ABSTRACT

Title of Dissertation: ANTIMICROBIAL RESISTANCE OF *LISTERIA MONOCYTOGENES* AND *ENTEROCOCCUS FAECIUM* FROM FOOD AND ANIMAL SOURCES

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The widespread use of antimicrobials in human and veterinary medicine, as well as in animal production has accelerated the development of drug resistance in a variety of pathogenic bacteria. *Listeria monocytogenes* and *Entercoccus faecium* are important Gram-positive pathogens of food safety and public health concern. But their mechanisms of antimicrobial resistance are relatively less clear than those in Gram-negative pathogens.

L. monocytogenes (n=167) recovered from deli meats, conventional and organic chicken, and conventional and organic fresh produce samples were characterized by serogrouping, DNA fingerprinting and antimicrobial susceptibility testing. The isolates belonged to five different serogroups. Percentages of resistance to ciprofloxacin, tetracycline, sulfonamide, and nalidixic acid were 1.8%, 9%, 73%, and 100%, respectively. The identification of potential serotype 4b from all food categories, especially from organic chicken products, raised a public health concern, because serotype 4b has been the number one serotype associated with clinical isolates.

Multiresistant *L. monocytogenes* strains were recovered from the food supply, including organic food products, suggesting our food supply may serve as the reservoir for multiresistant *L. monocytogenes* and the resistance genes. The PFGE and serogroup data also suggest the diverse sources of contamination.

E. faecium isolates (n=34), including 33 from seven poultry farms and one from an outpatient in Michigan, were studied by characterizing the quinupristin-dalfopristin (Q/D) resistant plasmids that carried *vatE*. Hybridization following restriction endonuclease digestion identified five different plasmid types. The *vatE*-carrying plasmid from the human isolate showed nearly identical hybridization patterns, following restriction endonuclease digestion, to a *vatE* carrying plasmid from an *E. faecium* recovered from a chicken farm. This study showed that a heterogeneous group of plasmids harbour *vatE* in a heterogeneous population of *E. faecium*. Some of the plasmids were obtained by *E. faecium* capable of infecting humans.

Q/D resistant *E. faecium* (n=28) with the MICs (minimal inhibitory concentrations) = 32 µg/ml were characterized by gene identification, conjugation, transformation, and *in vitro* transposon mutagenesis. *vatE* and *ermB* are responsible for high streptogramin resistance in most *E. faecium* isolates from poultry products but that the mechanisms of Q/D resistance among *E. faecium* isolates from poultry farms remain largely uncharacterized.

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by

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Part I. *Listeria monocytogenes*

Microbiology of *Listeria monocytogenes*

The genus *Listeria* consists of a group of Gram-positive bacteria of low G+C content closely related to *Bacillus, Clostridium, Enterococcus, Streptococcus, and Staphylococcus*. *Listeria* species are facultative anaerobic rods of 0.4 by 1 to 1.5 μm that do not form spores, have no capsule, and are motile at 10 to 25 ^{o}C (Ryser 1999). *Listeria* can grow in a wide range of temperatures (1-45 ^{o}C) with the optimum growth temperature of 30 ^{o}C. Glucose and glutamine are required as primary sources of carbon and nitrogen for growth (Premaratne, Lin et al. 1991). *Listeria* is microaerophilic, catalase-positive and oxidase-negative (Sergeant, Love et al. 1991; Ryser 1999).

Listeria species are isolated from a diversity of environmental sources, including decaying vegetation, soil, water, effluents, a large variety of foods, and the feces of humans and animals (Vazquez-Boland, Kuhn et al. 2001). On the basis of DNA-DNA hybridization, multilocus enzyme analysis, and 16S rRNA sequencing, the genus *Listeria* presently consists of six species: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi*. Within the genus *Listeria*, only *L. monocytogenes* and *L. ivanovii* are considered virulent. Only one species, *L. monocytogenes*, is a public health concern (Swaminathan, 2001). Human cases of *L. ivanovii* infection are rare (Cummins, Fielding et al. 1994). Most reported isolations of this species were from abortions, stillbirths, and neonatal septicemias in sheep and cattle (Sergeant, Love et al. 1991; Alexander, Walker et al. 1992; Chand and Sadana 1999)

Numerous methods have been used to identify *L. monocytogenes* to the species level. All *Listeria* species are phenotypically similar, but can be distinguished by the following tests: hemolysis, acid production from D-xylose, L-rhamnose, a-methyl-D-mannoside, and mannitol. Various commercial miniaturized culture or enzyme assays are now used for *Listeria* identification, since conventional culture procedures for identification are tedious and time consuming. These assays include API listeria, API 20 STREP, API ZYM, Micro-ID *Listeria*, etc. Classical phenotypic characteristics are used for routine identification of *Listeria* isolates. Genetic identification methods have also been developed, especially for identifying atypical isolates. These methods include 16S rRNA sequencing, sequence analysis of the 16S-23S internal transcribed spacer loci, ribotyping, random amplification of polymorphic DNA, repetitive element sequence-based PCR, multilocus enzyme electrophoresis (MEE), et al (Ryser 1999).

Subtyping of *L. monocytogenes*

Phenotypic subtyping methods for *L. monocytogenes* include serotyping, phage typing, and multilocus enzyme electrophoresis (MEE). Serotyping has proven its value for many years. It is a classical tool for strain differentiation of many foodborne pathogens, including *Salmonella*, *L. monocytogenes*, *Escherichia coli* and others. Thirteen serovars have been identified in *L. monocytogenes* by serotyping. Only three of them (1/2a, 1/2b, and 4b) are frequently isolated from clinical cases. Serovar 4b strains are responsible for 33 to 50% of sporadic human cases worldwide and for all major foodborne outbreaks since 1981 in the US (Swaminathan 2001).

The development of DNA-based subtyping methods has significantly improved our ability to differentiate *Listeria* species. The most commonly used molecular methods that provide accurate and discriminatory typing results for *L. monocytogenes* include ribotyping, pulsed-field gel electrophoresis (PFGE) and DNA-sequencing based subtyping (Wiedmann 2002). PFGE characterizes bacteria into subtypes by generating DNA banding patterns after restriction digestion of the bacterial DNA. Restriction enzymes commonly used for PFGE typing of *L. monocytogenes* include *Asc*I and *Apa*I. PFGE shows a high level of sensitivity for discrimination of *L.monocytogenes* strains, and is often considered the current gold standard for genomic DNA subtyping (Wiedmann 2002).

Ribotyping is also a DNA-based subtyping method in which bacterial DNA is initially cut into fragments by restriction enzymes. But the DNA fragments after restriction digestion are smaller compared with the PFGE method and the number of DNA pieces generated is 300-500 or even more. Automated ribotyping is often considered the DNA subtyping method of choice for many large-scale studies as well as for industrial application, as PFGE is more time-consuming and labor-intensive, and requires more personnel with a higher level of technical expertise (Wiedmann 2002).

Multilocus sequence typing (MLST) is a molecular subtyping approach that uses DNA sequencing of multiple genes or gene fragments to differentiate bacterial subtypes and to determine the genetic relatedness of isolates. The development of MLST approaches for *L. monocytogenes* is also aided by the fact that the virulence genes essential for listerial infection have been identified, thus providing good targets for DNA sequencing-based subtyping (Wiedmann 2002).

In summary, genetic subtyping methods have greater discriminatory power than conventional methods, but the use of multiple subtyping methods may further improve discriminatory power and may therefore be appropriate for epidemiological outbreak investigations. Serotyping, PFGE and ribotyping have been most widely used to characterize *L. monocytogenes* from both clinical and food sources (Wagner and Allerberger 2003).

Isolation of *L. monocytogenes* from food samples

Although *L. monocytogenes* is widely distributed, the numbers of the organism present in most environmental habitats are low. Isolation methods have therefore required that relatively large samples be selectively enriched to increase numbers to detectable levels and suppress the competing background microflora in the sample. Selective agents commonly used in selective enrichment/plating media include acriflavin, naladixic acid, lithium chloride, moxalactam, and phenylethanol. Among the most widely used procedures for detection of *L. monocytogenes* are protocols devised by the U.S. Department of Agriculture- Food Safety and Inspection Service (USDA-FSIS) for detection of *Listeria* in meats (McClain 1989; Cook 1999), and by the U.S. Food and Drug Administration (FDA) for detection of *Listeria* in dairy products (Lovett 1989; Hitchins 1995).

The prevalence of *L. monocytogenes* in foods has been studied worldwide. Contamination levels of *L. monocytogenes* in retail foods were 12.2% in minced beef, 20.6% in minced pork, 37% in minced chicken, and 25% in minced pork-beef mixture samples in Japan (Inoue, Nakama et al. 2000). An overall prevalence of 14.6% was

reported from 315 raw fish samples in the U.S. (Hoffman, Gall et al. 2003). The presence of *L. monocytogenes* in mixed meat amounted to 71.6% in a French study (Thevenot, Delignette-Muller et al. 2005). A study in Brazil revealed 35.6% of positive samples from surfaces, water, and poultry products in a poultry processing plant (Reiter, Bueno et al. 2005).

Two U.S. studies revealed 6.5% and 4.6% of prevalence in bulk tank milk samples, (Van Kessel, Karns et al. 2004; Jayarao and Henning. 2001) respectively. A low prevalence of *L. monocytogenes* (0.018%) was reported in pasteurized milk produced in the U.S. by a recent comprehensive survey (Frye and Donnelly. 2005).

A survey of *L. monocytogenes* in ready-to-eat foods in the U.S. revealed an overall prevalence of 1.82% with prevalences ranging from 0.17 to 4.7% among eight product categories: luncheon meats, deli salads, fresh soft "Hispanic-style" cheeses, bagged salads, blue-veined and soft mold-ripened cheeses, smoked seafood, and seafood salads (Gombas, Chen et al. 2003). *L. monocytogenes* was detected in 3% (88 of 2934) of ready-to-eat prepared salad vegetables in the United Kingdom in 2003 (Sagoo and Mitchell 2003).

Foodborne Listeriosis

Listeriosis is a serious infection caused by eating food contaminated with *Listeria*. *L. monocytogenes* is the only species in the genus *Listeria* that is of concern of human health. Healthy people rarely contract listeriosis, but the illness can be serious for the elderly, newborns, pregnant women and those with weakened immune systems. The disease symptoms vary and depend on the individual's susceptibility. Symptoms may include fever, fatigue, nausea, vomiting and diarrhea. The more serious forms of listeriosis can result in meningitis and septicemia. Listeriosis in pregnant women may

show flu-like symptoms; complications can result in miscarriage, stillbirth, or septicemia or meningitis in the newborn. In older children and adults, complications usually involve the central nervous system and blood stream, but may include pneumonia and endocarditis. Skin contact with *L. monocytogenes* can cause localized abscesses or skin lesions (Ryser 1999). In spite of the fact that foodborne illness caused by *L. monocytogenes* occurs less often than illness attributable to *Salmonella* or *Campylobacter*, the proportion of cases of listeriosis that are fatal is much higher than that associated with other foodborne bacterial pathogens (Mead, Slutsker et al. 1999). Each year, there are an estimated 2,500 cases of listeriosis in the US, with 500 deaths (Mead, Slutsker et al. 1999).

Transmission of human listeriosis was first shown to be foodborne in 1981 (Schlech et al. 1983). Foodborne transmission of *L. monocytogenes* is recognized as the main route of acquisition of the infection during epidemic and sporadic listerioses (Farber and Peterkin 1991; Pinner, Schuchat et al. 1992). Foods which are denoted as ready-to-eat (RTE) foods (e.g. deli meats, salads), unpasteurized dairy foods (cheese and milk), cured meats (e.g. hot dogs and undercooked chicken), and items such as prepared seafood salads and even raw and unprocessed meats have been common foods implicated in outbreaks of listeriosis (Schlech 2000). This has prompted the U. S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) to mandate a level of 'zero tolerance' (no detectable level) for *Listeria* in RTE foods (Swaminathan 2001). There have been several epidemic outbreaks worldwide implicating *Listeria* contaminated foods (Table 1).

Table 1 Summary of representative outbreaks of listeriosis.

Year	Location	No. infected	Mortality rate (%)	Food vehicle	Ref.
1981	Nova Scotia, Canada	41	34	Coleslaw	(Schlech, et al. 1983)
1983	Massachusetts, US	49	29	Pasteurized milk	(Fleming, et al. 1985)
1983-1984	Switzerland	57	32	Soft cheese	(Bula, et al. 1995)
1985	California, US	86	34	Mexican-style cheese	(Anonymous, 1985)
1989	Pennsylvania, US	36	44	Unknown	(Schwartz, et al. 1989)
1998	11 states, US	101	21	Hot dogs	(Anonymous, 1999)
2002	Northeastern US	46	22	Turkey, chicken deli meats	(Anonymous, 2002)

Incidence of antibiotic resistance in *Listeria* species

In general, *L. monocytogenes*, as well as other *Listeria* species, are susceptible to most antibiotics except cephalosporins and fosfomycin (Hof 1991; Hof, Nichterlein et al. 1997). Antibiotic resistance in *L. monocytogenes* was first reported in 1988 (Poyart-Salmeron, Carlier et al. 1990). A multiresistant strain of *L. monocytogenes* was first isolated in France in 1988 (Poyart-Salmeron, Carlier et al. 1990). Since then, *Listeria*

species isolated from food or the environment or in sporadic cases of human listeriosis have shown resistance to one or more antibiotics (Hadorn, Hachler et al. 1993; Franco Abuin, Quinto Fernandez et al. 1994; Charpentier, Gerbaud et al. 1995).

The drug of choice for treating listeriosis is ampicillin or penicillin G combined with an aminoglycoside, classically gentamicin. The combination of trimethoprim with a sulfonamide, such as sulfamethoxazole in co-trimoxazole, is considered to be a second-choice of therapy (Charpentier and Courvalin 1999). Unfortunately, gentamicin-resistant clinical strains of *L. monocytogenes* were reported in 1997 (Charpentier and Courvalin 1999; Walsh, Duffy et al. 2001). An *L. monocytogenes* strain resistant to ampicillin was identified in 1984 in the US (Rapp, Pershadsingh et al. 1984). Clinical strains of *Listeria* resistant to streptomycin, erythromycin, kanamycin, sulfonamide and rifampin were also reported from around the world (Poros-Gluchowska and Markiewicz 2003). Multiple drug resistance was also observed in strains isolated from foods in some countries (Poros-Gluchowska and Markiewicz 2003).

A study conducted in Northern Ireland (Walsh, Duffy et al. 2001) showed 0.6% of *L. monocytogenes* from retail foods was resistant to one or more antibiotics and resistance to tetracycline (6.7%) or penicillin (3.7%) was the most frequently observed resistance phenotype. Multi-drug-resistant strains were recovered from cabbage, environmental, and water samples in Texas, US (Prazak, Murano et al. 2002). Of 20 multi-drug-resistant isolates, 17 were resistant to penicillin. Gentamicin resistance was also observed. The detection of a foodborne strain of *L. monocytogenes* resistant to trimethoprim is of particular interest, since the trimethoprim-sulfamethoxazole combination is used in the

treatment of listeriosis, especially in patients allergic to the penicillins (Charpentier, Gerbaud et al. 1995).

Mechanisms of antibiotic resistance in *L. monocytogenes*

Antibiotic resistance in *L. monocytogenes* is mainly due to acquisition of three types of movable genetic elements: self-transferable plasmids, mobilizable plasmids, and conjugative transposons (Charpentier and Courvalin 1999). Efflux pumps were reported to be associated with fluoroquinolone resistance in *Listeria* (Godreuil, Galimand et al. 2003). Enterococci and Streptococci, in particular, represent a reservoir of resistance genes for *L. monocytogenes*. The gastrointestinal tract of humans is considered the most probable site where the acquisition, by *Listeria* spp., of conjugative plasmids and transposons from *Enterococcus-Streptococcus* takes place (Doucet-Populaire, Trieu-Cuot et al. 1991).

1. Antibiotic resistance mediated by conjugation

   Conjugation is the major mechanism used by *Listeria* to acquire antibiotic resistance. The conjugative transfer of plasmids and transposons carrying antibiotic resistance genes from *Enterococcus-Streptococcus* to *Listeria* and between *Listeria* species has been described by several studies (Perez-Diaz, Vicente et al. 1982; Flamm, Hinrichs et al. 1984; Vicente, Baquero et al. 1988).

   A broad-host-range plasmid, pIP510, first found in *Streptococcus agalactiae*, encodes resistance to chloramphenicol, macrolides, lincosamides and streptogramins. Plasmid pIP510 can transfer by conjugation from *S. agalactiae* to *L. monocytogenes*,

L. murrayi, and *L. grayi*. It replicates in *Listeria* to promote its own transfer between *Listeria* strains and back from *Listeria* to *Streptococcus* (Perez-Diaz, Vicente et al. 1982; Vicente, Baquero et al. 1988).

Tn916, initially discovered in *E. faecalis*, is a broad-host-range conjugative transposon, encoding resistance to tetracycline-minocycline. Transfer by conjugation of Tn916 was obtained at a frequency of 10^{-6} from *E. faecalis* to *L. innocua* (Vicente, Baquero et al. 1988). Conjugative transfer of the Tn916-related transposon Tn1545, initially found in *S. pneumoniae*, was obtained from *E. faecalis* to *L. monocytogenes* in vitro and in vivo (Doucet-Populaire, Trieu-Cuot et al. 1991; Poyart-Salmeron, Trieu-Cuot et al. 1992).

A broad-host-range plasmid of *E. faecalis*, pAMß 1, encoding resistance to erythromycin, was transferred to *L. monocytogenes* and back to *Enterococcus* (Flamm, Hinrichs et al. 1984). The conjugative transfer of a plasmid carrying the gene *vanA*, conferring resistance to glycopeptide antibiotics, from *E. faecalis* to *L. monocytognenes*, *L. ivanovi* and *L. welshimeri* was also described (Biavasco, Giovanetti et al. 1996).

Tetracycline resistance is believed the most frequent resistance trait in *L. monocytogenes* isolated from humans (Charpentier and Courvalin 1999). Conjugative plasmids and transposons originating from *Enterococcus-Streptococcus*, are responsible for the emergence of resistance to tetracycline in *L. monocytogenes* clinical isolates (Poyart-Salmeron, Trieu-Cuot et al. 1992). Plasmid pIP823, replicating by the rolling-circle (RC) mechanism, has a broad-host-range of both Gram-positive and Gram-negative bacteria. pIP823 was involved in high level

trimethoprim resistance in an *L. monocytogenes* isolate from France (Charpentier, Gerbaud et al. 1995). Chloramphenicol resistance can be conjugatively transferred through plasmids in the pC223 family (Charpentier and Courvalin 1999).

2. Active efflux of antibiotics

Antibiotic resistance mediated by efflux mechanisms was first reported in *L. monocytogenes* in 2000 (Mata, Baquero et al. 2000). The sequence of MdrL (multidrug efflux transporter of *Listeria*) protein is highly homological to the sequence of protein YfmO, a putative chromosomal multidrug efflux transporter of *B. subtilis*. An allele-substituted mutant of this gene in *L. monocytogenes* failed to pump out ethidium bromide and presented increased susceptibility to macrolides, cefotaxime and heavy metals.

Efflux pump Lde (*Listeria* drug efflux) is associated with fluoroquinolone resistance in clinical isolates of *L. monocytogenes* in France (Godreuil, Galimand et al. 2003). The Lde protein showed 44% homology with PmrA (pneumoniae multidrug resistance) of *Streptococcus pneumoniae*, which belongs to the major facilitator superfamily (MFS) of secondary multidrug transporters. The insertional inactivation of the gene *lde* results in increased susceptibility of fluoroquinolones in *L. monocytogenes* (Godreuil, Galimand et al. 2003).

Part II. Enterococci

Microbiology of Enterococci

Enterococci are Gram-positive cocci that occur singly, in pairs, or as short chains. More than 20 enterococcal species are now recognized. They are facultative anaerobes, tolerant to extremes in temperature, salinity, and pH and are among the most thermotolerant of the nonsporulating bacteria (Franz, Holzapfel et al. 1999). The optimum growth temperature is 35 ^{o}C with a growth range from 10 to 45 ^{o}C. They grow in broth containing 6.5% NaCl, and hydrolyze esculin in the presence of 40% bile salts. *E. faecium* strains have been able to survive heating to 65 ^{o}C for 20 min, 71 ^{o}C for 10 min, and 80 ^{o}C for 30 min (Kearns, Freeman et al. 1995). Thus, enterococci may survive some types of food processing, and have been implicated in food spoilage of processed cooked meat (Franz, Holzapfel et al. 1999).

Enterococci are a part of the natural flora in the intestinal tract of mammals and birds. Their existence in reptiles and insects has also been reported (Aarestrup, Butaye, et al 2002). *E. faecalis* is found more commonly than *E. faecium* in human feces (Noble 1978). The highest numbers of both species are found in the colon. In addition, enterococcal species can be found in soil, on plants, and in water. In water, they are generally considered as fecal contaminants and belong mainly to the species *E. faecium* and *E. faecalis*, but other species also can be recovered (Niemi, Niemela et al. 1993).

Several approaches have been used to identify enterococci to the species level, including classical phenotypic characteristics, analysis of WCP (whole-cell proteins) profiles, vibrational spectroscopic analysis, randomly amplified polymorphic DNA

(RAPD) analysis, sequencing analysis of the 16S rRNA gene, and DNA-DNA hybridization.

Enterococcal virulence

Enterococci are commensal organisms well suited to survival in intestinaland vaginal tracts and the oral cavity. However, as for most bacteria described as causing human disease, enterococci also possess properties that can be ascribed roles in pathogenesis. The virulence traits in enterococci include adherence to host tissue, invasion and abscess formation, resistance to and modulation of host defense mechanisms, secretion of cytolysins and other toxic products and production of plasmid-encoded pheromones (Gilmore, 2002). The resistance to antibiotics may also contribute to their virulence and make them effective opportunistic pathogens. Many of these enterococcal virulence traits, such as haemolysin-cytolysin production, the adhesion ability and the antibiotic resistance, have been shown to be transmissible by gene transfer mechanisms (Kreft, Marre et al. 1992; Chow, Thal et al. 1993; Gilmore, Segarra et al. 1994; Wirth 1994). The natural ability ofenterococci to readily acquire, accumulate, and share extrachromosomalelements encoding virulence traits or antibiotic resistance genes lends advantages to their survival under unusual environmental stresses and in part explains their increasing importance as nosocomial pathogens.

Antimicrobial resistance of enterococci

During the past several decades, enterococci have emerged as important nosocomial pathogens (Terpenning, Zervos et al. 1988; Murray 1990; Moellering 1992; Murray

2000). This importance is attributed primarily to the high degree of antimicrobial resistance that is exhibited by most enterococci.

The species responsible for most infections in the community, long-term care, and hospital settings is *E. faecalis*. *E. faecium*, intrinsically more resistant than *E. faecalis*, accounts for approximately 10% of enterococcal infections overall. *Enterococcus* is associated with a variety of different clinical infections, such as urinary tract infections, intra-abdominal, pelvic, and soft tissue infections, bacteremia and endocarditis (Malani et al 2002). Some uncommon infections, such as meningitis, hematogenous osteomyelitis, septic arthritis, and pneumonia, have also been diagnosed in clinical settings (Malani et al 2002).

There are at least two major mechanisms by which enterococci become multidrug-resistant: 1. intrinsic resistance to several antimicrobial agents; 2. acquired resistance via mobility of the resistance genes on plasmids and transposons, and chromosomal exchange. In addition, the environmental burden of antimicrobial utilization, colonization pressure, and nosocomial transmission of vancomycin resistant enterococci (VRE) are significant in many hospitals as well as in animal health industry (Mundy, Sahm et al. 2000).

Enterococci are intrinsically resistant to lactams (particularly cephalosporins and penicillinase-resistant penicillins), low concentrations of aminoglycosides, low concentrations of fluoroquinolones, clindamycin, and trimethoprim-sulfamethoxazole. They can also acquire resistance to high concentrations of ß-lactams, high concentrations of aminoglycosides, glycopeptides (vancomycin, teicoplanin), tetracycline, erythromycin,

high level concentrations of fluoroquinolones, rifampin, chloramphenicol, fusidic acid, and nitrofurantoin (Cetinkaya, Falk et al. 2000).

Acquired antimicrobial resistances by enterococci

Table 2 summarizes the acquired antibiotic resistance characteristics found in enterococci.

Table 2 Mechanisms of antimicrobial resistance in enterococci

Antimicrobial agents	Resistance mechanisms
Aminoglycosides (Gentamicin, Kanamycin, Streptomycin)	Enzymatic (production of aminoglycoside-modifying enzymes) AAC(6')-Ie + APH(2'')-Ia AAC(6')-Ii APH(2'')-Ib; APH(2'')-Ic; APH(2'')-Id APH(3'')-IIIa ANT(3')-Ia; ANT(4')-Ia; ANT(6')-Ia Alteration of the target (leading to decreased ribosomal binding)
Glycopeptides (Vancomycin, Teicoplanin)	Alteration of the target (modification of the peptidoglycan biosynthetic pathway)
ß-lactams (Penicillin, Ampicillin)	Alteration of the target (altered penicillin-binding proteins) Enzymatic (production of ß-lactamase)
Quinolones	Alteration of the target (changes to the sub-unit A of DNA gyrase)
Chloramphenicol	Enzymatic (production of chloramphenicol acetyl transferase)
MLS group (Macrolides: erythromycin, Lincosamides: clindamycin, Streptogramin B)	Enzymatic (production of methylating enzymes)

1. Aminoglycosides (high level resistance)

Amonoglycosides inhibit bacterial protein synthesis by binding irreversibly to the bacterial 30S ribosomal unit. Resistance to aminoglycosides in enterococci is defined by MICs for gentamicin (GEN) of = 1000 µg/ml and/or streptomycin (STR) of = 2000 µg/ml. The bifunctional enzyme AAC(6')-Ie + APH(2'')-Ia mediates high level resistances to all amonoglycosides (with the exception of streptomycin) and is also found in staphylococci and enterococci. High level resistance to aminoglycosides and/or ampicillin resistance compromise the combination therapy by ampicillin plus an aminoglycoside.

2. Glycopeptides

Glycopeptides inhibit the peptidoglycan biosynthesis of cell wall among Gram-positive bacteria by binding to the pentapeptide of the murein precursor, which inhibits the subsequent transglycosylation reaction by steric hindrance.

Vancomycin (VAN) or teicoplanin (TPL) are important therapeutic alternatives against multidrug-resistant enterococci and other Gram-positive bacteria. They are intended for treating patients infected with enterococcal strains with ampicillin resistance or if the patient possesses an allergy to penicillins. To date, two intrinsic and five acquired types of glycopeptide resistance are identified (Klare, Konstabel et al. 2003). These resistances, however, share the same mechanism: alteration in the pentapeptide of the murein precursor leads to no or a decreased binding of glycopeptides to their target. Among the glycopeptide resistance types, VanA is most

important and commonly identified in *E. faecium*. VanB is the second most important type.

3. Penicillins

The major antibacterial activity of penicillins is derived from their ability to inhibit a number of bacterial enzymes, namely, penicillin-binding proteins (PBPs), which are essential for peptidoglycan synthesis, thereby interfering with synthesis of the bacterial cell wall.

Penicillin G and, especially, ampicillin are valuable therapeutics in enterococcal infections. A study showed approximately 60-80% of the *E. faecium* strains are ampicillin-resistant whereas = 2% of the *E. faecalis* isolates possess this resistance (Williamson, le Bouguenec et al. 1985). The resistance mechanisms are mainly due to modifications in the penicillin-binding proteins (Williamson, le Bouguenec et al. 1985). Few cases also showed ampicillin resistance based on ß-lactamase production (Murray, Mederski-Samoraj et al. 1986; Murray 1992; Rice and Marshall 1992; Zscheck and Murray 1993; Tomayko, Zscheck et al. 1996).

4. MLS_B antibiotics

Resistance to MLS_B antibiotics (macrolides, lincosamides, and streptogramins of the B type) is mediated by the widespread *ermB* gene (methylation in bacterial 23S rRNA) or acetyltransferase, a hydrolase or an efflux pump mechanism. A detailed review will be presented later in the dissertation.

Enterococci and food

1. Presence of enterococci in foods

Due to their ubiquitous occurrence in nature, enterococci are not only associated with warm-blooded animals, but they also occur in soil, surface waters and on plant and vegetables, which determines their frequent finding in foods as contaminants. Indeed, enterococci commonly occur in large numbers in vegetables and foods of animal origin (Franz, Holzapfel et al. 1999). They can also contaminate finished products during food processing, especially if initially present in high numbers (Franz, Holzapfel et al. 1999).

2. Antimicrobial-resistant enterococci in foods

Antimicrobial-resistant enterococcal strains have been isolated from meat products, dairy products, fresh produce and the poultry production environment (Hayes, McIntosh et al. 2001; Giraffa 2002; Hayes, English et al. 2003; Johnston and Jaykus 2004). Antimicrobial-resistant enterococci can enter the human gut through consumption of contaminated foods (especially of animal origin). Food-associated enterococci could therefore be a reservoir for antimicrobial resistance genes. Once ingested, resistant strains can survive gastric passage and multiply, thus leading to sustained intestinal carriage.

Studies showed 73% of enterococcal isolates from Swedish retailed chicken, 55% from Danish chicken, 14% from Danish pork and 9% from Swedish pork were resistant to one or more antimicrobials, including tetracycline, erythromycin and vancomycin (Quednau, Ahrne et al. 1998). A U.S study reported 91% of *E. faecium*

isolates and 95% of *E. faecalis* isolates from retail meats were resistant to at least one antimicrobial agent (Hayes, English et al. 2003). In the same study, resistance to quinupristin-dalfopristin (Q/D), a human analogue of virginiamycin that is currently used in animal production in the U.S., was observed in 54, 27, 9, and 18% of *E. faecium* isolates from turkey, chicken, pork, and beef samples, respectively. In addition, Q/D resistance was found in 51 to 78% of *E. faecium* isolates from the chicken farm environment (Hayes, McIntosh et al. 2001). Antimicrobial resistance profiles of *enterococcus* species isolated from fresh produce were also characterized by Johnston and Jaykus (Johnston and Jaykus 2004). Of human clinical importance, *E. faecium* strains had a much higher prevalence of resistance to ciprofloxacin, tetracycline, and nitrofurantoin than *E. faecalis.* Thirty-four percent of the enterococcal isolates had multidrug-resistance patterns. Enterococci isolated from cheese in Europe were resistant to penicillin, tetracycline, chloramphenicol, erythromycin, gentamicin, lincomycin, rifampicin, fusidic acid and vancomycin (Teuber, Meile et al. 1999). Multidrug resistance was also observed in this study.

3. Presence, selection and spread of vancomycin-resistant enterococci (VRE) in foods

Among antibiotic resistant enterococci, the emergence, selection and spread of enterococci resistant to glycopeptide antibiotics vancomycin and teicoplanin in a hospital environment is of particular concern, because glycopeptide antibiotics often represent the last therapeutic option in the treatment of infections caused by enterococci. Although VRE have been most identified among hospitalized persons, several reports carried out in Europe and the US indicated that colonization with VRE

frequently occurs in the community, and that many animal, food and environmental reservoirs can serve as sources of VRE outside the health care setting.

4. Streptogramin resistance in *E. faecium*

With the increasing frequency of isolating vancomycin-resistant *E. faecium* (VREF), Q/D was approved for use for the treatment of VREF in 1999 in the U.S. and Europe (Soltani, Beighton et al. 2000; Soltani, Beighton et al. 2001). Q/D is a semisynthetic mixture of streptogramins A and B. Virginiamycin, another mixture of streptogramin A and B compounds, has been used in animal production for over two decades to control clostridial diseases and to promote the growth of commercial poultry. There have been numerous reports on the prevalence of Q/D resistance in *E. faecium*, which led to speculation that the use of virginiamycin in animal husbandry might contribute to the emergence of quinupristin-dalfopristin resistance among human isolates of Gram-positive pathogens (Hammerum, Jensen et al. 1998; Jensen, Hammerum et al. 1998). This was substantiated by the fact that streptogramin resistant *E. faecium* could be isolated from clinical samples even before the streptogramin combination Q/D was introduced for therapeutic purposes in European hospitals (Klare, Konstabel et al. 2003). As a result, virginiamycin use has been suspended in the European Union since July 1999 (Aarestrup, Seyfarth et al. 2001) but it is still used in the U.S.

The streptogramin antibiotics are comprised of two chemically distinct classes of cyclic molecules, type A and type B. Both groups of antibiotics are produced simultaneously in filamentous soil bacteria of the genus *Streptomyces* (Johnston,

Mukhtar et al. 2002). The combination of type A and B streptogramins is bactericidal against species of staphylococci and streptococci, and bacteriostatic against enterococci (Schmitz, Verhoef et al. 1999). Streptogramins act by binding to the ribosomal 50S subunit and interfering with peptidyltransferase activity. Despite structural differences, both type A and B streptogramins share separate but overlapping binding regions within the ribosomal P site (Nyssen, Di Giambattista et al. 1989).

Resistance to streptogramins was first reported in staphylococci in 1980 (el Solh, Fouace et al. 1980). Only resistance to the type A component is required for streptogramin resistance; however, resistance to both type A and B streptogramins is required for the high level resistance (el Solh, Fouace et al. 1980). There are mainly three mechanisms for streptogramin resistance in Gram-positive bacteria: ribosomal target modification, active efflux, and enzymatic inactivation (Johnston, Mukhtar et al. 2002). Modification of ribosomal RNA and ribosomal proteins is the most common mode of streptogramin resistance. Methylation of adenine residues is the most common type of target modification intermediated by an *erm*-encoded erythromycin-resistance methylase. Methylation of this residue results in a conformational change in the ribosome, creating inducible or constitutive cross-resistance to macrolides, lincosamides, and type B streptogramins (MLS_B). More than 20 *erm* genes have been described to date. This mechanism is prevalent among staphylococci and streptococci, but the *ermB* gene was also identified in *E. faecium* from chicken and turkey. Genes encoding active efflux pumps for streptogramin resistance are putative members of the ATP-binding cassette transporter superfamily. Resistance to type A

streptogramins is due to plasmid-borne *vgaA* and *vgaB* genes (Johnston, Mukhtar et al. 2002) and efflux of type B streptogramins is due to the presence of another ATP-binding transporter encoded by the *msrA, msrSA, msrB* genes, and *msrC* genes (Johnston, Mukhtar et al. 2002). Both *vga* and *msr* genes have been found mostly among *S. aureus* and coagulase-negative staphylococcal species, but the prevalence of *msrC* has also been reported in *E. faecium* isolates (Singh, Malathum et al. 2001; Werner, Hildebrandt et al. 2001). Inactivation of type A streptogramins is due to O-acetylation by five acetyltransferases designated Vat for virginiamycin acetyltransferase, first identified in 1993 (Allignet, Loncle et al. 1993). The genes encoding these enzymes include *vatA*, *vatB*, and *vatC* in staphylococci, and *vatD* and *vatE* in enterococci. All *vat* genes are plamid-borne among both staphylococci and enterococci and are often cotranscribed with *vga* and/or *vgb* genes on the same plasmid. A lyase, encoded by *vgbA* and *vgbB*, can cause streptogramin B inactivation but has only been identified among staphylococci. In addition to these three mechanisms, a new type of streptogramin A resistance has been observed among staphylococci but has not been found among *E. faecium*, characterized by an LS_A phenotype due to an unknown mechanism (Hamilton-Miller and Shah 2000).

Interestingly, these mechanisms can only explain Q/D resistance in about 30% of *E. faecium* strains resistant to Q/D. The resistance mechanisms to Q/D in over 70% of Q/Dresistant *E. faecium* isolates are currently unknown.

5. Antibiotic selective pressure in the food production environment

Despite the fact that hospitals are reservoirs for antimicrobial-resistant enterococci due to the use of antibiotics, the commercial animal husbandry that uses antibiotics as food additives may also be an important contributing factor. These antibiotics are or have been used in subtherapeutic doses. Cross resistances exist in related antibiotics therapeutically used in human or animal medicine, e.g., avoparcin (vancomycin, teicoplanin), virginiamycin S/M (quinupristin/dalfopristin), spiramycin, tylosin (erythromycin), and avilamycin (evernimicin). The use of some of the antibiotics as growth promoters creates selective pressure for the emergence of antimicrobial-resistant enterococci or their resistance genes, and both can be spread to other ecological habitats, including humans. Therefore, animals or animal production environments may serve as the reservoirs for those antimicrobial-resistant bacteria or resistance genes.

Antibiotic resistance gene transfer

It is now clear that the well-studied mechanisms known to be responsible for the spread of antimicrobial resistance genes among and between bacterial species are also responsible for the evolution of pathogenic potential, metabolic diversity, and perhaps even the operon structure of the genome itself (Gilmore 2002). Acquired resistances can occur if two prerequisites are present: genetic potential by the microorganisms (accumulation of mutations in DNA that finally leads to resistance or acquisition of transferable resistance genes from donor cells) and the antibiotic selective pressure. The transferable resistance genes arise from acquisition of "foreign" DNA by conjugation, transduction, or transformation. A variety of plasmids and transposons have been

identified and extensively characterized. Unfortunately, little is known about the role of bacteriophages and transduction in the evolution of this genus (Gilmore 2002).

Plasmids

Three classes of plasmids are known to be capable of replication in the enterococci: the rolling circle replicating (RCR) plasmids, the Inc18 plasmids, and the pheromone-responsive plasmids. The RCR and Inc18 plasmids are capable of replication in many Gram-positive bacteria, and some RCR plasmids are also capable of replication in Gram-negative species (del Solar, Moscoso et al. 1993). The replication of pheromone-responsive plasmids appears to be restricted to the enterococci, although plasmids with clearly related replicons are present in a variety of Gram-positive organisms.

1. RCR plasmids

   RCR plasmids are a group of small, high-copy plasmids that replicate via a rolling circle mechanism, which was originally described in the single-stranded DNA (ssDNA) coliphages. Originally identified in *S. aureus*, RCR plasmids have since been isolated from or shown to replicate in virtually every Gram-positive bacterial species (Gilmore 2002).

2. Inc18 plasmids

   The Inc18 plasmids include pIP501 from *S. agalactiae*, pSM19035 from *Streptococcus pyogenes*, and pAMß1 from *E. faecalis*. These plasmids are representative of a family of about 15 plasmids widely distributed among the low G+C Gram-positive bacteria, including several from the enterococci (Funnell 2004).

The plasmids are generally between 25 and 30 kb in size and are maintained at a relatively low copy number (< 10 copies/cell). Many are self-conjugative and can be transferred to and replicate in a wide range of Gram-positive species.

The emergence of resistance to gentamicin and vancomycin in enterococci in recent years has prompted investigations of the genetic elements carrying the related determinants. There are numerous reports of the involvement of plasmids encoding these resistance traits in a variety of enterococcal species, and many exhibit a broad host range (Funnell 2004). Leclercq et al (Leclercq, Derlot et al. 1989) were able to transfer a VanA resistance plasmid pIP819 from *E. faecium* to *Streptococcus sanguis*, *S. pyogenes*, *Lactobacillus lactis*, and *L. monocytogenes*, but not to *S. aureus*, whereas Noble et al (Noble, Virani et al. 1992) were able to transfer a vancomycin-resistance plasmid from *E. faecalis* to *S. aureus*.

3. Pheromone-responsive plasmids

The pheromone-responsive plasmids are a family of approximately 20 self-conjugative plasmids whose transfer functions are induced by peptide sex pheromones secreted by plasmid-free cells (Gilmore 2002). This class of plasmids seems to be confined to enterococci. They encode antibiotic resistance, bacteriocins, and hemolysins (Clewell 1993; Dunny, Leonard et al. 1995) and are commonly found in *E. faecalis*, whereas plasmids from *E. faecium* or other enterococci are not yet known to exhibit a pheromone response, although in some cases they exhibit a response when present in an *E. faecalis* host (Handwerger, Pucci et al. 1990; Heaton, Discotto et al. 1996).

The best-studied pheromone-induced plasmid transfer systems are pAD1, pCF10, and pPD1 (Grohmann, Muth et al. 2003). pAD1is a 59.3 kb hemolysin/bacteriocin plasmid that responds to the pheromone cAD1. The 65 kb pCF10 encodes tetracycline resistance and confers response to the pheromone cCF10. pPD1 is a 56 kb plasmid encoding bacteriocin production (Bac21), and its conjugative response depends on the pheromone cPD1.

Transposons

A number of individual transposons and several transposon classes have been described in enterococci. Enterococcal transposons generally fall into one of three classes: Tn3 family transposons, composite transposons, and conjugative transposons. The first two classes are widespread in the bacterial domain and their transposition mechanisms have been well described in *E. coli* and other Gram-negative bacteria. Conjugative transposons were initially discovered in *E. faecalis* and are now known to be widespread in Gram-positive and Gram-negative bacteria (Clewell 1993; Grohmann, Muth et al. 2003).

Conjugative transposons are mobile DNA elements that encode functions for intracellular transposition and intercellular conjugation. The first conjugative transposon identified was an 18 kb Tn916 transposon from *E. faecalis* (Franke and Clewell 1981). Tn916 and the closely related element Tn1545 from *Streptococcus pneumoniae* (Caillaud, Carlier et al. 1987; Courvalin and Carlier 1987) form the basis of a family of conjugative transposons with a broad host range (Rice 1998). All members of this family encode a

tetracycline determinant of the TetM type (Burdett 1991), and many of them also carry genes encoding resistance to additional antimicrobial agents.

Like the majority of conjugative transposons, Tn916 encodes a recombinase, Int, that belongs to the lambda integrase family, and carries an adjacent gene, *xis*, encoding a small basic protein that is analogous to lambda Xis protein. The *int* and *xis* genes are located at the left end of the transposon while the genes thought to be involved in conjugal transfer, *orf23* through *orf13*, are located at the right end (Senghas, Jones et al. 1988). The origin of transfer, *oriT*, defined as a DNA sequence that when cloned onto a plasmid allows the plasmid to be mobilized in trans by Tn916, together with the tetracycline resistance gene, *tetM*, are also located on the transposon.

Conjugative transposons and related elements move via a circular intermediate that is produced by excision of the integrated element from the donor chromosome. This circular intermediate then serves as a substrate for conjugation, and, in most cases, a single DNA strand of the intermediate is transferred from donor to recipient with transfer initiating at *oriT*. Once in the recipient, the circular intermediate reforms and then integrates into the recipient genome (Gilmore 2002).

In summary, the contribution of movable enterococcal elements to the emergence and dissemination of antibiotic resistance and enhanced virulence is clearly evident, and it appears that certain conjugation-related molecules may also contribute to pathogenicity.

By virtue of their ability to facilitate the exchange of genetic information, plasmids greatly enhance a bacterial species' ability to survive within the environment. In addition, both the pheromone-responsive plasmids and the broad-host range plasmids have been

implicated in the transfer of antibiotic resistance in the clinical setting. The broad host range of Tn916-like conjugative transposons suggests that they be important vectors for the dissemination of *tetM*-mediated tetracycline-minocycline resistance. And they may also play a role in disseminating non-*tetM* resistance genes (Caillaud, Carlier et al. 1987). Furthermore, the emergence and spread of vancomycin resistance among *E. faecium* strains in recent years has led to the discovery of a new group of transposons that bear significant similarities to Tn916. The prototype Tn5382 (Carias, Rudin et al. 1998) encodes vancomycin resistance. While enterococcal infections are generally not life-threatening, the genes for vancomycin resistance may be spread to bacteria that may cause fatal diseases (Grohmann, Muth et al. 2003). Moreover, the presence of a conjugative transposon in a donor may have consequences beyond the transfer of the element itself. In enterococci, some studies have suggested that the presence of a conjugative transposon in a donor cell promotes the exchange of unlinked chromosomal loci, even in the absence of transfer of the conjugative transposon itself (Torres, Korman et al. 1991).

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CHAPTER 1

Characterization of *Listeria monocytogenes* Isolated from Retail Deli Meats, Raw Chickens and Fresh Produce

Abstract

A total of 167 *Listeria monocytogenes* isolates were recovered from retail deli meats, raw chickens and fresh produce samples. Strain characterization was carried out by serogroup identification using PCR and genotyping using pulsed-field gel electrophoresis (PFGE). A broth microdilution method of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards, NCCLS) was followed to test the antimicrobial susceptibility of these isolates. Five *L. monocytogenes* serogroups were identified among the 167 isolates: 68 isolates (41%) belonged to serogroup 1/2b, 3b, 53 isolates (32%) to serogroup 4b, 4d, 4e, 43 isolates (26%) to serogroup 1/2a, 3a, 2 isolates (1.2%) from serogroup 1/2c, 3c and only 1 isolate (0.6%) to serogroup 4a, 4c. PFGE generated 120 patterns among the 167 isolates and correlated well with PCR serogrouping. Most *L. monocytogenes* isolates were resistant to sulfonamide (73%) and some were resistant to tetracycline (8.4%) and ciprofloxacin (1.8%). Tetracycline resistance was conjugatively transferable and *tet*(M) gene was identified from 14 tetracycline resistant isolates as well as their transconjugants. These findings indicate that *L. monocytogenes* present in food are diverse and resistance to one or more antibiotics is common. In addition, the presence of potential serotype 4b in all food categories raised a public health concern as serotype 4b has been the number one serotype associated with human listeriosis.

Introduction

The genus *Listeria* consists of a group of Gram-positive bacteria of low G+C content. Six species (*L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi)* have been identified within the genus. However, only *L. monocytogenes* and *L. ivanovii* are considered virulent. Human cases of *L. ivanovii* infection are rare (Cummins et al., 1994), whereas *L. monocytogenes* is an important foodborne pathogen (Swaminathan, 2001). Since foodborne listeriosis was first reported in 1981 (Schlech et al., 1983), numerous foodborne outbreaks of *L. monocytogenes* have been documented worldwide (Farber and Peterkin, 1991; Jacquet et al., 2002). It is estimated that approximately 2,500 cases of human illness and 500 deaths are caused by *L. monocytogenes* annually in the United States (Mead et al., 1999). The susceptible population to *L. monocytogenes* infection includes the elderly, newborns, pregnant women and immunocompromised individuals such as AIDS patients. Due to its ubiquitous distribution in the environment, *L. monocytogenes* has been isolated from a variety of food products. Meat, poultry, dairy, and vegetable products have all been implicated as vehicles of listeriosis (Schlech et al., 1983; Fleming et al., 1985; Schwartz et al., 1989; Bula et al., 1995).

Serotyping has been widely used to characterize *L. monocytogenes* (Miettinen et al., 1999; Wiedmann, 2002; Wagner and Allerberger, 2003). Although 13 serotypes have been identified in *L. monocytogenes*, only three of them (1/2a, 1/2b, and 4b) are most frequently isolated from clinical samples with serotype 4b causing the vast majority of human listeriosis (Raybourne, 2002; Borucki and Call, 2003). Serotype 1/2a is the most

prevalent serotype of *L. monocytogenes* in food. Therefore, serotyping may have value as a virulence screening test.

Pulsed-field gel electrophoresis (PFGE) characterizes bacteria into subtypes by generating DNA banding patterns after restriction digestion of the bacterial DNA. Restriction enzymes commonly used for PFGE typing of *L. monocytogenes* include *Asc*I and *Apa*I. PFGE shows a high level of sensitivity for discrimination, and is often considered the current gold standard for molecular subtyping of foodborne bacterial pathogens (Wiedmann, 2002).

L. monocytogenes, as well as other *Listeria* spp., are usually susceptible to a wide range of antibiotics (Hof et al., 1997). However, since the isolation of the first multiresistant strain of *L. monocytogenes* in France in 1988 (Poyart-Salmeron et al., 1990), *L. monocytogenes* strains resistant to one or more antibiotics have been described from food or the environment or in sporadic cases of human listeriosis (Hadorn et al., 1993; Franco Abuin et al., 1994; Charpentier et al., 1995). Standard therapy for listeriosis remains the administration of ampicillin or penicillin G combined with an aminoglycoside, such as gentamicin. A combination of trimethoprim with a sulfonamide, such as sulfamethoxazole in co-trimoxazole, is considered as a second-choice therapy (Charpentier and Courvalin, 1999). Antibiotics to which resistance has been developed by *L. monocytogenes* include tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulfonamide, trimethoprim, and rifampicin (Charpentier and Courvalin, 1999). Among these, tetracycline has been reported the most frequent resistance phenotype in *L. monocytogenes* (Charpentier and Courvalin, 1999). Quinolone resistance has been a big concern in bacteria of animal origin. That is why

tetracycline and quinolone drugs were also included in our antimicrobial susceptibility testing other than the drugs used for treatment of listeriosis. Since there is no resistant breakpoint available to date for *L. monocytogenes* antimicrobial susceptibility testing, we referred to the breakpoints for other Gram positive bacteria in this study.

Our study aimed at characterizing *L. monocytogenes* isolated from retail foods by serogroup identification and molecular subtying and investigating their antimicrobial susceptibility and the molecular determinants responsible for antimicrobial resistance.

Materials and methods

L. monocytogenes strains.

A total of 167 *L. monocytogenes* isolates recovered from retail foods in Florida and Greater Washington, DC area during the period of 2002 and 2003 were examined in the study. These included 91 from deli meats, 26 from conventional raw chickens, 45 from organic raw chickens, 3 from conventional fresh produce and 2 from organic fresh produce. Strain confirmation was carried out by Rapid' L. Mono selective agar plates (Bio-Rad, Hercules, CA) and real-time iQ check molecular-beacon PCR (Bio-Rad).

Serogroup identification and subtyping by PFGE.

A PCR method was followed to identify *L. monocytogenes* serogroups based on the fact that virulence gene evolution has paralleled that of somatic and flagellar antigens, the variation of which divides *L. monocytogenes* strains into different serogroups (Vines et al., 1992; Ericsson et al., 2000; Borucki and Call, 2003). The primers and assay conditions for PCR are described in Table 3 D1 and D2 primers differentiated serotypes 1/2b, 3b and all serotype 4 strains from serotypes 1/2a, 1/2c, 3a and 3c. Further differentiation between 1/2b, 3b and all serotype 4 strains was made using GLT primers. Serotypes 4a and 4c were identified with LM4B primers. Strains identified as belonging in 1/2a, 1/2c, 3a and 3c were further subtyped by using FlaA primers designed to differentiate serotypes 1/2a, 3a and 1/2c, 3c (Jinneman and Hill, 2001; Borucki and Call, 2003).

PFGE was performed to differentiate the isolates at the genomic level according to the PulseNet standardized protocol (Graves and Swaminathan, 2001). Briefly, genomic

DNA was prepared by mixing 240 µl of standardized cell suspension and 60 µl of 10 mg/ml lysozyme solution (Sigma, St. Louis, MO), followed by incubation at 37°C for 10 min. Sample plugs were digested with 25 U of *Asc*I at 37°C for 3 h or 160-200 U of *Apa*I at 30°C for 5 h (New England Biolab, Beverly, MA). Plugs were then loaded on 1% Seakem Gold agarose gel in 0.5× TBE (45mM Tris-45mM Borate-1mM EDTA) buffer and electrophoresed on a CHEF-DR III apparatus (Bio-Rad, Hercules, CA) using the following parameters: initial switch time, 4.0 s; final switch time, 40.0 s; run time, 22 h; angle, 120°; gradient, 6.0 V/cm; temperature, 14°C; ramping factor, linear. Gels were stained with ethidium bromide and visualized on a UV transilluminator.

Antimicrobial susceptibility testing.

Ampicillin, ciprofloxacin, gentamicin, nalidixic acid, penicillin G, sulfonamide, tetracycline and trimethoprim obtained from Sigma (St. Louis, MO, USA) were included in the susceptibility study. Antimicrobial stock solutions were prepared and stored in distilled water (ampicillin, ciprofloxacin, gentamicin, nalidixic acid, penicillin G and sulfonamide) or dimethyl sulfoxide (trimethoprim) or 50% ethanol (tetracycline). Concentrations of antimicrobial agents ranged from 0.5 to 8 µ g/mL (ciprofloxacin), 1 to 16 µg/mL (ampicillin and penicillin G), 2 to 32 µg/mL (gentamicin, nalidixic acid and tetracycline), 4 to 64 µ g/mL (trimethoprim) and 32 to 512 µg/mL (sulfonamide).

A broth microdilution method recommended by the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards, NCCLS) (2003) was used. Briefly, different concentrations of each of the eight antimicrobial agents diluted in Cation-adjusted Mueller Hinton broth (CAMHB; Difco,

Detroit, MI) were dispensed into a 96-well plastic microdilution tray. Since 50 µl of each antimicrobial agent and 50 µl of bacterial suspension were added to each well, the concentration of the antimicrobial agent made was at twice the desired final concentration in each well. The filled trays were sealed in plastic bags and immediately placed at -80 °C until use. CAMHB supplemented with lysed horse blood (LHB) (2-5% v/v) was used as growth medium and the inoculum density was 5×10^5 CFU/mL. The inoculated microtiter plates were incubated at 35 °C for 16-20 h. All experiments included quality control organisms *Enterococcus faecalis* ATCC29212, *E. faecalis* ATCC51299 and *Psedomonas aeruginosa* ATCC27853.

Characterization of tetracycline resistance.

PCR assays and DNA microarray were used to characterize 14 tetracycline-resistant *L. monocytogenes* isolates. DNA template for PCR was made by boiling overnight cultures of the *L. monocytogenes* isolates. Primers used to amplify the *tet*(L), *tet*(M) and *tet*(S) tetracycline resistance genes included: tetLF (ACTGGGTGAACACAGCCTTT), tetLR (CAGGAATGACAGCACGCTAA), tetMF (ACACGCCAGGACATATGGAT), tetMR (ATTTCCGCAAAGTTCAGACG), tetSF (CGCTACATTTGCGAGACTCA) and tetSR (GAATGCCACTACCCAAAGGA) (Call et al., 2003).

The DNA microarray was performed as previously described (Call et al., 2003) to identify tetracycline resistance genes in 7 *L. monocytogenes* isolates with MICs = 32 µg/mL. Genomic DNA was extracted with the DNeasy Tissue Kit (Qiagen, Valencia, CA) with modifications to increase the DNA yield from Gram-positive bacteria. Lysozyme (20 mg/ml) and RNase (250 ng/ml) dissolved in lysis buffer (20mM Tris•Cl, pH 8.0,

2mM sodium EDTA and 1.2% Triton X-100) were added to bacterial pellet collected from overnight culture in brain heart infusion (BHI, Difco, Detroit, MI) broth supplemented with tetracycline (16 µg/ml). The mixture was incubated at 37°C for 1 h followed by the procedures specified in the manufacturer's manual. Genomic DNA was then biotinylated and hybridized overnight to microarrays suitable for detecting 17 tetracycline resistance genes at 65°C. Hybridization signal was detected with the Tyramide Signal Amplification (TSA) kit (Perkin-Elmer Life Sciences, Boston, MA), Alexa Fluor 546 (Molecular Probes, Eugene, OR), and an arrayWoRx® scanner (Applied Precision, Issaquah, WA) (Call et al., 2003).

Conjugation experiments.

The 14 tetracycline-resistant *L. monocytogenes* isolates were used as donor strains in conjugation experiments to study tetracycline resistance gene transfer. *E. faecalis* JH2-2 (rif^r fus^r) was used as recipient strain. Conjugation was performed by the filter mating method described previously (Poyart-Salmeron et al., 1990) with modifications. Briefly, overnight cultures of the donor strains grown in BHI broth (Difco) containing tetracycline (16 µg/mL) and recipient grown in BHI (Difco) containing fusidic acid (50 µg/mL) and rifampicin (100 µ g/mL) were mixed (ratio, 1:1) in BHI broth (Difco). The mixture was then placed on a 0.45-µm-pore-size filter and incubated on BHI agar plates (Difco) at 37°C overnight. The filter was washed and vortex-mixed in BHI broth (Difco). The mating mixture was spread onto BHI agar (Difco) containing a combination of tetracycline (16 µ g/mL), fusidic acid (50 µ g/mL) and rifampicin (100 µ g/mL). Up to five potential transconjugants were purified on BHI agar containing appropriate antibiotics and resistance gene transfer by conjugation was confirmed by PCR and DNA microarray.

Conjugation frequencies were expressed as the numbers of transconjugants per recipient cell.

Results

Five serogroups were identified among the 167 *L. monocytogenes* isolates (Figure 1). Most isolates (164) belonged to serogroups 1/2b, 3b (40.7%), 4b, 4d, 4e (31.8%) and 1/2a, 3a (25.7%). Only three isolates (1.8%) were identified as serogroup 1/2c, 3c and 4a, 4c (Figure 1). Serogroup 1/2b, 3b was most frequently present in deli meats (51 of 91) and conventional chicken (13 of 25), followed by serogroup 1/2a, 3a (34 of 91) in deli meats and serogroup 4b, 4d, 4e (11 of 25) in conventional chicken. In the contrast, most organic chicken isolates (73.3%; 33 of 46) belonged to serogroup 4b, 4d, 4e, with serogroup 1/2a, 3a having the second highest number of isolates (7 of 46).

Out of 167 isolates tested, 120 PFGE profiles were identified. Isolates with identical PFGE patterns belonged to the same serogroup. For example, 23 *L. monocytogenes* from deli meats share same PFGE pattern as well as same serogroup file (1/2b, 3b). Three isolates from produce samples belonging to serogroup 4b, 4d, 4e had indistinguishable PFGE patterns. The patterns of *L. monocytogenes* obtained from one food category did not match with those from another food category except that in fresh produce samples, one isolate from an organic alfalfa sprout sample shared the same PFGE pattern with two isolates from conventional alfalfa sprout samples. These three isolates belonged to serogroup 4b, 4d, 4e. PFGE generated 46 distinct profiles among 91 isolates from deli meats.

Unique PFGE patterns were identified among the 71 chicken isolates (Figure 2). Many patterns, however, had only slight differences in either the number or the size of the bands. When comparing isolates from organic and conventional sources, there was no distinct PFGE pattern that separates the isolates according to their sources of

isolation, although some clusters with similar patterns were more frequently seen among *L. monocytogenes* recovered from conventional chicken isolates than those from organic chicken isolates, and vice versa.

The PFGE and serogroup results did not always match antimicrobial susceptibility profiles. Some isolates with the same serogroup and PFGE pattern exhibited distinct antimicrobial resistance profiles. For example, two isolates from deli meats with identical PFGE patterns and of the same serogroup (1/2a, 3a) showed resistance to nalidixic acid, and to nalidixic acid and ciprofloxacin, respectively. On the other hand, some isolates with distinct PFGE patterns and different serogroups shared a common antimicrobial resistance profile. For example, all isolates from conventional chicken were uniformly resistant to nalidixic acid and sulfonamide, whereas they belonged to unique PFGE subtype and five different serogroups. Overall, there did not appear to be any association between PFGE patterns and antimicrobial resistance profiles.

The results of antimicrobial susceptibility testing to ciprofloxacin, sulfonamide and tetracycline are shown in Table 4. The 167 *L. monocytogenes* isolates were all resistant to nalidixic acid, which was due to the use of nalidixic acid as a selective agent during isolation. Resistance to ciprofloxacin was found in three isolates from smoked ham, organic chicken and organic romaine lettuce, which accounted for 1.8% of the total isolates. All 14 tetracycline resistant isolates (8.4%) were recovered from deli meats. The majority of the isolates (73%) were resistant to sulfonamide. Resistance to at least two antimicrobials was observed in 137 strains (82%). The only strain with triple resistances was isolated from an organic chicken sample and belonged to serogroup 4b, 4d, 4e. It was resistant to ciprofloxacin, nalidixic acid and sulfonamide.

No resistance was observed to ampicillin, gentamicin, penicillin G or trimethoprim among the *L. monocytogenes* isolates. Even the lowest concentration of each of the four antimicrobials listed above (1 µg/mL ampicillin, 2 µg/mL gentamicin, 1 µg/mL penicillin G, and 4 µg/mL trimethoprim) showed an inhibition effect on bacterial growth, except that one strain from deli meats had a decreased susceptibility to ampicillin.

The minimum inhibitory concentrations (MICs) of 11 of 45 sulfonamide susceptible isolates were one dilution higher than those of the remaining 34 isolates and their distribution among different food categories were not significantly different. All sulfonamide susceptible isolates were from deli meats, organic chicken and produce. Three MIC values were obtained among ciprofloxacin susceptible-resistant strains: = 0.5 µg/mL, 1 µg/mL and 2 µg/mL. According to the NCCLS interpretive criteria for ciprofloxacin for *Enterococcus* and *Staphylococcus*, 135 were susceptible and 29 isolates were intermediate resistant to ciprofloxacin.

tet(M) was the only *tet* gene detected in the 14 tetracycline-resistant isolates by PCR. Seven of the 14 isolates were further tested by DNA microarray for 17 known *tet* genes. No tetracycline resistance genes other than *tet*(M) were identified. The 14 tetracycline resistant isolates showed transferable resistance by conjugation. The conjugation frequencies were about 10^{-5} transconjugants per recipient. *tet*(M) was identified in all the transconjugants by PCR. The transconjugants from three isolates were further tested by DNA microarray and no other *tet* genes were detected except *tet*(M). *tet*(M) was not identified in nine randomly selected tetracycline-susceptible *L. monocytogenes* (data not shown).

Discussion

Among 13 *L. monocytogenes* serotypes, 4b, 1/2a, and 1/2b are associated with most human illness, with serotype 4b being the most prominent. Previous studies have shown that serotype 1/2a is the most frequently isolated from food samples (Kathariou, 2002; Borucki and Call, 2003). Our data showed higher percentages of isolates belonging to serotypes 1/2b, 4b, and 1/2a compared to other serotypes in each food category, which was in agreement with previous studies (Gilot, 1996; Miettinen et al., 1999; Kathariou, 2002). However, the isolation rate of potential serotype 1/2a was lower than those of serotypes 1/2b and 4b, suggesting that the rank of each serotype may vary in different studies performed in various locations throughout the US. As serotypes 3a, 3b, 4d, and 4e are relatively rare from food isolates, presumably the isolates identified as serogroups (1/2a, 3a), (1/2b, 3b), and (4b, 4d, 4e) were serotypes 1/2a, 1/2b, and 4b, respectively. However, to determine their actual serotype identity, a conventional serotyping procedure is needed. One significant observation of this study was that many isolates (73.3%) from the organic chicken samples belonged to serogroup 4b, 4d, 4e (potentially serotype 4b), which is the most epidemiologically significant serotype of *L. monocytogenes* causing human listeriosis.

Although the serogroup and PFGE data had an excellent correlation in the study, they had little association with antimicrobial resistance profiles since they detect variations among bacteria based on the bacterial genome, whereas most antimicrobial resistance genes are carried on plasmids.

Tetracycline resistance has been the most frequently observed resistance phenotype (Charpentier et al., 1995; Charpentier and Courvalin, 1999; Walsh et al., 2001; Prazak et

al., 2002) among *L. monocytogenes* isolates since the first tetracycline-resistant strain was detected in 1988 in France (Poyart-Salmeron et al., 1990). The incidence of tetracycline-resistant *L. monocytogenes* in our study was 8.4%. All isolates were obtained from deli meats, some of which were turkey and beef. The relatively high incidence of tetracycline resistance in this food category may be due to the use of tetracycline in animal feed (Schroeder et al., 2002).

The gastrointestinal tract of humans and animals is believed the most probable site for *Listeria* species to acquire resistance genes via conjugative plasmids and transposons from *Enterococcus* and *Streptococcus* species (Doucet-Populaire et al., 1991). There have been six classes of tetracycline resistance genes (*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(P), and *tet*(S)) described in Gram-positive bacteria (Charpentier et al., 1995). However, only *tet*(L), *tet*(M), and *tet*(S) have been identified in *L. monocytogenes* (Poyart-Salmeron et al., 1992; Charpentier and Courvalin, 1999;). *tet*(M) and *tet*(S) confer resistance by ribosomal protection, whereas the *tet*(L) gene codes for a protein which promotes active efflux of tetracycline from the bacteria. *tet*(M) was originally described in streptococci (Burdett, 1986; Martin et al., 1986) and is common in both streptococci and enterococci. *tet*(L) is the second most common tetracycline resistance gene found in streptococci and enterococci and was detected in one clinical *L. monocytogenes* strain isolated from the UK (Poyart-Salmeron et al., 1992). *tet*(S) was initially identified in a clinical *L. monocytogenes* and subsequently isolated from *E. faecalis* (Charpentier et al., 1993; Charpentier et al., 1994). However, *tet*(M) is the only *tet* gene reported from food isolates of *L. monocytogenes* (Poyart-Salmeron et al., 1992; Charpentier et al., 1995; Cha rpentier and Courvalin, 1999; Pourshaban et al., 2002). The identification of *tet*(M) from the 14

tetracycline-resistant *L. monocytogenes* and the observation that the gene was readily transferable by conjugation supported the hypothesis that tetracycline-resistant *L. monocytogenes* from animal products may have obtained the *tet* genes on plasmids or transposons from other Gram-positive bacteria in animal GI tract.

Due to the wide spectrum of activity against most Gram-positive and Gram-negative bacteria, a combination of trimethoprim (TMP)-sulfamethoxazole (SMX) (cotrimoxazole) has been used for 30 years in human and veterinary medicine (Poros-Gluchowska and Markiewicz, 2003) TMP and SMX are two competitive inhibitors in the synthesis of dihydrofolic acid (DHF) and the transformation of DHF into tetrahydrofolic acid (THF), which then results in the inhibition of thymidine synthesis and DNA synthesis (Goldstein, 1999). Resistance to TMP has been described in one *L. monocytogenes* strain in France among 1040 strains of *Listeria* spp. from various sources (Charpentier et al., 1995). The resistance gene was identical to *dfrD* from *Staphylococcus haemolyticus*, which encodes S2DHFR (modification of antibiotic target) (Charpentier and Courvalin, 1997). In the current study, 122 isolates (73%) were resistant to SMX. To our knowledge, our study was the first report on SMX resistance in *L. monocytogenes.* Although TMP seems to be the most active agent in the TMP-SMX combination (Charpentier and Courvalin, 1999), given it is the second choice for the treatment of listeriosis among patients who are allergic to the penicillins (Jones and MacGowan, 1995), our data raised a concern about listeriosis treatment.

In summary, *L. monocytogenes* can no longer be thought to be uniformly susceptible to antibiotics active against Gram-positive bacteria. Continued surveillance of emerging

antimicrobial resistance among *L. monocytogenes* in both conventional and organic food production settings is needed to ensure public health.

Figure 1 Serogroup identification of *L. monocytogenes* isolates from retail foods

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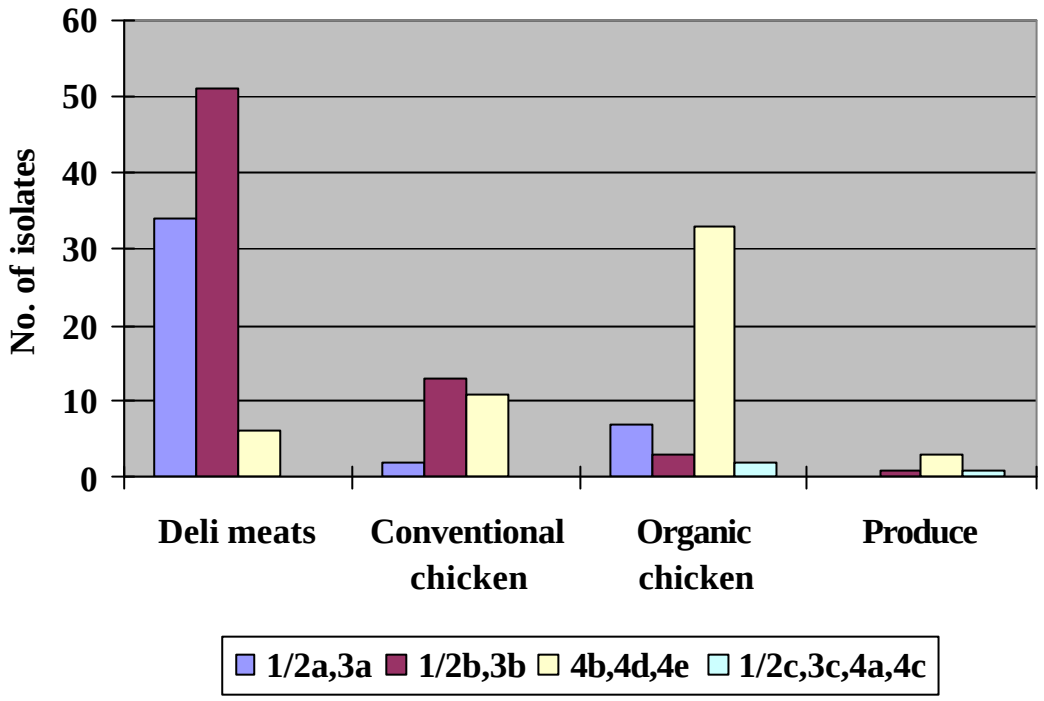

Figure 2 PFGE dendrogram (*Asc*I digestion) of *L. monocytogenes* isolates from conventional and organic chicken samples

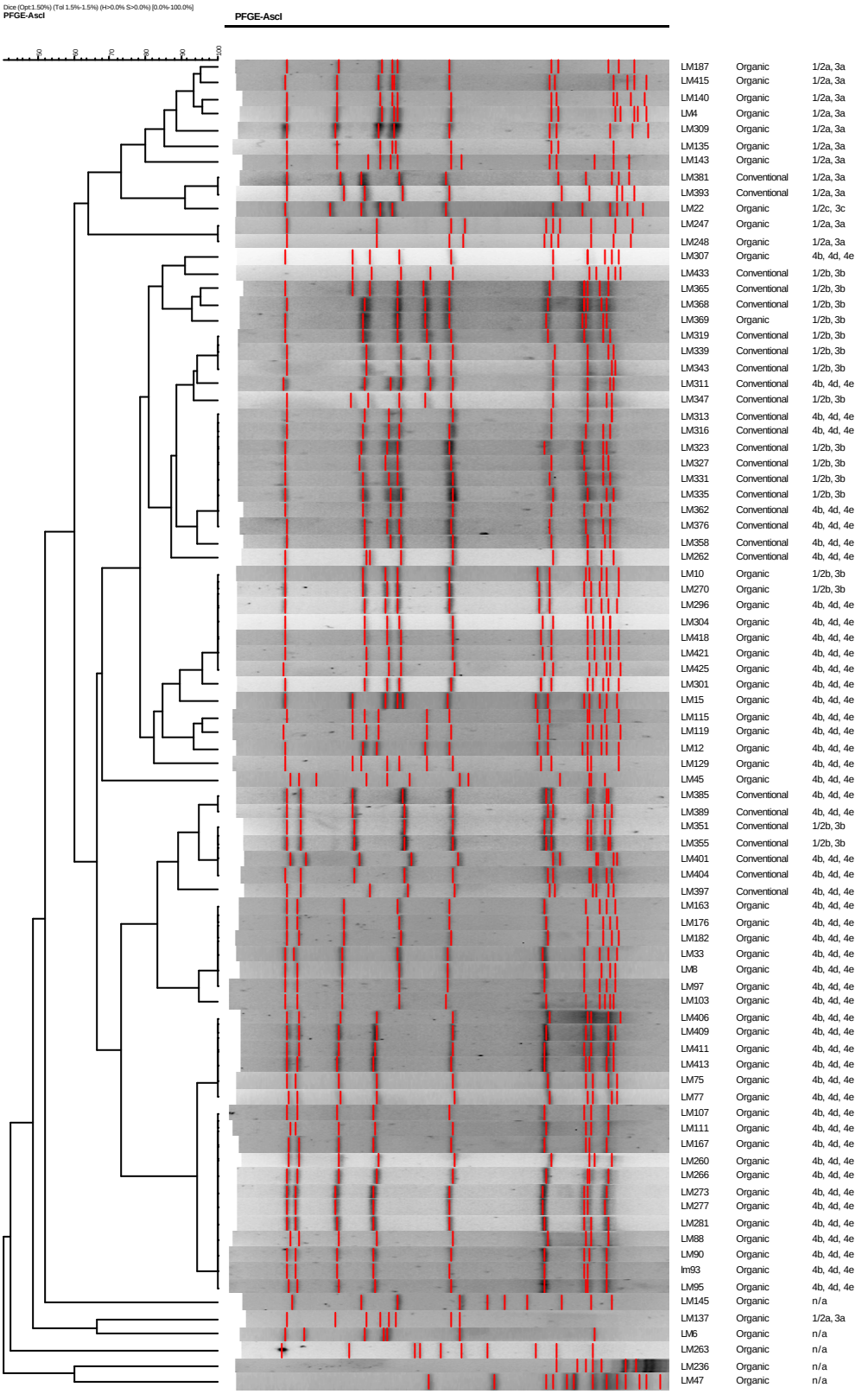
Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE-AscI
PFGE-AscI
100
90
80
70
60
50
40
LM187 Organic 1/2a, 3a
LM415 Organic 1/2a, 3a
LM140 Organic 1/2a, 3a
LM4 Organic 1/2a, 3a
LM309 Organic 1/2a, 3a
LM135 Organic 1/2a, 3a
LM143 Organic 1/2a, 3a
LM381 Conventional 1/2a, 3a
LM393 Conventional 1/2a, 3a
LM22 Organic 1/2c, 3c
LM247 Organic 1/2a, 3a
LM248 Organic 1/2a, 3a
LM307 Organic 4b, 4d, 4e
LM433 Conventional 1/2b, 3b
LM365 Conventional 1/2b, 3b
LM368 Conventional 1/2b, 3b
LM369 Organic 1/2b, 3b
LM319 Conventional 1/2b, 3b
LM339 Conventional 1/2b, 3b
LM343 Conventional 1/2b, 3b
LM311 Conventional 4b, 4d, 4e
LM347 Conventional 1/2b, 3b
LM313 Conventional 4b, 4d, 4e
LM316 Conventional 4b, 4d, 4e
LM323 Conventional 1/2b, 3b
LM327 Conventional 1/2b, 3b
LM331 Conventional 1/2b, 3b
LM335 Conventional 1/2b, 3b
LM362 Conventional 4b, 4d, 4e
LM376 Conventional 4b, 4d, 4e
LM358 Conventional 4b, 4d, 4e
LM262 Conventional 4b, 4d, 4e
LM10 Organic 1/2b, 3b
LM270 Organic 1/2b, 3b
LM296 Organic 4b, 4d, 4e
LM304 Organic 4b, 4d, 4e
LM418 Organic 4b, 4d, 4e
LM421 Organic 4b, 4d, 4e
LM425 Organic 4b, 4d, 4e
LM301 Organic 4b, 4d, 4e
LM15 Organic 4b, 4d, 4e
LM115 Organic 4b, 4d, 4e
LM119 Organic 4b, 4d, 4e
LM12 Organic 4b, 4d, 4e
LM129 Organic 4b, 4d, 4e
LM45 Organic 4b, 4d, 4e
LM385 Conventional 4b, 4d, 4e
LM389 Conventional 4b, 4d, 4e
LM351 Conventional 1/2b, 3b
LM355 Conventional 1/2b, 3b
LM401 Conventional 4b, 4d, 4e
LM404 Conventional 4b, 4d, 4e
LM397 Conventional 4b, 4d, 4e
LM163 Organic 4b, 4d, 4e
LM176 Organic 4b, 4d, 4e
LM182 Organic 4b, 4d, 4e
LM33 Organic 4b, 4d, 4e
LM8 Organic 4b, 4d, 4e
LM97 Organic 4b, 4d, 4e
LM103 Organic 4b, 4d, 4e
LM406 Organic 4b, 4d, 4e
LM409 Organic 4b, 4d, 4e
LM411 Organic 4b, 4d, 4e
LM413 Organic 4b, 4d, 4e
LM75 Organic 4b, 4d, 4e
LM77 Organic 4b, 4d, 4e
LM107 Organic 4b, 4d, 4e
LM111 Organic 4b, 4d, 4e
LM167 Organic 4b, 4d, 4e
LM260 Organic 4b, 4d, 4e
LM266 Organic 4b, 4d, 4e
LM273 Organic 4b, 4d, 4e
LM277 Organic 4b, 4d, 4e
LM281 Organic 4b, 4d, 4e
LM88 Organic 4b, 4d, 4e
LM90 Organic 4b, 4d, 4e
lm93 Organic 4b, 4d, 4e
LM95 Organic 4b, 4d, 4e
LM145 Organic n/a
LM137 Organic 1/2a, 3a
LM6 Organic n/a
LM263 Organic n/a
LM236 Organic n/a
LM47 Organic n/a

Table 3 PCR primers and working conditions used to serogroup *L. monocytogenes*

Serogroup	Primer set	Primer sequences (5'-3')	Product size (bp)	Anneal temp (°C)
1/2b, 3b, 4b, 4d, 4e, 4a, 4c	D1	F: CGATATTTTATCTACTTTGTCA R: TTGCTCCAAAGCAGGGCAT	214	59
1/2a, 1/2c, 3a, 3c	D2	F: GCGGAGAAAGCTATCGCA R: TTGTTCAAACATAGGGCTA	140	59
1/2a, 3a	FlaA	F: TTACTAGATCAAACTGCTCC R: AAGAAAAGCCCCTCGTCC	538	54
1/2b, 3b	GLT	F: AAAGTGAGTTCTTACGAGATTT R: AATTAGGAAATCGACCTTCT	483	45
4a, 4c	LM4B	F: CAGTTGCAAGCGCTTGGAGT R: GTAAGTCTCCGAGGTTGCAA	268	55

Table 4 Antimicrobial resistance profiles of *L. monocytogenes* isolates from different food categories

Source	No. of strains	No. of resistant strains (%)		
		Ciprofloxacin	Sulfonamide	Tetracycline
Deli meats	91	1 (1.1)	55 (60.4)	14 (15.4)
Conventional chicken	26	0 (0)	26 (100)	0 (0)
Organic chicken	45	1 (2.2)	37 (82.2)	0 (0)
Conventional produce	3	0 (0)	3 (100)	0 (0)
Organic produce	2	1 (50)	1 (50)	0 (0)
Total	167	3 (1.8)	122 (73)	14 (8.4)

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CHAPTER 2

Heterogeneity of *vat*(E) Carrying Plasmids in *Enterococcus faecium* Recovered from Human and Animal Sources

Abstract

A total of 34 quinupristin-dalfopristin (Q/D)-resistant *E. faecium* isolates (33 from 7 poultry farms and 1 from an outpatient) was analysed in this study. Q/D MICs (Minimal Inhibitory Concentration) of the *E. faecium* isolates ranged from 4 to 32 µg/ml. PCR detected the presence of *vat*(E) in all isolates. Using pulsed-field gel electrophoresis (PFGE), 14 distinct PFGE patterns were identified. The *E. faecium* isolate from human was distinguishable from the 33 farm isolates by PFGE. Southern hybridization localized the *vat*(E) gene to a single plasmid approximately 11 kb in size, and resulted in five plasmid hybridization types. The *vat*(E) carrying plasmid from the human isolate showed nearly identical hybridization patterns to a plasmid from *E. faecium* recovered on a chicken farm. This study showed that a *vat*(E), conferring resistance to Q/D, is carried on different plasmids in a heterogeneous population of *E. faecium*, some of which may be acquired by isolates capable of infecting humans.

Introduction

Enterococci are commensal bacteria in the human and animal intestinal tract; however, they have also become one of the major causes of hospital-acquired infections, including urinary tract infections, bloodstream infections, wound infections, and endocarditis (Giraffa, 2002; Klare et al, 2003). Two species, *Enterococcus faecalis* and *Enterococcus faecium*, have been isolated most frequently from human clinical cases, accounting for 85-90% and 5-10% of all infections, respectively (Simjee et al, 1999; Simjee et al, 2000). This is further complicated by the fact that enterococci are rapidly becoming resistant to virtually all the antimicrobial agents currently available in clinical medicine.

Quinupristin-dalfopristin (Q/D) (Figure 3), a semisynthetic mixture of streptogramin A and B compounds, was approved for the treatment of vancomycin-resistant enterococci (VRE) in the United States and Europe in 1999 (Soltani et al, 2000; Soltani et al, 2001). Virginiamycin is a streptogramin compound that has been used in animal feed as a growth promoter for more than two decades (Hammerum et al, 1998; Werner et al, 1998; Jensen et al, 2000; Soltani et al, 2000; Witte et al, 2000; Hayes et al, 2001; Hershberger et al, 2005). This raises the possibility that the use of streptogramins in animal feeds might select for streptogramin resistance among animal isolates, creating an environmental reservoir of resistance determinants that have the potential to transfer to human isolates.

Resistance to streptogramin A compound is required for *E. faecium* to develop streptogramin resistance, however, resistance to both A and B compounds can cause higher MICs (Minimal Inhibitory Concentrations). Resistance to streptogramin A in *E.*

faecium is conferred by either of two genes encoding an acetyltransferase, *vat*(D) or *vat*(E) (Johnston et al, 2002). *vat*(D) occurs in only a minority of the resistant *E. faecium* according to several reports from Europe (Hammerum et al, 1998; Jensen et al, 1998) and the prevalence of *vat*(E) is unknown (Soltani et al, 2000). By contrast, only *vat*(E) has been identified in *E. faecium* isolates in the United States (Simjee et al, 2002). In two recent studies, *vat*(E) was detected in 10 of 23 *E. faecium* isolates from chicken (Simjee et al, 2002), whereas no *vat*(E) was detected from the other study (Hayes et al, 2005). A study in France identified *vga*(A) conferring streptogramin resistance (Allignet et al, 1992), where pristina mycin, another antibiotic of the streptogramin class, has been used to treat human infections for over 20 years (Dancer et al, 2003).

A number of studies have investigated the clonal relatedness of vancomycin-resistant and high-level gentamicin-resistant enterococci recovered from human and animal sources (Zervos et al, 1986; Patterson et al, 1988; Woodford et al, 1993; van den Braak et al, 1998; Bischoff et al, 1999; Simjee et al, 1999). Some studies have also examined the relatedness of plasmids carrying *aac6'-aph2"* which confers high-level resistance to gentamicin (Simjee et al, 1999; Simjee et al, 2000). However, no reports have examined the clonal relatedness of *vat*(E) carrying plasmids. The aim of this study was to determine the clonal relatedness of *vat*(E) carrying plasmids in *E. faecium* recovered from human and animal sources by restriction endonuclease digestion and Southern hybridization.

Materials and methods

Bacterial strains

Thirty-four *E. faecium* isolates were collected from human and animal sources (Table 5. Thirty-three of the *E. faecium* were from 7 poultry farms (turkey farm n=19, chicken farm n=14) in Michigan and Indiana. One human isolate was recovered from an outpatient in Michigan. *E. faecium* CVM3001 was used as the *vat*(E) positive control (Hayes et al, 2005).

Determination of antimicrobial susceptibility of enterococci

Antimicrobial susceptibility of the 34 isolates were determined with the Sensititre Automated Antimicrobial Susceptibility System (Trek Diagnostic Systems, Westlake, OH) and interpreted according to the Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines for broth microdilution (Anonymous 2000), using 4 µg/ml as the resistant breakpoint. *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212 were used as quality control strains.

DNA extraction and PCR

Total bacterial DNA was extracted as described previously to be used as template for PCR (Simjee et al, 1999). The *vat*(E), *vat*(D) and *vga*(A) streptogramin resistance genes were amplified by PCR using oligonucleotide primers and PCR conditions previously described (Soltani et al, 2000). Primer sequences were: *vat*(E)-f (5'-ACT ATA CCT GAC GCA AAT GC-3'), *vat*(E)-r (5'- GGT TCA AAT CTT GGT CCG-3'), *vat*(D)-f (5'- GCT CAA TAG GAC CAG GTG TA-3'), *vat*(D)-r (5'- TCC AGC TAA CAT GTA TGG CG-3'), *vga*(A)-f (5'- AGT GGT GGT GAA GTA ACA CG-3'), *vga*(A)-r (5'- CTT GTC TCC TCC GCG AAT AC-3') (Soltani et al, 2000).

Pulsed-field gel electrophoresis (PFGE)

Preparation and digestion of genomic DNA, using *Sma* I restriction endonuclease (New England BioLabs, Beverly, MA), were performed as described previously (Simjee et al, 2000). Comparisons of the PFGE fingerprinting was made using computer assisted analysis (BioNumerics, Applied Maths, Austin, TX), and banding patterns were compared using Dice coefficients with a 1.5% band position tolerance. The interpretive criteria of Tenover et al (Tenover et al, 1995) were used to analyze the PFGE results for strain relatedness of the *E. faecium* isolates.

Plasmid isolation and restriction digestion

Plasmid DNA was extracted as described previously (Woodford et al, 1993; Simjee et al, 2000). Two strains of *Escherichia coli*, CVM 3013 and CVM 3014, harboring plasmids of known sizes were used as plasmid size markers. Electrophoresis of plasmid DNA was carried out in 1.0% agarose gels at 90 V for 1.5 h in 1X TBE buffer (89 mM Tris, 89 mM orthoboric acid, 2 mM EDTA (pH 8.0)) and visualized under UV light following ethidium bromide staining.

Plasmid DNA restriction digestions were done using *Hinc*II, *Hind*III, *Msl*I, *Ssp*I, *Ban*I, or *Nla*III according to the manufacturer's specifications (New England BioLabs). A simplified restriction map of *vat*(E) by these enzymes is shown in Figure 4. Digested plasmid DNA was separated by electrophoresis in 1.3% agarose gels at 90 V for 1.5 h. A digoxigenin (DIG) -labeled DNA ladder (Roche Molecular Biochemicals, Mannheim, Germany) was used as a molecular size marker.

Hybridization studies

The *vat*(E) probe (512 bp) used for Southern blot analysis was generated by PCR using primers *vat*(E)-f and *vat*(E)-r, and *E. faecium* CVM3001 as template. The PCR product was labeled with DIG according to the manufacturer's specifications (Roche Molecular Biochemicals). Digested or intact plasmid DNA was transferred from agarose gels to Hybond-N+ nylon membranes (Roche Molecular Biochemicals) using a Vacuum Blotting System (Amersham Pharmacia Biotech, Piscataway, NJ). The transferred DNA was fixed to the membrane using an ultraviolet cross linker (GS GeneLinker^{TM} UV Chamber; Bio-Rad, Hercules, CA) set at 150 μJ for 45 s. Hybridization was carried out at 40 ^{o}C overnight before washing the blot under conditions of high stringency (1×SSC for 15min × 2 at room temperature and 0.5×SSC for 15 min × 2 at 60 ^{o}C). Hybridized probe was detected using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Molecular Biochemicals).

Results

Antimicrobial susceptibility and PFGE profiles

The 34 *E. faecium* isolates had Q/D MICs = 4 µ g/ml (Table 5. The MICs were not very different between isolates from turkey and chicken farms. Three isolates from turkey farms had an MIC of 4 µ g/ml, whereas the MICs for the remaining 30 isolates from turkey and chicken farms were = 8 µ g/ml. The isolate from a human had an MIC > 32 µ g/ml. All 34 *E. faecium* isolates were *vat*(E) positive; whereas neither *vat*(D) nor *vga*(A) was detected in the isolates.

To determine whether a particular clone was responsible for the dissemination of Q/D resistant *E. faecium*, the isolates were analyzed by PFGE following *Sma* I-digestion of chromosomal DNA. PFGE results identified 14 distinct restriction patterns. A 'three-band difference' rule was employed to distinguish the isolates and the patterns obtained were allocated arbitrary designations of patterns 1-14 (Table 5). There was no distinct PFGE pattern that would separate the isolates according to their isolation sources. The *E. faecium* isolate from a patient in Michigan did not appear closely related to any of the *E. faecium* isolates from animals, all of which were isolated from animals in Michigan and Indiana.

Plasmid analysis

Plasmid analysis showed that the 34 strains carried 1 to 3 plasmids, varying in size from 8 kb to 35 kb, with an 11 kb plasmid present in all strains. Southern hybridization using the *vat*(E) probe localized the *vat*(E) gene exclusively to the 11 kb plasmid (data not shown).

To further analyze the *vat*(E) carrying plasmids, Southern hybridization studies were performed using a digoxigenin labeled *vat*(E) probe. The plasmids from all isolates were analyzed by restriction endonuclease digestion. The plasmid hybridization patterns were compared with those obtained from *E. faecium* CVM3001. Each restriction enzyme used gave up to four different patterns and allowed the plasmids to be allocated to arbitrarily assigned groups (Table 5). Five plasmid types were identified when the plasmid hybridization patterns generated by each individual enzyme digestion were combined (Table 6). No specific plasmid type was found in *E. faecium* isolates recovered exclusively from either turkey or chicken farms.

Digestion of plasmids by two enzymes, *Hinc*II and *Hind*III, which did not cut within the gene, produced two plasmid patterns after Southern hybridization. Neither of those patterns was identical to that from the control plasmid. *Msl*I, an enzyme that cut once within *vat*(E) gene, identified four different patterns among all the plasmids after digestion. The *Msl*I fragments of *vat*(E) were 161 bp and 484 bp. Plasmid type M3, identified in most of the isolates, was distributed in isolates from both turkey and chicken farms. The plasmid patterns identified in the human isolate (pattern M1) and one isolate from a broiler house (M2) shared one band of about 500 bp. Both M1 and M2 were distinguishable from the patterns of the other 32 isolates (Table 5, lane 9 and 11 in Figure 5). A similar situation held true for the patterns identified in these two isolates after *Ban*I digestion, in which case two bands (900 bp and 177 bp, patterns B1 and B2) were shared (lanes 13 and 16 in Figure 5). *Ban*I recognized two cutting sites within the *vat*(E) gene, which generated three fragments of *vat*(E) with 238 bp, 177 bp, and 230 bp in size.

One single plasmid pattern (N1) was identified for all 34 *E. faecium* isolates after *Nla*III digestion. Although *Nla*III has two cutting sites on the *vat*(E) gene, generating three fragments (234 bp, 314 bp, and 97 bp) after digestion, the second cutting site was outside the probe sequence. As a result, only two bands were obtained after hybridization with the 97bp fragment not recognized by the probe. There were two *Ssp*I cutting sites on *vat(*E) generating 87 bp, 195 bp, and 363 bp fragments. Restriction digestion of plasmids by *Ssp*I from all the isolates showed two different types, pattern S1 and pattern S2. Thirty-three isolates belonged to S2, whereas plasmid types identified in isolates from a human (CVM 17885) and broilers (CVM 18747) were the only ones assigned to S1.

Discussion

Streptogramins are important therapeutic agents in both veterinary and human medicine. Q/D was introduced into human medicine in 1999 by an accelerated approval track through the FDA to combat the increasing incidence of vancomycin-resistant *Enterococcus* (VRE) bacteremias, as well as of *Staphylococcus aureus* and *Streptococcus pyogenes* skin and soft tissue infections (Johnston et al, 2002). Streptogramin resistance among *E. faecium* has been reported in countries where streptogramins have never been used in human medicine or have only been introduced recently (Klare et al, 2003). Resistance to Q/D is increasingly being reported among enterococcal isolates obtained from poultry and swine farms where virginiamycin has been used in animal feed as a growth promoter (Simjee et al, 2002). This suggests that the use of streptogramins in animal feed may have selected for streptogramin resistance mechanisms among animal isolates, creating an environmental reservoir of resistance determinants that could have the potential to transfer to human enterococcal isolates.

In this study, the MICs of all isolates were =4 µ g/ml and were not significantly different between isolates recovered from turkey farms and chicken farms. However, the single human isolate examined had an extremely high MIC of >32 µg/ml. The fact that 14 different PFGE patterns were identified from 34 isolates confirmed that the *vat*(E) gene was not confined to a single *E. faecium* clone disseminated between different poultry species. The *E. faecium* isolate from a human had a unique PFGE pattern distinguishable from those observed in the isolates from farms. While this study was limited by the availability of a single *vat*$(E)^+$ human *E. faecium* isolate, these data suggest

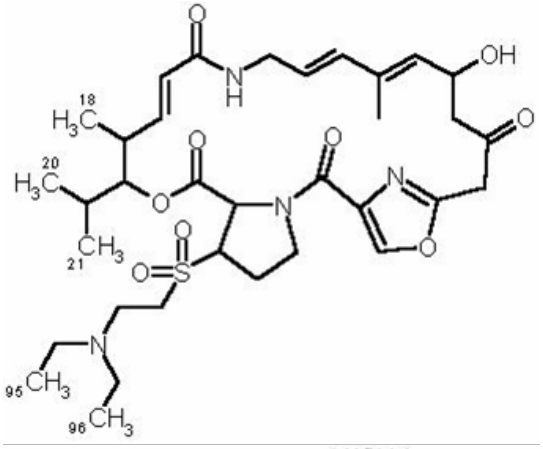
that the dissemination of streptogramin-resistant *E. faecium* could not be explained by the clonal spread of a particular strain unique to an animal species, and that the farm isolates were not responsible for the human enterococcal infection. This is not unexpected in the genus *Enterococcus* considering that the acquisition and spread of VRE in the USA mainly occur in the hospital, whereas there have been no reports on clonal transmission of VRE from farm animals to human patients (Malathum et al, 1999).

To our knowledge, the present study is the first report of a *vat*(E) carrying plasmid recovered from a *E. faecium* isolated from a human in the USA. The current study also examined whether *vat*(E) found on plasmids recovered from poultry and human Q/D-resistant *E. faecium* comprised a homologous or heterogeneous plasmid group. Plasmid typing was employed to determine if a single plasmid type was responsible for the dissemination of the *vat*(E) between different Q/D resistant *E. faecium* clones. The plasmid background from the human isolate was similar to that from a chicken farm isolate based on plasmid typing study. Southern analysis using four different restriction enzymes showed identical plasmid hybridization patterns that were unique to these two strains. Along with comparable MIC values for these strains, this suggests that these plasmids are recently evolved from a common *vat*(E)$^+$ ancestor.

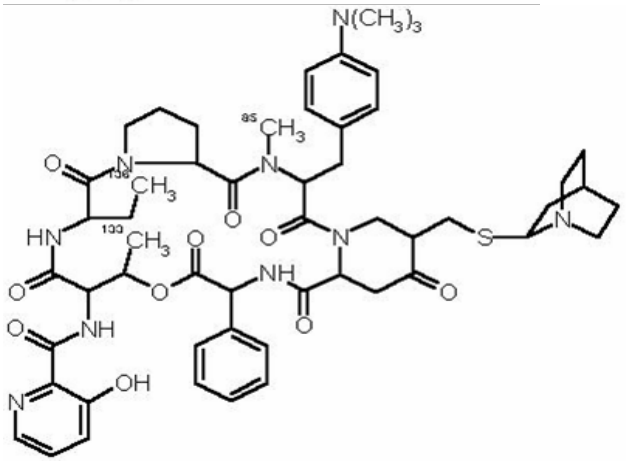
In conclusion, this study shows that the *vat*(E) is being maintained on a heterogeneous group of plasmids in the poultry production environment. A *vat*(E) containing plasmid of human origin showed similar plasmid profiles to a chicken farm *vat*(E) containing plasmid. Further studies are warranted to determine the risk factors for dissemination of *vat*(E) mediated streptogramin resistance and characteristics of transfer,

such as transfer frequency, co-transfer of other resistances, and ability of these enterococci to cause infection in humans.

Figure 3 Molecular structure of quinupristin and dalfopristin



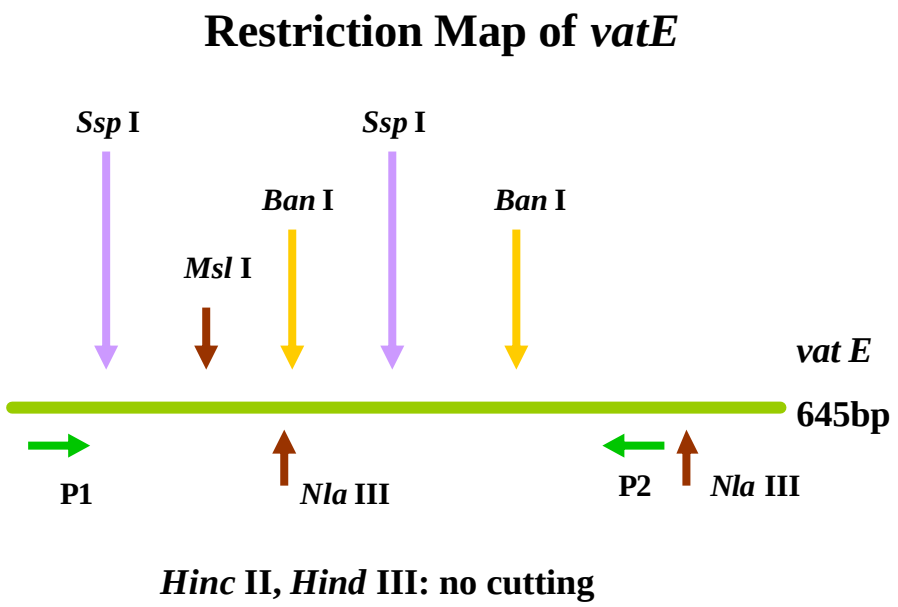
Dalfopristin



Quinupristin

Figure 4 Restriction map of *vat*(E) by six enzymes used in this study

.



P1 and P2 were PCR primers used to amplify the *vat*(E) gene and the amplicon was labeled as probe for hybridization study.

Figure 5 Southern hybridization profiles of *E. faecium* plasmids hybridized with digoxigenin-labelled *vat*(E) probe.

Lanes 1 and 20: digoxigenin-labeled DNA ladder; Lanes 2, 5, 12: controls (*E. faecium* CVM 3001 digested by *Hinc* II, *Hind* III, and *Ban* I, respectively). The remaining lanes show the restriction patterns of plasmid DNA from *E. faecium* tested (also see Table 1). Lane 3: C1; Lane 4: C2; Lane 6: D1; Lane 7: D2; Lane 8: M3; Lane 9: M1; Lane 10: M4; Lane 11: M2; Lane 13: B1; Lane 14: B4; Lane 15: B3; Lane 16: B2; Lane 17: N1; Lane 18: S2 ; Lane 19: S1.

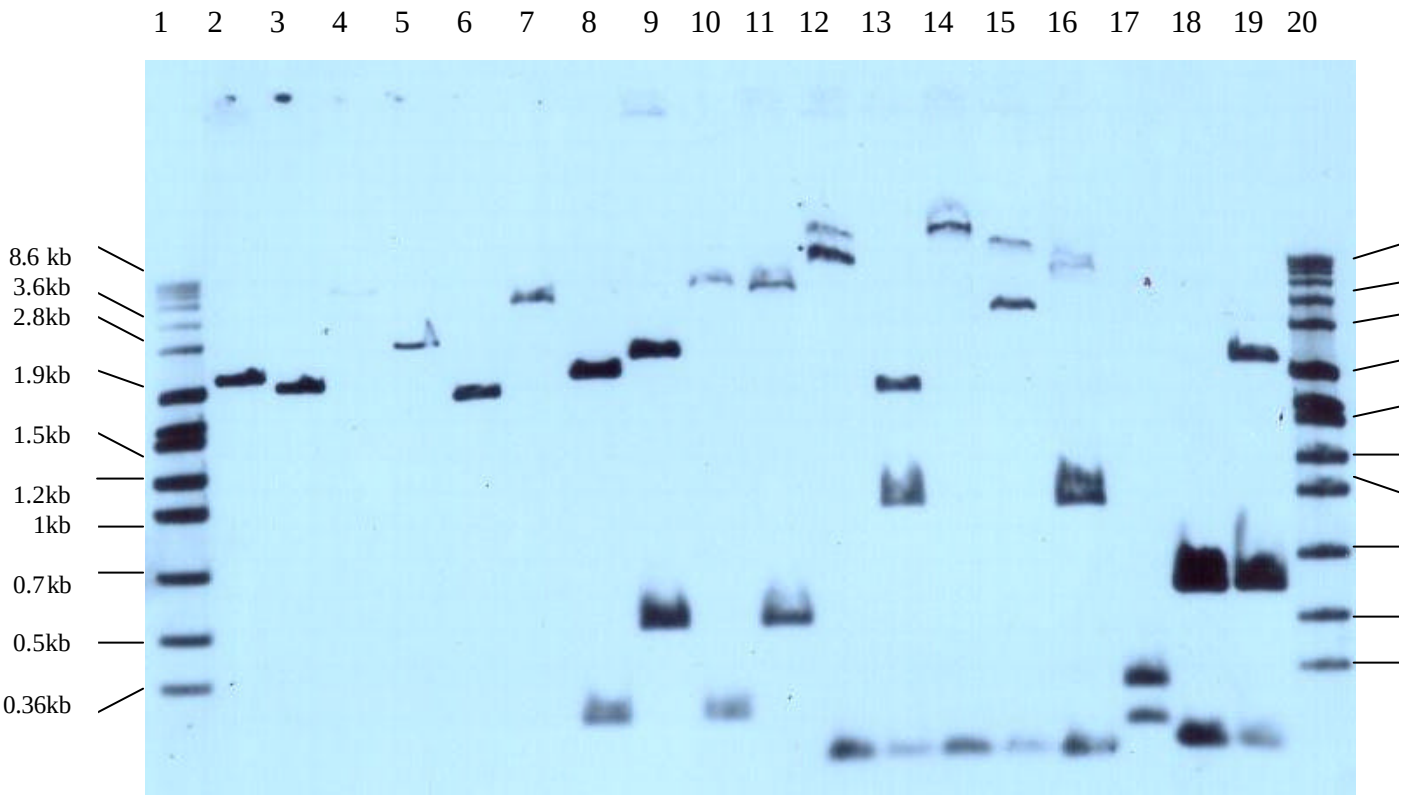

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Table 5 Quinupristin-dalfopristin (Q/D) MICs, pulsed-field gel electrophoresis (PFGE) profiles and plasmid types of 34 *E. faecium* strains tested.

Strain	MIC (μg/ml)	PFGE profile	Plasmid pattern						Source
			*Hinc*II	*Hind*III	*Msl*I	*Ban*I	*Nla*III	*Ssp*I	
17885	>32	14	C1	D1	M1	B1	N1	S1	Human
18747	16	3	C1	D1	M2	B2	N1	S1	Chicken
18468	16	11	C1	D2	M3	B3	N1	S2	Turkey
18471	16	13	C1	D2	M3	B3	N1	S2	Turkey
18475	16	16	C1	D2	M3	B3	N1	S2	Turkey
18717	8	6	C1	D2	M3	B3	N1	S2	Chicken
18725	16	3	C2	D1	M3	B3	N1	S2	Chicken
18726	16	3	C2	D1	M3	B3	N1	S2	Chicken
18457	8	4	C2	D1	M3	B4	N1	S2	Turkey
18458	8	3	C2	D1	M3	B4	N1	S2	Turkey
18459	4	8	C2	D1	M3	B4	N1	S2	Turkey
18460	8	2	C2	D1	M3	B4	N1	S2	Turkey
18462	8	6	C2	D1	M3	B4	N1	S2	Turkey
18463	8	7	C2	D1	M3	B4	N1	S2	Turkey
18464	8	5	C2	D1	M3	B4	N1	S2	Turkey
18467	4	10	C2	D1	M3	B4	N1	S2	Turkey
18470	8	12	C2	D1	M3	B4	N1	S2	Turkey
18472	16	2	C2	D1	M3	B4	N1	S2	Turkey
18474	16	2	C2	D1	M3	B4	N1	S2	Turkey
18477	8	5	C2	D1	M3	B4	N1	S2	Turkey
18478	16	5	C2	D1	M3	B4	N1	S2	Turkey
18479	16	1	C2	D1	M3	B4	N1	S2	Turkey
18481	8	6	C2	D1	M3	B4	N1	S2	Turkey
18710	8	1	C2	D1	M3	B4	N1	S2	Chicken
18711	8	1	C2	D1	M3	B4	N1	S2	Chicken
18712	8	2	C2	D1	M3	B4	N1	S2	Chicken
18719	>16	8	C2	D1	M3	B4	N1	S2	Chicken
18720	8	9	C2	D1	M3	B4	N1	S2	Chicken
18736	8	1	C2	D1	M3	B4	N1	S2	Chicken
18749	8	1	C2	D1	M3	B4	N1	S2	Chicken
18750	8	2	C2	D1	M3	B4	N1	S2	Chicken
18751	>16	3	C2	D1	M3	B4	N1	S2	Chicken
18754	8	6	C2	D1	M3	B4	N1	S2	Chicken
18461	4	1	C2	D1	M4	B4	N1	S2	Turkey

Table 6 Plasmid hybridization patterns of *E. faecium* in this study

Type	No. of isolates	Restriction enzymes						Sources
		Hinc II	*Hind* III	*Msl* I	*Ban* I	*Nla* III	*Ssp* I	
I	1	C1	D1	M1	B1	N1	S1	Human
II	1	C1	D1	M2	B2	N1	S1	Chicken
III	4	C1	D2	M3	B3	N1	S2	Turkey Chicken
IV	27	C2	D1	M3	B4	N1	S2	Turkey Chicken
V	1	C2	D1	M4	B4	N1	S2	Turkey

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CHAPTER 3

Prevalence of Streptogramin Resistance Genes among *Enterococcus faecium* Isolates Recovered from Poultry Production Environments in the US

Abstract

Quinupristin/dalfopristin, a streptogramin complex, has been approved to treat human infections due to vancomycin-resistant *Enterococcus faecium*. However, the development of streptogramin resistance in enterococci has greatly compromised effectiveness of this antimicrobial agent. In this study, we investigated the prevalence of streptogramin resistance genes among 28 *E. faecium* isolates recovered from poultry products and poultry production environments in Maryland, Georgia and Tennessee. All 28 isolates were highly resistant to streptogramin with Minimal Inhibitory Concentrations (MICs) = 32 μg/ml. The presence of known streptogramin resistance genes (*vatD*, *vatE*, *msrC*, *vgaA*, *vgaB*, *vgbA*, and *vgbB*), as well as the macrolide resistance gene, *ermB*, was examined by PCR in all isolates. The *vatE* gene was detected in 50% (14/28) of the isolates, followed by *ermB* gene in 39.3% (11/28). Nine isolates had both *vatE* and *ermB* genes, whereas none of the eight genes was identified in 11 other isolates. Streptogramin resistance was conjugatively transferable in 20 *E. faecium* isolates. These results indicate that *vatE* and *ermB* are responsible for high streptogramin resistance in most *E. faecium* isolates from poultry products and environments and that the resistance mechanisms within this population remain largely uncharacterized.

Introduction

Enterococci are important nosocomial pathogens. Multiple antimicrobial resistance has severely compromised treatment of patients with enterococcal infections in the US. Quinupristin/dalfopristin, a semisynthetic mixture of the compounds streptogramins A and B, was approved to treat vancomycin-resistant *E. faecium* infection in 1999 by the US Food and Drug Administration (Henney 1999). Virginiamycin, another mixture of streptogramin A and B compounds, has been used in chickens, turkeys, swine, and cattle production for growth promotion for over two decades (McDermott, Cullen et al. 2005). It is also used to prevent necrolic enteritis caused by *Clostridium perfringens* in chickens, to prevent coccidiosis in chickens and turkeys, to treat and control swine dysentery, and to reduce the incidence of liver abscesses in cattle (Hershberger, Donabedian et al. 2004).

There have been numerous reports on the prevalence of Q/D resistance in *E. faecium*, which led to speculation that the use of virginiamycin in animal husbandry might contribute to the emergence of Q/D resistance among human isolates of Gram-positive pathogens (Hammerum, Jensen et al. 1998; Jensen, Hammerum et al. 1998). More significantly, streptogramin-resistant *E. faecium* was isolated from clinical samples before Q/D was introduced for therapeutic purposes in European hospitals (Klare, Konstabel et al. 2003; Hershberger, Donabedian et al. 2004). As a result, the use of virginiamycin in animal production has been suspended in the European Union since July 1999 (Aarestrup, Seyfarth et al. 2001).

The Q/D resistance mechanisms (Figure 8) include: 1. Ribosomal protection by an *erm*-encoded erythromycin-resistance methylase. This mechanism confers *E. faecium* resistance to streptogramin B component; 2. Efflux pump. *vgaA* and *vgaB* genes encode

an ATP-binding transporter to confer resistance to streptogramin A, but *vgaA* and *vgaB* have only been identified in staphylococci. Another efflux pump gene is *msrC*, which is prevalent, but not intrinsic, in *E. faecium*. *msrC* encodes resistance to the streptogramin B component. 3. Enzymatic inactivation. Five acetyltransferases have been shown to inactivate streptogramin A. These enzymes are encoded by *vatA*, *vatB*, and *vatC* in staphylococci, and *vatD* and *vatE* in Enterococci. Inactivation of streptogramin B is due to a lyase, encoded by *vgbA* and *vgbB*, which have only been identified in staphylococci.

Although much has been learned over the last decade about the epidemiology of nosocomial enterococci, additional information on the epidemiology of antimicrobial-resistant *E. faecium* and their resistance mechanisms is still needed. Herein, we investigated the molecular mechanisms of Q/D resistance among *E. faecium* isolates recovered from poultry products and poultry production environments. The transferability of the resistance genes was also studied.

Materials and methods

Bacterial strains.

A total of 28 *E. faecium* isolates were recovered from poultry products and poultry production environments in Maryland, Georgia and Tennessee, including 11 from chicken breast, 10 from ground turkey, and 7 from poultry litter (Table 7).

Determination of antimicrobial susceptibility of enterococci.

Antimicrobial susceptibility of the 28 isolates and their transconjugants were determined using the Sensititre Automated Antimicrobial Susceptibility System (Trek Diagnostic Systems, Westlake, OH) and interpreted according to the National Committee for Clinical Laboratory Standards guidelines for broth microdilution (NCCLS 2003). *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212 were used as quality control strains.

DNA extraction and PCR studies.

Total bacterial DNA was extracted by boiling four to six colonies in 200 µL of sterile water for 10 min in MicroAmp 96-well plates (Applied Biosystems, Foster City, CA). Plates were centrifuged at 2500 rpm for 3 min in a Centra-CL3 centrifuge (Thermo IEC, Needham Heights, MA) and the supernatant was transferred to another plate for storage at -20 ^{o}C. Ten microliters of DNA were used for PCR. Alternatively, plasmid DNA was extracted as described previously (Woodford, Morrison et al 1993) and used as template for PCR. All PCR primers and working conditions are listed in Table 8. Degenerate primers (strep) were used to detect genes likely to encode streptogramin A resistance (Soltani, Beighton et al 2001).

Conjugation experiments.

All 28 Q/D -resistant *E. faecium* isolates were used as donor strains in conjugation experiments to study resistance gene transfer. *E. faecium* GE-1 (rif^r fus^r) was used as the recipient strain. Conjugation was performed by the filter mating method as described previously (Simjee, White et al. 2002) with some modifications. Briefly, overnight cultures of the donor strains grown in BHI (Brain Heart Infusion, Difco, Detroit, MI) broth containing Q/D (4 µg/ml) and recipient grown in BHI with fusidic acid (50 µg/ml) plus rifampicin (100 µg/ml) were mixed (ratio, 1:1) in BHI. The mixture was then collected on a 0.45-µm-pore-size filter and incubated on BHI agar plates (Difco, Detroit, MI) at 37°C overnight. The filter was washed and vortex-mixed in BHI. The mating mixture was spread onto BHI agar containing a combination of Q/D (4 µg/ml), fusidic acid (50 µg/ml) and rifampicin (100 µg/ml). Up to 5 potential transconjugants were purified on BHI agar containing appropriate antibiotics. Conjugation frequencies were expressed as the numbers of transconjugants per recipient cell.

Transposon construction.

1. Generation of the insert. The transposon used in this study was constructed by cloning an erythromycin resistance gene into a transposon construction vector EZ::TN pMOD-2<MCS> (EPICENTRE, Madison, WI). An *erm* gene was amplified from plasmid pAT18 (Trieu-Cuot et al. 1990; Trieu-Cuot et al. 1991) using primers *erm*-F: 5' CGC GGA TCC GAA GCA AAC TTA AGA GTG TGT TGA and *erm*-R: 5' CGG GGT ACC GTA GGC GCT AGG GAC CTC TT). The restriction recognization sites of *BamH*

I and *Kpn* I were added to the 5' end of each primer, respectively. In addition, to facilitate the restriction digestion, an extra 3-base-sequence was added before endonuclease restriction sites (see the nine underlined bases in the primers). PCR conditions included: 94 °C 10min, 35 cycles of 94 °C 1 min, 45 °C 1 min, 72 °C 1 min, and a final extension at 72 °C for 10 min. The desired amplicon of 1193 bp was electrophoresed and visualized under UV light. A sequential restriction digestion of the PCR product with *Kpn*I and *BamH*I was carried out by setting up a *Kpn*I digestion reaction that was incubated at 37 °C overnight followed by a *BamH*I digestion that was incubated at 37 °C for 2 h. The digested *erm* gene fragment was gel purified with a Qiagen gel purification kit (Qiagen, Valencia, CA).

2. Treatment of the transposon construction vector. The transposon construction vector EZ::TN pMOD-2<MCS> was treated using the same procedure for the gene insert. A 2545bp fragment was gel purified with a Qiagen gel purification kit.

3. Cloning of the insert into EZ::TN pMOD-2<MCS>. The ligation reaction was carried out at 25 °C for 2 h. Molarity ratio of insert:vector was 4:1. To avoid recircularized vector from being introduced into a competent *E. coli* recipient, the *SmaI* restriction site present between *Kpn*I and *BamH*I on the multiple cloning site of pMOD-2 was used. After ligation, the mixture was digested by *Sma*I at 25 °C for 2 h before electroporation. An *E. coli* K12 was used as the recipient for electroporation. The electroporation procedure was followed as previously described (Sambrook, 1989). Electroporants were selected on Luria-Bertani agar (Difco, Detroit MI) supplemented with erythromycin (150 µg/ml).

4. Isolation of the transposon. The pMOD-2 with the *erm* gene insert was isolated from *E. coli* K12 using a plasmid mini prep kit (Roche Molecular Biochemicals, Mannheim, Germany). The transposon was amplified with PCR primers provided by the manufacturer (Epicentre). The primer sequences were: FP-1 (5' ATTCAGGCTGCGCAACTGT) and RP-1 (5' GTCAGTGAGCGAGGAAGCGGAAG). PCR conditions were: initial denaturation of 94 °C 2 min, followed by 30 cycles of 94 °C 30 sec, 60 °C 45 sec and 72 °C 90 sec, and a final extension time of 6 min at 72 °C.

Plasmid isolation from Q/D resistant *E. faecium* (CVM 23090)

E. faecium CVM 23090 was recovered from chicken breast and has a Q/D MIC of 32 µg/ml. The Q/D resistance was conjugatively transferable, but no known resistance genes were identified in this strain. Plasmid was isolated from CVM 23090 using a high pure plasmid isolation kit (Roche Molecular Biochemicals) with modifications. Briefly, bacterial culture was obtained by incubating CVM 23090 in brain heart infusion (BHI) broth (Difco) supplemented with Q/D (4 µg/ml) at 37 °C overnight. Lysozyme (10 mg/ml) was added into cell pellet after centrifugation of the overnight culture. The mixture was incubated at 37 °C for 1 h followed by the procedures specified in the manufacturer's manual.

Electroporation of *Enterococcus faecalis* TX5332

E. faecalis TX5332 (Singh, 2005) is an *lsa* (lincosamide and streptogramin A resistance) disruption mutant of *E. faecalis* OG1RF (plasmid free). The mutant carries a kanamycin resistance marker. The MIC of TX5332 to Q/D is 0.75 µg/ml (breakpoint 4 µg/ml).

Electrocompetent TX5332 was made by the following procedures: An overnight culture of TX5332 was prepared by inoculating 5 ml of M17 medium supplemented with kanamycin (2000 µg/ml) from a single colony. The overnight culture was diluted 1:40 into 200 ml fresh M17 medium and incubated until culture OD_{600} reached 0.4-0.6. Cells were pelleted at 6000 rpm for 10 min at 4 ^{o}C and resuspended in 10 aliquots of 1ml ice cold 10% glycerol. Cells were centrifuged at 13000 rpm for 1 min at 4 ^{o}C and resuspended in 500 µl of lysozyme solution (25 µg/ml of lysozyme dissolved in 10mM Tris pH8.0, 20% sucrose, 10mM EDTA and 50mM NaCl). The mixture was incubated at 37 ^{o}C for 20 min followed by 3 washing steps with 1 ml ice cold electroporation buffer (0.5M sucrose and 10% glycerol). After the final washing step, cell pellet was resuspended 200 µl of electroporation buffer/tube and split into 40 µl aliquots before storing at -80 ^{o}C. Electroporation was carried out with a Gene Pulser (Bio-Rad). The electroporation parameters were: 16 kV/cm, 200O, 25 µF, and the time constant between 4.0 and 4.7 milliseconds. Immediately after the electroporation, the cell suspension was mixed with 0.96 ml of ice cold SGM17MC (M17 supplemented with 10% sucrose, 20mM $MgCl_2$ and 2mM $CaCl_2$) and incubated at 37 ^{o}C for 2 h. Finally, 100 µl aliquots of the transformed cells were spread on selective M17 agar with kanamycin (2000 µg/ml) and Q/D (4 µg/ml) added and incubated at 37 ^{o}C up to 48 h. Plasmid from the electroporants were isolated and used for transposon insertion reactions.

Transposon insertion reactions

A transposon insertion reaction was set up with the plasmid and the EZ::TN Transposase (Epicentre, Madison, WI) following the manufacturer's instructions. The reaction was

incubated at 37 °C for 2 h followed by adding 1µl EZ::TN 10× Stop solution that was heated for 10 min at 70 °C. The amount of 1 µl of the transposition mixture was used for electroporation into TX 5332 and the electroporated cells were plated on selective M17 agar with erythromycin (150 µg/ml) and kanamycin (2000 µg/ml) added and incubated at 37 °C up to 48 h.

Replica plating for selection of clones carrying novel Q/D resistance determinants.

Colonies grown on selective agar plates after transpon insertion reaction were replica plated on M17 agar with erythromycin (150 µg/ml) and kanamycin (2000 µg/ml) supplemented, and on M17 agar with erythromycin (150 µg/ml), kanamycin (2000 µg/ml), and Q/D (4 µg/ml) supplemented. The colonies that were grown on the plate without Q/D but failed to grow on the plate with Q/D added were picked and streaked to purify. The target Q/D resistance determinant was amplified and sequenced using the primers provided by the transposon construction vector kit (Epicentre). The process of the *in vitro* transposon mutagenesis used in this study is shown in Figure 6.

Results and discussion

Antimicrobial susceptibility profile

Multiple antimocribial-resistant *E. faecium* was identified (Table 7, Figure 7). All 28 *E. faecium* isolates showed high Q/D resistance with MICs = 32 µg/ml. Erythromycin resistance was found in 53.6% (15/28) of the isolates. All but one strain were resistant to tetracycline. The percentages of resistance to penicillin and ciprofloxacin were 60.7% (17/28) and 28.6% (8/28), respectively.

Quinupristin/dalfopristin resistance gene identification

Q/D is a mixture of the semi-synthetic streptogramin A and B compounds. Only resistance to the A component is required for streptogramin resistance; however, resistance to both A and B components may result in higher level of resistance (el Solh, Fouace et al. 1980). *vatD* and *vatE* confer resistance to streptogramins by enzymatic inactivation of the streptogramin A component, whereas the resistance mechanisms of *vgaA* and *vgaB* are due to their active efflux of the streptogramin A component. To date, *vgaA* and *vgaB* have only been reported in staphylococci (Allignet, Loncle et al. 1993; Allignet and El Solh 1997). Ribosomal target modification, however, is the most common mode of streptogramin resistance (Johnston, Mukhtar et al. 2002). An *erm* (erythromycin-resistance methylase) gene product can cause a conformational change in the ribosome and result in cross-resistance to macrolides, lincosamides, and the streptogramin B component (MLS_B). *vgbA* and *vgbB* confer resistance to streptogramin B by the mode of enzymatic inactivation. *msrC* is prevalent in *E. faecium* and encodes an

efflux pump. The resistance mechanism by *msrC* is also due to its inactivation of the streptogramin B component (Simjee, White et al. 2002).

Our initial attempt to amplify the above eight known resistance genes was unsuccessful by using total DNA as the template for PCR. When plasmid DNA was extracted and used as PCR template, *vatE* was identified in 50% of the *E. faecium*. In addition, *ermB* was detected in 39.3% of the isolates. However, we were unable to detect *vatD* or any of the other streptogramin resistance genes by PCR. Degenerate PCR was performed to test for the streptogramin A resistance gene in isolates without any known resistance gene detected, but the gene was not found. The presence of *vatE* in half of our strains explained the high MICs to streptogramins. *vatE* has long been known to be carried on plasmids, which is of greater public health concern, since plasmids can transfer to human pathogens or to the resident human microflora.

Transferability of antimicrobial resistance

Twenty isolates were able to transfer their Q/D resistance after conjugation with the frequencies ranging from 10^{-8} to 10^{-5} transconjugants/recipient. This indicated that some streptogramin resistance determinants were carried on transferable elements, such as self-transferable plasmids or conjugative transposons.

In addition to Q/D resistance, all the transconjugants had tetracycline susceptibility profiles identical to the respective donor strains. Erythromycin resistance from seven *E. faecium* isolates transferred to the recipient cell after conjugation and the transconjugants showed identical MIC levels to erythromycin as their donor strains. We were also able to detect the presence of *vatE* and *ermB* genes in the seven transconjugants by PCR amplification. Resistance to other antimicrobial agents varied between the

transconjugants examined. Neither the *vatE* nor *ermB* gene was detected in transconjugants from three strains that had either *vatE* or *ermB* gene.

A study carried out in Denmark (Jensen 2000) showed a linkage of *vatE* and *ermB* in 74% of streptogramin-resistant *E. faecium* in Europe. In our study, 32% (9/28) of the *E. faecium* isolates had both *vatE* and *ermB*. Out of the nine strains, seven *E. faecium* were able to transfer both *vatE* and *ermB* to the recipient cell after conjugation. The other two strains failed to transfer their resistance by conjugation. The *vatE-ermB* linkage was identified in one of the seven strains by amplifying the highly conserved genetic element that contains both *ermB* and *vatE* and mediates resistance to streptogramins and macrolides.

Based on the transferability of the Q/D resistance, together with the fact that gene was amplified from plasmid DNA, it is possible that some *E. faecium* isolates had resistance genes carried on self-transferable plasmids, whereas some resistance genes were present on non-conjugative or non-mobile elements.

The failure to detect any known streptogramin resistance genes in 12 *E. faecium* in our study suggests that novel genes may be responsible for resistance to the streptogramin A component. The resistance was conjugatively transferable in nine strains, suggesting the resistance determinant was carried on either a conjugative plasmid or transposon. Our study also revealed three strains that had streptogramin resistance determinants present on non-conjugative or non-mobile elements. Although active efflux of streptogramin A has only been found in streptococci, it is possible that a staphylococcal homologue is present in the *E. faecium* isolates.

Exploration of novel resistance determinant(s)

Transposon mutagenesis is a useful molecular tool to randomly insert into DNA targets, and therefore, to make insertion mutants. It has been widely used in both Gram-positive and Gram-negative microorganisms. However, there are mainly two obstacles in *E. faecium* that make transposon mutagenesis not as useful in *E. faecium* as in many other bacteria. First, many *E. faecium* strains, if not all, are not transformable by electroporation or the transformation efficiency is very low. In addition, *E. faecium* is notoriously resistant to multiple antimicrobial agents, which makes a lot of the antibiotic resistance markers carried on transposon vectors useless for genetic studies. We bypassed the electroporation by doing an *in vitro* transposon mutagenesis involving incubating transposons and the plasmid that carries Q/D resistance determinant followed by introducing the transposon inserted plasmid into a Q/D susceptible *E. faecalis* mutant (TX5332). Also, an erythromycin resistance marker, to which the *E. faecium* strain (CVM23090) was susceptible, was cloned into our transposon isolated from a transposon construction vector EZ::TN pMOD-2.

Selection of clones carrying potential resistance gene was not successful in our study due to poor electroporation efficiency using the plasmid from CVM23090. Since a control plasmid pAT18 was successfully transformed into the recipient strain TX5332 (data not shown), the plasmid from the CVM23090 may not be suitable for electroporation or the recipient itself is not an ideal one for this plasmid. So, an alternative to identify the resistance determinant(s) would be to construct a plasmid library and perform DNA sequencing. Or, an *lsa* (lincosamide and streptogramin A resistance) disruption mutant of *E. faecalis* JH2-2 can be made as specified for the construction of *E. faecalis* OG1RF mutant TX5332 (Davis et al, 2001; Singh and Murray,

2005) and used as the recipient for electroporation, because we observed a higher electroporation rate with JH2-2 than OG1RF (data not shown).

In summary, a high prevalence of *vatE* was detected from Q/D-resistant *E. faecium* with high MICs. The data also suggest that *E. faecium* from poultry may serve as a reservoir of multiple resistance genes. Continuing measures are needed to encourage judicious use of antimicrobials and to prevent the spread of resistant pathogens and genes in animal production environments.

Figure 6 Flow chart of *in vitro* transposon mutagenesis used in this study

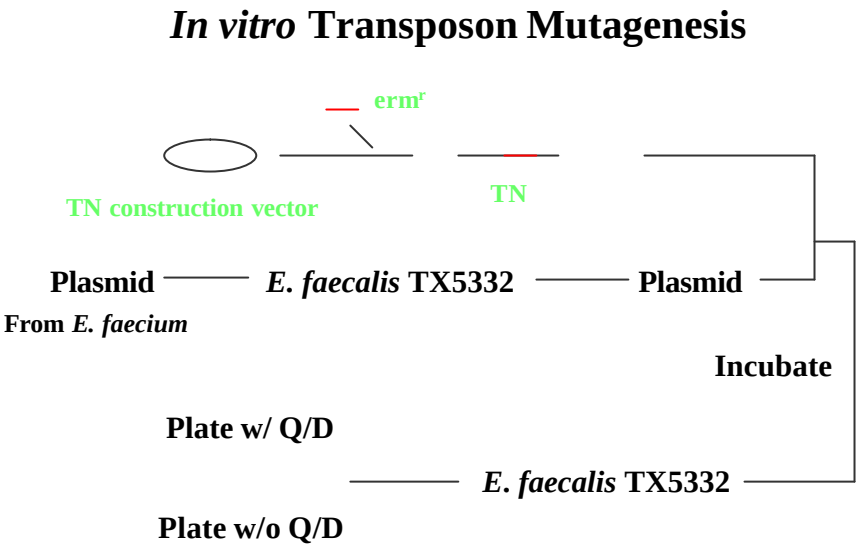

Figure 7 Multidrug-resistant *E. faecium* recovered in this study

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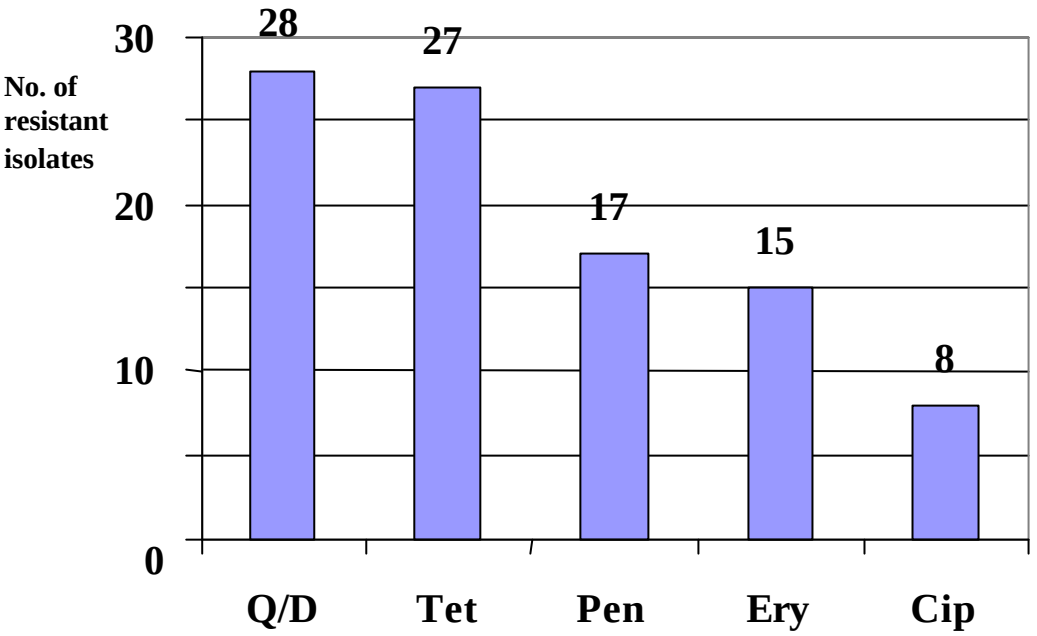

Figure 8 Mechanisms of Q/D resistance in bacteria

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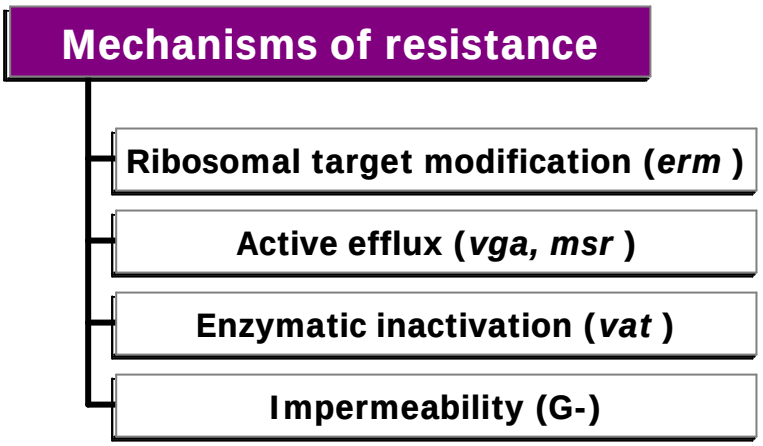

Table 7 Streptogramin resistance gene distribution among *E. faecium* strains in this study#.

Isolate ID	Source	State	*vatE*	*ermB*	Conjugation	STR*	ERM*	TET*	PEN*	CIP*
19924	Chicken breast	MD	+	+	+	32	>8	>32	4	1
19930	Ground turkey	MD	-	-	+	32	=0.5	>32	4	4
19962	Chicken breast	MD	+	+	+	32	>8	>32	8	1
19963	Chicken breast	MD	+	+	+	32	>8	>32	4	1
19965	Chicken breast	MD	+	+	+	32	>8	>32	4	1
19975	Ground turkey	MD	+	-	-	32	>8	>32	>16	0.5
20001	Chicken breast	MD	+	+	-	32	>8	>32	8	2
20527	Ground turkey	TN	-	-	+	32	=0.5	>32	16	4
20565	Ground turkey	TN	-	-	-	32	=0.5	>32	>16	4
20993	Chicken breast	GA	-	-	+	32	2	>32	4	2
21164	Chicken breast	MD	-	+	-	32	>8	>32	16	1
21200	Chicken breast	MD	-	-	+	32	=0.5	>32	>16	2
21209	Ground turkey	MD	+	+	-	32	>8	>32	16	4
21212	Ground turkey	MD	+	-	+	32	4	>32	16	2
21242	Ground turkey	MD	+	-	-	32	>8	>32	>16	2
21244	Ground turkey	MD	+	-	+	32	4	>32	>16	2
21245	Ground turkey	MD	+	+	+	32	>8	>32	16	2
22319	Chicken breast	GA	+	+	+	32	>8	>32	4	1
23090	Chicken breast	MD	-	-	+	32	2	>32	8	2
23105	Ground turkey	MD	-	+	+	32	>8	>32	16	4
23485	Chicken breast	TN	+	+	+	32	>8	>32	2	1
127	Litter		-	-	+	32	1	>32	16	4
540	Litter		-	-	+	32	8	32	16	2
551	Litter		+	-	+	>32	8	>32	8	2
556	Litter		-	-	-	32	1	>32	32	4
2000	Litter		-	-	+	>32	0.12	32	32	2
2019	Litter		-	-	-	32	1	0.5	32	4
2763	Litter		-	-	+	32	1	>32	16	2

Numbers in colors are resistance MICs.

* Breakpoints: streptogramin 4 μ g/ml, erythromycin 8 μ g/ml, tetracycline 16 μ g/ml, penicillin 16 μg/ml, ciprofloxacin 4 μg/ml.

Table 8 Primer sequences and PCR conditions used for streptogramin resistance genes

Primer	Sequence (5'-3')	Product size	PCR conditions	Reference
vatD	F: GCT CAA TAG GAC CAG GTG TA R: TCC AGC TAA CAT GTA TGG CG	271 bp	94 °C 10min; 35 cycles of 94 °C 1 min, 55 °C 1 min and 72 °C 1 min; 72 °C 10min.	Soltani, 2001
vatE	F: ACT ATA CCT GAC GCA AAT GC R: GGT TCA AAT CTT GGT CCG	511 bp	94 °C 10min; 30 cycles of 94 °C 25s, 52 °C 40s and 72 °C 50s; 72 °C 6min.	Soltani, 2001
msrC	F: TAT AAC AAA CCT GCA AGT TC R: CTT CAA TTA GTC GAT CCA TA	410 bp	94 °C 10min; 35 cycles of 94 °C 1 min, 52 °C 1 min and 72 °C 1 min; 72 °C 10min.	Hayes, 2005
vgaA	F: AGT GGT GGT GAA GTA ACA CG R: CTT GTC TCC TCC GCG AAT AC	659 bp	94 °C 10min; 35 cycles of 94 °C 1 min, 55 °C 1 min and 72 °C 1 min; 72 °C 10min.	Soltani, 2001
vgaB	F: TGA CAA TAT GAG TGG TGG TG R: GCG ACC ATG AAA TTG CTC TC	576 bp	94 °C 10min; 35 cycles of 94 °C 1 min, 52 °C 1 min and 72 °C 1 min; 72 °C 10min.	Soltani, 2001
vgbA	F: TAC AGA GTA CCC ACT ACC GA R: TCA ATT CCT GCT CCA GCA GT	569 bp	94 °C 10min; 35 cycles of 94 °C 1 min, 52 °C 1 min and 72 °C 1 min; 72 °C 10min.	Soltani, 2001
vgbB	F: CAG CAG TCT AGA TCA GAG TGG R: CAT ACG GAT CCA TCT TTT CC	728 bp	94 °C 10min, 60 °C 2 min; 30 cycles of 72 °C 20s, 95 °C 20s and 60 °C 20s; 72 °C 1 min.	Soltani, 2001
ermB	F: CAT TTA ACG ACG AAA CTG GC R: GGA ACA TCT GTG GTA TGG CG	424 bp	94 °C 10min; 25 cycles of 94 °C 1 min, 52 °C 1 min and 72 °C 1 min; 72 °C 10min.	Soltani, 2001
strep	F: ATHATGAAYGGIGCIAAYCAYMGIATG R: ICCDATCCAIACRTCRTTICC	150 bp	95 °C 5min; 35 cycles of 40 °C 2 min, 72 °C 90s and 95 °C 30s; 40 °C 4min, 72 °C 12min.	Soltani, 2001

SUMMARY

Both *L. monocytogenes* and *E. faecium* are ubiquitous in the environment, which gives them opportunities to enter the food chain. Despite the fact that *L. monocytogenes* has been thought to be susceptible to most antimicrobial agents used in the clinical environment, multiresistant strain of *L. monocytogenes* has emerged since 1988 when the first antimicrobial-resistant *L. monocytogenes* strain was identified in France. The most important clinical feature of enterococcal infections is the high prevalence of resistance to numerous antimicrobials. Enterococci are known to be intrinsically resistant or tolerant to many antimicrobials, with *E. faecium* more often resistant than *E. faecalis*. The research described in this dissertation focused on antimicrobial resistance of *L. monocytogenes* and *E. faecium* from food and animal sources. One hypothesis examined is that the use of antimicrobial agents in the agricultural environment selects for resistant isolates of *L. monocytogenes* and *E. faecium*. We tested this hypothesis by performing:

I. Characterization of *L. monocytogenes* isolated from retail foods and investigation of their antimicrobial susceptibility profiles (chapter 1).

II. Characterization of *E. faecium* isolated from poultry products and poultry production environment (chapters 2 and 3).

In terms of *E. faecium*, with the development of the resistance to a newly approved antimicrobial agent (quinupristin/dalfopristin), in-depth research is needed to study the resistance mechanisms as well as the genetic background of resistance gene. That has been the driving force ofour following studies on *E. faecium*:

III. Study of the genetic background of the streptogramin resistance gene in *E. faecium* isolated from poultry production environment (chapter 2).

IV. Study of the prevalence of the streptogramin resistance determinants in *E. faecium* with high MICs and exploration of novel streptogramin resistance genes in *E. faecium* (chapter 3).

In chapter 1, 167 *Listeria monocytogenes* isolates obtained from retail deli meats, raw chicken and fresh produce were characterized by PFGE, serogrouping, and antimicrobial susceptibility testing. The data indicate the great diversity of *L. monocytogenes* and the presence of antimicrobial-resistant *L. monocytogenes* strains in food supply, including organic food products. *L. monocytogenes* is cpable of acquiring antimicrobial resistance genes from foreign sources through movable genetic elements such as transposons and plasmids. The common sources of resistance genes for *L. monocytogenes* appear to be enterococci and streptococci. Emergence and dissemination of antimicrobial resistance in *L. monocytogenes* may have significant future clinical implications for the treatment of listeriosis.

The overall incidence of serotype 4b in clinical cases is higher than that of other serotypes of *L. monocytogenes*, whereas serotype 1/2a is the leading serotype recovered from food samples. The discrepancy may suggest that strains of serotype 4b are more virulent to humans than other serotypes, although other possibilities, including transmission by routes other than contaminated foods, cannot be excluded.

Whereas the transmission of enterococci in the clinical environment stems from fecal contamination of hospital surfaces, the most common vehicle of exposure of healthy

persons is thought to be the consumption of contaminated food. Public concern over the use of the streptogramin antimicrobial virginiamycin in poultry and other food animal production environments following the approval of Q/D to treat resistant human *E. faecium* infections underscores the importantce in understanding the genetic background behind the phenotypic resistance observed to streptogramin antimicrobials.

In chapter 2, 34 Q/D-resistant *E. faecium* isolates (33 from 7 poultry farms and 1 from an outpatient) were characterized by pulsed-field gel electrophoresis (PFGE) and the *vat*(E) carrying plasmids from all isolates were further typed by southern hybridization. Non-human sources have been increasingly suspected as a revervoir for some antimicrobial-resistant bacteria. In our study, the *E. faecium* from human was clonally distinguishable from the isolates from poultry farms, which was not surprising in the genus of *Enterococcus*, because there has been no report so far on transmission of *Enterococcus* spp. from food animals to human patients. However, poultry farms still serve as reservoir for resistance genes and the genes have potential to spread to other bacterial species or genera. Southern blot is a useful molecular tool to characterize the genetic background of the antimicrobial resistance genes due to some advantages to this application, such as time, cost and availability. But regarding the accuracy of the data, southern blot can never compete with DNA sequencing. The purpose of the study, as well as time and cost should always be considered to choose an efficient experimental method.

To our knowledge, the present study is the first report of a *vat*(E) carrying plasmid recovered from a human *E. faecium* isolate in the US. The plasmid background was much similar to that from a chicken farm isolate based on plasmid typing study. We could assume that the plasmid entered the food chain via chicken carcass and eventually to the

consumer. In a follow up study, we were no longer able to recover that strain from the patient suggesting a transient colonization of the resistant strain in the human gut and that, more importantly, the chicken strain did not transfer the plasmid to a human commensal enterococci. All these observations are very important for development of a risk assessment model to quantify the public health risk attributable to the use of streptogramin in food producing animals. The MIC for human isolate was extremely high (> 32µ g/ml), while the MIC for chicken farm isolate was 16 µg/ml. We could therefore assume that human isolate also acquired other mechanisms responsible for streptogramin resistance other than *vatE* gene from a different source.

In chapter 3, we investigated the antimicrobial resistance profiles of the *E. faecium* isolates recovered from poultry products and poultry production environment in Maryland, Georgia and Tennessee. The work demonstrated the magnitude of resistance to a number of antimicrobials that are used in clinical medicine or in agriculture. Resistance to Q/D, erythromycin, tetracycline, penicillin, and ciprofloxacin was observed. An increased prevalence of Q/D resistance is not unexpected given the length of time that the analogue virginiamycin has been used in poultry production. No vancomycin resistance was found strengthening the argument that enterococcal resistance to vancomycin in the clinical environment is due to the used of vancomycin in that environment.

Our work also examined all known streptogramin resistance mechanisms that have been described to date. This information is critical to begin to evaluate the potential risk that the use of related antimicrobials in animal agriculture poses to the treatment of disease among the human population. It was also demonstrated in this work that a significant proportion possess uncharacterized mechanisms of resistance.

Future work should continue to investigate the mechanisms behind the large degree of uncharacterized ressitance to streptogramin antimicrobials. Molecular technologies to monitor antimicrobial resistance will be critical tools to monitor the epidemiology of specific resistance elements.

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