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Intrinsic and acquired resistance mechanisms in enterococcus

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Enterococci have the potential for resistance to virtually all clinically useful antibiotics. Their emergence as important nosocomial pathogens has coincided with increased expression of antimicrobial resistance by members of the genus. The mechanisms underlying antibiotic resistance in enterococci may be intrinsic to the species or acquired through mutation of intrinsic genes or horizontal exchange of genetic material encoding resistance determinants. This paper reviews the antibiotic resistance mechanisms in *Enterococcus faecium* and *Enterococcus faecalis* and discusses treatment options.

Introduction

Enterococci are Gram-positive, facultative anaerobic organisms characterized by their ability to grow at 6.5% NaCl concentrations and at high pH and to hydrolyze bile-esculin and L-pyrrolidonyl-B-naphthylamide (PYR). Formerly considered members of Lancefield group D streptococcus, DNA homology studies suggested that they are a distinct genus. Enterococci were previously considered commensal organisms of little clinical importance, but have emerged as serious nosocomial pathogens responsible for endocarditis and infections of the urinary tract, bloodstream, meninges, wounds and the biliary tract, among others.[1] Recent surveillance data indicate that the enterococcus is the third most commonly isolated nosocomial pathogen (12% of all hospital infections), behind only coagulase-negative staphylococcus and *Staphylococcus aureus*.[2] The rise in prevalence of enterococcal infections in humans is influenced to some degree by the ability of enterococci to escape the action of our most commonly used antibiotics. The influence of antibiotics is most directly seen on the extent to which enterococci colonize the gastrointestinal tract. Animal data have clearly shown the relationship between exposure to parenteral antibiotics, especially extended-spectrum cephalosporins and agents with potent activity against anaerobic bacteria, and high level gastrointestinal colonization by ampicillin-resistant *Enterococcus faecium*.[3] The relationship between colonization and subsequent infection is also established. Along with increasing antimicrobial resistance, the acquisition of virulence factors and the ability of enterococcus to form biofilms have also contributed to the rise in nosocomial prevalence.[4]

This paper reviews the mechanisms underlying antibiotic resistance in enterococci, both intrinsic (universally found within the genome of the species) and acquired (through acquisition of new genetic material or through sporadic mutations to intrinsic genes). Interspecies differences will be addressed as they arise throughout the paper. This paper will additionally provide an overview of current treatment strategies for enterococcal infections. Focus will be on *Enterococcus faecalis* and *E. faecium*, as these two species account for the overwhelming majority of human enterococcal infections (**Table 1**).

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Intrinsic Resistance

β-lactams and cephalosporins. Growth of most bacteria depends upon enzymatic linkage of pentapeptide precursor molecules into a peptidoglycan cell wall. The enzymes responsible for these cross-linking reactions are referred to as penicillin binding proteins (PBPs) because β-lactams (structural analogs of pentapeptide precursors) bind covalently and disrupt normal cell wall growth.[5] Attachment of β-lactam agents to PBPs results in impaired cell wall synthesis and, in most cases, programmed cell death via creation of reactive oxygen species.[6] Enterococci express low-affinity PBPs (PBP5 in *E. faecium*, PBP4 in *E. faecalis*) that bind weakly to β-lactam antibiotics. As a result, minimum inhibitory concentrations (MICs) for penicillins are typically 2–8 μg/ml for *E. faecalis* and 8–16 μg/ml for *E. faecium*,[7] much higher than MICs for streptococci and related Gram-positive organisms that do not contain chromosomally-encoded low-affinity PBP genes.[8] At the population level, enterococcal MICs have increased over time.[9,10] Galloway-Pena et al.[11] demonstrated two distinct clades of *E. faecium*. These clades have PBP5 enzymes that vary in affinity, a result of differences in amino acid sequence and transcriptional regulation. Overproduction of non-mutated low-affinity PBPs represents a relatively rare mechanism by which enterococci express low-level resistance to penicillins.[7,12]

Early studies by Jawetz et al.[13] indicated that enterococci were not killed by penicillin when exposed to drug concentrations in the range of the MIC (a phenomenon known as tolerance). Tolerance in *E. faecalis* has been attributed to removal of reactive oxygen species by the enzyme superoxide dismutase.[14] In other

Table 1. Mechanisms of resistance to *E. faecium* and *E. faecalis*

Antibiotic resistance	Mechanism of resistance	Associated enzyme	Phenotype	Intrinsic, sporadic or associated MGE	Host range	References
Aminoglycosides						
	Low cell wall permeability	-	Low-level aminoglycoside resistance, synergy preserved	Intrinsic	*E. faecalis*	8–10
	Ribosome mutation	-	High-level aminoglycoside resistance with MIC > 128,000 μg/ml	Sporadic	*E. faecalis* *E. faecium*	42
	Aminoglycoside-modifying enzyme (AME)	Aac(6′)-Ii	Low-level tobramycin and kanamycin resistance	Intrinsic	*E. faecium*	11
	AME	Aph(3′)-IIIa	Low-level kanamycin resistance	pJH1	*E. faecium*	43
	AME	Ant(4″)-Ia	Low-level resistance to kanamycin, tobramycin, amikacin and neomycin	pIP810	*E. faecium*	44
	AME	Aph(2″)-Ia-Aac(6′)Ie	High-level gentamicin resistance	Tn*5281*	*E. faecalis* *E. faecium*	34
	AME	Aph(2″)-Ib	High-level gentamicin resistance	Unknown	*E. faecium*	136
	AME	Aph(2″)-Ic	High-level gentamicin resistance	pYN134	*E. faecalis* *E. faecium*	38
	AME	Aph(2″)-Id	High-level gentamicin resistance	Unknown	*E. faecium*	40
	AME	Ant(6′)-Ia	High-level streptomycin resistance	Tn*1546*, Inc.18, Tn*5382*	*E. faecalis* *E. faecium*	37, 137, 138
	AME	Ant(3″)-Ia	High-level streptomycin resistance	pR538–1	*E. faecium*	37, 41
	Ribosome-modifying methyltransferase	EfmM	Tobramycin and kanamycin resistance	Intrinsic	*E. faecium*	12
β-lactams and cephalosporins						
	PBP4/5 production	-	Low-level penicillin resistance; moderate to high-level cephalosporin resistance	Intrinsic	*E. faecalis* *E. faecium*	3
	PBP4/5 point mutation	-	High-level ampicillin and imipenem resistance	Sporadic	*E. faecalis* *E. faecium*	25–27
	Altered cell wall	L,D-transpeptidase	β-lactam resistance	Intrinsic	*E .faecium*	139, 140
	Destruction of β-lactam ring	β-lactamase on *bla* genes	β-lactam resistance	Tn552 and others	*E. faecalis* *E. faecium*	4
Glycopeptides	Synthesis of alternative cell wall	VanA, VanH, VanY, VanX, VanR, VanS	Resistance to vancomycin +/− teicoplanin depending on the phenotype	Tn*1546*, Inc.18	*E. faecalis* *E. faecium*	47
Lincosamides	ABC-efflux pump	Lsa	Resistance to clindamycin, streptogrammin A and B	Intrinsic	*E. faecalis*	14
	ABC-efflux pump	MsrC	Low-level resistance to streptogramin B compounds	Intrinsic	*E. faecium*	16
	ABC-efflux pump	VgaD	Streptogramin A resistance	Putative transposon	*E. faecium*	83
	Acetyltransferase	VatD (SatA)	Streptogramin A resistance	Putative transposon	*E. faecium*	77
	Acetyltransferase	VatH	Streptogramin A resistance	Putative transposon	*E. faecium*	83
	Acetyltransferase	*VgbA*	Streptogramin B resistance	Unknown	*E. faecium*	79
	Acetyltransferase	VatE (SatG)	Streptogramin A resistance	Unknown	*E. faecium*	78, 141
	Altered ribosome	ErmA	MLS_A phenotype	Tn*554*	*E. faecalis* *E. faecium*	142
	Altered ribosome	ErmB	MLS_B phenotype	Tn*917*, Tn*1545*	*E. faecalis* *E. faecium*	16, 20

Table 1. Mechanisms of resistance to *E. faecium* and *E. faecalis* (continued)

Antibiotic resistance	Mechanism of resistance	Associated enzyme	Phenotype	Intrinsic, sporadic or associated MGE	Host range	References
Linezolid	rRNA point mutations	G2576T, G2505A, L4(F101L)	Linezolid resistance	Sporadic	*E. faecalis* *E. faecium*	89–91
	Methylated rRNA	Cfr	Linezolid, lincosamides, streptogramin A resistance	pEF-01	*E. faecalis* *E. faecium*	94
Daptomycin	Altered membrane-bound protein	Cardiolipin sythetase	Contributes to Daptomycin resistance through an unknown mechanism	Sporadic	*E. faecalis* *E. faecium*	104, 105
	Altered membrane-bound protein	GdpD	Daptomycin resistance, effect is amplified in combination *liaF* mutation	Sporadic	E. faecalis E. faecium	105
	Altered membrane-bound protein	LiaF	Daptomycin resistance when combined with *gdpD* mutation	Sporadic	*E. faecalis* *E. faecium*	105

Gram-positive species, downregulation or absence of a two-component signal transduction VncR/S autolytic system also contributes to penicillin tolerance,[15] but this mechanism has not been demonstrated in enterococcus. Tolerance may be induced when penicillin is administered by pulsed-dosing. As such, penicillin-naive enterococcal strains may appear susceptible in vitro but develop tolerance after exposure to the drug.[16,17]

Aminoglycosides. Both *E. faecium* and *E. faecalis* are intrinsically resistant to clinically achievable concentrations of aminoglycosides. In *E. faecalis*, MICs vary for the aminoglycosides, with the greatest degree of resistance seen to streptomycin (MIC up to 500 μg/ml). Intrinsic resistance in *E. faecalis* is attributed to an inability of the aminoglycoside to enter the cell (where they act by inhibiting ribosomal protein synthesis), as demonstrated in experiments by Moellering and colleagues in the early 1970s.[18,19] When enterococci were exposed to radiolabeled aminoglycoside with or without penicillin, higher intracellular aminoglycoside concentrations were achieved in the presence of the cell wall synthesis inhibitor. The combination of cell wall active agents and aminoglycosides also resulted in bactericidal activity (bactericidal synergism). These studies provide physiologic context to the long-standing observations of improved clinical outcomes with aminoglycoside-penicillin combination therapy.[20]

Some enterococci also express chromosomally-encoded enzymes that increase the MIC of aminoglycosides and prevent synergism. Ubiquitous among *E. faecium*, the aminoglycoside 6' acetyltransferase [AAC(6')-Ii] confers resistance to tobramycin with MICs as high as 1000 μg/ml and to kanamycin.[21] Additionally, an *efmM*-encoded m^5C methyltransferase in *E. faecium* confers low-level resistance to dibekacin, tobramycin and kanamycin.[22] EfmM methylates the 16S rRNA resulting in a sterically-hindered ribosome target site.[22]

Intrinsic enzyme-mediated high-level resistance to neither gentamicin nor streptomycin has been described in enterococci. As such, these drugs retain synergistic activity in enterococci and have consequently emerged as the drugs of choice to achieve synergism in severe infections caused by either *E. faecium* or *E. faecalis*.[23]

Lincosamides and streptogramins. *E. faecalis* are intrinsically resistant to clindamycin (a lincosamide), quinupristin (streptogramin B class) and dalfopristin (streptogramin A class) through activity conferred by expression of the *lsa* gene. *lsa* is related structurally to ATP-binding cassette (ABC)-efflux pumps, suggesting drug efflux as a possible mechanism,[24] and was found in 180/180 strains of *E. faecalis* and 0/189 other enterococcus species, suggesting the gene is intrinsic to *E. faecalis*.[24] In general, for clinical resistance to quinupristin-dalfopristin to occur, the bacteria must be resistant to both streptogramin A and streptogramin B. *E. faecium* harbors a different putative ABC-efflux pump encoded by the intrinsic *msrC* gene.[25] This gene, a close relative of *msrA* and *msrB* in staphylococci, confers low-level resistance (MIC 1–2 μg/ml) to streptogramin B compounds, explaining the elevated quinupristin-dalfopristin MICs seen when *E. faecium* acquires a separate determinant that confers streptogramin A resistance alone.

Trimethoprim-sulfamethoxazole. Most bacteria lack the ability to absorb folate from the environment and as such require de novo folate synthesis in order to produce nucleic acids. The antibiotic combination trimethoprim-sulfamethoxazole inhibits two sequential steps in the tetrahydrofolate synthesis pathway, thereby inhibiting folate synthesis and synergistically killing a broad spectrum of bacterial species. Enterococci are unusual in that they can absorb folic acid from the environment, bypassing the effects of trimethoprim-sulfamethoxazole.[26] Therefore, in vitro testing of enterococcal susceptibility to trimethoprim-sulfamethoxazole in a media devoid of folate will yield a susceptible result.[27] Despite this apparent in vitro susceptibility, trimethoprim-sulfamethoxazole is ineffective in treating serious enterococcus infections.[28,29]

Acquired Resistance

Acquired resistance in enterococci (that which is not intrinsic to the species) can occur through sporadic mutations or through acquisition of foreign genetic material. Horizontal gene exchange among enterococci occurs through the transfer of pheromone-sensitive or broad host range plasmids, or through the movement of transposons. With few exceptions, multiple plasmids and transposons can be identified in clinical strains. These elements may interact with each other and with the bacterial chromosome to form composite mobile elements. For recent reviews of the plasmids and transposons in enterococci, refer to Palmer[30] and Hagstead,[31] respectively.

Pheromone-responsive plasmids are found predominantly in *E. faecalis*. Chromosomally encoded lipoprotein fragments ("pheromones") released by recipient cells are sensed by nearby donor cells and stimulate production of aggregation substance (Asa1, PrgB and others), encoded by the plasmid.[32] Aggregation substance interacts with enterococcal binding substance (EBS) on the surface of the recipient cell and stimulates recipient-donor contact that promotes conjugation.[30] These plasmids transmit genetic information in a highly efficient manner between *E. faecalis* strains (10^{-3}/donor cell during 4 h of mating), but are largely restricted to this species. pRUM plasmids in *E. faecium* are similar to pheromone-responsive plasmids in *E. faecalis* in that they transfer at a high frequency but exhibit a narrow host range.[31]

In contrast, broad host range plasmids are capable of transferring genetic information to other gram-positive and even gram-negative species,[30] but at a lower frequency (10^{-7}/donor cell during 4 h of mating) than pheromone-responsive plasmids. Transfer of these plasmids requires close contact between cells. Inc.18-type plasmids are well-known broad host range plasmids that have been implicated in the transfer of vancomycin resistance determinants to *S. aureus* in recent years.[33]

Three types of transposons are responsible for most gene mobility in enterococci, Tn*3* family transposons, composite transposons, and conjugative transposons.[31] The prototypical Tn*3* family transposons are Tn*917* [conferring macrolide, lincosamide and streptogramin$_B$ resistance (MLS_B)] and Tn*1546* (conferring glycopeptide resistance), whereas the prototypical conjugative transposon is Tn*916*, which confers resistance to minocycline and tetracycline.[34] Composite transposons can readily be formed by the interaction of related IS elements that are liberally sprinkled throughout the genome of most clinical enterococcal strains. The movement of these IS elements not only confers mobility to resistance genes, but it promotes cointegration of plasmids with other plasmids and with the bacterial chromosome.

β-lactams. Enterococci may develop increased resistance to penicillins through acquisition of β-lactamases or PBP4/5 mutations. Plasmid-mediated *bla* genes (encoding β-lactamases) were first described in *E. faecalis* in 1983.[35] Since that time, enterococcal β-lactamase production has been rare and described predominantly in *E. faecalis*. The *bla* genes in enterococcus are identical to those in *S. aureus*[8] and are often encoded by remnants of staphylococcal β-lactamase transposon Tn*552*.

High-level penicillin resistance in *E. faecium* is most commonly associated with accumulation of point mutations in the penicillin binding region of PBP5.[5] A variety of point mutations have been described in both *E. faecium*[36,37] and *E. faecalis*.[38] Although these point mutations likely originated de novo in individual bacteria under selective pressure from antibiotics, chromosome-to-chromosome transfer of low affinity *pbp5* genes has been documented in vitro and likely contributes to the dissemination of high-level penicillin resistance in *E. faecium*.[39]

Ceftaroline and ceftobiprole, fifth generation cephalosporins, have activity against enterococcus, but may be prone to emergence of resistance with increased clinical use. Clark et al.[40] subjected *E. faecalis* to serial passages of ceftaroline and identified two resistant isolates (one with an MIC of 8 μg/ml and the other with an MIC of 32 μg/ml). Ceftobiprole shows good in vitro activity against *E. faecalis* with no reports of resistance to date,[41,42] but is ineffective against penicillin-resistant clinical strains of *E. faecium*.[43,44]

Aminoglycosides. While intrinsic mechanisms result in low-level aminoglycoside resistance, acquisition of mobile genetic elements typically underlies high-level aminoglycoside resistance in both *E. faecium* and *E. faecalis*. Ensuing MICs range from 2,000 μg/ml to as high as 128,000 μg/ml.[1] Among the genes that encode high-level resistance, the most concerning are those that result in gentamicin and streptomycin resistance because these antibiotics are used for synergistic therapy of serious enterococcal infections.

High-level gentamicin resistance most frequently occurs through acquisition of a bifunctional gene encoding APH(2'')-Ia-AAC(6')-Ie.[45] These enzymes inactivate gentamicin (and structurally related aminoglycosides) by phosphorylation at the 2'hydroxy position of gentamicin and simultaneous acetylation of the 6'hydroxy position of the other aminoglycosides.[46,47] The modified antibiotic is no longer capable of binding to its target on the 30S ribosomal subunit and thereby loses antibacterial activity. Strains that contain *aph(2'')-Ia-aac(6')-Ie* are clinically resistant to all aminoglycosides except for streptomycin.[48] The *aph(2'')-Ia-aac(6')-Ie* gene is most commonly flanked by IS*256* in a composite transposon designated Tn*4001* in *S. aureus* and Tn*5281* in *E. faecalis*.

Several other genes have been identified that confer gentamicin resistance, including *aph(2'')-Ic*, *aph(2'')-Id* and *aph(2'')-Ib*.[49–51] In comparison with *aph(2'')-Ia-aac(6')-1e*, these genes are minor contributors to gentamicin resistance in enterococci. Their prevalence varies by geographical region. Importantly, MICs for enterococci harboring *aph(2'')-Ic* may be as low as 256 μg/ml, an MIC which would be interpreted as gentamicin-susceptible by labs that use an MIC of 500 μg/ml as a cut-off to determine high-level gentamicin resistance. Despite the lower MIC, bacteria expressing these enzymes are resistant to the synergistic activity of cell wall active agents and gentamicin.[48] Thus, in geographical area where *aph(2'')-Ic* is present, laboratories should be alerted to lower the threshold MIC for gentamicin to enhance detection of enterococci that would be resistant to synergy.

High-level resistance to streptomycin occurs most commonly through enzymatic modification of the antibiotic or by single point mutations to the ribosome. Two well-described adenylyltransferases, Ant(6')-Ia and Ant(3'')-Ia, are capable of inactivating streptomycin (and structurally related aminoglycosides).[48,52] Enterococci can also develop ribosomal mutations that result in streptomycin resistance. Whereas resistance caused by aminoglycoside-modifying enzymes (AME) will typically have MICs in the 4,000 to 16,000 μg/ml range, ribosomal mutations result in MICs of 128,000 μg/ml.[53]

Other acquired AMEs have been identified in enterococci, including Aph(3')-IIIa, an aminoglycoside phosphotransferase that confers resistance to kanamycin[54] and Ant(4'')-Ia, a nucleotidyltransferase that confers resistance to tobramycin, amikacin, neomycin and kanamycin.[55] As these enzymes do not confer gentamicin or streptomycin resistance, they are of less clinical significance.

Glycopeptides. The acquisition of glycopeptide resistance by enterococci has been an epidemiological and antimicrobial dilemma for the past 25 years. First described in 1988, glycopeptide-resistant enterococci (GRE) have since emerged as a major cause of nosocomial infections. The majority of GRE infections are attributed to *E. faecium*, although glycopeptide resistance occurs in *E. faecalis* and other enterococcus species as well. Currently in the United States, an estimated 30% of clinical enterococcus isolates are resistant to glycopeptides.[56]

Vancomycin acts by binding to the D-ala-D-ala terminus of the pentapeptide precursor, thereby inhibiting cell wall synthesis. Glycopeptide-resistant organisms modify these pentapeptide precursors, replacing the terminal D-ala with D-lac or D-ser. These modified cell wall precursors bind glycopeptides with 1,000-fold lower affinity than do normal precursors. To create the modified precursors at least seven enzymes are required. Using the VanA cassette as a model, these enzymes are VanA, H, X, Y, Z, R and S (**Fig. 1**). Initially, cellular pyruvate is converted to d-lactate by the VanH dehydrogenase. The VanA ligase then ligates D-ala to D-lac. Host enzymes ligate D-ala-D-lac to the tripeptide precursor, yielding the low affinity pentapeptide precursor. Full resistance to glycopeptides, however, requires not only construction of the altered precursor, but also elimination of normal precursors.[33,57,58] VanX hydrolyzes D-ala-D-ala to its constituent amino acids, which allows D-ala-D-lac to be the sole substrate for cell wall synthesis.[59] VanY hydrolyzes the terminal D-ala from any normal pentapeptide precursor, rendering it useless for normal cell wall construction.[60] The mechanism by which VanZ augments resistance is unknown, but when present it confers decreased susceptibility to teicoplanin.[61] Additional open reading frames VanW and VanV have been described on the VanB operon; their functions also are not yet known.[62]

Expression of the genes for VanA, H, X, Y and Z are all regulated by VanR and VanS, a two-component sensor-transducer system that is part of the VanA operon within Tn*1546*. While the specific regulatory factors are not known, the presence of glycopeptides in the environment results in activation of VanS through autophosphorylation. Activated VanS then phosphorylates

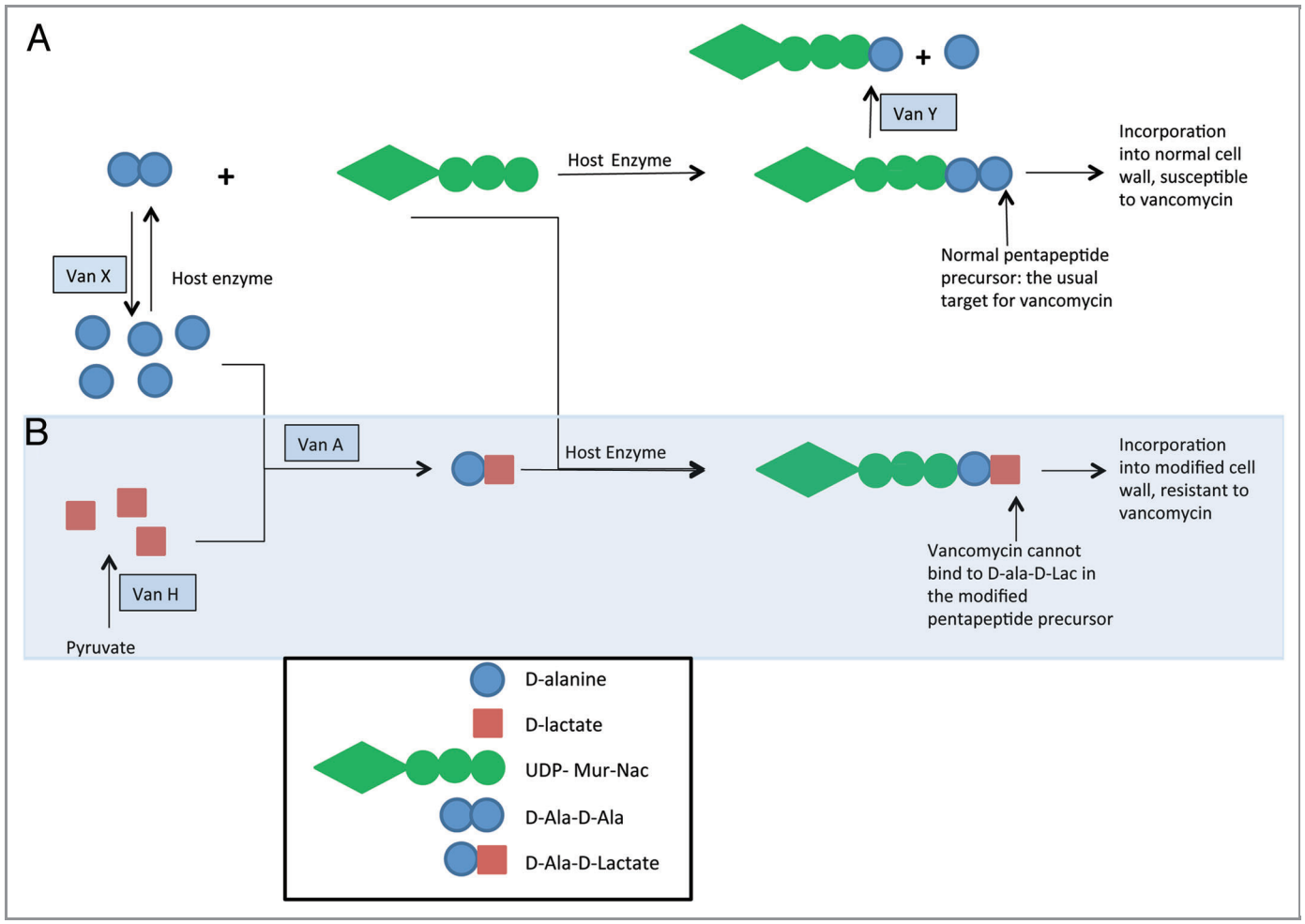

Figure 1. An illustration of the VanA resistance mechanism as it relates to normal cell wall synthesis. The top pathway denotes normal cell wall synthesis, and the mechanisms by which VanX and VanY disrupt this pathway. The shaded pathway denotes construction of a modified cell wall that is resistant to vancomycin. Adapted from Gold et al.[58]

VanR. Phosphorylated VanR increases VanH, A, X, Y and Z transcription through interaction with specific promoter regions. VanR also interacts with its own promoter region, augmenting VanR and VanS transcription.[63] Clinical strains that harbor the VanA operon but contain deletions in VanR and VanS genes have been isolated and are susceptible to both vancomycin and teicoplanin. This suggests that VanR activity is required for the full expression of the VanA operon.[64,65]

VanA and VanB operons are by far the most prevalent in human GRE infections. In the VanA phenotype, the enterococcus is resistant to both vancomycin and teicoplanin. In the VanB phenotype, vancomycin but not teicoplanin induces resistance resulting in a vancomycin resistant, teicoplanin-susceptible phenotype; however, constitutive expression (which may be selected by teicoplanin exposure) results in resistance to both compounds. VanC resistance is intrinsic to *E. gallinarum* and *E. casseliflavus*. A total of nine resistance operons have been described. They may be grouped by their ligase activity. Operons that encode D-lac ligases result in high-level resistance with MICs $>$ 256 μg/ml (VanA, VanB, VanD and VanM) while operons that encode D-ser ligases result in low-level resistance with MICs 8–16 μg/ml (VanC, VanE, VanG, VanL and VanN).[31,66–69] Of the low-level resistance phenotypes, only VanN has been shown to be transferable.

Horizontal transfer of the Van genes occurs through a variety of mechanisms. VanA is mobilized on Tn*3*-family transposon Tn*1546*. Tn*1546* is found on both non-conjugative and conjugative plasmids. Inc.18 plasmids are broad host range plasmids that have been implicated in the transfer of the VanA operon to methicillin-resistant *S. aureus*.[33] Vancomycin-resistant *S. aureus* (VRSA) has been found in clinical settings in a handful of cases. Werner et al.[70] demonstrated in vitro that interspecies transfer of Tn*1546* is relatively uncommon compared with intraspecies transfer. It appears that while broad host-range plasmids can transfer between species, their stability within different species varies. As such, broad host range plasmids containing an intact copy of Tn*1546* may transfer resistance to staphylococci more stably, since the transposon can transfer to replicons within the staphylococcal strain that are stable. Staphylococcal variants that have acquired broad host range plasmids with Tn*1546* variants that have lost their ability to transfer through deletion of or insertion into the transposition genes will exhibit an unstable phenotype due to the instability of the plasmid in the staphylococcal milieu.[71] Additionally, in vitro studies demonstrating transfer of Tn*1546* from enterococcus to *S. aureus* have occurred in *E. faecalis*.[72,73] Sequence homology has been observed between plasmids found in VRSA isolates and GRE isolates taken from VRSA infected patients, with the most overlap occurring with *E. faecalis* isolates.[74] Compared with *E. faecium*, VanA-containing *E. faecalis* are relatively uncommon in the clinical setting. If *E. faecalis* is a more effective (but less common) donor than *E. faecium,* then this may help to explain why VanA in staphylococci is rare. VanB is most often carried on a carried on Tn*5382*/*1549* or related conjugative transposons. VanB carrying transposons have been identified in pheromone-sensitive and conjugative plasmids.[30,31]

The complex enzymatic pathways that confer glycopeptide resistance predate the emergence of GRE in the late 1980s. *E. gallinarum* and *E. casseliflavus* exhibit innate low-level resistance through a chromosomally-encoded VanC operon[75] and have been implicated as a source of the genes seen in other Van phenotypes.[76,77] Additionally, a number of soil and bowel organisms have been identified as harboring VanB genes and may have played a role in the transfer of glycopeptide-resistance genes to *E. faecium*.[78-82] Enterococci are increasingly recognized as belonging to two distinct clades, one that predominates in the hospital environment and another within the community. These clades differ genetically, and may have diverged between 300,000 to a million years ago.[83] The nosocomial clade has acquired virulence and resistance determinants that confer a selective advantage in this setting. Acquisition of the VanA cassette in the late 1980s likely conferred further advantage that contributed to the observed increase in prevalence of infections due to *E. faecium*.

Streptogramins. The streptogramin B/A combination quinupristin-dalfopristin is one of two antibiotics approved by the FDA for treatment of infections caused by vancomycin-resistant *E. faecium*. Because *E. faecalis* are intrinsically resistance to streptogramins, the majority of genes that confer horizontally-transferable resistance have been isolated from *E. faecium*. Between 1 to 12% of *E. faecium* isolates are resistant to streptogramins.[84,85] There are three mechanisms by which acquired genetic elements cause streptogramin resistance: acetylation of the antibiotic, efflux of the antibiotic, and dimethylation of the 23S rRNA target site. To date, 12 genes that cause streptogramin resistance have been described in enterococci, although additional genes have been described in staphylococci and streptococci.

The widespread use of virginamycin, a veterinary streptogramin A compound, was associated with extensive resistance among enterococci isolated from farm animals and agricultural sewage. Consequently, quinupristin-dalfopristin resistance is most common in environmental samples, although the prevalence in nosocomial infections with resistance is increasing.[84] Enzymatic acetylation of streptogramin A compounds was the first resistance mechanism described in the class. Virginamycin acetyltransferase genes *vatD*, *vatE* and *vatH* are among the streptogramin resistance genes with probable veterinary origins. *vatD* and *vatE* (formerly called *satG*) have been isolated from plasmids alongside *erm* and *vgbA* genes (described below) that reduce susceptibility to streptogramin B—thus providing full resistance to quinupristin-dalfopristin.[86–89] One plasmid has been identified with both *vatD* and the VanA operon,[90] resulting in resistance to both vancomycin and quinupristin (but not dalfopristin) when expressed in recipient cells in vitro. VatH may be seen in conjunction with another streptogramin acetyltransferase, VgbA, the only known acetyltransferase with activity against streptogramin B in enterococci.[88] All of the above acetyltransferase genes have been isolated exclusively from *E. faecium*, with the exception of *vatE* which has been isolated from *E. faecium* and from *E. faecalis* in a veterinary setting.[91]

The ABC-efflux channel VgaD also plays a role in acquired streptogramin resistance, independent of the intrinsic ABC-efflux

channels encoded by *lsa* genes in *E. faecalis* and *msrC* gene in *E. faecium* (described above). VgaD has been described only in *E. faecium*. *vgaD* was found on a plasmid with *vatH*, both of which confer only streptogramin A resistance.[92] To date, no other acquired streptogramin efflux pumps have been described.

Perhaps the best understood mechanism of streptogramin resistance is dimethylation of the 23S rRNA.[25] This resistance mechanism, which confers the MLS_A or MLS_B phenotype occurs through acquisition of either the *ermA* or *ermB* genes on broad host range plasmids such as pAMβ1. If these plasmids also contain *vatE* or *vatD* genes, then they confer resistance to quinupristin-dalfopristin when acquired by a recipient cell.

Linezolid. Prior to FDA approval in 2000, reports of linezolid resistance in enterococci existed but were rare. The emergence of linezolid resistance occurred slowly and only in sporadic cases associated with prolonged exposure.[93] The industry-sponsored LEADER trial has monitored linezolid efficacy from 2004 to 2009 and has found yearly resistance rates between 0.49 and 1.83%.[56] In contrast, Pogue et al.[94] found linezolid resistance in 20% of GRE samples from the University of Pittsburgh Medical Center. Only 25% of isolates in their study were associated with prior linezolid exposure, suggesting clonal spread.

Linezolid is a first-in-class oxazolidinone, an entirely synthetic class of antibiotics that binds to the initiation complex and inhibits protein synthesis. Most bacteria, including the enterococci, have multiple copies of the genes encoding 23S rRNA. *E. faecalis* have four copies of the gene[95] and *E. faecium* six copies.[96] In theory, the presence of multiple gene copies makes resistance from sporadic mutations less likely because the unaffected gene copies would mask the effect of the mutated gene. However, recombination between susceptible and resistant copies (referred to as "gene conversion") will yield strains with multiple mutated copies under persistent linezolid selective pressure. In clinical isolates, a mutation in one *E. faecium* rRNA gene conferred an MIC of 8–16 μg/ml. The same mutation in > 3 rRNA genes conferred an MIC between 64–128 μg/ml.[96] A variety of point mutations that confer linezolid resistance have been identified, the most common of which is G2576T. In the most recent LEADER study results (2009), the G2576T mutation was identified in all eight of the linezolid-resistant enterococci strains isolated in the United States. Four of the eight strains found in this study were isolated in Louisville, KY and appeared clonally related.[56,97] Other sporadic point mutations have been associated with linezolid resistance, including G2505A and L4 (F101L).[98-100]

In 2006, the transferable *cfr* gene was identified in *S. aureus* as the source of resistance to linezolid, lincosamides and streptogramin A compounds, among others.[101] *Cfr* encodes an rRNA methyltransferase that modifies an adenosine in the linezolid-binding region on the 23S rRNA, preventing antibiotic binding. It is hypothesized that the *cfr* gene emerged from animal strains of bacteria that were exposed to natural compounds with an rRNA binding site similar to linezolid.[101,102] In 2011, *cfr* was identified in an *E. faecalis* strain (designated EF-01) from a cattle farm in China.[103] In this strain, the gene was located on a plasmid (pEF-01) and flanked by IS*1216*, suggesting transposability. This was the first enterococcus harboring *cfr* to be reported in the literature, although human isolates of *E. faecalis* and *E. faecium* with *cfr* were reported in a 2010 abstract.[103] Overall, linezolid resistance remains rare in enterococci.

Daptomycin. Daptomycin is a lipoprotein with bactericidal activity against enterococci. While not FDA approved for treatment of GRE, it is often used by clinicians for this purpose.[104-106] The epidemiology of daptomycin resistance in enterococcus (defined as MIC > 4 μg/ml) was recently reviewed by Kelesidis et al.[107] Rates of daptomycin resistance in this study were approximately 0.6% (111 daptomycin resistant isolates/17,084 enterococcus isolates total). In general, *E. faecium* is more likely than *E. faecalis* to express daptomycin resistance, although resistance has been reported in both species. The increased prevalence of daptomyicin resistance in *E. faecium* may reflect increased use of daptomycin with this species compared with *E. faecalis*, which is usually susceptible to penicillins. Daptomycin resistance appears to be less common in North America than in Asia or Europe.[107]

Daptomycin incorporates itself into the cell membrane of Gram-positive organisms in the presence of physiologic calcium concentrations and promotes leakage of intracellular potassium into the extracellular space, resulting in cell death by destruction of the transcellular potassium gradient.[108-110] Normal cell membrane polarity is required for daptomycin intercalation. In staphylococci, alteration of the cell membrane charge by virtue of modification of cell membrane lipoproteins has been associated with reduced daptomycin susceptibility.[111] A number of genes have been described in staphylococci that contribute to daptomycin resistance,[112] none of which have been identified in enterococcus to date. While the mechanism of daptomycin resistance in enterococcus remains unresolved, several reports have elucidated gene mutations associated with enterococcus daptomycin resistance.[113,114]

Palmer et al.[113] created three daptomycin-resistant strains by exposing *E. faecalis* to increasing daptomycin concentrations until stable resistance was identified. They then performed complete genome sequencing of the strains before and after emergence of daptomycin resistance and identified seven gene mutations. Of the seven mutations observed in this study, only EF1797 and EF0631 gene mutations were identified in all three resistant strains. EF1797 encodes a putative membrane protein that may be involved in phosphatidylserine and sphingolipid synthesis, but its function has yet to be determined. EF0631 encodes a putative cardiolipin sythetase (*cls*), a transphosphatidylase involved in the synthesis of the cell membrane protein cardiolipin. One specific mutation in this gene, R218Q, was found in two of the resistant strains and occurs in the presumed active domain of the EF0631 enzyme. Through comparison with a DNA sequence database, the authors identified one other *E. faecalis* strain with an EF0631 frameshift mutation, but this isolate had a daptomycin-susceptible phenotype.

In a similar study, Arias et al.[114] compared the nucleotide sequence and cell membrane proteins of *E. faecalis* isolates before and after the development of daptomycin resistance in a patient with enterococcus bacteremia. Genome sequencing of the

resistant strain revealed three mutated genes: *cls*, *gdpD* and *liaF*. A cardiolipin synthetase mutation was identified but when the mutant gene was placed in daptomycin-sensitive enterococcus strain the MIC did not change. The same *cls* mutation was observed in other *E. faecalis* and *E. faecium* strains resistant to daptomycin. The other two gene mutations, *gdpD* (glycerophosphoryl diester phosphodiesterase) and *liaF* (lipid II cycle-interfering antibiotic protein), did have an impact on MICs when reconstituted in the daptomycin-susceptible strain. The *liaF* mutation increased the MIC from 1 to 4 μg/ml. The *gdpD* mutation did not increase the MIC, but the combination of both proteins increased the MIC to 12 μg/ml. Mutations in both *gdpD* and *liaF* were also identified in other resistant strains of *E. faecalis* and *E. faecium*, but were not demonstrated in the Palmer study. Thus, cardiolipin synthetase, GdpD and LiaF are cell membrane proteins associated with daptomycin resistance. Given that a number of different membrane-associated proteins have been linked to reduced daptomycin susceptibility in staphylococci, it seems likely that more genes conferring enterococcal resistance to daptomycin will be identified in the future.

Tigecycline. Tigecycline, a novel glycylcycline antibiotic, gained FDA approval in 2005 for complicated intra-abdominal infections, skin and soft tissue infections, and community-acquired pneumonia. It has been used off-label to successfully treat MRSA and GRE infections.[115] Typical tigecycline MICs for enterococcus range from 0.125 μg/ml to 0.25 μg/ml, while MICs $>$ 0.5 μg/ml are considered resistant. Early surveillance studies of tigecycline showed no cases of resistant enterococcus,[116] although two case reports of *E. faecalis* strains with MICs of 2 μg/ml and 6 μg/ml, respectively, have been described.[117,118] A more recent study from Taiwan reviewed antimicrobial resistance among 219 VRE isolates and found two isolates with a tigecycline MIC of 0.5, and one isolate with an MIC of 1, with a trend toward increasing tigecycline MIC over time.[119] The mechanism of tigecycline resistance in enterococcus is unknown. In staphylococcus, tigecycline resistance is mediated by a novel family of efflux pumps,[120] but these genes have not been demonstrated in enterococcus.

Other antibiotics. Resistance occurs to other antibiotics including macrolides, tetracyclines, chloramphenicol, fosfomycin, rifampin and quinolones. These resistance mechanisms will not be described in this review, as resistance to these antimicrobial agents is so common that they are seldom involved in treatment of enterococcus infections.

Management

Because of the differences in resistance patterns between *E. faecium* and *E. faecalis*, it is imperative to differentiate the pathogen to the species level and perform susceptibility testing on strains isolated from patients with clinical infections. Treatment of enterococcal infections depends upon (1) the species, (2) the resistance patterns present in the clinical isolate and (3) the location and severity of the infection. Uncomplicated enterococcal infections may be adequately treated with monotherapy, whereas severe infections such as endocarditis benefit from a synergistic regimen.

In uncomplicated, fully susceptible *E. faecalis* and *E. faecium* infections, ampicillin remains the preferred therapy. In the uncommon presence of β-lactamase, combination with a β-lactamase inhibitor such as sulbactam may improve outcomes. When complicated infections such as endocarditis occur in susceptible enterococcal infections, an aminoglycoside should be added to a cell wall active agent for synergistic killing, as has been the standard for almost 60 years.[20] Among aminoglycosides, only gentamicin and streptomycin should be considered for synergistic therapy. Historically, twice-daily or three times daily aminoglycoside dosing regimens have been used. In streptococcus infections, once-daily aminoglycoside dosing was shown to be effective in humans.[121] For enterococcal endocarditis, though, once-daily aminoglycoside dosing has only been studied in animal models with evidence for[122–124] and against[125,126] its use. Differences between these studies likely reflect the pharmacodynamic and pharmacokinetic differences between the animal models. In humans, the efficacy of once-daily aminoglycoside dosing has not been established for enterococcus infections. As such, guidelines continue to recommend three times daily dosing for gentamicin and twice-daily dosing for streptomycin.[127] In the presence of high-level penicillin resistance, synergy has been observed in animal models with the combination of aminoglycosides and other cell wall active antibiotics including vancomycin or daptomycin.[128]

In the instance of complicated enterococcal infections resistant to high-levels of gentamicin and streptomycin, an alternative agent must be used for synergistic activity. Despite relative resistance to both agents, the combination of ceftriaxone and ampicillin has been shown to be efficacious in animals.[129] In case reports and prospective case-series, 56 patients have been treated with this combination with success rate of 71.4% (40/56);[130-132] although these numbers may reflect publication bias. The presumed benefit of ceftriaxone-ampicillin combination therapy is attributed to full saturation of PBPs 2–4, which cannot be achieved with either agent alone. By inhibiting all PBPs, the bacteria have no alternative enzyme with which to build a cell wall. In in vitro and animal model studies, similar synergistic bactericidal activity has been shown with other cell wall active combination therapies, including ceftriaxone-fosfomycin[133] and ampicillin-imipenem,[134] but not with ampicillin-ertapenem.[135]

Treatment of glycopeptide-resistant enterococcus. Vancomycin-resistant enterococci pose particular problems for treatment because the strains which harbor VanA and VanB resistance are also typically resistant to other classes of antibiotics. While only linezolid and quinupristin-dalfopristin have FDA approval for treatment of GRE infections, other antimicrobial agents including daptomycin, tigecycline, fosfomycin, quinolones, tetracyclines and new fifth generation cephalosporins exhibit in vitro activity and have been used with success in individual cases. In uncomplicated cases, monotherapy based upon the antibiotic susceptibility profile is appropriate. In complicated cases such as endocarditis, the ideal therapy for GRE has not been determined.

Both linezolid and quinupristin-dalfopristin have been shown to be efficacious in treatment of complicated GRE infections and are FDA approved for this indication.[56,136] Linezolid has been

used for GRE endocarditis in both *E. faecalis* and *E. faecium*, both with and without additional agents. To date, it has not been shown that combination therapy is more efficacious than monotherapy in this setting. Because linezolid is not bactericidal, treatment of GRE endocarditis with linezolid remains controversial.[23] Quinupristin-dalfopristin can be efficacious against *E. faecium*, but should not be used to treat *E. faecalis* due to the intrinsic presence of *lsa*-mediated resistance (described above). When using quinupristin-dalfopristin for treatment of severe or complicated GRE infections, combination therapy may be necessary although the optimal choice and dose of adjunct antibiotic has yet to be determined. Several studies have compared linezolid to quinupristin-dalfopristin. In a small, single-center study, Chong et al.[137] found increased resistance and increased number of days of bacteremia in patients treated with quinupristin-dalfopristin. Several other studies also reported more resistance to quinupristin-dalfopristin than linezolid,[56,138] suggesting that of the two linezolid may be superior for GRE treatment.

Use of daptomycin for GRE infections, particularly endocarditis, is appealing because of its bactericidal activity against enterococci.[104] While monotherapy may be adequate in many GRE infections, daptomycin failure has been reported.[106,139] Arias et al.[139] reported subsequent response to the combination of daptomycin/gentamicin/ampicillin after failing daptomycin monotherapy, which may be explained by a synergistic effect of the triple therapy. Daptomycin synergy has been described in vitro with ampicillin, cephalosporins, imipenem, rifampin and gentamicin.[140-143] As with quinupristin-dalfopristin, there may be a role for adjunct antibiotics to achieve a synergistic effect, although appropriate agents and dosing regimens have not been adequately evaluated in humans.

Tigecycline has also been used off-label for treatment of GRE. Cai et al.[115] performed a meta-analysis of randomized trials to evaluate the use of tigecycline for GRE infections. While efficacy of treatment was no different for tigecycline monotherapy than other empirical regimens, there were significantly more adverse events and a non-significant trend toward higher mortality. As such, tigecycline monotherapy should not be considered as first line for treatment of GRE infections. Tigecycline demonstrates in vitro synergism with a number of other agents, including rifampin and daptomycin, although these combinations have not been evaluated beyond small case series in humans.[144]

Future Directions

Over the years enterococci have demonstrated the potential to harbor and transfer resistance genes and as such have become an important clinical pathogen. A better understanding of resistance mechanisms to daptomycin and tigecycline is needed and will aid in the prediction and prevention of epidemiologic spread. Several new drugs are emerging as potential options for GRE treatment. Ceftaroline has been shown to be more efficacious than linezolid in animals and may play a larger role in the future.[145] Additionally, arbekacin, which is not currently available in the United States, has demonstrated synergistic killing in combination with penicillins even in the presence of high-level gentamicin and streptomycin resistance.[146] A number of antibiotic combinations, including those mentioned in the management section, have shown in vitro synergistic activity and are promising as potential treatment modalities for complicated GRE infections, but must first be evaluated more rigorously in humans. The novel glycopeptide oritavancin is currently under investigation and shows promise in treating GRE infections. In addition, non-antimicrobial pharmacotherapy targeted at specific virulence factors (such as anti-adhesions) may play a preventative or therapeutic role in the management of enterococcal infections. Future directions of research must focus on development of new antimicrobial agents. Finally, efforts must continue to prevent development of antibiotic resistance and spread in the enterococci through infection control and antibiotic stewardship programs.

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