

Characterization of Vancomycin-Resistant *Enterococcus faecium* Isolated from Swine in Three Michigan Counties[∇]||

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Vancomycin-resistant enterococci are a major cause of nosocomial infections but are rarely found in humans in the community and have not been identified in food animals in the United States. We evaluated a total of 360 fecal specimens from humans and their animals being raised for exhibit at three county fairs in Michigan. Fecal samples from 158 humans, 55 swine, 50 cattle, 25 horses, 57 sheep, 14 goats, and 1 llama were obtained and plated onto Enterococcosel agar containing 16 µg/ml of vancomycin. Vancomycin-resistant *Enterococcus faecium* (VREF) was isolated from six pigs but not from humans or any animal other than pigs. All six VREF isolates had a MIC to vancomycin of ≥256 µg/ml and contained the *vanA* gene. Pulsed-field gel electrophoresis (PFGE) patterns of the six VREF isolates were ≥80% similar. Multilocus sequence typing (MLST) revealed sequence type 5 (ST5) (*n* = 2), ST6 (*n* = 3), and ST185 (*n* = 1), which are *E. faecium* sequence types belonging to clonal complex 5 (CC5). These findings show the dissemination of VREF strains among pigs in three Michigan counties. This is the first report of VRE found in food animals in the United States.

Enterococci are a major cause of morbidity and mortality in hospitalized patients in the United States. In a 2006–2007 report to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, *Enterococcus* species were the second most common pathogen in U.S. hospitals (29). Although enterococci are commensal organisms in the guts of most animals, their intrinsic resistance to many antimicrobial agents and their ability to efficiently acquire antibiotic resistance determinants make them a particular threat in the hospital setting, causing serious wound infections, bacteremia, and endocarditis (44, 66). Widespread use of vancomycin and extended-spectrum cephalosporins in U.S. hospitals likely contributed to the emergence and dramatic increase of vancomycin-resistant enterococci (VRE) over the past 20 years (18, 35). Vancomycin resistance is most commonly found in *Enterococcus faecium* and is encoded by the *vanA* gene cluster carried on the mobile genetic element Tn1546 (4). Antimicrobial agents, including several classes of antibiotics used in humans (e.g., β-lactams, cephalosporins, tetracyclines, macrolides, and streptogramins), are used on U.S. farms for growth promotion as well as therapeutically, and many studies have reported antibiotic-resistant enterococci in food animals and retail meat in the United States (16, 17, 26–28, 43, 51, 60). Glycopeptides, however, have never been permitted for use on farms in the United States. VRE have not been isolated from food animals or retail meat in the United States and have only rarely been found in companion

animals, the environment, and humans without hospital exposure in the community (10, 42, 48, 52).

In Europe, the glycopeptide avoparcin was used for growth promotion on farms for many years. When it was found that VRE could commonly be isolated from food animals, retail meats, and humans in the community, the use of avoparcin for growth promotion was banned, first in Denmark in 1995, and later in the entire European Union in 1997 (1, 5, 6, 36, 56, 57, 59). Despite the high rate of VRE in farm animals and humans in the community, nosocomial infections with VRE in Europe were not common and still occur at a lower rate than in U.S. hospitals. This has been attributed to less use of vancomycin and extended-spectrum cephalosporins in European hospitals (35). Recently, there have been reports of increasing numbers of infections with VRE in hospitalized patients in European countries (3, 21, 34, 37, 46, 64). A specific clonal complex of *E. faecium* strains, clonal complex 17 (CC17), has been responsible for outbreaks in hospitals worldwide, including the United States (7, 38, 39, 45, 64). The development of ampicillin resistance, possession of virulence genes such as *esp* and *hyl*, and the acquisition of vancomycin resistance gave *E. faecium* strains from CC17 the advantages needed to adapt to the hospital environment (49, 50, 63, 64). In a recent study, a functional collagen adhesion gene (*acm*) was also described as a likely contributor to the increased virulence of CC17 strains causing serious infections, such as endocarditis (45). Although *E. faecium* strains in CC17 are associated with epidemicity in hospitals, there has been a report of a CC17 strain in swine from Portugal (20). Conversely, several studies in Europe have reported finding swine-adapted *E. faecium* CC5 strains in humans (3, 11, 12, 20, 23, 30, 33).

In this study, we surveyed members of 4-H organizations from three counties in Michigan and evaluated human and animal fecal samples in order to determine whether vanco-

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mycin-resistant enterococci were present in these populations.

MATERIALS AND METHODS

Study design and sample collection. Three county 4-H organizations were selected for this study because of their active animal husbandry programs. In these programs, 4-H members personally raised one or perhaps a few animals for exhibit at the July or August 2008 county fair. Adolescents, parents, and other adult supervisors participating in these 4-H organizations were invited to complete a risk factor survey, submit a fecal specimen from themselves, and submit a fecal specimen from the farm animal (bovine, porcine, equine, caprine, or camelid) to which they had the most contact. In instances where there was close contact with a second farm species, a fecal specimen was also obtained from that animal. Study participants were monetarily compensated for their contribution. The study was reviewed and approved by the Michigan State University Institutional Review Board. Study subjects completed a survey regarding animal exposure, hospitalization, current or previous diarrheal illness, use of antibiotics, and other sources of enteric pathogens. Risk factors were analyzed for association with VRE results by using the mid-P exact test, prevalence ratio with 95% confidence limits, and Student's *t* test.

Samples were placed in Cary-Blair transport medium (MCC, Torrance, CA) and maintained on ice or refrigerated until reaching the laboratory (within 3 to 4 days), where they were kept at -20°C until processing.

Isolation, identification, and antimicrobial testing of enterococci. Fecal samples were plated onto Enterococcosel agar (Becton Dickinson, Cockeysville, MD) containing $16\ \mu\text{g/ml}$ of vancomycin and incubated for 48 h at 37°C . Distinct morphological colony types displaying esculin hydrolysis were isolated, plated on Trypticase soy agar II (TSAIL) (Becton Dickinson, Cockeysville, MD), and confirmed as enterococci using standard biochemical reactions (54). Antimicrobial susceptibility testing for vancomycin, ampicillin, ciprofloxacin, gentamicin, linezolid, erythromycin, tetracycline, and quinupristin-dalfopristin were determined by E strip (bioMerieux, Solna, Sweden) using CLSI guidelines (9).

Detection of glycopeptide resistance genes and virulence genes *esp* and *hyl*. PCR was performed to detect the presence of the glycopeptide resistance genes *vanA* and *vanB* by using a previously described method (8). Primers for amplification of genes for the virulence determinants enterococcal surface protein (*esp*) and hyaluronidase (*hyl*) were described previously (58).

Analysis of Tn1546. Vancomycin-resistant *Enterococcus faecium* (VREF) isolates were evaluated for polymorphisms in glycopeptide resistance determinant Tn1546. PCR was performed to amplify an internal fragment of the *vanX* gene by using primers from a previous study (31). The resulting 424-bp amplicon was digested with restriction enzyme DdeI (New England BioLabs, Beverly, MA) to determine whether a previously described base pair variant at position 8234 was present (32). Previously described primers were used to detect the presence of IS1251 and to determine whether IS1216V was combined with the IS3-like element in the left end of Tn1546 (25, 31).

PFGE. Genomic DNA was prepared in agarose plugs, digested with SmaI (New England BioLabs, Beverly, MA), and run on a CHEF-DR III (Bio-Rad Laboratories, Hercules, CA), as described previously (14). BioNumerics software version 3.5 (Applied Maths, Kortrijk, Belgium) was used to calculate the percent similarity (Dice coefficient) of SmaI pulsed-field gel electrophoresis (PFGE) banding patterns. Isolates were considered related if their PFGE banding patterns were $\geq 80\%$ similar.

MLST. Multilocus sequence typing (MLST) was performed to determine the evolutionary relationship between isolates using a previously described method (30). Fragments of the seven housekeeping genes from *Enterococcus faecium* (*adk*, *atpA*, *ddl*, *gyd*, *gdh*, *purK*, and *pstS*) were amplified, purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), sequenced using the BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems, Warrington, United Kingdom), and analyzed on an ABI 3100 sequencer (PE Applied Biosystems). The eBURST V3 program, which can be accessed at <http://efaecium.mlst.net>, was used to assign a sequence type (ST) to each isolate according to its allelic profile (19).

RESULTS

Recovery of vancomycin-resistant enterococci. Fecal specimens were collected from humans ($n = 158$), swine ($n = 55$), cattle ($n = 50$), horses ($n = 25$), sheep ($n = 57$), goats ($n = 14$), and llama ($n = 1$). Vancomycin-resistant *Enterococcus faecium*

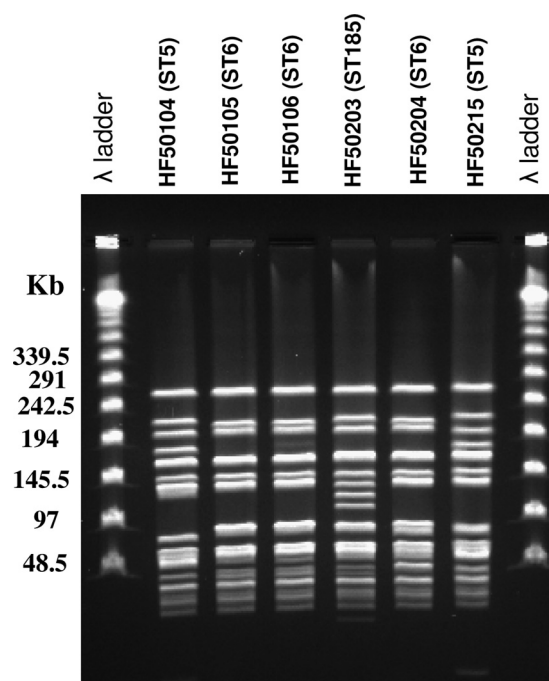


FIG. 1. PFGE of SmaI-digested genomic DNA from six *vanA*-containing *Enterococcus faecium* isolates from swine in three Michigan counties.

isolates were recovered from six pigs (10.9%). Four pigs were from county 1 (two were pen-mates), one was from county 2, and one was from county 3. Vancomycin-resistant *E. faecium* isolates were not recovered from humans, sheep, cattle, horses, goats, or the llama. There were no vancomycin-resistant *Enterococcus faecalis* strains recovered in this study.

Antimicrobial susceptibilities. The six VREF isolates had MICs to vancomycin of $\geq 256\ \mu\text{g/ml}$. All VREF isolates were also resistant to erythromycin and tetracycline and had intermediate resistance to quinupristin-dalfopristin. All six VREF isolates were susceptible to ampicillin, ciprofloxacin, gentamicin, and linezolid and did not produce β -lactamase.

Glycopeptide resistance genes and virulence genes *esp* and *hyl*. All six VREF isolates contained the *vanA* gene. All isolates were negative for the virulence genes *esp* and *hyl*.

Tn1546 analysis. Analysis of Tn1546 for each isolate revealed a G-to-T mutation at position 8234 in the *vanX* gene. IS1216V combined with the IS3-like element at the left end of Tn1546 was detected in all isolates. IS1251 was not detected in any of the VREF isolates.

PFGE and MLST. PFGE patterns of SmaI restriction fragments were $\geq 80\%$ similar (Fig. 1 and 2). MLST analysis yielded sequence type 5 (ST5) ($n = 2$), ST6 ($n = 3$), and ST185 ($n = 1$). These sequence types belong to *E. faecium* clonal complex 5 (CC5).

4-H participant interviews. No owners were hospitalized within the previous 6 months; however, 2 of the 6 owners of VRE-positive pigs had outpatient treatment in the previous 6 months, and 1 resided with a health care worker who cared for human patients. During the previous 75 days, 3 of 49 owners of VRE-negative pigs and 2 of 6 owners of VRE-positive pigs had used antibiotics. One person with a VRE-positive pig had used

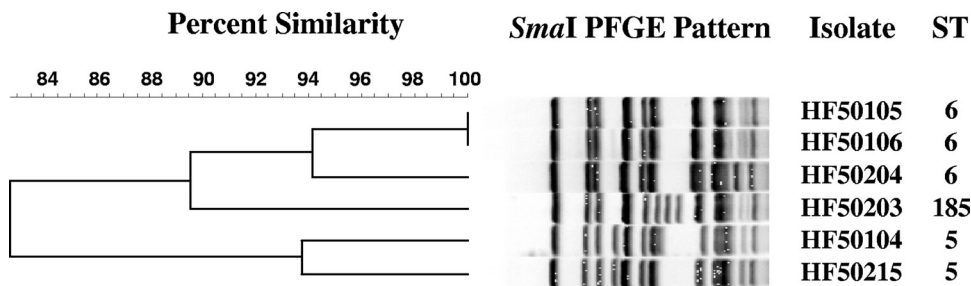


FIG. 2. Dendrogram showing comparisons of *Sma*I PFGE patterns for six *vanA*-containing *Enterococcus faecium* isolates from swine in three Michigan counties.

amoxicillin and azithromycin 60 days previously, and the second had used cephalexin 60 days previously. Two of 49 owners of VRE-negative pigs received amoxicillin and 1 received Kefzol 60 days previously. Antibiotic use in owners was associated with having a VRE-positive pig at $P = 0.047$ by the mid-P exact test, with a prevalence ratio of 5.0 (1.2 to 21). One of the VRE-positive pigs received ceftiofur, an extended-spectrum cephalosporin, within 30 days of sampling. One VRE-negative pig received ceftiofur, and two received both oxytetracycline and tylosin. No statistical association was found between VRE in pigs and their exposure to antibiotics in the previous 30 days ($P > 0.75$ in all instances). There was no association between VRE carriage and a history of diarrhea in the pigs during the previous 14 days ($P = 0.62$).

DISCUSSION

This study provides the first description of high-level vancomycin-resistant enterococci isolated from food animals in the United States. We found related strains of VREF in six pigs from three Michigan counties, indicating clonal transmission was at least partially responsible for the dissemination of vancomycin resistance. The *vanA*-containing *E. faecium* from swine in this study were part of CC5, which is widely disseminated in swine in Europe (12, 20, 47). The prevalence of *E. faecium* CC5 strains has not been described in the United States; however, it may be a predominant clone of *E. faecium* in swine in the United States as well. Characteristics of CC5 strains are *in vitro* susceptibility to ampicillin, possession of *purK* allele 9, and lack of virulence genes. Conversely, VREF from the hospital setting in the United States and elsewhere, particularly strains belonging to CC17, are resistant to ampicillin, have *purK* allele 1, and often contain multiple virulence determinants, such as *esp*, *hyl*, and *acm* (37, 45, 49, 64). Selective pressure from the environment, such as farm, community, or hospital, likely plays a significant role in determining the characteristics of VREF (41, 63, 64). It has been suggested that the CC5 lineage of *E. faecium* may have a fitness advantage in swine and could spread more readily than other, less adapted strains between swine and potentially between swine and humans (47). Many studies have shown that it is possible for the same strains of enterococci to infect both humans and farm animals (3, 6, 13, 23, 33, 53, 56). If large numbers of CC5 strains acquire vancomycin resistance, this could pose a health threat to humans since this also suggests the potential for the occurrence of vancomycin-resistant enterococci in food. It is

therefore important to determine the prevalence of CC5 strains in humans and farm animals in the United States and to continue to assess the extent to which these strains have acquired vancomycin resistance.

Enterococci are remarkable in their ability to disseminate antimicrobial resistance by a variety of routes. Since the *vanA* gene cluster is on a mobile genetic element, Tn1546, horizontal transmission of vancomycin resistance between different strains of enterococci occurs commonly. Many studies have demonstrated the transmission of Tn1546 between animals and humans (2, 32, 61, 65). There is often polymorphism in Tn1546, and these variations can be of use in tracing the source of VREF. There have been numerous descriptions of insertion sequences integrated into Tn1546, such as IS1216V, IS1251, IS1476, and IS1542 (22, 25, 31, 40, 65). Researchers in Denmark found a common base pair variation in *vanX* in which the base at position 8234 was either a G (as in the control VanA strain BM4147) or a T (31). They later evaluated 271 VREF isolates from pigs, poultry, and humans in several countries and found that all poultry and 64% of humans had a G at position number 8234 in *vanX*, while nearly all (97%) of pigs and 36% of humans had the variant T (32). Therefore, pigs and poultry had different variations of Tn1546, while humans had both types. The six VREF isolates in this study also had the variant T at position 8234 in *vanX*. Insertion sequences (IS) are commonly found in bacterial genomes and may facilitate transfer of resistance genes. IS1216V is commonly found in enterococci from both human and animal sources and can sometimes be present in multiple copies. IS1216V has also been found with an IS3-like element at the left end of Tn1546 (24). IS1251 is an insertion sequence which has been detected mainly in clinical isolates of VRE from the United States (15, 24, 25, 31). In this study, we found VREF from swine which shared many characteristics with VREF from swine in European studies (20, 23, 30–32, 47). All strains were part of *E. faecium* clonal complex 5, had the T variant of *vanX*, were positive for IS1216V linked to the IS3-like element at the left end of Tn1546, and were negative for IS1251.

There is clear evidence that with an increase in the consumption of antimicrobial agents by humans or animals, there is a resultant increase in antimicrobial resistance. It is likely that the use of avoparcin on farms in Europe resulted in high levels of VREF in farm animals and eventually humans in the community (5). Although it has been established that the use of antimicrobial drugs for growth promotion and therapeutic treatment in food animals selects bacteria among the normal

intestinal flora of animals, whether it poses a risk to human health remains controversial. In this study, we found VREF in pigs that were not given glycopeptides. We found no association between antibiotic consumption in pigs and colonization with VREF; however, 2 of the 6 (33%) owners of pigs positive for VREF and 2 of the 49 (4%) owners of pigs negative for VREF received antibiotics within 60 days prior to specimen collection. We were not able to determine how the swine in this study acquired VREF; however, since the use of antimicrobial agents that lack *in vitro* activity against enterococci will create selective pressure to increase the emergence and dissemination of vancomycin-resistant enterococci, there is a need to prevent the misuse and overuse of these agents in both food-producing animals and humans.

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