minnesota*	1, 2, 5	2, 5	2, 4				

Table C.2 Complete list of all serotypes tested in this study with multiplex PCR and microarray in the blind study. The numerator indicates total of number of isolates that were correctly identified molecularly, and the denominator indicates total number tested with serotypes identified by traditional antibody tests. An * indicates serotypes previously unscreened by STM and STY multiplex PCR assays.

Serotype	Multiplex PCR Blind Study	Microarray Blind Study
Agona	5/5	2/2
Anatum	4/6	
Berta	2/2	2/2
Bovismorbificans	2/2	2/2
Braenderup	2/2	2/2
Brandenburg	2/2	2/2
Chester	2/2	2/2
Cholerasuis*	2/2	
Derby	3/3	
Dublin	7/7	3/3
Enteritidis	1/1	1/1
Hadar	2/2	2/2
Heidelberg	2/2	2/2
Infantis	5/5	
Javiana	2/2	2/2
Kentucky*	3/4	
London*	1/1	
Mbandaka	3/3	1/1
Meleagridis*	4/4	
Minnesota*	3/3	3/3
Montevideo	20/20	1/1
Muenster*	8/8	
Muenchen	3/3	1/1
Newport	7/7	3/3
Ohio	2/2	2/2
Oranienburg	3/3	1/1
Orion*	4/4	
Paratyphi B	2/2	2/2
Poona 2	2/2	2/2
Reading*	3/3	2/2
Saintpaul	3/4	1/2
Stanley	2/2	2/2
Tennessee*	2/2	
Thompson	2/2	2/2
Typhimurium	6/6	2/2
Uganda*	8/8	4/4
Weltevreden	1/2	1/2
Westhampton	0/2	0/2
Total	**135/142**	**52/56**

Table C.3 List of primers used in STV multiplex PCR assay.

Gene	NCBI accession no.	Primer	Primer sequence (5'→ 3')	Amplicon size (bp)	Reference
spvC	M64295	SPVC-1 SPVC-2	ACTCCTTGCACAACCAAATGCGGA TGTCTTCTGCATTTCGCCACCATCA	571	[12]
invA	M90846	INVA-1 INVA-2	ACAGTGCTCGTTTACGACCTGAAT AGACGACTGGTACTGATCGATAAT	244	[12]
PT4	AF370716	PT4 F PT4 R	GGCGATATAAGTACGACCATCATGG GCACGCGGCACAGTTAAAA	225	[27]
sseL	AE008002	*sseL* F *sseL* R	TTCCGCGACAACCGACCTTTCTAA TTCTTGAACCAGACCTTGCGTTGC	169	This study
STM 7	AE008795	STM2150F STM2150R	CATAACCCGCCTCGACCTCAT AGATGTCGTGAGAAGCGGTGG	101	[27]

Table C.4 (Supplementary Data) 70mer probes used in this study for serotyping *Salmonella enterica*.

Assay	70mer Oligo	Primer sequence	NCBI accession no.
STM 1	STM 1 0716F_441-510 A	TGCGGCTACGCCCTTGCTGATAAAGGGATTGATACACGACTCATACAGGACTATCTGGGGCACAGGAATA	AE008729
	STM 1 0716F_416-485 B	ACATCCTCATATGCTGCGACATGCCTGCGGCTACGCCCTTGCTGATAAAGGGATTGATACACGACTCATA	
STM 2	STM 2 1350F_1077-1146 A	ATGAACACCGATGGTTATGCCGCCACCGGTGTAGAAATTAAAATCGTGGATGAAGATCGCAATACGCTTC	AE008758
	STM 2 1350F_373-442 B	AATGTCAGGCTAAAATATTCTTCGCCCCCACCGTGTTCAAACAGAATCGTCCGGTCGATCTTATCCTTCC	
	STM 2 1350F_768-837 C	TTTTTAATCGGGGCGCGTAGCGTATTGCTGGACATCTTTACCCCAGAAGCCTGCCTTACCTTATTAGCGC	
	STM 2 1350F_407-476 D	GTTCAAACAGAATCGTCCGGTCGATCTTATCCTTCCGCTACAAAATCAACTGCGCCATCTGACGCATATT	
	STM 2 1350F_1005-1074 E	TTGAGTATTTACGGTTCTACAGAAAGTTCTCCACACTCGATGGTTAATCTGGGTGATTCGACTTCACGCA	
STM 3	STM 3 0839F_461-530 B	TGAAGTTGAGGATATCGAAGGCGTATGGATGCGTACCTATGGTGCTGATTGCTTTGGGCTACCAGATTTC	AE008735
	STM 3 0839F_255-324 D	GATACTTCACCTTTAGAGCAATATGTTGCGTTGGCTGTGGTTGCAGGTGCATTAAGTAACATGGGGGCTG	
	STM 3 0839F_111-180 E	CATGTAATATCGATGGTTGGATTTAAAACGCCTTACCCTCAGGAATCAATCGAACAATGCGTCGCGCCAG	
	STM 3 0839F_424-493 G	TCCCGCTGACCTCTCTATTTTGTGGATTTGTGAAATATGAAGTTGAGGATATCGAAGGCGTATGGATGCG	
STM 4	STM 4 4525F_1081-1150 C	GTTCTTTACCAAAGGCACGGTCACCAATCCGCATCAGGATAAAAACTGCACCGATGACGTGTGGGTGTAT	AE008913
	STM 4 4525F_82-151 D	CTCCTATCAAAACTACGTCAATGAACTCGCCTCGCTGCTGTTTTTGAAAATGTGCAAAGAGACCGGCCAG	
STM 5	STM 5 4538F_666-735 A	CCCATTACGTTTGGTACGGGAGAAGCGAAAACCCATGTTCAGGACATTATTAACGACATCATGCCTTGTC	AE008913
	STM 5 4538F_635-704 B	GATGACGGCCTCCATGATTGATATCACCATTCCCATTACGTTTGGTACGGGAGAAGCGAAAACCCATGTT	
	STM 5 4538F_285-354 C	GTGGCGATGGAGGAGAAAAATAGCCAGCAGAAAGAGATGGACGCCAGTTCTATCGATAACGTCAAAGCGT	
	STM 5 4538F_691-760 D	CGAAAACCCATGTTCAGGACATTATTAACGACATCATGCCTTGTCTGCTGCCGTTAATTAGCTTCGCCAT	
STY1	STY 1 0312R_281-350 A	TGTGGGCTATGACTCTCCTTCTGTTGGTGCTACGGATATCTGGGGATTATTTTCCGTCAGTCCGAAAACA	AL627266
	STY 1 0312R_92-161 B	ATCAAATATCCCGGTGTTTGATTTGGCTAAATTAAATCAGGAAGGGCTTGCCGAGACACAGGCTCAGGCA	
STY 2	STY 2 0346F_384-453 A	ATGGAAGTGACTCTGGGAGGACGGTCACTGACCACCACCAATTCTGTACTGGAAGCTAAAACCCTGTTCC	AL627273
	STY 2 0346F_149-218 B	GGTTCAGAAGGATATTACCGTCACTGCCAATATTGACAGTACACTTGAACTGCTGCAGGCCGATGGTTCA	
	STY 2 0346F_178-247 C	ATATTGACAGTACACTTGAACTGCTGCAGGCCGATGGTTCATCCCTCCCGTCGACTATGAAGCTGGATTT	
	STY 2 0346F_97-166 D	TTATTGCCGCAGCTGTGGCATTGGCCACCGTTTATTCTTTTTCTGTTTCTGCGGTTCAGAAGGATATTAC	
STY 3	STY 3 2299F_19-88 A	ATGGGAGCGTTTGGGTTCCTTGGATCACGACTTACATCCTACTTCGAAAGTCGACATACTGTGATTGGCT	AL627273
STY 4	STM 4 3845F_92-161 A	TCCTTTTCGTGTGTGGTGGCAAAGTCGATGTACGTGCACCAATTCCCCCCAGTTTTAGGGATAGACTACT	AE008879
	STM 4 3845F_63-132 B	TAACTTCAATGTTGATATATCTCATCGTCTCCTTTTCGTGTGTGGTGGCAAAGTCGATGTACGTGCACCA	
STY 5	STY 5 2349F_122-191 A	AAAAATTCCAGTTTTGGCCAGCCAGGGCGAACAGCTTTACAAGACCCAAAAGTATGCCAAAGCACTCGAC	AL627273
	STY 5 2349F_490-559 B	AGGTTAATTTTCAGGGTTACTGGTTCGGTTTAATGGGGATCTACTTCGGCCCCAATATCGGCGAGTTCTA	
STV 1	*spvC* 3364-3436	GAAAAATAATTTCAACTCCTTGCACAACCAAATGCGGAAGATGCCGGTATCCCACTTTAAAGAGGCGCTG	D14490
STV 2	*invA* 78-147	ACCTACCTATCTGGTTGATTTCCTGATCGCACTGAATATCGTACTGGCGATATTGGTGTTTATGGGGTCG	U43273
	invA 2 1885-1950	TTATTGATTGCACATAAAGATCTTGTCCTCCTTACGTCTGTCGATGTCCGTCGATTTATTAAGAAA	
STV 3	PT4 3452-3521	AAATTTTGTGGTGCGGTGCCTGGTGCCTCCAGGTGACATTAACCAGTTAACAATTAATGCCGACTTAAAC	AF370716
	PT4 2 3425-3494	ATCATAGCCCCTCCATTTCTGGTAAATAAATTTTGTGGTGCGGTGCCTGGTGCCTCCAGGTGACATTAAC	
	PT4 3 3492-3561	AACCAGTTAACAATTAATGCCGACTTAAACCACCCATACTGATTCAGGGAGTTTTAACTGTGCCGCGTGC	
STV 4	*sseL* 18937-19006	TTTACAGAACAATGTACCCAACGGCTGTGGTCTATTTTGTTACCATACAATTCAACTCTTATCGAATGCC	AE008802
	sseL 2 18702-18771	GCACTCAGCTACTTGAAAAGATTGCTCAATCAGGATTATCTCACAATGAAGTCTTCCTGGTAAATACAGG	
STV 5	STM 7 2150F_315-384 E	CTGCAACCGCAGGGAGCAACGGATTGTATTGCGTTAAAAGATGTCGTGAGAAGCGGTGGCTATACTTTTA	AE008795

Appendix D - Effects of two oxytetracycline dosing regimens on horizontal transfer of antimicrobial resistance plasmids in an *in vitro* pharmacodynamic model

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Short Title: Antimicrobial resistance transfer in an *in vitro* pharmacodynamic model

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Keywords: *in vitro* pharmacodynamic model, antimicrobial resistance, horizontal gene transfer, oxytetracycline

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Abstract

Objective – To evaluate the impact of oxytetracycline exposure on the horizontal transfer of an antimicrobial resistance plasmid.

Study model – *In vitro* pharmacodynamic model.

Procedures - Mixed populations of plasmid donor (*Salmonella*) and recipient bacteria (*E. coli*) were assigned to one of two simulated oxytetracycline dosing regimens (High peak concentration

– short elimination half life / Low peak concentration – long elimination half life) or untreated control. Donor, recipient and transconjugant (*E. coli* that has acquired the plasmid) bacteria populations were quantified by plating on selective bacterial growth media at 12, 24 and 36 hours following oxytetracycline administration.

Results - The ratio of transconjugant to donor bacteria was significantly reduced in the oxytetracycline exposed replicates compared to the controls at 12 hours. At the 24 and 36 hour timepoints, the high concentration treatment was not significantly different from its respective control (p=0.24 and 0.98, respectively), while the comparison of the low concentration treatment to its control approached significance at both timepoints (p=0.057 and 0.062, respectively). The oxytetracycline concentration at these timepoints (12 hours in the high concentration regimen and all 3 timepoints in the low concentration regimen) were in excess of the minimum inhibitory concentration of the recipient bacteria.

Conclusions and Clinical Relevance - These observations suggest that transfer of antimicrobial resistance plasmids can be suppressed *in vitro* by oxytetracycline exposures above the minimum inhibitory concentration of the recipient bacteria.

Abbreviations

HC-SHL	High Concentration – Short Half Life
LC-LHL	Low Concentration – Long Half Life
IVPM	*in vitro* pharmacodynamic model

Introduction

The remarkable adaptive abilities of bacteria have lead to the phenomenon of antimicrobial resistance. Just 30 years ago, we were "closing the book on infectious diseases"[1].

Today, we face a global pandemic of re-emerging infectious diseases brought around, in part, by antimicrobial resistance[2]. Three key factors have been recognized in the emergence of bacterial resistance: mutation, bacterial genetic exchange, and selective pressure in health care and community settings[3]. Of these factors, the primary focus has been investigating methods to decrease the selective pressure within health care and the community by focusing on prudent drug use[4,5,6]. The importance of prudent antimicrobial use has also been recognized in veterinary medicine as evidenced by the American Veterinary Medical Association's policy statement on judicious therapeutic use of antimicrobials[7]. For veterinarians, the prevention of antimicrobial resistance has important implications in our obligations to both public health and the successful treatment of our patients. This is particularly true in production animal medicine where these goals are often depicted as contradictory in the public debate.

The roles of mutation and dosing strategies to minimize resistance development have been studied extensively, giving rise to theories such as the Mutant Prevention Concentration[8,9,10,11]. There is also a plethora of literature in regard to the mechanisms of bacterial gene exchange[12]; however, relatively little has been published regarding the influence of drug exposure on the rates of conjugative transfer[13].

The issue of antimicrobial resistance has resulted in a paradigm shift with regard to investigation of antimicrobial therapeutics. Since the late 1990's, researchers have sought dosing regimens that impart not only clinical efficacy, but also minimize the development of antimicrobial resistance. Consistent with these goals, the objective of this research was to evaluate the impact of oxytetracycline exposure on the horizontal transfer of an antimicrobial resistance plasmid in an IVPM. Our hypothesis was that different oxytetracycline exposures would result in differential frequency of plasmid transfer between bacterial species. Results of

this study provide information for the development of oxytetracycline dosing regimens that minimize the horizontal transfer of antimicrobial resistance genes.

Materials and Methods

Bacterial Species/Strains

In this study, we used a clinical isolate of *Salmonella enterica* subspecies *enterica* serovar Typhimurium strain number 5678 containing a 100kB type A conjugative plasmid that had been used in previous plasmid transfer experiments[14]. This low copy number plasmid contains a bla_{CMY-2} ESBL gene (with resistance to ampicillin, ceftriaxone, and cefoxitin) as well as resistance markers for chloramphenicol, streptomycin, sulfisoxazole, trimethoprim-sulfamethoxazole, and tetracycline. The resistance gene that supplies the resistance to tetracycline/oxytetracycline is present on an IS26-like portion of the plasmid and was shown by PCR amplification to be positive for the *tetA* efflux pump (data not shown). Monitoring of transconjugants indicated that this plasmid transfers all resistance genes, as is shown by the ability to screen on ampicillin. Additional tests indicated that other antibiotic markers were present as well (data not shown). The recipient bacterium, *E. coli* C600N, is a laboratory strain that carries a chromosomally encoded resistance marker for nalidixic acid. Aliquots of stock bacteria were stored separately at -80º C.

Determination of Minimum Inhibitory Concentration (MIC)

The minimal inhibitory concentration for each of the study bacteria was determined by modification of CLSI recommended procedures[15]. Fresh cultures of donor and recipient bacteria were separately grown overnight on a rotary shaker (37º C, 150 rpm) in ten milliliters of fresh

Luria-Bertani (LB) broth. Ten microliters of a standardized bacterial suspension (OD_{600} =0.03) were pipetted into 11 mL of (LB) broth[A]. Aliquots of the bacterial suspension (100 µL) were added to a 96 well plate[B]. One hundred microliters of oxytetracycline solution had been previously added to the wells, so that the final concentrations tested were: 0, 1, 5, 25, 50, 75, 100, 125, 150, 175, 200, 250, 500, 750, and 1000 ng/mL. Testing was done in duplicate for the donor and recipient strains.

The plates were placed in an incubated spectrophotometer[C] set to read absorbance at 600 nm with readings taken hourly. The inhibitory concentration was determined to be the lowest concentration at which optical density readings were reduced.

Experimental Stock Culture

Single frozen aliquots of the *Salmonella* and *E. coli* were thawed at room temperature. The bacteria were streaked for isolation on LB agar plates[D]. A single colony of each bacteria was transferred to separate flasks containing 10 mL of sterile brain-heart infusion broth[E] and incubated overnight at 37º C on a rotary shaker[F]. The entire 10 mL of the overnight cultures were transferred into separate flasks containing 90 mL of sterile brain-heart infusion broth. Both bacterial cultures were grown to logarithmic phase growth (OD_{A600} = 0.6)[G]. Once the desired optical densities were reached, the *Salmonella* and *E. coli* were transferred to syringes[H] in a 1:5 volumetric ratio. Five mL of the bacterial mixture was inoculated into each central reservoir of the IVPM systems. A one hour growth interval was observed in the systems prior to oxytetracycline administration.

In vitro Pharmacokinetic Model (IVPM)

Variations of the model used in this study have been described by others[16,17,18,19]. The model consists of a 500 mL central reservoir (CR)[I], a 4 L fresh media reservoir[J], a 4 L waste collection reservoir[K], reservoir caps[L] and connecting tubing[M] (Figure 1). All components were autoclaved prior to system assembly. For each experimental run, four of the above described systems (two designated oxytetracycline treatments and two associated controls) were assembled. During the experiment, the central reservoir of each system was housed in an incubator[N] at 37 C. The central reservoir media was constantly stirred by use of a stir rod/plate[O] setup.

Fresh media pump speed was based on equation 1, to achieve a specified half-life. Waste media pump speed was set slightly faster, with the exit port set just above the CR fluid line to maintain a constant CR volume. Dual pump heads were used to control the fresh and waste media flow for a given treatment and the associated control arm. To eliminate residual effects in the samples, a single CR port was designated for bacterial inoculation and oxytetracycline dosing. A separate port was designated for bacterial and antimicrobial sampling. Three replicates of each treatment (High Concentration / Low Concentration) and the associated control were conducted.

Equation 1: Pump calculations in the IVPM

$$T_{\frac{1}{2}}(\mathrm{hr}) = \frac{0.693 \times \mathrm{Volume}_{\text{Central Reservoir}}\,(\mathrm{mL})}{\text{Pump speed}\,\left(\mathrm{mL}/\mathrm{hr}\right)}$$

The two dosing regimens were modifications of intravenous[20] and intramuscular[21] dosing of oxytetracycline in swine. The intravenous dose was designed so that initial peak concentrations were above 1000 ng/mL and time within the 150-1000 ng/mL range would be minimized. The intramuscular simulation was designed so that drug concentrations would

remain within the 150-1000 ng/mL range for a comparatively longer period of time. Each of the three (3) replicate runs included: HC-SHL, HC-SHL control, LC-LHL, and LC-LHL control.

Antimicrobial Treatment

Analytical grade oxytetracycline as the hydrochloride salt was diluted in fresh brain-heart infusion broth prior to dosing in the IVPM. Corrections for salt and purity were made to achieve oxytetracycline concentrations outlined in Table 1.

Bacterial Quantification & Determination of Plasmid Transfer

Samples were collected from the central reservoir of the IVPM at 12, 24 and 36 hours after oxytetracycline administration. The entire sample (approximately 1mL) was centrifuged at 5,000 x g for 10 minutes. The supernatant was pipetted into cryovials and stored at -70º C for oxytetracycline quantification as described below.

The bacterial pellet was resuspended with phosphate buffered saline (PBS), then serial dilutions of the bacterial resuspension were made to the 10^{-8} dilution. Transconjugant bacteria were quantified by duplicate plating of 50 µL of the -1, -4, -6 and -8 dilutions on Hektoen enteric agar containing 50 µg/mL of ampicillin and 12 µg/mL nalidixic acid. *Salmonella* (the donor) were quantified by plating 50 µL of the -4, -6 and -8 dilutions on Hektoen enteric agar containing 50 µg/mL of ampicillin. *E. coli* (the recipient) were quantified by plating 50 µL of the -4, -6 and -8 dilutions on Hektoen enteric agar containing 12 µg/mL nalidixic acid. Colony-forming unit counts were determined as by Equation 2.

Equation 2: Colony forming unit calculation for transconjugant, donor and recipient bacteria

$$\mathrm{CFU}/_{\mathrm{mL}} = \text{Number of colonies} \times 20 \times \frac{1}{\text{Dilution Factor}}$$

Equation 3: Calculation of Transconjugant (TC) ratio

$$\text{TC ratio} = \frac{\text{Transconjugant Bacteria } \left(^{\text{CFU}}/_{\text{mL}}\right)}{\text{Donor Bacteria } \left(^{\text{CFU}}/_{\text{mL}}\right)}$$

Oxytetracycline Quantification

Sample preparation

All chemical reagents were analytical grade. Briefly, 50 µL of internal standard (doxycycline[P] 2,000 ng/mL) was added to 50 µL of sample. The analyte and internal standard were extracted using 10 µL of concentrated phosphoric acid[Q] followed by the addition of 150 µL of deionized water. The samples were loaded on HLB solid phase cartridges[R] for the extraction procedure. Following the first elution with 5:95 (methanol:water), the columns were washed with 500 µL of 5:95 (Methanol:Water) and dried under high vacuum (≈ 20 inches Hg) for 10 minutes. Oxytetracycline was recovered from the SPE cartridge using a methanol wash (300 µL). A 150 µL sample was then transferred to a HPLC vial and stored at 4°C in the sample carousel until analysis

HPLC/MS/MS methods

Quantitiation of oxytetracycline was performed using high performance liquid chromatography and triple quadrupole mass spectrometry (HPLC/MS/MS). Chromatographic separation was achieved using a gradient elution of 100% (0.2% glacial acetic acid[S] in H_2O) moving to 5% (0.2% glacial acetic acid in H_2O):95% (0.2% glacial acetic acid in acetonitrile[T]) on a HPLC system[U] with a C_{18} analytical column[V]. Injection volume was 2 µL and flow rate was 0.35 mL/min with a total run time of 5 minutes. Retention times for oxytetracycline and doxycycline (IS) were

2.12 and 2.15 minutes, respectively. Mass spectrometry utilized an electrospray ionization source[W]. The instrument[X] was set to operate in positive ion mode. Transitions were monitored at m/z 461 → 426 for oxytetracycline and m/z 445 → 321 for doxycycline (internal standard). The standard curves were prepared daily and consisted of 7 non-zero points ranging from 20 to 14,000 ng/mL.

The run was accepted if the concentrations of the standards were within 15% of the expected concentration and the fit of the curve was at least 0.99. Two low (350 ng/mL) and two medium (6000 ng/mL) quality controls were run; one low QC was more than 20% different from the expected value. The accuracy and coefficient of variation of the remaining QC samples were ± 19% and ± 17%, respectively.

Corrections for Protein Binding

Protein binding was determined in brain-heart infusion broth by ultrafiltration / centrifugation. Triplicate 200 µL aliquots of the low (20 ng/mL), medium (500 ng/mL) and high (14000 ng/mL) calibration solutions from the standard curve were pipetted into centrifugal filtration vials[Y]. The vials were centrifuged at 14,000 x g for 30 minutes. The preparation and quantification procedures were as detailed previously with the following exception: the standard curve for protein binding estimate was fit with a quadratic equation ($R^2 = 0.9998$) consisting of 6 points across the range of concentrations. Accuracy of the standards was within ± 3% of expected concentration. The analytic run consisted of only the standard curve and 9 ultrafiltered samples. Protein binding was calculated using equation 4.

Equation 4: Calculation of protein binding

$$\text{Protein Binding} = 1 - \left[\frac{\text{Concentration In Ultrafiltered Sample}}{\text{Concentration in Known Standard}}\right]$$

For filtered samples at the low concentration, the centrifuged / filtered sample concentrations were above the LOD but below the LLOQ. For these samples, concentration was calculated by dividing the area ratio of the sample by the area ratio of the standard and multiplying by the known concentration of the standard.

Equation 5: Protein binding correction for oxytetracycline in brain – heart infusion broth

$$\%\,\textbf{protein bound} = -0.066 \times \ln(\textbf{measured oxytetracycline concentration}) \times 0.587$$

Statistical Analysis

Statistical analyses were performed using a commercial software package[Z]. Transconjugant (TC) ratios were logarithmically (base 10) transformed prior to statistical analysis. The lower limit for the calculation of the transconjugant ratio is detailed in equations 6-8. The TC ratios were analyzed by timepoint using one-way ANOVA with treatment as the independent variable. Significant treatment differences were further evaluated by use of two-way contrast statements. Statistical significance was set *a priori* at the p=0.05 level.

Equation 6: Upper limit of quantification for donor

$$\textbf{ULOQ (CFU)} = 200 \times 20 \times 10^{8} = 4 \times \frac{10^{10}\ \textbf{CFU}}{\textbf{mL}}$$

Equation 7: Lower limit of quantification for transconjugants

$$\textbf{LLOQ (CFU)} = 1 \times 20 = 20\ {}^{\textbf{CFU}}/_{\textbf{mL}}$$

Equation 8: Lower Limit for TC ratio

$$\text{TC ratio}) = \frac{\textbf{Transconjugant LLOQ}}{\textbf{Donor ULOQ}} = \frac{20}{4 \times 10^{10}} = 5 \times 10^{-10})$$

Results

Determination of Minimum Inhibitory Concentration

Bacterial growth reductions were generally seen following 8-12 hours of incubation. The oxytetracycline MIC for the *E. coli* (recipient bacteria) was 125 ng/mL. The MIC for the *Salmonella* (donor bacteria) was 60,000 ng/mL.

Oxytetracycline Protein Binding in brain-heart infusion broth

Protein binding in brain-heart infusion broth was non-linear between 20 and 14,000 ng/mL (Figure 2). For concentrations > 700 ng/mL, protein binding was estimated at less than 15% and was not corrected. For the Low concentration dosing regimen, one concentration measurement was above 700 ng/mL (751), but for consistency, all data for this regimen were corrected for protein binding according to equation 5. For the High concentration dosing regimens, the 12 hour samples were above 700 ng/mL and the 36 hour samples were below the LLOQ of the assay, so no corrections were applied. The 24 hour sample for all High concentration replicates was corrected for protein binding.

Transconjugant Ratio Determination

Four replicate-timepoints in antimicrobial treated regimens had transconjugant ratios of zero (no transconjugant colonies). These values were mathematically set equal to the lower limit of the TC ratio as detailed in equation 6-8 above. Three of these time points were at 12 hours (2 LC-LHL and 1 HC-SHL), while the fourth time point was a LC-LHL replicate at 36 hours (Table 1).

The mean transconjugant ratios for the two control simulations were not significantly different at any of the three timepoints. At 12 hours, the transconjugant ratios for the High Concentration and Low Concentration treatments were statistically less than their respective controls ($p=0.01$ for both contrasts). At the 24 and 36 hour timepoints, the High Concentration treatment was not significantly different from its respective control ($p=0.24$ and 0.98,

respectively), while the comparison of the Low Concentration treatment to its control approached significance at both timepoints (p=0.057 and 0.062, respectively). The transfer rate comparison of the High concentration to Low concentration treatment was not significantly different at 12, 24 or 36 hours (p= 0.65, 0.70, and 0.13), respectively. Results are presented graphically in figure 3.

Oxytetracycline Concentrations

In the HC-SHL simulations, average free (non-protein bound) oxytetracycline concentrations were approximately 750 and 65 ng/ml at 12 and 24 hours, respectively. Antimicrobial concentrations were below the limit of detection at 36 hours for these dosing simulations.

In the LC-LHL simulations, average free concentrations were 515, 383, and 225 ng/ml for the 12, 24 and 36 hour timepoints, respectively. Table 1 summarizes these results.

Discussion

There is a growing body of *in vitro* evidence suggesting that the development of antimicrobial resistance within a population of bacteria can be suppressed by extrapolating the pharmacokinetic/pharmacodynamic indices associated with clinical efficacy[22,23,24]. The primary focus of these studies have been on the outgrowth of resistant bacteria: either originating from genetic mutation or pre-existing within the population. In contrast, the study reported here focuses on the development of antimicrobial resistance due to emergence of resistant organisms following the acquisition of a horizontally transferred plasmid. The importance of this mechanism of resistance development in bacterial populations has been discussed previously[2,25,26].

The IVPM is an ideal laboratory tool to study the most basic interactions between antimicrobials and pathogens. A limitation to the one-compartment IVPM utilized here is the dilution effect on the bacteria caused by inflow of fresh media to the central reservoir. Because the transconjugant ratios of the HC-SHL and LC-LHL controls were not significantly different at any time during the experiments, the effect of dilution rate was considered negligible. Another limitation of the IVPM under the described conditions is the favorable advantage given to the bacterial population. The type of growth media, the constant inflow of nutrients and removal of waste products, inoculum size, timing of treatment and temperature at which the experiments are conducted, and lack of a functional immune system give every conceivable advantage to the bacterial pathogen. For the present study, conjugative events may also be favored (compared to filter mating studies) given the absolute number of donor and recipient bacteria present in the culture system, and the constant stirring present in the IVPM. Results of the present study should be interpreted as the "best case" scenario for plasmid transfer given the *in vitro* conditions of the experiment.

The transfer rates in the present study are in agreement with the conjugative rates found by Showsh and Andrews[27]. Using two *Bacillus* strains, the authors reported filter mating transfer rates ranging from 1×10^{-1} to $<1 \times 10^{-8}$, when the donor was pre-grown in the presence of tetracycline (10 μg/mL). These authors also demonstrated that at low tetracycline concentrations (during mating) the conjugative frequency was enhanced. Although transfer rates noted in the previous work were achievable in our research, the conclusions are quite different with regard to the effects of drug exposure on plasmid transfer. The conclusions from Showsh and Andrews suggest that tetracycline exposure during either the pre-growth or mating period increases conjugation frequency, while in the present study oxytetracycline exposure suppressed transfer

rates. The previous authors hypothesized that the enhanced conjugation was a direct effect on the donor strain, not antibiosis of the recipients.

In a paper by Torres *et al*[28], conjugal transfer rates were also shown to be enhanced in the presence of tetracycline for *E. faecalis.* Filter matings were performed at static concentrations (10 µg/mL) of tetracycline, as compared to the dynamic pharmacokinetics in liquid culture described here. Due to the static drug exposures in the Showsh *et al.* and Torres *et al.* and the dynamic drug exposures in the present study it is difficult to make direct comparisons of the results. In fact, it could be argued that in the studies by Showsh *et al.* and Torres *et al.* the oxytetracycline exposures were not true static exposures due to oxytetracycline degradation. Loftin *et al.*[29] demonstrated that static concentrations of oxytetracycline at 35°C – pH 7, degraded with a half-life of 19 hours. Stability estimates from the product monograph report a half-life of 26 hours under similar conditions[30]. This is in agreement with experiments in our laboratory that demonstrated an average half-life of 24 hours for 'static' concentrations (Lubbers, *et al.*, unpublished data). Failure to account for the actual drug exposure limits the conclusions that can be drawn from static concentration experiments. To the author's knowledge, there have been no studies investigating the effects of oxytetracycline on conjugative plasmid transfer with simulated *in vivo* antimicrobial exposures.

Previous static concentration experiments (Peterson and Narayanan, unpublished data) with the donor and recipient bacteria used here had shown that plasmid transfer was most efficient at concentrations up to 1000 ng/mL. The results presented here show that conjugation was suppressed when antimicrobial concentrations exceeded the inhibitory concentration of the recipient strain. In contrast to the previous cited research (Torres *et al,* Showsh *et al*), at no time points did oxytetracycline exposure enhance the conjugation rates. The discrepancy may be a

result of differences in either the laboratory conditions (filter vs. liquid culture) or the drug exposure profiles (static vs. dynamic).

The use of the transconjugant ratio has been described previously and was calculated using Equation 3 above[31]. Because the transconjugant ratio is a hybrid of two individual measures, both require examination to make inferences about the ratio. One limitation of this study was that the plating procedures were not normalized for sample volume, however the use of the transconjugant ratio rather than actual numbers of transconjugant bacteria accounts for this. However, the *Salmonella* population in the control replicates was numerically greater than in the treated replicates. The potential impact on the transconjugant ratio due to changes in the *Salmonella* population alone is approximately 0.5 log decrease for the oxytetracycline exposed populations. The decrease in the transconjugant ratio seen here was driven by relative fewer transconjugants formed in the treated populations, not by a comparative increase in the number of donor organisms (See Figures 4 and 5).

The results presented here show suppression of conjugative transfer in both oxytetracycline treated systems at 12 hours when compared to the respective controls ($p = 0.01$). Although not statistically significant, the transconjugant ratio in the low concentration regimens was numerically suppressed (compared to controls) at the 24 and 36 hour time points ($p = 0.057$ and 0.06, respectively). This suppression was not present at 24 or 36 hours in the high concentration regimen ($p = 0.24$ and 0.98, respectively). Taken together, transconjugant ratios were suppressed at all times when corresponding oxytetracycline concentrations were above the MIC of the recipient bacteria (Figure 3 and Table 1). Two observations merit discussion: the peak rate of plasmid transfer occurred early in the time course of the study and the development of transconjugant bacteria were suppressed by exposure to oxytetracycline. Both observations

can be related to effects of bacterial growth. If conjugation is a function of bacterial growth, then a change in bacterial growth due to either the inherent growth properties of the bacterial population (stationary / death phase) or the induction of bacterial stasis due to the presence of an antimicrobial, would suppress horizontal gene transfer as was seen in the present study for the treated bacterial populations.

It is inappropriate to use low power estimates to infer differences where statistical significance was not obtained. However, numerical differences within relatively low powered studies are legitimately used as an incentive for further studies with greater power. Using the difference in means and standard deviations in the low concentration regimens at 24 and 36 hours, 3 replicates of each treatment gives a study power of approximately 0.57 or a probability of > 0.4 for a false negative result. These findings provide preliminary insight into the relationship between drug exposure and the development of antimicrobial resistance due to horizontal gene transfer. Future studies are needed to investigate other antimicrobial-pathogen combinations and to validate these findings *in vivo.*

[A] BD, Franklin Lakes, NJ
[B] Corning Costar 96 well plates, Sigma Aldrich, St. Louis, MO
[C] SpectraMax 190, Molecular Devices, Sunnyvale, CA
[D] BD, Franklin Lakes, NJ
[E] Sigma Aldrich, St. Louis, MO
[F] Gallenkamp orbital incubator, Sanyo-Gallenkamp, Loughborough, Liecestershire, UK
[G] Spectronic 20D+, Thermo Scientific Corp., Waltham, MA
[H] Monoject 12 mL regular luer syringe, Kendall, Mansfield, MA
[I] Bellco Technologies, Vineland, NJ
[J] Thermo Fisher scientific, Pittsburgh, PA
[K] Thermo Fisher scientific, Pittsburgh, PA
[L] Fibercell systems, Fredrick, MD
[M] Masterflex L/S 13 Platinum cured silicon tubing, Cole Parmer, Vernon Hills, IL
[N] NuAire IR Autoflow, NuAire, Inc., Plymouth, MN
[O] IKA Big Squid, Sigma Aldrich, St. Louis, MO
[P] Doxycycline hyclate, Sigma-Aldrich, St. Louis, MO

[Q] O-Phosphoric acid, Fisher Scientific, Pittsburgh, PA
[R] HLB 10 mg, Waters Corp., Milford, MA
[S] Glacial Acetic acid, Fisher Scientific, Pittsburgh, PA
[T] Acetonitrile, Fisher Scientific, Pittsburgh, PA
[U] Shimadzu LC-20AD, Shimadzu Scientific – North America, Columbia, MO
[V] Sunfire C18, Waters Corp., Milford, MA
[W] Turbo-Ionspray Atomospheric Pressure Ionization Source, MDS Analytical Technologies, Concord, ON
[X] Sciex API 4000, MDS Analytical Technologies, Concord, ON
[Y] Microcon YM-10m, Millipore Corp., Bedford, MA
[Z] SAS 9.3.1, SAS Institute, Cary, NC

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Figures and Tables

Figure D.1 Schematic diagram of the in vitro pharmacodynamic model.

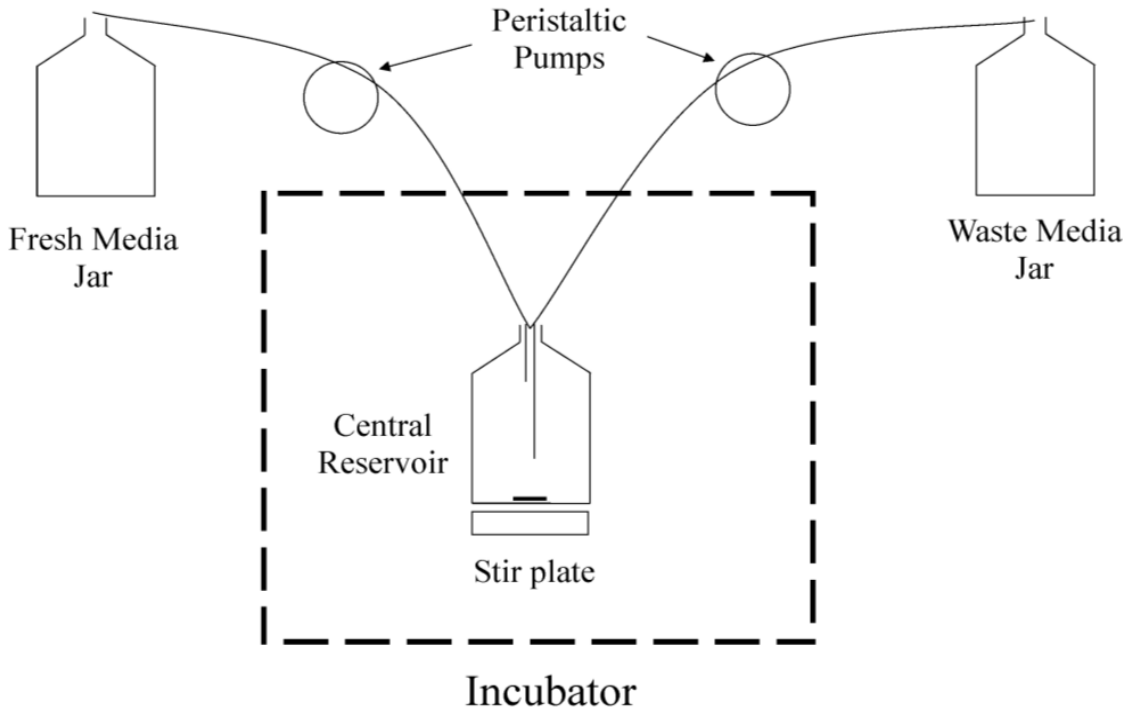


Figure D.2 Oxytetracycline protein binding in brain-heart infusion broth.

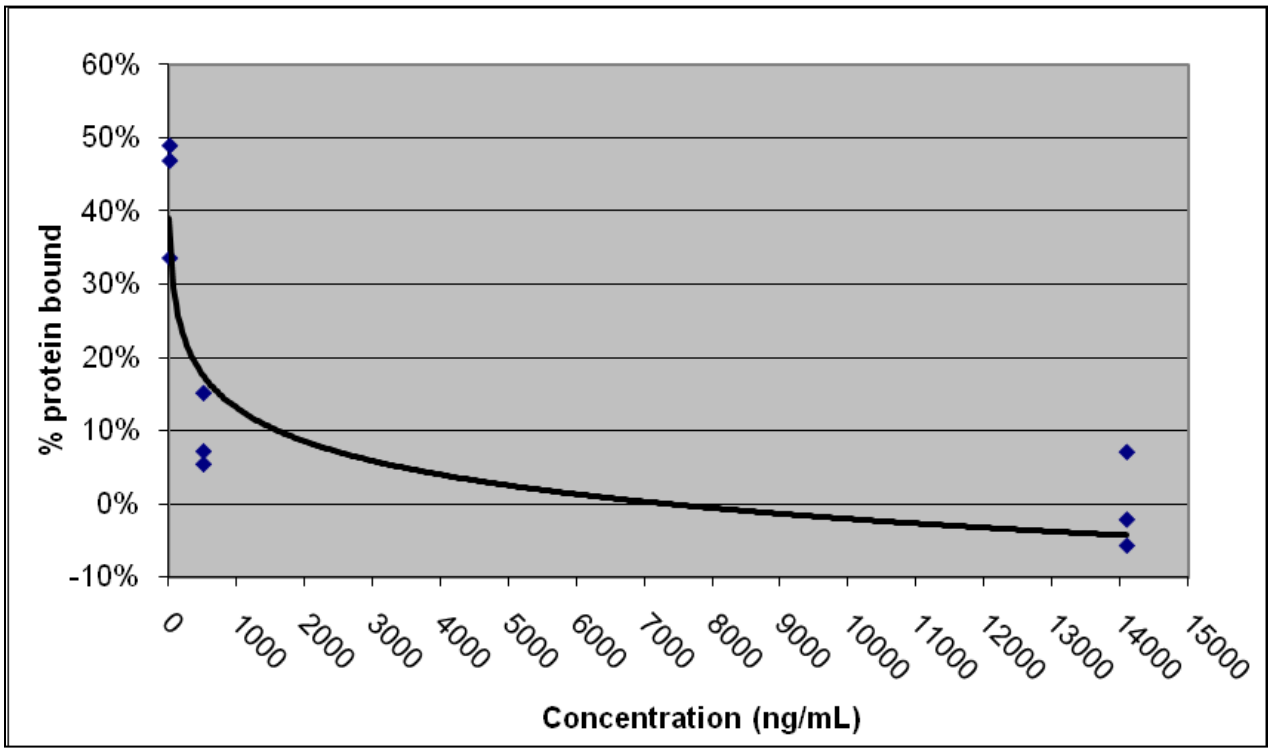


Figure D.3 Log transconjugant ratios for antimicrobial treated and control regimens over time.

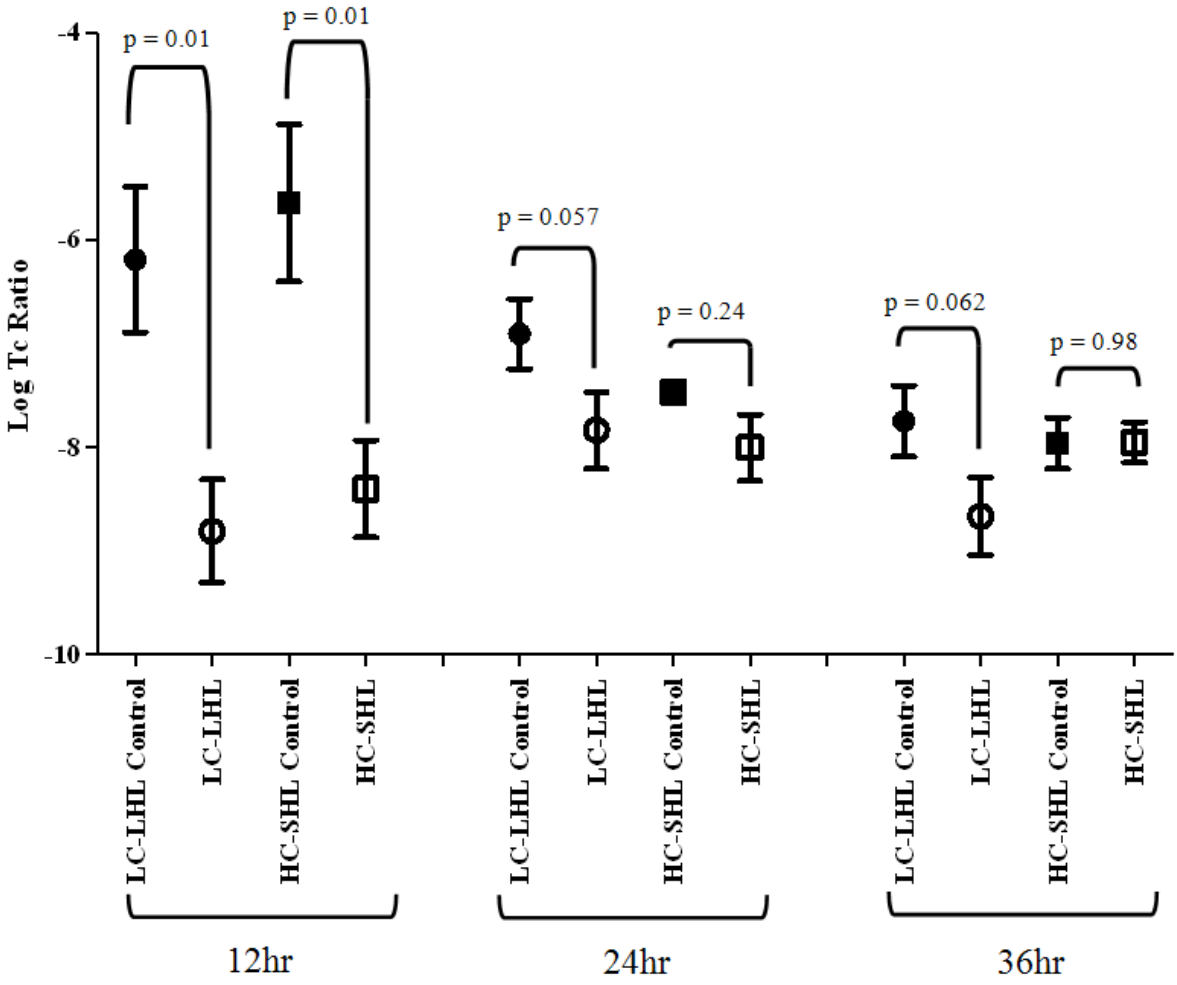


Figure D.4 Colony Forming Unit /mL counts for *Salmonella* [Donor Bacteria].

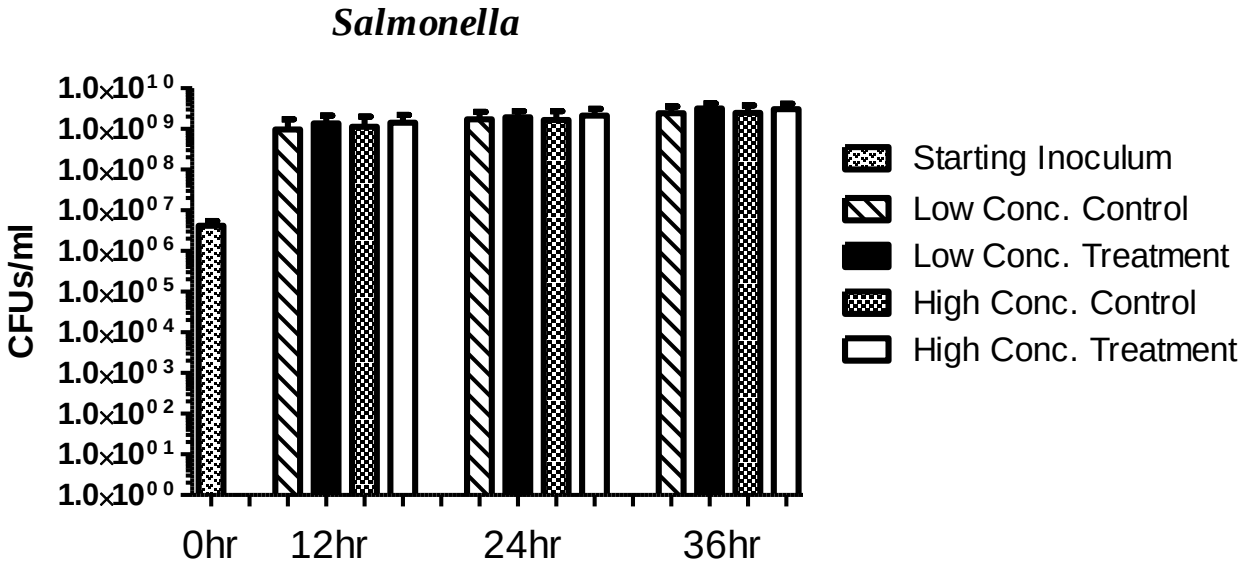
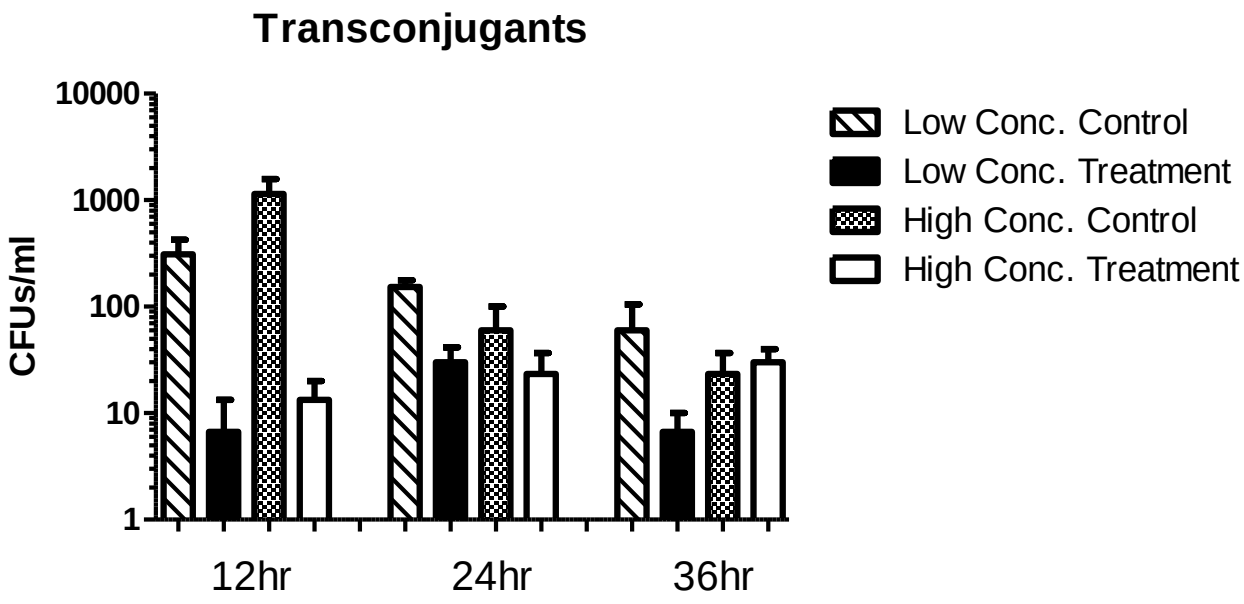


Figure D.5 Colony Forming Unit/mL counts for transconjugant bacteria.



Appendix E - Genetic variations in Shiga toxin-producing abilities of bovine and human *Escherichia coli* O157:H7

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Summary

Cattle are a primary reservoir of *Escherichia coli* O157:H7, a major foodborne pathogen. The organism causes hemorrhagic colitis which can lead to serious complications, including hemolytic-uremic syndrome. Although *E. coli* O157:H7 is widely prevalent in cattle and cattle environments, the number of human cases remain relatively low, suggesting possible strain diversity and differences in virulence between human and bovine strains. Shiga toxins, Stx1 and Stx2, are the major virulence factors. Differences in Stx2 production between human and bovine strains have been demonstrated previously, and isolates possessing the stx_2 gene, but not producing Stx2 (toxin non-producing [TNP] strains) have been identified. In this study, 150 isolates (56 human, 94 bovine) were tested by PCR for *stx2* upstream regions associated with TNP and the *Q933* gene, which has been previously associated with toxin production. A reverse passive latex agglutination test was used to evaluate 107 isolates (50 human, 57 bovine) for Stx1 and Stx2 production. The percentages of human and bovine isolates positive for presence of the TNP regions were similar (57.1% and 53.1%, respectively), while a higher percentage of human isolates was positive for *Q933* gene (89.3% vs. 54.3%). Stx2 production of ≥1:8 was found in

86.0% of human isolates compared to 26.3% of bovine isolates. Bovine isolates with the presence of the TNP regions were associated with significantly lower Stx2 production ($p < 0.05$), while the *Q933* gene was associated with higher Stx2 production ($p < 0.05$). However, the presence of the TNP region was not associated ($p > 0.05$) with low Stx2 production in human isolates. Therefore, *Q933* was a better indicator of high Stx2 production by human and bovine isolates and may be a useful screening method to assess their potential to cause human disease.

Introduction

Shiga-toxin producing (STEC) *Escherichia coli* O157:H7 remains an important cause of food-borne disease, resulting in an estimated 73,000 illnesses annually (Mead et al., 1999). *Escherichia coli* O157:H7 infections in humans cause hemorrhagic colitis which can lead to serious complications, including hemolytic-uremic syndrome (HUS). Cattle are asymptomatic reservoirs for *E. coli* O157:H7 (Renter and Sargeant, 2002; Gyles, 2007; Hussain, 2007) and either direct or indirect contamination of food products by cattle feces has been implicated in numerous outbreaks (Mead and Griffin, 1998; Rangel et al., 2005; Hussain, 2007). Although *E. coli* O157:H7 is frequently isolated from cattle, with reported prevalence estimates from 0 to 41.5% (Lejeune et al., 2004; Callaway et al., 2006; Gyles, 2007), the overall number of human infections remains relatively low despite the low infectious dose of the organism (Tuttle et al., 1999; Strachan et al., 2001). The apparent discrepancy between high prevalence in cattle and their environment and the relative rarity of human infections suggest possible strain diversity and differences in virulence between human and bovine strains (Boerlin et al., 1999; Baker et al., 2007).

Studies have indicated that bovine and human *E. coli* O157:H7 strains come from separate lineages with the bovine lineage less likely to cause disease or spread effectively from cattle to humans (Kim et al., 1999; Dowd and Williams, 2008). Differences among genotypes of Shiga toxin-encoding bacteriophage insertion sites found in bovine and human isolates also exist (Besser et al., 2007). Among several virulence factors, Shiga toxins 1 (Stx1) and 2 (Stx2), which are cytotoxic to vascular endothelial cells, are crucial to infections in humans (Besser et al., 2007; Gyles 2007); *stx*$_2$ positive O157:H7 isolates are five times more likely to be associated with severe disease than negative isolates (Boerlin et al., 1999). Similarly, Stx2 is about 1,000 times more toxic to microvascular endothelial cells of human kidneys than Stx1 (Gyles, 2007). *Escherichia coli* O157:H7 isolates of bovine origin generally appear to produce lower amounts of toxin than isolates of human origin (Richie et al., 2003; Baker et al., 2007).

Several studies have suggested possible factors affecting *stx*$_2$ gene expression resulting in altered Stx2 production. A study by Koitabashi et al. (2006) evaluated *E. coli* O157 strains, primarily from non-clinical sources that carried the *stx*$_2$ gene, but produced little or no Stx2 due to a nonfunctional promoter sequence in the *stx* region. A PCR assay (TNP-PCR) was developed to detect these toxin non-producing (TNP) strains, and subsequently it was determined that the lack of toxin production was attributable to both a nonfunctional *stx*$_2$ promoter as well as a weak antitermination activity of the Q protein (Koitabashi et al., 2006). A similar study utilizing *E. coli* O157:H7 isolates from human clinical cases and asymptomatic individuals found that strains producing low or no Stx2 were most often associated with asymptomatic carriers and regulation of toxin production appeared to be influenced by both a mutation in the promoter region of the *stx*$_2$ gene, as previously reported (Koitabashi et al., 2006), and absence of the *Q933* gene (Matsumoto et al., 2008). The *Q933* gene was associated with higher levels of Stx2

production and was more often identified in isolates from clinically ill humans than from healthy cattle (Lejeune et al., 2004). Although these studies have looked at genetic differences among STEC strains and resulting variations in virulence, further investigation into alterations in distribution or regulation of the *stx* genes is necessary (Pradel et al., 2001; Besser et al., 2007).

The objectives of this study were to 1) assess the frequency of occurrence of toxin non-producing strains (Koitabashi et al., 2006) and *Q933* genes among human clinical and bovine isolates, and 2) assess the amount of Stx2 produced for isolates with different genetic profiles and from different sources.

Materials and Methods

A total of 150 *E. coli* O157:H7 isolates were used: Fifty-six isolates were from human clinical cases reported between May 2002 and November 2004 in Kansas and 94 isolates were from cattle (91 fecal, 2 environmental, 1 carcass) collected between May 2001 and June 2007 from Kansas and Nebraska. Human isolates were provided by the Kansas Department of Health and Environment, while bovine isolates were selected from an isolate bank of over 2,000 bovine isolates. In addition, for bovine isolates, pulsed-field gel electrophoresis (PFGE) analyses were available for 58 isolates and all had different PFGE patterns (Sargeant et al., 2006). For bovine isolates without available PFGE patterns, no isolates collected on the same day from the same or adjacent pens were used. Presence or absence of stx_1 and stx_2, *Q933* and *Q21* genes was determined by PCR according to previously published methods (Fagan et al., 1999; Lejeune et al., 2004). Primers for all reactions are listed in Table 1.

TNP-PCR

Isolates stored in protect beads were cultured on blood agar plates and DNA extracted by boiling cells in distilled water from a single colony. The toxin non-producing PCR (TNP-PCR) procedure described by Koitabashi et al. (2006) was performed on all human and bovine isolates. Briefly, four separate PCR reactions (TNP-A, TNP-B, TNP-C and TNP-D) were carried out with seven primers (Biosynthesis Inc., Lewisville, TX) listed in Table 1. The reaction mastermix contained 0.25 µM of both forward and reverse primers, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (all Promega, Madison, WI) as well as 1 µL sample DNA for a total volume of 20 µL. The four PCR reactions yielded amplicons of 458 bp, 694 bp, 268 bp, and 549 bp, respectively. An isolate was considered TNP-PCR positive if expected amplicons were observed in all four reactions (Koitabashi et al. 2006).

In order to simplify detection of the region from the *q* gene to the *stx*$_2$ gene previously targeted by the TNP-PCR procedure, a new PCR procedure (NM-PCR) was developed utilizing a new set of primers (NMf and NMr, Table 1). Using Vector NTI Suite 8.0 (Invitrogen, Carlsbad, CA) and BLAST (NCBI, Bethesda, MD) software, a 604 base pair region of the approximately 1,650 bp TNP-PCR target was identified and confirmed as highly conserved among O157:H7 strains. Mastermix, as previously listed, with 1 µL (0.5 µM) of each forward and reverse primer was used for a total reaction volume of 20 µL. A Takara Thermal Cycler (Takara Bio Inc., Takara, Japan) was used for all PCR reactions with initial denaturation at 96ºC for 5 min, 30 amplification cycles with conditions dependent on primers used (Table 1) and a final extension round for 7 min at 72ºC. All PCR products were separated by gel electrophoresis using 1.5% agarose gel in 1X Tris-Borate-EDTA (TBE; Sigma-Aldrich, St. Louis, MO) buffer and stained with ethidium bromide to allow visual confirmation of amplicons by ultraviolet transillumination.

Stx1 and Stx2 Assays

Fifty human isolates and 57 bovine isolates where tested for Stx1 and Stx2 production using a commercially available reverse passive latex agglutination kit (Oxoid VTEC-RPLA Toxin Detection Kit, Remel, Lenexa, KS) according to manufacturer's instructions. In order to minimize the possibility of testing clonal isolates, multiple human samples submitted from the same county within thirty days and with the same gene profile (*stx*$_1$ and *stx*$_2$, *Q933* and *Q21*) were excluded from the toxin assay. Briefly, isolates were cultured overnight on brain-heart infusion agar at 37°C. Colonies were then suspended in 1 mL 0.85% sodium chloride solution containing 5,000 U of polymixin B. The suspensions were adjusted to an absorbance of 1.5 at 600 nm wavelength and incubated for 30 minutes, shaking periodically. Samples were then centrifuged for 20 min at 4,000 rpm. A serial two-fold dilution of a 25 µL aliquot of the supernatant was performed using a 96-well V-bottom microtitre plate and test reagents were added according to manufacturer's directions. Results were read after 24 hours. The toxin titer was defined as the reciprocal of the highest dilution of the test sample that produced an agglutination reaction. Toxin titers $\leq$ 1:2 were considered negative.

Statistical Analysis

Data analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC). For all proportions, binomial exact 95% confidence intervals (CI) were calculated. Agreement between PCR methods for toxin non-production (TNP-PCR and NM-PCR) was measured using a kappa statistic (Dohoo et al., 2003). Variables assessed for potential association with Stx1 production included presence of the *stx*$_2$ gene and the source of isolate (human or bovine), while

variables of interest for Stx2 production included presence of the *stx*$_1$ gene, source of isolate, TNP-PCR and/or NM-PCR results, and presence of *Q933* gene. Separate multivariable cumulative logistic regression models using generalized estimating equations (Proc Genmod, SAS v9.1), with the Stx1 or Stx2 titers as ordinal dependent variables, were built using a series of previously described model building steps (Dohoo et al., 2003). An initial screening step was performed to evaluate unconditional associations between outcomes and potential variables of interest. Each variable associated with an outcome ($P < 0.2$) was then added into a multivariable model, and then removed using backwards selection until all variables were significant ($P < 0.05$). First order interactions between main effects were subsequently entered into the model and removed by backwards selection until all remaining variables and interactions were significant ($P < 0.05$). Two-sided significance testing was used for hypothesis testing.

Results

Proportions of *stx*$_1$, *stx*$_2$, and *Q933* in the human and bovine isolates used in this study are shown in Table 2. Of the 56 human isolates used, 1.8% (0.1-9.6%) were positive for *stx*$_1$ gene only, 58.9% (45.0-71.9%) were positive for only *stx*$_2$ gene and 39.3% (26.5-53.2%) were positive for both genes. For the 94 bovine isolates analyzed, 6.4% (2.4-13.4%) were positive for *stx*$_1$ gene only, 44.7% (34.4-55.3%) were positive for only *stx*$_2$ gene and 48.9% (38.5-59.5%) were positive for both genes. The *Q933* allele was found in 87.5% (75.9-94.8%) of human isolates compared to 54.3% (43.7-64.6%) of bovine isolates (Table 2).

Occurrence of TNP-PCR positives was similar for both human (57.1%; 43.2-70.3%) and bovine isolates (51.1%; 40.5-61.5%). Also, results were similar across both human (57.1%; 43.2-70.3%) and bovine isolates (52.1%; 41.6-62.5%; Table 2) for presence of NM-PCR positive

region. The kappa value for the previously published TNP-PCR method and the new NM-PCR procedure was 0.936. Overall, results for the two PCR methods were consistent, with discrepancies only for three bovine isolates out of 94 tested. Of the three isolates, two were positive for NM-PCR and negative for TNP-PCR and the third isolate was negative for NM-PCR and positive for TNP-PCR. The three isolates were from different pens from two feedlots and were genetically dissimilar (< 95% Dice similarity) according to PFGE analyses.

The Stx1 and Stx2 toxin assay results for human and bovine isolates are shown in Tables 3 and 4, respectively. Based on previously listed criteria, six human isolates were not tested for toxin production. Of the human isolates tested, 18 (36.0%; 22.9-50.8%) had Stx1 toxin titer > 1:2 (mode 1:128, range 1:16 to 1:128) while 44 (88.0%; 75.7-95.5%) had Stx2 toxin titer ≥ 1:4 (mode 1:32, range 1:4 to 1:128). For the bovine isolates tested, 34 (59.7%; 45.8-72.4) isolates were positive for Stx1 toxin titer > 1:2 (mode 1:64, range 1:4 to 1:128) while only 15 (26.3%; 15.5-39.7%) isolates had Stx2 toxin titer ≥ 1:4 (mode 1:16, range 1:8 to 1:64). Overall, 36.0% (22.9-50.8%) of human isolates produced Stx1 toxin of ≥ 1:8 compared to 54.4% (40.7-67.6%) of bovine isolates. However, 86.0% (73.3-94.2%) of human isolates produced Stx2 toxin of ≥ 1:8 compared to only 26.3% (15.5-39.7%) of bovine isolates. Only one bovine isolate was positive for Stx2 at 1:64 titer and none at 1:128, while 10 human isolates were positive at 1:64 and 6 were positive at 1:128.

For high Stx2 producing isolates (titer ≥ 1:8), 24 human isolates were TNP-PCR and NM-PCR positive (55.8%; 39.9-70.9%) while 4 bovine isolates were positive by TNP-PCR and NM-PCR (26.7%; 7.8-55.1%). Titers for Stx2 ranged from 1:8 to 1:128 (mode 1:32) for human isolates while toxin titers were1:8 for two and 1:16 for the remaining bovine isolates. Of the three isolates with disparate TNP-PCR and NM-PCR results, TNP-PCR incorrectly identified

one bovine isolate as toxin non-producing (Stx2 = 1:8) while NM-PCR incorrectly identified one bovine isolate as toxin non-producing (Stx2 = 1:8) and another isolate lacking the *stx*$_2$ gene as toxin non-producing positive.

Of the *Q933* positive isolates tested for toxin production, only 15.9% (7.9-27.3%) had Stx2 titers ≤ 1:2 while 82.5% (70.9-90.9%) produced Stx2 toxin at levels ≥ 1:8. Of the *Q933* positive isolates producing low levels of toxin (titer < 1:8), 8 were bovine and 2 were human isolates. In contrast, 12 bovine isolates and 40 human isolates were positive for the *Q933* gene and produced high amounts (titer ≥ 1:8) of Stx2 toxin (Table 3 and Table 4). Of 6 human isolates that had Stx2 toxin titers of 1:128, 5 (83.3%; 35.9-99.6%) were *Q933* positive.

In regard to Stx1 production, the only variable significantly associated with altered toxin production was presence of the *stx*$_2$ gene which resulted in significantly ($P < 0.01$) lower Stx1 titers. In the initial unconditional models for Stx2 toxin, all variables were significantly associated with production. In the multivariable model, presence of the *stx*$_1$ gene ($P < 0.05$), isolates from bovine sources ($P < 0.01$), and isolates positive for NM-PCR ($P < 0.05$) were associated with significantly lower Stx2 toxin production while presence of the *Q933* gene ($P < 0.01$) was positively associated with Stx2 toxin production. No interaction terms were significant

Discussion

A number of isolates from both human and bovine sources possess a *stx*$_2$ gene, but produce low or undetectable levels of toxin (Zhang et al., 2005; Koitabashi et al., 2006; Dowd et al., 2008; Matsumoto et al., 2008). Whether this decreased toxin production is due to differences in transcriptional or translational regulations has not been established (Besser et al., 2007). Two

primary lineages of *E. coli* O157:H7 have been reported, of which lineage I strains produce more Stx2 than lineage II strains and are more often associated with human disease (Dowd et al., 2008). Differences in conserved genomic regions encoding suspected virulence factors have been hypothesized, including a possible hemolysin activation protein and a suspected iron transport system in lineage I strains (Kim et al., 1999; Steel et al., 2007). However, recent work indicates that differences in virulence between the lineages may result from absence or incomplete functioning of late-regulatory machinery needed for Stx2 production in lineage II isolates (Dowd et al., 2008).

In an earlier study by Koitabashi et al. (2006), 41 *E. coli* O157 isolates, primarily from cattle sources were evaluated for presence of the stx_2 gene with little or no Stx2 production. In these toxin non-producing (TNP) strains, the *Q* gene appeared highly homologous to the Φ21 phage, but not that of the 933 phage, leading to poor antiterminator activity of Q protein which is essential for strong transcription. Therefore, the lack of toxin production by these TNP strains was attributed to both a nonfunctional stx_2 promoter as well as weak antitermination activity of the Q protein (Koitabashi et al., 2006). The low toxin and toxin-non producing isolates have also been identified by a study using 68 human *E. coli* O157:H7 strains (Lejeune et al., 2004). Our study examined toxin production for both human and bovine isolates. In addition, new primers were designed (NM-PCR) to identify toxin non-producing strains. Overall, TNP-PCR and NM-PCR performed equally well, with identical results for all human isolates, but with differing results for just 3 out of 94 bovine isolates. There was a high level of agreement between TNP-PCR and NM-PCR methods, and the NM-PCR procedure was equally accurate in identifying toxin non-producing strains. Therefore, a similarly performing NM-PCR assay requiring a single

PCR reaction was definitely advantageous compared to using four separate PCR reactions required for TNP-PCR assay.

A recent study by Matsumoto et al. (2008) using *E. coli* O157:H7 isolates from human clinical cases and asymptomatic carriers supported previous findings that stx_2 gene expression in isolates producing low or no measurable Stx2 was caused by mutation in the stx_2 promoter region. Substitution of the *Q21* gene in place of the *Q933* gene also seemed to affect Stx2 toxin production, although isolates producing high levels of toxin with only the *Q21* gene present were reported. Of the 56 high toxin producing strains identified, 96.4% carried the *Q933* gene which was similar to the *Q933* gene frequency of 87.5% among human isolates in our study and the study by LeJeune *et al*. (90%). In addition to presence of the *Q21* gene, all low or no toxin producing strains possessed the $stx_{2vh\text{-}a}$ gene variant (Matsumoto et al., 2008). While differences in frequency of stx_2 gene variants have been previously reported among isolates from human and animal origin (Beutin et al., 2007), further work is needed to determine whether differences in Stx2 toxin production exist among all stx_2 gene variants.

For the bovine isolates used in this study, presence of the TNP region, as identified by NM-PCR, was associated with lower toxin production as previously reported (Koitabashi et al., 2006). Due to the high level of agreement between TNP- and NM-PCR methods, one would expect TNP-PCR to also be associated with decreased Stx2 production. However, this was not demonstrated; possibly because of inadequate sample size. Although interactions between source of isolate (bovine/human) and NM-PCR result were not statistically significant in regards to Stx2 production, the relationship between positive TNP- or NM-PCR results appeared to be a better predictor of decreased or no production of Stx2 toxin for bovine isolates than for human isolates. For the human isolates tested by both PCR methods (TNP and NM), 48.0% (33.7-

62.6%) were misclassified for low Stx2 toxin production (toxin titer actually ≥ 1:8) compared to 7.0% (1.9-17.0%) of bovine isolates. Screening of additional isolates for the targeted sequence is needed to further elucidate differences in the ability to predict toxin production between human and bovine isolates. In contrast to other studies, a marked difference in Stx1 toxin production between bovine and human isolates was not observed (Boerlin et al., 1999).

In this study, as previously reported, presence of the *Q933* gene appeared to be associated with higher levels of Stx2 production (Lejeune et al., 2004; Ahmad and Zurek, 2006) and was found more often in isolates from clinically ill humans than from healthy cattle (Lejeune et al., 2004). Although it has been hypothesized that variations in the frequency of *Q933* in bovine *E.coli* O157:H7 strains exist among countries and regions, the occurrence of *Q933* for both human and bovine isolates was similar to previous findings in isolates from the United States, Scotland, Australia, and Japan (Lejeune et al., 2004; Matsumoto et al., 2008).

A limitation of this study was that isolates with neither stx_1 nor stx_2 were not available for comparisons. In order to minimize the possibility of testing clonal isolates, some human isolates were not tested for Shiga toxin production. Because bovine isolates were chosen non-randomly to decrease the likelihood of testing clonal strains, it is possible that the differences between the frequency of gene occurrence or virulence of isolates from human and bovine sources were due to selection bias rather than any physiological variation. However, our finding that presence of the *Q933* gene was associated with higher levels of Stx2 production agree with the study of Lejeune et al., (2004). Additionally, details regarding further testing of clinical samples were not available due to confidentiality concerns; so for seven human isolates with Stx2 titer < 1:8, we were unable to determine if they were the sole pathogen recovered or if additional pathogens were involved.

Overall, bovine isolates produced lower amounts of Stx2 than human clinical isolates. These findings are consistent with several previous studies which have observed higher pathogenicity in isolates from human clinical cases than those from clinically healthy cattle (Baker et al., 2007). Our findings support the suggestion that only a subset of *E. coli* O157:H7 strains from cattle have the potential to cause human clinical disease (Kim et al., 1999; Baker et al., 2007; Besser et al., 2007). In our study, presence of the toxin non-producing region, as detected by the NM-PCR method, was associated with decreased Stx2 toxin production while presence of the *Q933* gene was associated with increased Stx2 toxin production. Our data indicate that while both methods were acceptable predictors of toxin production for bovine isolates, presence of the *Q933* gene appeared to be a better predictor of Stx2 production for human isolates. However, further work is needed to clarify underlying regulatory mechanisms for Stx2 toxin production and sites at which the toxin non-producing region and the *Q933* gene may alter expression of the stx_2 gene in both human and bovine isolates.

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Tables

Table E.1 Primer sequences and running conditions for toxin non-producing (TNP) and new method (NM), *Q933*, *stx*$_1$ and *stx*$_2$ PCR reactions.

PCR assay	Primer sequence	Denaturation temp.	Annealing temp.	References
TNP-A	F 5'CCATGAGCAAATGATGATTG3' R 5'TTTAGTTCTCTTATGCCCAC3'	94° C	55° C	Koitabashi et al., 2007
TNP –B	F 5'CTAAATTCATGGAGAGCGTG3' R 5'TTAACGTCAGGCACAAAGAG3'	94° C	55° C	Koitabashi et al., 2007
TNP –C	F 5'AACCGGAAACGTGTAGAG3' R 5'TTAACGTCAGGCACAAAGAG3'	94° C	55° C	Koitabashi et al., 2007
TNP-D	F 5'GAACATATCAAAATCAGGC3' R 5'GGGAATAGGATACCGAAG3'	94° C	55° C	Koitabashi et al., 2007
NM	F 5'CGCATGGGTTTATTCAGGTC3' R 5'GTTGCTCATTTGCTCAACGA3'	94° C	55° C	This study
Q933	F' CGGAGGGGATTGTTGAAGGC3' R' CCGAAGAAAAACCCAGTAACAG3'	94° C	52° C	Lejeune et al., 2004
stx$_1$	F 5'ACACTGGATGATCTCAGTGG3' R 5'CTGAATCCCCCTCCATTATG3'	95° C	58° C	Fagan et al., 1999
stx$_2$	F 5'CCATGACAACGGACAGCAGTT3' R 5'CCTGTCAACTGAGCAGCACTTTG3'	95° C	58° C	Fagan et al., 1999

Table E.2 Proportions of *Q933* gene and toxin non-producing region determined by toxin non producing (TNP) and new method (NM) PCR in bovine and human isolates of *Escherichia coli* O157:H7

Source	Number of isolates	*Q933* positive	Toxin non-producing region	
			TNP-PCR positive	NM-PCR positive
Bovine				
stx_1^+/stx_2^+	46	34	16	16
stx_1^+/stx_2^-	6	1	0	1
stx_1^-/stx_2^+	42	16	32	32
Total isolates	94	51	48	49
Human				
stx_1^+/stx_2^+	22	20	5	5
stx_1^+/stx_2^-	1	1	0	0
stx_1^-/stx_2^+	33	28	27	27
Total isolates	56	49	32	32
Total bovine and human isolates	150	100	80	81

Table E.3 Shiga toxin 1 production by human and bovine isolates in relation to toxin non-producing (TNP) region or *Q933* gene.

Toxin titer	Number of isolates tested	*stx*$_1$ positive	*Q933* positive	TNP-PCR positive	NM-PCR positive
Human					
≤2	32	1	27	25	25
16	1	1	1	0	0
32	3	3	2	1	1
64	4	4	4	1	1
128	10	10	9	3	3
Total isolates	50	19	43	30	30
Bovine					
≤2	23	0	4	19	20
4	3	3	1	0	0
8	4	4	2	2	0
16	5	4	3	2	2
32	7	7	4	4	5
64	9	9	6	5	6
128	6	6	0	2	2
Total isolates	57	33	20	34	35

Table E.4 Shiga toxin 2 production by human and bovine isolates in relation to toxin non-producing (TNP) region or *Q933* gene.

Toxin titer	Number isolates tested	*stx*$_2$ positive	*Q933* positive	TNP-PCR positive	NM-PCR positive
Human					
≤ 2	6	6	2	5	5
4	1	1	1	1	1
8	3	3	3	2	2
16	9	9	9	4	4
32	15	15	13	10	10
64	10	10	10	6	6
128	6	6	5	2	2
Total isolates	50	50	43	30	30
Bovine					
≤ 2	42	36	8	30	31
8	4	4	2	2	2
16	9	9	8	2	2
32	1	1	1	0	0
64	1	1	1	0	0
Total isolates	57	51	20	34	35

Impacts

- Human clinical strains of *E. coli* O157:H7 produced more Shiga toxin 2 (Stx2) than strains isolated from cattle.
- The percentages of human and cattle isolates positive for the nonfunctional promoter region for Stx2 were similar, while a higher percentage of human isolates was positive for *Q933* gene, which encodes for a protein with weak antitermination activity.
- Presence of *Q933* was a better indicator of high Stx2 production by *E. coli* O157:H7 than the nonfunctional promoter region and may be a useful screening method to assess potential of cattle strains to cause human disease.

Appendix F - Microarray Protocol

Microarray Labeling Protocol (In the Dark)

1. Extract genomic DNA and Nanodrop. The readings should be between 1.7 and 2.0 for both 260/280 and 260/230 readings.
2. Start with between 1.0 and 1.5ug of genomic DNA, and use reagents from Invitrogen BioPrime Plus Array CGH Genomic Lableing System.
3. To an amber tube in the dark add:

   Alexa Flour 555 or 647 Panomer : 20ul

   Genomic DNA approx 1.5ug : X ul

   Sterile water : q.s. to 42.5ul

4. Incubate at 95degC for 10 minutes and cool on ice for 5 minutes.
5. On ice, add:

   10X Nucleotide Mix 555 or 647: 5ul
   Cy3 or Cy5 dCTP (Amersham) : 1.5ul *Optional*
   Exo-Klenow Fragment : 1ul

6. Mix gently and centrifuge.
7. Put into sealed film canister and incubate for 2 hours in the dark.
8. Optional: add 5ul of Stop Buffer (0.5M EDTA), or, if necessary, store overnight in -20degC.
9. Generally, before you start fragment purification, you can start slide preps (see below).
10. Purify labeled fragments by using provided columns. Can also use Qiagen PCR purification column.
11. For Invitrogen kit, add 200ul of Binding Buffer B2 to fragments.
12. Add to spin column and centrifuge for 1 minute at 10K x g.
13. Discard flow through.
14. Add 650ul of Wash Buffer W1 with EtOH added.
15. Centrifuge at 10K x g for 1 minute and discard flow through.
16. Spin again with caps open for 3 minutes at 12K x g to dry membrane.
17. Add column to a new amber tube.
18. Add 15ul of Elution Buffer E1 and incubate for 1 minute at RT.
19. Centrifuge at 11K x g for 2 minutes.
20. Repeat elution steps 17-18.
21. Nanodrop with “microarray” feature to determine dye incorporation efficiency.

Slide Preparation

1. After printing, store slides in dark, and keep dry.
2. When ready to use, crosslink at 600mJ in UV crosslinker.
3. Prehyb slides in blocking solution (0.1% BSA, 5X SSC, 1% SDS) at 42^{o}C for one hour with shaking.
4. Spin dry slide either on slide spinner or in 50ml tube in the centrifuge at approx 4000 RPM.
5. Add elevated coverslips over oligo fields.
6. Slides are now ready for hybridization mix.

Preparation of Hybridization Mix (In the Dark)

1. After fragments are purified and nanodropped, dry down the volume to 14ul in a speedvac. Adjust with H2O as necessary.
2. Add 15ul of 2X Hybridization Mix, and 1ul of appropriate 25mer.
3. Heat at 80degC for 5 minutes and then cool on ice.
4. Spin down to get condensation off the sides of the tubes.
5. Add directly to slides so that it travels under the coverslip.
6. Hybridize in chambers overnight at 42degC in the dark.

Slide Washing (In the Dark)

1. Remove slides from hybridization chambers, and without disturbing coverslips transfer them to slide wash container with first wash.
2. Agitate slightly under Wash 1 solution until coverslips float off.
3. Wash 10 minutes in 10X SSC + 0.2% Sarkosyl.
4. Wash 10 minutes in 10X SSC.
5. Wash 10 minutes in 0.2X SSC.
6. Quickly dip in distilled H2O and spin dry.
7. Read on slide reader.

Appendix G - RNA Extraction Protocol

RNA Extraction Protocol

1. Centrifuge tubes at 5k x g for 10min
2. Pour off RNA later
3. Add 1mL of TRIzol and vortex
4. Incubate at RT for 5min
5. Add 200uL of Chloroform and mix
6. Centrifuge at 12k x g for 15min at 4degC
7. Remove aqueous phase and add to a new tube
8. Add 500uL of isopropyl alcohol
9. Incubate samples at RT for 10min
10. Centrifuge at 12k x g for 10min at 4degC
11. Remove supernatant
12. Wash with 1mL of 75% EtOH
13. Vortex and centrifuge at 7.5k x g for 5min at 4degC
14. Remove EtOH and allow samples to dry
15. Resuspend in 50uL of RNase-Free H2O

Appendix H - Southern Blot Protocol

Southern Blot Protocol

1. Depurination:

   Pour 0.25M HCl onto the gel surface and leave for 20 minutes. Check every few minutes that the gel surface is still covered, adding more 0.25M HCl with a transfer pipette if necessary. At the end of 20 minutes, remove the HCl with a transfer pipette.

2. Denaturation:

   1.5M NaCl 87.66g

   0.5M NaOH 20.00g

   In 1L Milli-Q H2O.

   Increase vacuum to 50cm H2O. Pour onto gel and denature for 20 minutes. Remove the solution with a transfer pipette

3. Neutralization:

   1.0M Tris (base) 121.10g

   2.0M NaCl 116.88g

   In 1L Milli-Q H2O.

   Adjust to pH 5.0

1. Pour onto gel and neutralize for 20 minutes. Remove the solution with a transfer pipette.
2. Transfer DNA as per standard method using 20X SSC for transfer buffer
3. Crosslink DNA to nitrocellulose by using the quick hyb feature on crosslinker
4. Store in 20X SSC at 4degC until used
5. DIG label DNA by adding 1ug to 16uL total volume of H2O
6. Denature DNA by heating at 95degC for 10min and cooling on ice
7. Add 4uL of DIG-High Prime (vial 1) to DNA mixture
8. Incubate o/n at 37degC

9. Stop reaction in morning by adding 2uL of 0.2M EDTA
10. Prepare pre-hyb solution by adding 128mL (in two 64mL quantities) to DIG Easy Hyb Granules (bottle 7)
11. Dissolve by stirring immediately for 5min at 37degC
12. Pre-heat DIG Easy Hyb solution to 42degC
13. Add 13uL of DIG-labeled probe to a separate tube and denature by heating at 95degC for 5min and then cool on ice
14. Add the 13uL to 25mL of DIG Easy Hyb solution that has been pre-heated
15. Pour off pre-hyb solution and add probe mixture
16. Incubate 4hrs to o/n at 47degC (for blaCMY2)
17. After incubation, wash 2 x 5min in 2xSSC, 0.1% SDS at 15-25degC
18. Wash 2 x 15min in 0.5x SSC, 0.1% SDS that was warmed to 65-68degC
19. Membrane is now ready for antibody detection
20. Make the following solutions:

1. **Maleic Acid** 0.1M, 0.15M NaCl
   Add 11.6g of Maleic acid and 8.7g of NaCl to 700mL of H2O
   Adjust pH to 7.5 with NaOH (this will take a lot of NaOH)
   QS the final volume to 1L
2. **Wash Buffer:** 0.1M Maleic acid, 0.15M NaCl, and 0.3% Tween 20
   Take 500mL of Maleic acid solution (pH 7.5) and add 1.5mL of Tween 20
3. **Detection Buffer:** 0.1M Tris, 0.1M NaCl
   Add 6.05g Tris and 2.9g of NaCl to 500mL of H2O
   Adjust pH to 9.5
4. **Blocking Solution:** Add 225mL of Maleic Acid to 25mL of Blocking Solution (vial 6)
5. **Antibody Solution:** Add 20mL of blocking solution to 4uL of anti DIG antibody (vial 4)
6. **Color Substrate Solution:** Add 200uL of NBT/BCIP (vial 5) to 10mL of Detection Buffer
7. **TE Buffer:** 10mM Tris-HCl, 1mM EDTA pH 8.0

21. After hybridization and stringency washes, rinse the membrane briefly (1-5 min) in Wash Buffer
22. Incubate for 30min in 100mL of Blocking Buffer
23. Incubate for 30min in 20mL of Antibody Solution
24. Wash 2 x 15min in 100mL of Washing Buffer
25. Equilibrate 2-5min in 20mL of Detection Buffer
26. Incubate in 10mL of Color Substrate Solution in the dark for up to 16hrs
27. To stop the reaction, add H2O or TE