Journal of Animal and Veterinsry Advances 5 (10): 855-862, 2006
© Medwell Online, 2006

Distinguishing Closely-Related Streptogramin-Resistant *Enterococcus faecium*: SNPs in One Specific 16-23S rRNA Intergenic Region

[1]Shane C. Burgess, [1]Timmothy S. Cummings, [2]Anneke M.T. Steegh, [1]Larry A. Hanson,
[3]Donna C. Sullivan[c] and [3]Rathel L. Nolan
[1]Department of Basic Sciences, College of Veterinary Medicine,
Mississippi State University, Box 6100, MS 39762-6100, USA
[2] Department of Applied Sciences, Fontys University of Professional Education,
Biology and Medical Laboratory Science. The Netherlands
[3]Division of Infectious Diseases, University of Mississippi Medical Center,
2500 North State Street, Jackson, MS 39216, USA

Abstract: Human infections caused by Enterococcus species are increasing; they are the third most common cause of nosocomial infections. *E. faecium,* though a relatively rare Enterococcal nosocomial infection, is very commonly resistant to multiple antibiotics. Quinupristin/Dalfopristin, a Streptogramin, is an antibiotic of "last resort" for treating multi-antibiotic-resistant *E. faecium.* However, *E. faecium* isolates are now becoming resistant to Quinupristin/Dalfopristin. The source of Streptogramin-resistant *E. Faecium* is controversial. Although medical environments are a potential source, Streptogramin-resistant Enterocci are present in livestock fed Streptogramin growth-promoters. However, *E. faecium* subspecies are suggested to be predominantly host-species specific and thus livestock may not be a reservoir for Streptogramin-resistant *E. faecium* that infect humans. To resolve this issue, sensitive methods to quantify Streptogramin-resistant *E. faecium* relatedness are essential. We designed a rapid sensitive, specific, molecular diagnostic technique, targeting one *E. faecium* 16S/23S intergenic spacer region to distinguish between *E. faecium* at the nucleotide level. This region has no obvious evolutionary-conservation pressure and has been used to quantify relatedness in other bacteria. We analyzed streptogramin-resistant *E. faecium* from chickens from one grower house, on one farm, to quantify nucleotide polymorphism between isolates that would be expected to be closely related. We detected single nucleotide polymorphisms in four out of ten Streptogramin-resistant *E. faecium* isolated from this single source. The chicken-derived *E. faecium* sequences were more similar to each other than they were to the reference human *E. faecium* strain. Our method will be useful for studies of *E. faecium* ecology, pathogenicity and antibiotic resistance.

Key words: Sequence analysis, single nucleotide polymorphism, antibiotic resistance

INTRODUCTION

Over the last twenty years the number of nosocomial infections due to *Enterococcus* species has increased. *Enterococci* are the third most common cause of nosocomial infections after *Staphylococcus* and *Escherichia coli*[1]. Initially, most human Enterococcal infections were successfully treated with antibiotics. Predictably in response, however, Enterococcal antibiotic-resistance is increasingly common. Data collected between 1995 and 1997 from over 15,000 Enterococcus isolates showed less than 2% of the *Enterococcus faecalis* isoaltes were resistant to ampicillin and vancomycin, whereas most *E. faecium* isolates were resistant to Ampicillin (83%) and Vancomycin (52%)[2-4]. Most concerning is Enterococcal resistance to one of the last resort streptogramin antibiotics, quinupristin/dalfopristin[1,5-11]. Although *E. faecalis* is the most common cause of human Enterococcal infections (79%), streptogramin-resistance more often occurs in *E. faecium* infections[7,12]. The source of and/or reservoir for Streptogramin antibiotic-resistant *E. faecium* is controversial.

Before 1990, streptogramin-resistant *E. faecium* were thought to be present only in hospitals where streptogramin antibiotics had been used for many years. Notably though, streptogramin-resistant Enterococci are also present in livestock fed the streptogramin growth-promoting antibiotic virginiamycin. Virginiamycin is commonly used in livestock feed in the USA. The use

Corresponding Author: Shane C. Burgess, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Box 6100, MS 39762-6100, USA

of virginiamycin in poultry feed, in particular is suggested to directly threaten human health[9,13-18]. Certainly streptogramin-resistant *E. faecium* have been isolated from poultry and poultry products[9,13,14,16,18-20].

However, *E. faecium* subspecies are suggested, after analysis by Amplified Fragment Length Polymorphism (AFLP), to be predominantly host-species specific[21]. Therefore, regardless of antibiotic-resistance, the *E. faecium* that colonize human gastro-intestinal tracts could be different from those *E. faecium* in livestock. The potential corollary is that steptogramin-resistant *E. faecium* livestock subspecies could be no threat to human health. The question remains, do *E. faecium* and/or streptogramin-resistant *E. faecium* transfer from livestock to humans? To resolve these issues sensitive diagnostic methods, which can quantify *E. faecium* relatedness, are needed for epidemiological studies.

Unfortunately neither AFLP[17,21], Pulse Field Electrophoresis (PFGE)[22] nor ribotyping[23], which have all been used to study the epidemiology of *E. faecium* previously, sensitively quantify the relatedness of bacterial isolates at the single nucleotide level. Our primary aim was to design a rapid, sensitive, specific and high through-put molecular diagnostic tool to distinguish between *E. faecium* at the nucleotide level. Our secondary aim was to compare closely related streptogrammin-resistant *E. faecium* isolates to each other and to the reference *E. faecium* TX16[24,25], that has had its genome sequenced.

Poultry, especially in rural areas of intensive poultry production, are an excellent model for testing both the host-species specificity of *E. faecium* and also of quantifying the risk that livestock fed Streptogramins pose to humans. We collected *E. faecium* isolates from a commercial poultry facility that had used Virginiomycin in the production cycle in Mississippi (USA). Only those isolates that were streptogrammin-resistant were analyzed. We sampled chickens from one grower house on one farm because we wanted to quantify nucleotide polymorphism between isolates that would be expected to be closely related. We targeted one *E. faecium* 16S/23S intergenic spacer region. This region has no obvious evolutionary-conservation pressure and has been used to quantify relatedness in other bacteria[26,27]. In *E. faecium* isolated from this single chicken flock we were able to detect Single Nucleotide Polymorphisms (SNPs) in this one 16S/23S intergenic spacer region. Notably, all chicken-derived *E. faecium* sequences were more similar to each other than they were to the genome-sequenced reference human *E. faecium* strain TX16. Our method should be a valuable addition to future studies on molecular epidemiology of *E. faecium* ecology, pathogenicity and antibiotic resistance.

MATERIALS AND METHODS

Chickens: We sampled 50 randomly-selected commercial broiler chickens immediately post mortem (Cobb 500; 45 days-of-age i.e. end of the production cycle) derived from a single commercial broiler house. During the production cycle the chickens were housed on built-up pine shaving litter and had been fed three diets, each with an antibiotic growth promoter (starter diet, bacitracin, hatch until 18 days-of-age; grower diet, bacitracin, 19 to 32 days-of-age; withdrawal diet, virginomycin, 10g $Ton1^{-1}$, 33 to 45 days-of-age). The chickens had also been fed the ionophore coccidiostat Salinomycin (Alpharma, Fort Lee, NJ; starter diet 45 g/ton; grower diet 60 g ton^{-1}) . Nipple drinkers (1 nipple:12 chickens) provided water ad libertum to the chickens.

Streptogramin-resistant E. faecium isolation: Sterile rayon swabs (FoodTechSource™, Vernon Hills, IL) were used to take samples directly from each chicken's cloaca. Immediately after sampling, each swab was used to inoculate a tube of selective enterococcal broth as described[9]. Selective Enterococcal broth consists of bile esculin azide broth with quinupristin/dalfopristin (4 μg mL^{-1}) and ampicillin (2 μg mL^{-1}). Ampicillin makes the broth more selective for *E. faecium* than for E. faecalis. The broths were returned to the University of Mississippi Medical Centre and incubated for 48 h at 37°C. Ten il of each broth was then subcultured in modified Ford's agar antibiotic quinupristin/dalfopristin (4 μg mL^{-1}) and ampicillin (2 μg mL^{-1}). After 48 h incubation at 37°C, all colonies that were morphologically typical of *E. faecium* were Gram stained and spot-tested with pyrrolidonyl arylamidase reagent to determine whether they were Enterococci. Susceptibility to quinupristin/dalfopristin was determined by E-test and Kirby-Bauer disc susceptibility testing following NCCLS criteria (Wayne, PA, USA).

Ten chickens had quinupristin/dalfopristin-resistant *E. faecium*. One colony from each chicken was picked using a sterile tooth pick and cultured in selective Enterococcal broth (1 mL; quinupristin/dalfopristin [4 μg mL^{-1}], ampicillin [2 μg mL^{-1}]; 48 h, 37°C). These cultures were then centrifuged (1000g) the supernatants removed, the *E. faecium* pellets frozen (-70°C) and then shipped to the College of Veterinary Medicine, Mississippi State University on dry ice.

DNA isolation: DNA was first isolated, using Bactozol™ exactly as described by the manufacturer (Molecular Research Center Inc., Cincinnati, OH), from one culture after scraping a sample of the frozen pellet using a sterile pastette,. This DNA was used for PCR optimization and

test sequencing. However, because our aim was to make the assay rapid and suitable for a high through-put molecular epidemiology, we next isolated DNA by adding scrapings (~50 uL) of the frozen cultures to 100ul Milli-Q H_2O heating to 98°C, 30 min. The samples were centrifuged (1,000g; 5 minutes) and the supernatant removed to a fresh tube and ethanol precipitated (500 uL, 100% EtOH, 4°C, 1 h, centrifuged 10,000 g, 10 min). The pelletes were then washed twice using 70% EtOH and air dryed before resuspending in 50 µL Milli-Q H_2O.

Target sequence identification: The 16S and 23S rRNA DNA sequences are highly conserved between bacterial species; within species they are identical. In contrast, the intergenic regions between the16S and 23S rRNA genes, with the exception of the two specific tRNA genes, have no known evolutionary pressure to maintain sequence conservation. *E. faecium* has multiple 16S and 23S rRNA genes. To identify the16S rRNA and 23S rRNA encoding DNA regions of *E. faecium*, we first derived core consensus 16S rRNA and 23S rRNA DNA sequences, using CLUSTAL-W[28], from representative bacterial 16S rRNA and 23S rRNA DNA sequences (*Acetobacter*, *Aeromonas*, *Escherichia*, *Shigella*, *Salmonella*, *Vibrio* species). We then Blast[29] searched the minimally-annotated *E. faecium* geneome sequence (E. Faecium TX16[24,25]GenBank ID: AAAK00000000, US Department of Energy Joint Genome Institute, http://www.jgi.doe.gov/JGI_microbial/html/index.html) using our derived consensus 16S rRNA and 23S rRNA DNA sequences. We arbritraily chose one intergenic region that contained tRNA-isoleucine and tRNA-alanine genes. These tRNA genes were identified by BLAST searching the *E. faecium* 16S-23S intergenic sequence that we had chosen against Baccilli genome sequences (US National Center for Biotechnology Information).

Polymerase chain reaction: Because it was crucial for us to amplify only one and the same 16S-23S intergenic region, two sets of primers were designed. Primer set 1 (16SFOR, TAGTAGCTTAACCTTCGG; 16SREV, ATGGTGGGTTAGAGCAGC; predicted amplicon 401 bp) was designed to be complimentary to part of the 16S rRNA gene sequence and the tRNA-isoleucine gene. Primer set 2 (23SFOR, ATATGGTGGAGCTATGC and 23SREVTACTTCCTGCACGATTAGACG; predicted amplicon 414 bp) was designed to be complimentary to part of the 23S rRNA gene sequence and the tRNA-alanine gene. When used in the PCR, these primer sets amplified two overlapping amplicons (predicted total length 611 bp Fig. 1a). Together these amplicons allowed

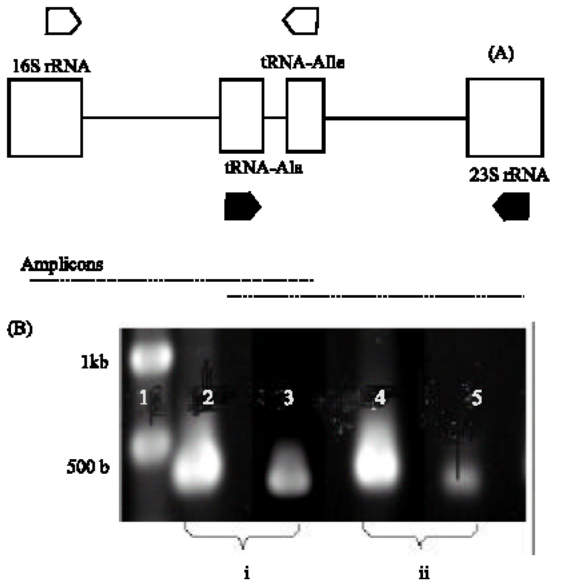

Fig. 1: a. Schematic of targeted *E. faecium* 16S-23S region PCR. PCR primer oligonucleotides were designed to produce overlapping amplicons. B. Image from agarose gel of PCR amplicons. Template DNA produced using (i) Batozol™ (Molecular Research Center Inc., Cincinnati, OH) and (ii) one example of the hot-water DNA preparation (see materials and methods) were PCR amplified with similar efficiently and specifically. Lane 1 = DNA ladder (New England Biolabs, Beverly, MA); lanes 2 and 4 = "primer set 2", which primes amplification of the tRNA-Ala – 23SrRNA amplicon; lanes 3 and 5 = "primer set 1", which primes amplification of the 16SrRNA – tRNA-Ile amplicon

us to sequence one specific 16S-23S intergenic (non-coding) region of 395 bp (Fig. 2).

A 96-well Mastercycler Gradient Thermocycler (Brinkmann Instruments, Westbury, NY) and high fidelity Taq polymerase (Expand High Fidelity PLUS PCR System, Roche Diagnostics Corporation, Indianapolis, IN) were used throughout. All reactions were 25 µL and used 1 uL of the template DNA. The PCR conditions were first optimized, using the DNA extracted using Bactozol™ to (a) Primer set 1: 94°C 5 min +35 (94°C, 1 min; 52°C, 30 sec, 74°C 1min) + 72°C, 10 min, Mg^{2+} 50 mM and (b) Primer set 2: 94°C 5 min +35 (94°C, 1 min; 72°C, 30 sec, 74°C 1 min) + 72°C 10 min, Mg^{2+} 50 mM. Six uL of each PCR was run on an agarose gel (1.2%, Promega Biosciences Inc., San Luis Obispo, CA), stained using Gelstar (BioWhittaker Molecular Applications, Rockland, ME) and visualized using a Chemi 5500 gel-documentation-system (Alpha Innotech Corporation, San Leandro, CA). The PCR

A.

TAGTAGCTTAACCTTCGGCCGTGTACGCTTAGTCACTTAACCTCACAACCCGA
AGGTGTTTCACTTCGTGCTGTAAGCATTTGAGAGACTCTCGGATCACTTTATT
AAAAGCAATCCGAGTGTTTTCGAATTTTCAGCTTGTTCCAGATTGTTAAAGAG
CAAAATATTTCACAACATGCTGATTTCTCAGTATGTTCTGAAATATGGTGGAG
CTATGCGGGATCGAACCGCAGACCTCCTGCGTGCAAAGCAGGCGCTCTCCCA
GCTGAGCTATAGCCCCATGACTTTACTGCAATACCTTATTACCACCGTCCGCT
TCACACAGGAAGGGAGTTGGTAGGCCTGAGTGGACTCGAACCACCGACTTCA
CCCTTATCAGGGGTGCTGCTCTAACCCACCAT

B.

ATATGGTGGAGCTATGCGGGATCGAACCNGCAGACCTCCTGCGTGCAAAGCA
GGCGCTCTCCCAGCTAGAGCTATAGCCCCATGACTTTACTGCAATACCTTATT
ACCACCGTCCGCTTCACATCAGGCAAGGGAGTTGGTAGGCCTGAGTGGACTC
GAACCACCGACTTCACCCTTATCAGGGGTGCTGCTCTAACCCACCATGTAGCT
ACAAGCCTGCATAAGGTATTTGCTCGTGACTACTTATCAGACTAATCTGTGTG
AGCACTACACAATATTTCGTATCTTCTAGGTAAGGAGGTGATCCAACCGCTA
GGTTACCCCTACGGTTACCTTGTTACGACTTCACCCCAGTCATGAATCACAAA
GTGGTAAGCGCCCTCCCAAGGAAAGCGTCTAATCGTGCAGGAAGTA

C.

TAGTAGCTTAACCTTCGGCCGTGTACGCTTAGTCACTTAACCTCACAACC
CGAAGGTGTTTCACTTCGTGCTGTAAGCATTTGAGAGACTCTCGGATCACTT
TATTAAAAGCAATCCGAGTGTTTTCGAATTTTCAGCTTGTTCCAGATTGTTAA
AGAGCAAAATATTTCACAACATGCTGATTTCTCAGTATGTTCTGAAATATGG**T**
GGAGCTATGCGGGATCGAACCGCAGACCTCCTGCGTGCAAAGCAGGCG
CTCTCCCAGCTGAGCTATAGCCCCATGACTTTACTGCAATACCTTATTACCA
CCGTCCGCTTCACACAGGAAGGGAGTTGG**TAGGCCTGAGTGGACTCGAAC**
CACCGACTTCACCCTTATCAGGGGTGCTGCTCTAACCCACCATGTAGCTA
CAAGCCTGCATAAGGTATTTGCTCGTGACTACTTATCAGACTAATCTGTGTGA
GCACTACACAATATTTCGTATCTTCT**AGGTAAGGAGGTGATCCAACCGCTA**
GGTTACCCCTACGGTTACCTTGTTACGACTTCACCCCAGTCATGAATCAC
AAAGTGGTAAGCGCCCTCCCAAGGAAAGCGTCTAATCGTGCAGGAAGTA

Fig. 2: Sequences of both PCR amplicons, derived from *E. faecium* TX16 genome sequence ([refs] GenBank ID: AAAK00000000, US Department of Energy Joint Genome Institute, http://www.jgi.doe.gov/JGI_microbial/html/index.html). A. 16SrRNA-tRNA-Ile amplicon. PCR primer oligonucleotides underlined. Overlapping region in grey. B. tRNA-Ala-23SrRNA amplicon. PCR primer oligonucleotides underlined. Overlapping region in grey. C. Compiled sequence showing consensus 16SrRNA, tRNA-Ile, tRNA-Ala and 23SrRNA sequences (in that order) in bold. Consensus sequences compiled from representative bacterial 16S-23S rRNA DNA sequences (*Acetobacter, Aeromonas, Escherichia, Shigella, Salmonella, Vibrio* species) as described in materials and methods

was then done on all 10 DNA samples isolated by boiling.

Sequencing and sequence comparison: The PCR amplicons were sequenced directly. The primers used for sequencing were those used for the appropriate PCR amplification. Two separate sequencing reactions were done on each PCR amplicon (one from each direction). PCR products were prepared for sequencing using the Montage single-sample centrifugal filter PCR clean-up columns following the manufacturer's directions (Millipore, Billerica, MA). The sequencing reaction was done using the DTCS quick start kit for dDye terminator cycle sequencing following the manufacturer's directions (Beckman Coulter, Inc., Fullerton, CA). The following were mixed in each reaction 8 il DTCS Quick Start Master Mix, 1.6 μL (2 pmol μL^{-1}) sequencing primer, 15 ng template and MilliQ water to a total volume of 20 μL. The sequencing reactions were done using the Mastercycler Gradient Thermocycler (30 [96°C, 20 s; 50°C 20s; 60°C, 4 min]). Five μL of a sequencing stop-solution (2 μL 3M NaOAc, 2 μL 100 mM Na_2EDTA, 1 μL 20 mg μL^{-1} glycogen) was then added to each tube. The reactions were then ethanol-preciptated and washed twice using 70% EtOH. Each reaction was resuspended in 40 μL of sample loading solution (Beckman Coulter),overlaid with

mineral oil (Beckman Coulter) and the sequences analyzed using a CEQ™ 8000 genetic analysis system capillary DNA sequencer (Beckman Coulter). Sequences from each of the ten *E. faecium* isolates were manually compiled and then compared using Clustal-W.

RESULTS AND DISCUSSION

Despite using virginiamycin during the production cycle, we could only isolate Streptogramin-resistant *E. faecium* from ten out of the 50 chickens that we swabbed. This in itself was surprising. These broiler chickens lived in high-density (0.75-0.85 sq ft/bird) on pine litter. The method that we used for isolating *E. faecium* from our chicken swabs was identical to the accepted method for isolating *E. faecium* from humans and it was done in the same laboratory by the same individual (DCS) who routinely isolates *E. faecium* from human samples. We expected that if we isolated Streptogramin-resistant *E. faecium* at all, it would have colonized all of the chickens that we sampled.

We specifically wanted to simplify our DNA isolation procedure to minimize both costs and time. We were able to get clean PCR amplicons from DNA isolated using the simple boil-preparation (Fig. 1b).

Because we wished to compare bacteria from the same host species and from the same environment, we needed to design the most sensitive test possible. DNA sequencing of a specific part of the genome, from different isolates, provides the ultimate in sensitivity for unambiguous identification. Other methods of analysing the genome such as ribotyping, PFGE and AFLP detect changes in nucleotide sequence in only very small parts of the genome. Until recently, ribotyping, PFGE and AFLP were more attractive than DNA sequencing because they were considered less expensive. However, declining sequencing costs, high-throughput protocols and automation now makes DNA sequencing practical; especially considering the increase in information provided by DNA sequencing. With this in mind, we designed our method to be a practical and minimally-expensive molecular method to rapidly identify *E. faecium* isolates at the nucleotide level. We aimed to design a method that could be automated, be high-throughput and be done by unskilled staff. No special DNA isolation procedure or reagents were used.

Despite sequencing only one specific 16S-23S intergenic region from only 10 Streptogramin-resistant *E. faecium* isolates from chickens housed together on a single farm, we identified eight SNPs in total in four out of the ten isolates (Fig. 3). All isolates with SNPs had two or more. With the exception of one isolate that had an A instead of a T in the tRNA-Ile gene, all of the SNPs occurred in the intergenic regions. Five SNPs were

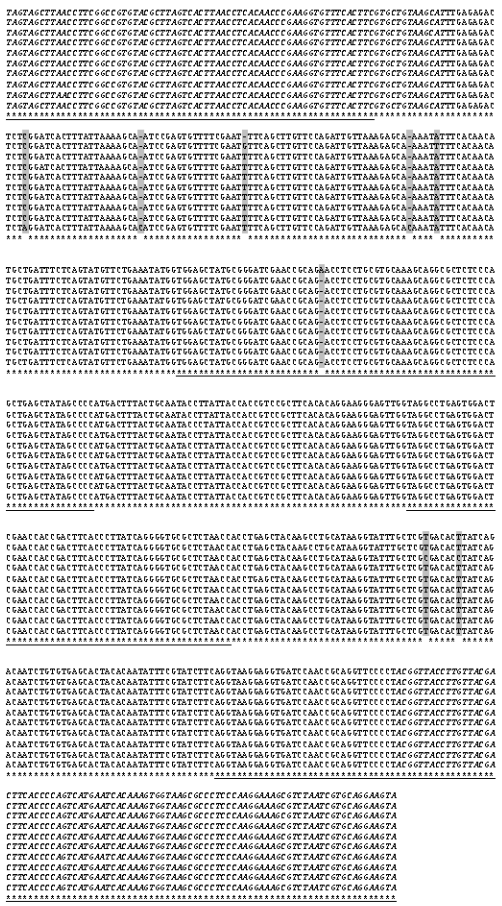

Fig. 3: Comparison 16S-23S rRNA sequences from ten streptogramin-resistant *E. faecium* isolates from chickens. Accession numbers: AY591324-8. 16SrRNA, tRNA-Ile, tRNA-Ala and 23SrRNA sequences (in that order) underlined (*). Sequences in italics are areas where only a single strand sequence was available for comparison between all ten isolates; no single nucleotide polymorphisims (SNPs) were present in these areas. The remaining sequence was compiled from at least two sequences from opposite directions. Eight SNPs (highlighted in grey) were identified in total. No SNPs were present in the 16S rRNA incomplete sequence, tRNA-Ala sequence and 23S rRNA incomplete sequence. One out of the eight SNPs was present in the tRNA-Ile sequence; five SNPs were presnet in the 141 bases that comprise the 16SrRNA to tRNA-Ile intergenic sequence (3.5%); two SNPs were present in the 92 bases that comprise the tRNA-Ala to 23SrRNA intergenic sequence (2.2 %). No SNPs were present in the tRNA-Ile to tRNA-Ala intergenic sequence

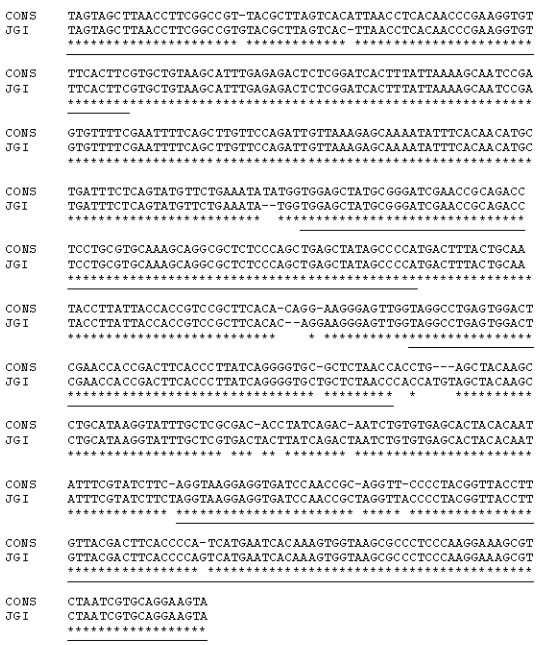

Fig. 4: Comparison of the consensus sequence (CONS) derived from the ten chicken streptogramin-resistant *E. faecium* with the sequence derived from *E. faecium* TX16 genome sequence ([refs] GenBank ID: AAAK00000000, US Department of Energy Joint Genome Institute, http://www.jgi.doe.gov/JGI_microbial/html/index.html). 16SrRNA, tRNA-Ile, tRNA-Ala and 23SrRNA sequences (in that order) underlined (*). Twenty-five single nucleotide polymorphisims (SNPs) were identified in total. Two SNPs were identified in the 16S rRNA incomplete sequence and the 16SrRNA to tRNA-Ile intergenic sequences; five SNPs were identified in the tRNA-Ile to tRNA-Ala intergenic sequence; one SNP was identified in the tRNA-Ala sequence; twelve SNPs were identified in the tRNA-Ala to 23S rRNA sequence and three SNPs were identified in the 23S rRNA incomplete sequence.

present in the 141 bases that comprise the 16SrRNA to tRNA-Ile intergenic sequence (3.5%); two SNPs were present in the 92 bases that comprise the tRNA-Ala to 23SrRNA intergenic sequence (2.2 %). No SNPs were present in the tRNA-Ile to tRNA-Ala intergenic sequence. We are confident of these SNPs because they were consistently present in all DNA sequencing reads (2 or 4 reads depending on where the SNPs were) that covered the 16S-23S region that we amplified.

One question that this result raises is did streptogramin-resistance occur on up to four occasions in our 10 chickens independently or did it occur once, with mutations in the intergenic regions occurring after acquisition of streptogramin-resistance? Furthermore, our dataset does not address whether streptogramin-resistance was transferred horizontally.

Because we identified SNPs in Streptogramin-resistant *E. faecium* from a small and isolated chicken population sampled at one time point, this suggests that this intergenic region is highly variable. The currently-accepted genetic paradigm would predict even more polymorphisms to be present from isolates that are geographically, temporally and/or ecologically distinct. Further analysis of our data set supports this prediction. We compared the sequence from the *E. faecium* TX16 human isolate fully-sequenced at the JGI (the current reference *E. faecium* genome) with a consensus sequence we derived from the 10 isolates that we sequenced (Fig. 4). Twenty-five nucleotides differed between our consensus sequence and the JGI sequence. Two SNPs were identified in the 16S rRNA incomplete sequence and the 16SrRNA to tRNA-Ile intergenic sequences; five SNPs were identified in the tRNA-Ile to tRNA-Ala intergenic sequence; one SNP was identified in the tRNA-Ala sequence; twelve SNPs were identified in the tRNA-Ala to 23S rRNA sequence and three SNPs were identified in the 23S rRNA incomplete sequence. It is notable that the 16S, 23S and tRNA genes themselves were well conserved between our isolates and TX16. The differences between the JGI reference *E. faecium* and our consensus sequence are far greater than the differences between the 10 isolates that we sequenced.

Arguably, given that this intergenic region is so variable, it is not surprising that our chicken isolates were more similar to each other than to TX16. Also our data does not rule out the possibility that, as previously suggested[21], poultry (livestock) *E. faecium* may be a separate subspecies or "genogroups" from those colonizing humans. Our primary aim was to establish a method that could be used to help answer this and similar questions about the ecology of *E. faecium*. Although we have proven the method, our dataset does not allow us to draw conclusions about *E. faecium* ecology.

The initial dataset that we present suggests that sequencing one 16S-23S may be adequate for molecular epidemiology of *E. faecium*. Furthermore, the method can easily be modified to increase the sequence coverage of regions with probable low sequence conservation by including more 16S-23S regions. This could be done using our standard 16S and 23S PCR primer oligonucleotides simply by designing PCR primer oligonucleotides to the different pairs of tRNA sequences. Others have quantified the relatedness of *E. faecium* by sequence comparison of house-keeping genes and/or virulence genes ([30] and

reviewed in[23]). We chose the alternative approach of sequencing a region that we considered to have no obvious evolutionary conservation pressure. Our reasoning was based on paradigm; any DNA sequence that encodes for a functional product (i.e. a gene) has its sequence constrained by the function of its product. Because DNA sequence primarily determines gene-product structure, only small fractions of the sequence are mutable without a change in that structure with an associated loss or degradation of gene-product function. Furthermore, only mutations that allow conservation of nucleotides (when the final gene-product is RNA) or amino acids may be allowable. Targeting genes constrains the numbers of possible SNPs per unit sequence length and also risks assuming relatedness when identical mutations are actually due to convergent evolution (i.e. because of the limited options for changes in sequence due to selection of gene function). In our study this would be streptogramin resistance. It is most likely that the evolution of *E. faecium* streptogramin-resistance occurred independently many times and yet only a small number of genetic changes account for this resistance.

In addition to its power to detect differences in closely related isolates, our simple methodology may be an affordable addition to Strepogramin-resistant *E. faecium* molecular epidemiology studies that have minimal equipment and no molecular biologist on staff. The entire molecular protocol can be done on a single thermocycler. With the present-day abundance of affordable sequencing facilities, the PCR clean-up and sequencing need not even be done in-house. Specifically, our method should be useful for confirming *E. faecium* species specificity and thus, whether *E. faecium* genogroups are truly zoonotic. Furthermore, the submission of sequences to the NCBI provides ready accessible database for diagnostic laboratories worldwide and provides the potential for immediate direct comparisons of *E. faecium* isolates.

ACKNOWLEDGEMENT

This study was done as part of an Undergraduate Traineeship placement for AS at MSU August 2002-December 2002. The authors acknowledge the help of M. Ruddis, L. M. Ford and L. A. Shack. This project was funded by the CVM MSU and UMMC.

REFERENCES

1. Peters, J., K. Mac, H. Wichmann-Schauer, G. Klein and L. Ellerbroek, 2003. Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. Int J Food Microbiol., 88: 311-314.
2. Linden, P.K. and C.B. Miller, 1999. Vancomycin-resistant enterococci: the clinical effect of a common nosocomial pathogen. Diagn Microbiol Infect Dis., 33: 113-120.
3. Huycke, M.M., D.F. Sahm and M.S. Gilmore, 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg Infect Dis., 4: 239-249.
4. Gold, H.S., 2001. Vancomycin-resistant enterococci: Mechanisms and clinical observations. Clin Infect Dis., 33: 210-219.
5. Van Den Bogaard, A.E., N. London and E.E. Stobberingh, 2000. Antimicrobial resistance in pig faecal samples from the Netherlands (five abattoirs) and Sweden. J. Antimicrob Chemother, 45: 663-671.
6. Goff, D.A. and S.J. Sierawski, 2002. Clinical experience of quinupristin-dalfopristin for the treatment of antimicrobial-resistant gram-positive infections. Pharmacotherapy, 22: 748-758.
7. Jones, R.N., C.H. Ballow, D.J. Biedenbach, J.A. Deinhart and J.J. Schentag, 1998. Antimicrobial activity of quinupristin-dalfopristin (RP 59500, Synercid) tested against over 28,000 recent clinical isolates from 200 medical centers in the United States and Canada. Diagn Microbiol Infect Dis, 31: 437-451.
8. Linden, P.K., R.C. Moellering, Jr., C.A. Wood, S.J. Rehm, J. Flaherty, F. Bompart and G.H. Talbot, 2001. Treatment of vancomycin-resistant Enterococcus faecium infections with quinupristin/dalfopristin. Clin Infect Dis., 33: 1816-1823.
9. McDonald, L.C., S. Rossiter, C. Mackinson, Y.Y. Wang, S. Johnson, M. Sullivan, R. Sokolow, E. DeBess, L. Gilbert, J.A. Benson, B. Hill and F.J. Angulo, 2001. Quinupristin-dalfopristin-resistant Enterococcus faecium on chicken and in human stool specimens. N. Engl. J. Med., 345: 1155-1160.
10. Murray, B.E., 2000. Problems and perils of vancomycin resistant enterococci. Braz J Infect Dis, 4: 9-14.
11. Solanti, M., D. Beighton, J. Philpott-Howard and N. Woodford, 2000. Mechanisms of resistance to quinupristin-dalfopristin among isolates of enterococcus faecium from animals, raw meat and hospital patients in western Europe. AAC., pp: 433-436.
12. Winston, L.G., D.R. Bangsberg, H.F. Chambers, S.C. Felt, J.I. Rosen, E.D. Charlebois, M. Wong, L. Steele, J.L. Gerberding and F. Perdreau-Remington, 2002. Epidemiology of vancomycin-resistant Enterococcus faecium under a selective isolation policy at an urban county hospital. Am. J. Infect Control, 30: 400-406.

13. Klare, I., H. Heier, H. Claus, G. Bohme, S. Marin, G. Seltmann, R. Hakenbeck, V. Antanassova and W. Witte, 1995. Enterococcus faecium strains with vanA-mediated high-level glycopeptide resistance isolated from animal foodstuffs and fecal samples of humans in the community. Microb Drug Resist, 1: 265-272.
14. Hammerum, A.M., V. Fussing, F.M. Aarestrup and H.C. Wegener, 2000. Characterization of vancomycin-resistant and vancomycin-susceptible Enterococcus faecium isolates from humans, chickens and pigs by RiboPrinting and pulsed-field gel electrophoresis. J. Antimicrob Chemother, 45: 677-680.
15. Johnson, J.A., S. Qaiyumi, L.L. English, J.H. Hayes, D.G. White, S.W. Joseph and D.D. Wagner, 2000. Comparison of Steptogramin-resistant Enterococcus faecium from poultry and humans. In: Annual AVMA (American Veterinary Medical Asso.) Conference Ed.^Eds.), Vol. p.^pp. Abstract AAAP section.
16. Sorensen, T.L., M. Blom, D.L. Monnet, N. Frimodt-Moller, R.L. Poulsen and F. Espersen, 2001. Transient intestinal carriage after ingestion of antibiotic-resistant Enterococcus faecium from chicken and pork. N. Engl. J. Med., 345: 1161-1166.
17. Borgen, K., Y. Wasteson, H. Kruse and R.J. Willems, 2002. Vancomycin-Resistant Enterococcus Faecium (VREF) from Norwegian poultry cluster with VREF from poultry from the United Kingdom and The Netherlands in an amplified fragment length polymorphism genogroup. Applied Environ Microbiol., 68: 3133-3137.
18. Chen, H.Y., R.L. Hill, M. Kirk, M.W. Casewell and D. Beighton, 2002. Differential antimicrobial susceptibility between human and chicken isolates of vancomycin-resistant and sensitive Enterococcus faecium. Intl. J. Antimicrob Agents, 19: 39-46.
19. Bager, F., M. Madsen, J. Christensen and F.M. Aarestrup, 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant Enterococcus faecium on Danish poultry and pig farms. Prev. Vet. Med., 31: 95-112.
20. Bruinsma, N., R.J. Willems, A.E. Van den Bogaard, M. Van Santen-Verheuvel, N. London, C. Driessen and E.E. Stobberingh, 2002. Different levels of genetic homogeneity in vancomycin-resistant and-susceptible Enterococcus faecium isolates from different human and animal sources analyzed by amplified-fragment length polymorphism. Antimicrob Agents Chemother, 46: 2779-2783.
21. Willems, R.J., J. Top, N. Van Den Braak, A. Van Belkum, H. Endtz, D. Mevius, E. Stobberingh, A. Van Den Bogaard and J.D. Van Embden, 2000. Host specificity of vancomycin-resistant Enterococcus faecium. J. Infect Dis, 182: 816-823.
22. Morrison, D., N. Woodford, S.P. Barrett, P. Sisson and B.D. Cookson, 1999. DNA banding pattern polymorphism in vancomycin-resistant Enterococcus faecium and criteria for defining strains. J. Clin. Microbiol, 37: 1084-1091.
23. Brisse, S., V. Fussing, B. Ridwan, J. Verhoef and R.J. Willems, 2002. Automated ribotyping of vancomycin-resistant Enterococcus faecium isolates. J. Clin. Microbiol., 40: 1977-1984.
24. Arduino, R.C., K. Jacques-Palaz, B.E. Murray and R.M. Rakita, 1994. Resistance of Enterococcus faecium to neutrophil-mediated phagocytosis. Infect Immun., 62: 5587-5594.
25. Arduino, R.C., B.E. Murray and R.M. Rakita, 1994. Roles of antibodies and complement in phagocytic killing of enterococci. Infect Immun., 62: 987-993.
26. Graham, T., E.J. Golsteyn-Thomas, V.P. Gannon and J.E. Thomas, 1996. Genus- and species-specific detection of Listeria monocytogenes using polymerase chain reaction assays targeting the 16S/23S intergenic spacer region of the rRNA operon. Can J. Microbiol., 42: 1155-1162.
27. Graham, T.A., E.J. Golsteyn-Thomas, J.E. Thomas and V.P. Gannon, 1997. Inter- and intraspecies comparison of the 16S-23S rRNA operon intergenic spacer regions of six Listeria spp. Intl. J. Syst. Bacteriol., 47: 863-869.
28. Higgins, D.G. and P.M. Sharp, 1988. Clustal: A package for performing multiple sequence alignment on a microcomputer. Gene., 73: 237-244.
29. Altschul, S.F., W. Gish,, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. J. Mole. Biol., 215: 403-410.
30. Homan, W.L., D. Tribe, S. Poznanski, M. Li, G. Hogg, E. Spalburg, J.D. Van Embden and R. Willems, 2002. Multiulocus sequence typing scheme for Enterococcus faecium. J. Clin. Microbiol., 40: 1963-1971.