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Vancomycin-Resistant Enterococci: A Review of Antimicrobial Resistance Mechanisms and Perspectives of Human and Animal Health

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Vancomycin-resistant enterococci (VRE) are both of medical and public health importance associated with serious multidrug-resistant infections and persistent colonization. Enterococci are opportunistic environmental inhabitants with a remarkable adaptive capacity to evolve and transmit antimicrobial-resistant determinants. The VRE gene operons show distinct genetic variability and apparently continued evolution leading to a variety of antimicrobial resistance phenotypes and various environmental and livestock reservoirs for the most common *van* genes. Such complex diversity renders a number of important therapeutic options including ''last resort antibiotics'' ineffective and poses a particular challenge for clinical management. Enterococci resistance to glycopeptides and multidrug resistance warrants attention and continuous monitoring.

Keywords: vancomycin-resistant enterococcus, van operons, van genes, public health, emerging pathogen, zoonosis

Introduction

Enterococcus species are natural inhabitants of the environment and an essential component of the intestinal microbiota of healthy humans and animals.[1,2] To date, over 50 different enterococcal species have been described.[3,4] Species most frequent within the human intestines are *Enterococcus faecalis*, and to a lesser extent *Enterococcus faecium*, whereas the most common species in various food animals are *E. faecium* together with *Enterococcus cecorum*, *E. faecalis*, and to some extent *Enterococcus hirae*.[4–6] Enterococci are also opportunistic pathogens associated with serious and life-threatening infections to humans such as urinary tract infections, sepsis (blood stream infections), and endocarditis.[7–10] *E. faecalis* and *E. faecium* account for the majority of human enterococcal infections, and a leading cause of hospital-acquired and multidrug-resistant infections.[1,11,12]

Enterococci became recognized as important major nosocomial pathogens due to their natural-intrinsic resistance to several antimicrobials (*e.g.*, penicillin, ampicillin, and most cephalosporins) and capacity to quickly acquire virulence and multidrug resistance determinants.[13–18] In fact, enterococci can rapidly develop resistance following the clinical introduction of antimicrobial agents, including last resort antimicrobials used to treat glycopeptide and multidrug resistance (such as quinupristin-dalfopristin, linezolid, daptomycin, and tigecycline).[13,18–20]

Since the first reports of vancomycin-resistant enterococci (VRE) in the 1980 s,[21,22] epidemiological studies have demonstrated serious health and economic impacts from VRE-associated infections and persistent colonization in human medicine.[8,23–27] Alternatively, VRE are rare causes of infection in animals, and infrequently encountered in companion animals.[2,28–30]

The genetic and molecular basis of vancomycin resistance have been described with evidence that VREs may act as reservoirs and sources of other antimicrobial-resistant genes. In this article, glycopeptide and multidrug enterococcal resistance referred to by the historic term vancomycin-resistance enterococcus (VRE) and their associated clinical phenotypic/genotypic (Genotypes and phenotypes of VREs are referred to as Van. Genes, clusters, and operons of VREs are referred to as *van*) characteristics are reviewed and discussed. Also, the impact of the use and past-use of certain antimicrobial agents on the evolution and emergence of VRE are discussed.

Glycopeptides Resistance in Enterococci

The main mechanism of glycopeptide resistance (*e.g.*, vancomycin) in enterococci involves the alteration of the peptidoglycan synthesis pathway, specifically the substitution of D-Alanine-D-Alanine (D-Ala-D-Ala), to either D-Alanine-D-Lactate (D-Ala-D-Lac) or D- Alanine-D-Serine (D-Ala-D-Ser).[31,32] Such alterations can lead to variable expressions of

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glycopeptide resistance. For example, the respective altered D-Ala-D-Lac and D-Ala-D-Ser leads to less binding affinity of glycopeptide drugs compared to the normal cell wall precursors D-Ala-D-Ala; ~1000-fold decreased binding affinity for D-Ala-D-lac and ~7-fold for D-Ala-D-Ser.[33–35] The ability to induce such alterations is related to several genes harbored on mobile genetic elements and/or chromosomally encoded regions of different *Enterococcus* species.[18,32] The latter mechanisms appear to underlie most of the vancomycin-resistant phenotypes and the variable composition of Van-related forms of resistance; this distinction may provide insights into the different levels (low-level vs. high-level) of resistance to glycopeptides.[34,36,37]

The *van* Resistance Genetic Determinants of *Enterococcal spp*.

To date, operons related to vancomycin resistance for enterococci are described as *vanA*, *-B*, *-C*, *-D*, *-E*, *-G*, *-L*, *-M*, and *N* (Fig. 1). These are distinguishable by their degree of reduced susceptibility (*i.e*., resistance) to the glycopeptides, transferability, and inducibility (Table 1).[12,18,31,32,34,37–50] An additional operon (*vanF*) has also been described but thus far only in *Paenibacillus popilliae*.[51] This *vanF* variant has a high similarity to the amino acid sequences of the *vanA* operon, and as such *P. popilliae* has been suggested as a possible origin for vancomycin resistance in enterococci.

Most VRE outbreaks in human populations are attributed to the *vanA* and *vanB* gene clusters,[52,53] both of which have also been identified in various colonized animals and environmental materials.[54–56] These are complex distinct genetic determinants and the most globally widespread *van*-operons.[57–60]

The *vanA* operon is associated typically with transposons (Tn), such as Tn*1546*, involving two genes for the transposition of the element (*orf1* and *orf2*), and one gene related to teicoplanin resistance (*vanZ*) (Fig. 1).[61–63] The *vanA* gene cluster includes seven open reading frames transcribed from two separate promoters. The regulatory apparatus is encoded by the *vanR* (response regulator) and *vanS* (sensor kinase) two-component system, which are transcribed from a common promoter, while the remaining genes are transcribed from a second promoter.[18,31] Gene products that specifically modify the production of peptidoglycan precursors include *vanH* (dehydrogenase that converts pyruvate to lactate) and *vanA* (ligase that forms D-Ala-D-Llac dipeptide).

An essential part of the vancomycin resistance phenomenon is that the production of the normal D-Ala-D-Ala end of the pentapeptide does not continue. This is resolved by the *vanX* and *vanY* genes where *vanX* (dipeptidase that cleaves D-Ala-D-Ala) hydrolyzes and thereby interrupts the production of the pentapeptides, and *vanY* (D, D-carboxypeptidase) cleaves the pentapeptides that might still be produced. There can be variations in the composition of this vancomycin resistance operon due to insertion of IS elements and these variants are described as *vanA*-like elements.

The typical *vanB* operon has a similar genetic backbone to the *vanA*. High-level vancomycin resistance has been reported within *vanB* subtypes and designated as *vanB1-3*

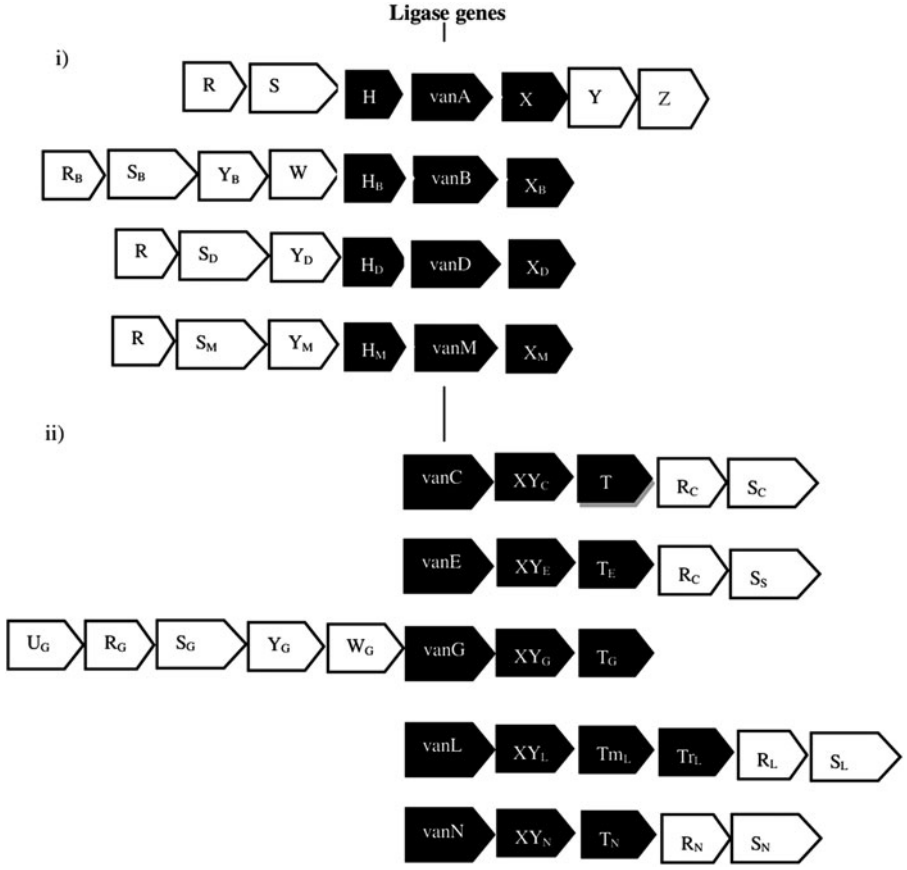

FIG. 1. Schematic diagram of van operons in Enterococci. Operons are aligned at the ligase genes and aligned according to the substitute peptidoglycan precursors as following: **(i)** D-Ala-D-Lac operons (A, B, D, & M) **(ii)** D-Ala-D-Ser operons (C, E, G, L, & N). D-Ala-D-Lac, D-Alanine-D-Lactate; D-Ala-D-Ser, D- Alanine-D-Serine; *vanU*, (U) transcriptional activator; *vanR*, (R) response regulator; *vanS*, (S) sensor histidine kinase; *vanW*, (W) unknown function; *vanH*, (H) dehydrogenase; *vanT*, (T) serine racemase; $vanT_m$, (T_m) serine racemase membrane domain; $vanT_r$, (T_r) serine racemase racemase domain; *vanY*; (Y) carboxypeptidase; *vanX*, (X) dipeptidase; *vanXY*, (XY) dipeptidease/carboxypeptidase; *vanZ*, (Z) unknown function but involved in teicoplanin resistance. *➨ Genes encode for proteins essential for expression of glycopeptides resistance (*i.e*., required genes for expressed VAN-resistance).

Table 1. Summary of the Phenotypic and Genotypic Characteristics of the Alphabet VREs Operons

Van-operon	*Common carrier spp.*	*Degree level of resistance vancomycin vs. teicoplanin*	*Phenotypic expressions*	*Location & mobility*	*Refs. (No)*
vanA	*E. faecium* *E. faecalis*	High for both	Inducible	Chromosome Transferable	18,32,38
vanB; vanB1, B2, B3	*E. faecalis* *E. faecium*	High-variable to vancomycin Susceptible to teicoplanin	Inducible	Chromosome Transferable	18,31,32,39,40
vanC; vanC1, C2, C3, C4	*E. gallinarum* *E. casseliflavus* *E. flavescens*	Low to vancomycin Susceptible to teicoplanin	Constitutive Inducible —	Chromosome —	12,34,41,42
vanD; vanD1, D2, D3, D4, D5	*E. faecium*	Low to high for both	Constitutive Inducible	Chromosome —	43–45
vanE	*E. faecalis*	Low-moderate to vancomycin Susceptible to teicoplanin	Inducible	Chromosome —	32,34,46
vanG; vanG1, G2	*E. faecalis*	Low to vancomycin Susceptible to teicoplanin	Inducible	Chromosome Transferable	47
vanL	*E. faecalis*	Low to vancomycin Susceptible to teicoplanin	Inducible	Chromosome —	48
vanM	*E. faecium*	High for both	Inducible	Unknown Transferable	49
vanN	*E. faecium*	Low to vancomycin Susceptible to teicoplanin	Constitutive	Plasmid Transferable	50

VRE, vancomycin resistance enterococci.

with *vanB-2* as the most prevalent genotypes worldwide.[40] The transfer of *vanB* resistance alleles occurs through the acquisition and/or exchange of transposons such as Tn*1547*, Tn*1549*, and Tn*5382*.[38,64,65] The conjugative *vanB* transposon, known as Tn*1549* is widely prevalent among VanB-type enterococci and other gram-positive bacteria.[66] This is mainly a chromosomal transposon and less frequently found on plasmids.[67,68]

The genetic organization of *vanB* is similar to that of *vanA*, in that it contains two distinct promoters transcribing seven open reading frames, but there are some important differences (Fig. 1). For example, *vanB* encodes a two-component signaling system (named $vanR_B$ and $vanS_B$) that is considerably different from that encoded in *vanA*. Furthermore, *vanB* encodes homologs of *vanH* and the D-Ala-D-Ala ligase (encoded by *vanB*), and the peptidases (*vanX* and *vanY*). However, *vanB* lacks a homolog of *vanZ*, and instead encodes a protein named *vanW*, whose role in resistance is not fully understood.[18]

In contrast, the *vanC* operon is genetically different from *vanA* and *vanB*, and typically ''less virulent'' enterococci than those carrying inducible *vanA* and *vanB* gene clusters.[34,41,42] The *vanC*-resistant subtypes, *vanC-1*, *vanC-2*, and *vanC-3*, are known to be intrinsically present in *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens*, respectively,[12] and sometimes seen as markers for these enterococcal species. The *vanC*-2 gene cluster in *E. casseliflavus* demonstrates a composition similar to the *vanC*-1 cluster in *E. gallinarum*.[32,42] More recently, a *vanC-4* subtype was also described with 93–95% nucleotide homology with *vanC-2/-3*.[41]

The *vanD* operons are exclusively chromosomal and similar to *vanA* and *vanB*.[43–45,69–72] This operon represents a number of different combinations of mutations and furthermore, different enterococcal strains demonstrate a constitutive resistance phenotype resulting from different mutations within the operon regulators. These various modifications have led to a wide range of subtypes and resistance phenotypes.[73] Nevertheless, the *vanD* is described sparsely among various enterococci species, and can also be carried by the *vanC E. gallinarum*.[74]

The *vanE* gene cluster has been described in a few *E. faecalis* strains from North America and Australia.[46,75,76] The *vanE* cluster resembles the *vanC1*, which occurs naturally in *E. gallinarum* (Fig. 1). Studies have suggested that a cluster within an integrase gene of the *vanE- E. faecalis* may have evolved with the acquisition of this operon; however, reports have not determined the transfer-ability of this gene cluster.[75] *Enterococcus faecalis* possessing a *vanG* cluster have been described and two subtypes were identified.[47,77,78] In contrast to the other *van* D-Ala-D-Ser operons (*i.e.*, *vanE*, *vanC vanL* and *vanN*), this operon has been shown to be transferable via a conjugative plasmid from *E. faecalis*.

The set of *van* genes was expanded recently after the discovery of *vanL*, *vanM*, and *vanN* operons. The *vanM* is genetically and phenotypically similar to *vanA*, *vanB*, and *vanD*, whereas both *vanL* and *vanN* are similar to *vanC* (Fig. 1). The *vanL* gene cluster exhibits 49–51% sequence identity to the *vanE* and *vanC* ligases. However, this VRE *E. faecalis* isolate did not demonstrate either a transfer or conjugation ability, suggesting that the *vanL* gene cluster is chromosomally encoded.[48] The *vanM* operon has been described in *E. faecium* VRE isolates and demonstrated a close gene arrangement to *vanD* and *in vitro* transferable resistance by conjugation.[49] The *vanN* operon is the most recently identified gene cluster described in *E. faecium*.[50] This is a similar operon to *vanG*, but more unique since it has also been reported to be transferable via mobile genetic elements, and only in *E. faecium*. In general, the D-Ala-D-Ser operons are frequently chromosomally encoded but some of the latest added operons (*i.e.*, G and N) are transferable in *E. faecalis* and *E. faecium* respectively, and only exceptionally.

Phenotype Versus Genotype Expression of Vancomycin Resistance in Clinical Enterococcal Species

VRE phenotypes are defined by the expression of vancomycin-related resistance and virulence factors, regardless of the genes expressed but mainly related to their clinical characteristics.[79] The VanA and VanB phenotypes, and most recently the VanC, appear to be clinically the most important, and capable of conferring high-levels of resistance to vancomycin and teicoplanin; however, the VanA phenotype appears remarkably more virulent.

Motile enterococci, such as *E. gallinarum*, *E. casseliflavus* deserve special mention in phenotype designations, since the VanC phenotypes were previously less frequent causes of human clinical infections and confer a low-level of resistance to vancomycin, but not to teicoplanin.[12,14] While the global incidence of VanC phenotypes remains relatively low, including a lower risk of mortality compared to VanA and VanB,[80] there has been a rise in VanC-type outbreaks reported in humans.[81–85] For example, several human bacteremia cases have been identified as hospital-acquired VRE-C-type *E. gallinarum* and *E. casseliflavus* infections, of which a few cases failed to respond to therapeutic interventions with fatal consequences.[84] Moreover, a study involving 13 Greek human hospitals demonstrated that the VanC phenotype (*i.e.*, C1, C2, and C3) was the most prevalent, representing 57% of VRE isolates followed respectively by VanA and VanB phenotypes.[86] A Swiss study identified the VanC genotype in 98% of 296 clinical VRE strains isolated from patients of which 290/296 VRE isolates were of fecal origin.[87] This is in contrast to the USA, where the VanC genotype is very rare compared to the dominant VanA and VanB genotypes. In addition, recent reports from other regions such as North Africa have revealed a low prevalence of clinical VanC enterococci in comparison to VanA type.[88]

The VanC phenotype is known to be constitutively expressed; however, *E. casseliflavus* and *E. gallinarum* have been reported to harbor inducible resistance types as well.[89] Furthermore, *E. gallinarum/casseliflavus* have been found to carry other *van* gene clusters (*i.e.*, *vanA, vanB* and *vanD*) and express high-level vancomycin resistance.[90–94] VanC *E. gallinarum* have been detected in clinical isolates from humans and farm animals, carrying *vanC-1* and *vanA* genes, and showing a high-level of resistance to glycopeptides and linezolid.[81,82,92,95–98] *E. gallinarum* can capture the genetic determinants of high-level glycopeptide resistance and transfer these determinants to other enterococci (*i.e.*, *E. faecium*).[90,93,99] The emergence of high-level resistance to glycopeptides among less common motile enterococci, mainly *E. gallinarum*, suggests a need for increased awareness for detection and proper characterization of these microorganisms.[100,101]

Geno-Geographic Characteristics of the VRE and Enterococcal Clones

Both *E. faecium* and *E. faecalis* are distinct among enterococci and recognized as a major reservoir of acquired glycopeptide and multidrug-resistance (MDR) by carrying the most common reported *van* genes.[1,8] A systematic review and meta-analysis involving Iran and few European countries revealed a close rate of prevalence between countries of VRE infections in humans and ranges between 8–13%, largely from *E. faecalis* carriers.[102] Whereas in the USA, *E. faecium* is the carrier of 95% of recovered VRE isolates[103] and the main clinical reservoir of the VanA and VanB genotypes in Europe, Northern and Latin America, and Southeast Asia.[14,52,53,63,104] Alternatively, *E. faecalis* has been frequently associated with *vanG*, *L* and *E* genes (Table 1). The other *van* genes remain sparsely reported and neither responsible for most VRE clinical infections nor outbreaks in human medicine.

The VanA genotype is the most common among the reported *E. faecium* and *E. faecalis* isolates worldwide from humans and animals but less frequently reported in other enterococci species such as *E. gallinarum*, *Enterococcus hira*, and *Enterococcus durans*.[18,105] For instance, a recent Iranian study describing a collection of 160 clinical enterococci reported the rate of VRE strains at 19%, largely *E. faecium* species dominantly carrying *vanA* genes.[104]

On the other hand, the VanB genotype is reported more frequently in fewer countries such as Australia, Sweden, and Germany.[106–108] Such variability in genotypes has been attributed to the different antimicrobial use patterns between different countries. For example, in Australia the VanB-VRE is the dominant *E. faecium* from human cases, but has neither been found in animals nor frequently in normal human feces.[109] However, indistinguishable *vanB* elements have been found in anaerobic commensal bacteria from human faeces, and might be a source of VanB resistance to enterococci.[110] In addition, *vanB* genetic determinants have been detected in VanC enterococcal species,[111] and *vanC* genes in *E. faecalis* and *E. faecium* from human infections.[36,112–114] This supports the argument that glycopeptides resistance cannot be predicted only by the VRE genotype, particularly the motile species.[100]

Elucidating further about VRE and enterococcal clinical outbreaks requires different molecular techniques to assess patterns and clonality of genomic DNA as well as subspecies phylogenetic relationships of enterococci. The application of such methods in recent years (*i.e.*, amplified fragment length polymorphism, restriction endonuclease analysis, CRISPR analysis, multi-locus sequence typing (MLST) and whole genome datasets have separated *E. faecium* into two major clades designated as clades A and B.[11,115–121] Clade A are mostly of clinical origin and associated with human-hospital infections and further divide into two subtypes: clade A1, which include epidemic hospital strains, and clade A2, which include animal and sporadic human infections. Whereas, clade B strains are of nonclinical origin and associated with community.[117,120,122]

The genome of clade A1 are characterized by harboring a greater abundance of virulence and antibiotic resistance genes compared to non-A1 lineages.[4] Clade A1 or clonal complex *E. faecium* 17 (CC17, a MLST designation) is a global polyclonal cluster of hospital adapted clones with the potential to cause invasive disease and adapted to the hospital environment and GIT colonization. This clone contains the well characterized global sequence type (ST) 17 and the descendant variants; ST16, ST78, ST63, ST64, and ST174.[122] The global clonal complex *E. faecium* CC17 is frequently characterized as multidrug resistant, including vancomycin, and typically carrying specific virulent gene markers, especially the hyaluronidase gene (*hyl*) and the

enterococcal surface protein encoding gene (*esp)*, as well as the *IS16* insertion-element. These elements characterize CC17 clonal strains and associated with hospital acquired and MDR enterococcal infections.[88] Alternatively, *E. faecalis* are less restricted and some clones such as CC2, CC40, and CC87 are shared between hospitals and the community.

The global increase in MDR *Enterococcus faecium* strains has been linked to the emergence of lineage 78. Isolates belonging to sequence type 117 (ST117) within the lineage 78 are increasingly identified from clinical isolates in many European health institutions.[123] Other countries such as Australia have experienced the emergence of new and rapid spread of *E. faecium* clones within the clade A1 lineage replacing the common ST17 type by the ST203 type as the main cause of blood stream infections and a rising proportion of *vanB* VRE.[120] Genomic analysis of these particular new emergent clones revealed 40 unique genes including Tn*1549* indicating a rapid change in the epidemiology and genomics of VRE in this country.

Genomic and epidemiological investigations of VRE are sparse from the under-developed regions, such as North African. Recent studies from such regions have reported novel information. For instance, a study from Tunisia has documented the prevalence of VanA - *E. faecium* from clinical and hospital sources at 5%, carrying *purK* and *esp* gene, and the IS16 element, but without the *hyl* gene.[88] Furthermore, e-BURST analysis of these strains has identified a new emergent clone ST910 as the dominant type and not belonging to the global CC17, suggesting a rapid evolution in this country.[88]

A previous study from this North African country reported frequent sequence types ST18 and ST80 belonging to CC17 among clinical *van*A *E. faecium*, all carrying the *IS16* element, but lacking *hyl* and *esp* genes.[124] Similar variability was also reported from a Brazilian study describing VRE-*E. faecium* strains from colonized hospital patients showing ST412 as the most shared, belonging to CC17, harboring the *IS16*, Tn*1546*, *esp*, and *acm* genes, but lacking the *hyl* gene.[125] These results contrast previous reports from Brazil and suggesting interspecies horizontal transfer of genetic and resistant elements between the enterococci.[125]

Global VRE and VSE isolates, both from human (*i.e.*, hospital and community-acquired) and nonhuman (*i.e.*, environment and animals) sources have revealed that hospital enterococcal isolates are related closely to/or descending from the MLST single clonal-complex (CC), CC17.[23,126] Further analysis of *E. faecium* demonstrated that the major hospital clones ST78 and its single locus variants ST192 and ST203 group belong in a different BAPS group than ST17 and ST18.[115,127,128] This indicates that hospital-associated *E. faecium* have not recently evolved from a single ancestor and consequently the designation of CC17 as a hospital-selected clonal complex may not be entirely accurate.

Also, the highly prevalent *E. faecium* hospital clones are almost absent in the community,[129] but this is not the case for *E. faecalis*. Based on MLST, there are seven most prevalent *E. faecalis* clones among clinical and outbreak-associated isolates ($n=355$), including ST6, ST9, ST16, ST21, ST28, ST40, and ST87, accounting for only 37% of the hospital-derived isolates (http://efaecalis.mlst.net/).[130] These are found frequently in the community, farm animals and food products while the major hospital-derived *E. faecium* clones are found rarely in the community (8% of 513 nonhospital isolates).[30,131,132]

The complete genomic sequence of *E. faecium* was completed in 2012 and in-depth studies analyzing the genomics of the significant enterococci are still relatively small.[133] Most recent molecular epidemiological studies and genomic analysis using WGS have revealed discriminating information about the genomic characteristic and elements of enterococci involved in virulence and antimicrobial resistance.[121] However, knowledge is still incomplete, where future prospective analysis should rely on combining the current and future genomic insights with the subsequent functional and phenotypic change of the pathogen population and dynamics.

Insights into Animal-Associated VRE and Public Health Issues

Previously, the emergence of VREs in food-animal production systems has been largely attributed to the widespread use of sub-therapeutic avoparcin (a glycopeptide for animal use) for growth-promotion throughout Europe and other countries in the mid-1990 s.[134–141] In fact, the isolation of different VRE genotypes, particularly VanA-VRE, from the community, animal faeces, sewage and raw meats, suggested that VREs colonized and spread as a result of the use of animal growth promoters (esp. avoparcin) and not human hospital-use alone.[57,142–144]

On the contrary, other countries like Canada and United States have never approved avoparcin and not reported VRE in animals until 2008.[18,37] In North America, VREs are one of the major hospital acquired infections attributed largely to the clinical use of vancomycin in humans.[145,146] In addition, the unique epidemiological distribution of VREs in Australia has been attributed to the combination of extensive use of vancomycin in humans and avoparcin as a growth promotor in the animal industry.[4,147]

Avoparcin, as a growth promoter, was banned in the EU in 1997 as detailed in the Commission Directive 97/6/EC. Despite the subsequent reduction of VRE prevalence from animal-derived products and fecal samples of humans,[148] VRE-related human infections and outbreaks have increased in the EU since 1999[149–154] as well as the prevalence of VRE in asymptomatic human carriers in Europe.[155,156] While the ban of antimicrobial growth promoters likely played an important role in reducing VRE carriers in animals, and in some cases a remarkable decrease in the incidence of VREs and glycopeptide-resistant *Enterococcus* species, the consequences of such previous livestock practices may not be reversible.[143,148,157]

In Norway, VREs were still easily found (96–98% proportion of positive samples) from poultry farms exposed previously to avoparcin after more than a year and a half after the ban, whereas none were isolated from the unexposed poultry and swine farms.[158] Moreover, 3 years after the ban of avoparcin, VRE were isolated from previously exposed and unexposed poultry farms (99% and 11% of the respective grouped-studies farms) as well as from farmers (18% and 1% from farmer samples from exposed and unexposed farms).[159,160] Recent data from Europe revealed that VRE-VanA genotypes were detected in Danish poultry production 15 years after the ban of avoparcin and found in 47% of fecal samples with variable resistant phenotypes and

diverse *E. faecium* clones.[161] In Italy and Germany, the frequency has been reduced, however, VREs can still be recovered from different farm animals, poultry meat and pork products, and fecal samples of healthy persons,[148] including clonally related *vanA* VREs.[105] Similar results have also been reported in Taiwan, where a ban of avoparcin in year 2000 resulted in a notable decreased VRE prevalence in chicken but persisted in the same populations.[162]

Prior to the European avoparcin ban, high rates of VRE carriage were also reported in dogs.[163] A subsequent Dutch study, performed 5 years after the avoparcin ban, reported no VRE in a 100 dogs samples.[164] Other studies have found that healthy dogs and cats can be colonized by VRE.[165–167] In addition, VRE isolates from dogs demonstrate similar genetic lineages to hospital-acquired infections in humans.[168–170] Furthermore, VRE carrying *vanA* have been described in healthy horses in Italy, Poland, and Hungary.[171]

When VRE contaminated meat was found in retail outlets as well as the fecal flora of humans, in countries with a heavy use of avoparcin,[172,173] it was quickly concluded that the case against growth-promoting antibiotics, especially avoparcin, was proven.[157,174,175] However, experiences gained after the ban have revealed some doubts as to the route of possible transmission of VRE determinants between animals and humans. For example, despite higher VRE carrier frequencies in animals in Europe, nosocomial VRE infections in humans were lower in Europe than in the United States [176] even though avoparcin was never used in animals in North America.

Initial reports from 10 to 20 years ago indicated that it has been difficult to prove a food-borne infection route to humans directly and the few examples published of identical VREs between humans and animals resulted from direct animal contact, rather than meat consumption.[173] Experimental infection of humans with animal VRE strains resulted in transient colonization only.[177,178] Nevertheless, evidence obtained by using different molecular typing techniques, indicates strong correlations between VREs or genetic determinants in animals and humans (Table 2).[23,65,130,131,166,177–199] These correlations between animal and human VRE carriage does not necessarily demonstrate causality, but that several reservoirs exist, including wild animals and plants.[200]

Alternative theories have been proposed to explain VRE persistence among food producing animals, despite the avoparcin ban.[161] In Denmark, the use of the macrolide, tylosin, in pigs was suggested to co-select for VREs since the genes encoding the two resistances are located on the same plasmid.[201,202] For example, *in vitro* studies have shown that *ermB* and *vanA* genes were located on the same transferable DNA elements, most likely large plasmids. A similar co-selection for VRE persistence has been proposed via tetracycline resistance coded by the *tet*(M), but transferability has not been easily demonstrated.[135,201] Also, a conjugative plasmid conferring acquired copper resistance, via the *tcrB* gene, has been shown to be strongly correlated with macrolide [*erm*(B)] and glycopeptide resistance (*vanA*) genes in *Enterococcus faecium* isolates from pigs, whereby all genes can share the same conjugative plasmid.[143,204]

Another theory is that the plasmid addiction systems located on the same plasmid as the *vanA* gene would force the bacteria to retain the resistance.[204] Essentially, the plasmid addiction system is the strict natural selection of plasmid-encoded gene(s) that is required for the viability of the bacterium.[205]

Nosocomial human-related VREs tend to be more clonal populations persisting in hospitals, and related to human consumption of antimicrobials or hospital environments.[101,206] What appears to be important differences between the VRE subpopulations are other factors that determine spread and virulence. For instance, screening admitted patients for colonized VRE has significantly reduced nosocomial VRE outbreaks and distinguished the community-acquired VRE cases.[206]

In addition, epidemic hospital-acquired strains of vancomycin-resistant *E. faecium* from the USA, Europe, and Australia often have certain virulence genes, *esp*[207] and *hyl* genes,[208] which are not found in nonepidemic or animal isolates, food or sewage.[209] These genes, are part of a putative pathogenicity island and considered to be a marker for nosocomial-epidemicity that contributes to the acquisition of antimicrobial resistant genes and spread of vancomycin-resistant *E. faecium* isolates in hospitals.[210] Thus, the administration of glycopeptides for humans and previous use as feed additive growth promoters in animals are important factors in the emergence of vancomycin resistance in enterococci,[103,147] but other virulence factors appear necessary before these subpopulations are represented in human clinical infections.[211]

Since year 2000, VRE rates in human clinical isolates have increased in numerous European countries despite glycopeptide resistance declining in nonhospital reservoirs.[212] The acquisition of vancomycin resistance by a distinct subpopulation of hospital-acquired ampicillin-resistant *E. faecium* strains has been the main factor responsible for the spread and dissemination. Clones belonging to the CC17 lineage are frequently characterized by ampicillin and fluoroquinolone resistance, and harboring the putative virulence genes *esp* and *hyl*.[23,213–215] As late as 2016, there was a notable increase hospitalized human patients with VRE infections in Danish hospitals (DANMAP, 2016), mostly due to *vanA E. faecium*. In Europe, new reports of VRE are still being reported (*e.g.*, West Balkan regions), as *Enterococcus faecalis* and *E. faecium*, both as VanA and VanB phenotypes.[216]

It is possible that a proportion of European human hospitalized VRE cases stem from environmental or food sources either through bacterial contamination or indirectly, via gene transfer.[217,218] This may in-part explain the different VRE subpopulations with variable levels of virulence associated with human clinical infections. For example, molecular typing of clinical VRE isolates from European hospitalized patients tend to be heterogeneous with respect to their genotypic fingerprints,[176,207] whereas in the USA homogeneity is more common.[219] Other human VRE subpopulations have emerged, either as community-acquired VRE that are frequently susceptible to amoxicillin or gentamicin, or nosocomial VRE, which in contrast are also resistant to amoxicillin/gentamicin.[220]

Emergence of MDR Among *Enterococcus* spp.

The emergence of multidrug resistant enterococci to the newer drugs including last resort antibiotics is worrisome and of global concern (www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en). These have variable susceptibility to even less antimicrobial agents

Table 2. Summary of the Evidence of Links Between VRE or Enterococcal spp. of Animal Origin Versus Human Origin Isolates

Molecular study	*Unit of comparison*	*Results*	*Refs. (No.)*
Cohort study	*E. faecium*	Transient colonization with *E. faecium* of animal origin can persist within the intestines of healthy humans not receiving antimicrobial agents for between 4 to 30 days.	177,178
PFGE	*E. faecium* (Animal vs. human origin)	Similar to highly similar VRE PFGE profiles (Animal isolates vs. Human)	179,180
AFLP	*E. faecium* (Animal vs. Human origin)	Different VRE strains from hospitalized versus nonhospitalized human strains. Hospitalized human VRE strains were similar to dogs, cats, and some veal calves. Nonhospitalized human VRE strains were similar to pigs.	181
MLST	*E. faecium* (Animal vs. Human origin)	Initial reports of VRE (*E. faecium*) strains were host-specific; for example, hospitalized humans VREs clustered in clonal complex (CC)17, whereas animal *E. faecium* isolates belonged to other sequence types and clonal clusters.	23,182
MLST	*E. faecium* animal isolates	Dogs can be a reservoir of VRE (*E. faecium*) belonging to STs (*e.g.*, ST17) related to human clinical infections. Pig: found CC17: ST132 (part of CC17) Chicken meat: found *vanB2* ST17 *E. faecium* Rabbit meat: found *vanA* ST78 *E. faecium* Pig: *vanA E. faecium* isolates CC5 common to pigs and found in human urinary tract infections and fecal samples from nonhospitalized humans.	65,166,183–185
MLST	*E. faecalis* (Animal vs. Human origin)	Hospitals outbreaks related to MLST clonal complexes (CC2, CC9, and CC87). CC2 (ST6) also detected in pigs.	131,186
MLST and PFGE	*E. faecalis*	ST16 *E. faecalis* isolates have been detected in pigs, poultry, healthy humans, and hospitalized patients with endocarditis. ST116 was found in *vanA E. faecalis* ST116 isolates found in turkey meat, nonhospitalized humans, and a patient. *E. faecalis* isolates of ST40 and ST97 detected in pigs and endocarditis patients.	130,131,186–190
PCR, sequencing	*E. faecium*	Indistinguishable variants of Tn*1546* transposons found in isolates of human and nonhuman origin. Point mutation in *vanX* (G to T) at position 8234 in Tn*1546*, specifies VREs of pig origin (*e.g.*, T mutation), and poultry origin (*e.g.*, G mutation). Both types were found among human *E. faecium* isolates.	191–195
Gene transfer experiment, with gnotobiotic mice/rats.	*E. faecium*	High frequency transfer of *vanA* from animal origin *E. faecium* to human origin *E. faecium*. *vanA* of chicken origin was transferred to a CC17 recipient (obtained from a patient with sepsis) within the intestines of cephalosporin-treated mice.	196–198
In vivo conjugation experiment	*E. faecium*	Transfer of *vanA* from an *E. faecium* isolate of animal origin to an *E. faecium* isolate of human origin (non-CC17 type) within the intestines of healthy human volunteers.	199

VRE, vancomycin-resistant enterococci; PFGE, pulsed field gel electrophoresis; AFLP, amplified-fragment length polymorphism; MLST, multilocus sequence typing; PCR, polymerase chain reaction; CC, clonal complex; ST, sequence type.

and increasingly associated with significant morbidity and mortalities in human medicine. The concern is further complicated by the genetic intra-ability of enterococci to exchange resistance determinants and/or transfer to other Gram-positive organisms such as staphylococci and streptococci.[221] Generally, oxazolidinone-linezolid, daptomycin, quinupristin/dalfopristin (QD), and tigecycline are invaluable antibiotics frequently used to treat serious infections and therapeutic complications caused by multidrug resistant enterococcal infections, VRE and MRSA.[222,223]

Linezolid, daptomycin, and tigecycline have been increasingly utilized over the past decade as last-line therapeutics, to combat MDR enterococci and staphylococci, however, clinical isolates with reduced susceptibility have emerged.[221,224–226] Oxazolidinone-linezolid was approved in 2000 and widely used, but the emergence of resistance is reportedly associated with its therapeutic and extended applications for VRE as well as vancomycin-susceptible enterococci cases. Linezolid-resistant enterococci (LRE) is of greater concern and increasingly encountered over the past decade although at a low prevalence of less than 1% among the enterococci and staphylococci.[223,225] However, new generation oxazolidinones (*i.e.*, Tedizolid phosphate) has been recently approved, which demonstrated better efficacy against clinical MDR Gram-positive pathogens such as MRSA, VRE, and LRE.[225]

Resistance (or reduced susceptibility) to the oxazolidinone-linezolid is frequently reported to be mediated either by acquisition of the chloramphenicol–florfenicol resistance (*cfr*) gene or point mutations within the ribosomal complex system (23S rDNA or ribosomal protein genes).[225] Linezolid resistance is also linked to *optr*A gene, which recently was described in humans, food producing animals and products, from various global regions (*i.e.*, China and Denmark).[218] Acquisition of the *cfr* gene is often carried on transferrable mobile genetic determinants, associated with resistant infections in livestock and reduced susceptibility to a broad range of drugs (*i.e.*, Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A). Furthermore, a study from Germany has recently documented a *cfr* like-gene named *cfr* (B) variant gene locus in clinical strains of *E. faecium* with 99.9% identical sequence to a *cfr*-like gene from *Clostridium difficile*.[223] This new variant gene was found on both mobile and/or extra-chromosomal DNA. Recently, *E. faecium* was reported from food producing animals harboring a novel plasmid coding for multidrug resistance, including Linezolid.[227]

In addition, tigecycline-resistant *E. faecium* is also emerging, but the precise mechanism is unclear. A recent genomic analysis of clinical enterococci expressing tigecycline-resistant revealed tetracycline-resistant determinants; tet(L)- encoded efflux pumps and tet(M)-encoded ribosomal protein in the expression of such resistance.[226] Another recent German investigation analyzing the spread of Tn*1549*-*vanB*-genotype resistance revealed the dissemination was attributed to the exchange of large chromosomal fragments between positive and negative *vanB* enterococci isolates thus providing a better understanding of the spread of multidrug-resistant pathogens.[108]

In summary, VRE and enterococcal MDR infections are increasingly reported from humans with distinct geographic differences. The *vanA*-VRE are more commonly associated with poultry than other food animals (*e.g.*, cattle, swine), with some geographic differences. On the other hand, VanC and VanN-VRE phenotypes have been detected frequently from farm and companion animals as well as meat products destined for human consumption (*i.e.*, chicken, pigs, cow, and horses).[228–230] Other reports have also shown the unusual presence of different *van* genes in *E. casseliflavus* isolated from farm animals.[231] Thus, animal microflora may play an important role in the development and emergence of novel and evolved geno/phenotypes of glycopeptide and multidrug resistances in different enterococcal strains as well as other bacterial strains.[232,233] Nevertheless, the zoonotic risk of different resistant organisms including VREs from farms and livestock has not been fully defined.[234]

Enterococci of foodborne origin are not known as a direct source of resistant enterococci in humans, but could pose a risk to transfer resistance determinants (*e.g.*, *van* genes) to human-adapted enterococcal strains. For example, human colonization by VRE of animal origin has been documented including trans-conjugant transfer of *van* genes between enterococcal species within the human intestinal microflora.[199,235,236] Also, the documentation of *in vitro* interspecies conjugal transfer of vancomycin resistance and MDR enterococcal phenotypes of environmental origin indicates the potential risk of these sources as well as the diverse ability to transfer such resistance determinants to human pathogens.[237] Antimicrobial-resistant *E. faecalis* and *E. faecium* of animal origin have demonstrated the ability to acquire resistance genes and act as donors of such genes.[238,239] Such complex genetic exchanges and acquisitions might explain the continuing emergence and evolution of enterococcal antimicrobial resistance.

Conclusions and Prospective Challenges

Enterococcus species colonize and survive in widely different hosts and conditions. As part of the healthy intestinal flora and environment, enterococci are part of the cycle of continuous exposure to prophylactic/metaphylactic and therapeutic antimicrobial agents in human and animal hosts, which has likely contributed to their ability to acquire and develop unique profiles of virulence and antimicrobial resistance.

In contrast to vancomycin-resistant *E. faecalis* and *E. faecium*, other species such as *E. casseliflavus* or *E. gallinarum* have attracted less attention, despite associations with environmental and livestock sources as well as human infections and outbreaks. This suggests a need for continuous monitoring of a broader range of enterococcal species as well as other vancomycin-resistant genes (*e.g.*, VanC genotypes).

The role of animals and food products in the human VRE population dynamics needs to be further defined. While many aspects of VRE are well known to human and public health, both veterinary and agricultural awareness of these ongoing issues is sparse. It may have been a premature conclusion that the animal involvement of VRE issues was resolved after the ban of avoparcin. The collection of evidence suggests that animal-associated vancomycin-resistant *E. faecalis* and *E. faecium* as direct sources of human infections is unlikely. The role of animal-associated vancomycin-resistant *E. casseliflavus*, *E. gallinarum* and others needs to be further defined.

Food animals can be reservoirs of *van* genes of clinical importance to human-related infections (*e.g.*, *vanA*, *vanC*), including novel *van* genes. Further detailed sequencing of animal-associated *van* genes compared to clinical human VRE isolates could help to better define the significance of animal reservoirs. Transient colonisation of animal-associated VRE can lead to the transfer of *van* genes to human adapted enterococci strains with relevant virulence factors. The amplication of animal VRE reservoirs can be related to antimicrobial use patterns, as demonstrated by the previous massive consumption of avoparcin, with major spillover effects on human populations.

Persistence of *van* genes in animal populations has proven to be more challenging to resolve than previously thought. The potential co-selection of VREs in animals based on other antimicrobial consumptions (*e.g.*, macrolides, tetracyclines) is worthy of further investigations. In contrast to humans, VRE infections in animals are rare, and infrequently seen in companion animals. Given the importance of animal reservoirs then VRE screening should be part of national antimicrobial resistance surveillance systems for indicator bacteria. Since the ban on avoparcin, veterinary medicine has demonstrated that the needs of animal health can be served without glycopeptide antimicrobials. As such, the class of glycopeptide antimicrobials should be reserved for human medicine.

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None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

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