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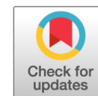
MECHANISMS OF RESISTANCE



Prediction of Antimicrobial Resistance in Clinical *Enterococcus faecium* Isolates Using a Rules-Based Analysis of Whole-Genome Sequences

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ABSTRACT *Enterococcus faecium* is a major cause of clinical infections, often due to multidrug-resistant (MDR) strains. Whole-genome sequencing (WGS) is a powerful tool to study MDR bacteria and their antimicrobial resistance (AMR) mechanisms. In this study, we used WGS to characterize *E. faecium* clinical isolates and test the feasibility of rules-based genotypic prediction of AMR. Clinical isolates were divided into derivation and validation sets. Phenotypic susceptibility testing for ampicillin, vancomycin, high-level gentamicin, ciprofloxacin, levofloxacin, doxycycline, tetracycline, and linezolid was performed using the Vitek 2 automated system, with confirmation and discrepancy resolution by broth microdilution, disk diffusion, or gradient diffusion when needed. WGS was performed to identify isolate lineage and AMR genotype. AMR prediction rules were derived by analyzing the genotypic-phenotypic relationship in the derivation set. Phylogenetic analysis demonstrated that 88% of isolates in the collection belonged to hospital-associated clonal complex 17. Additionally, 12% of isolates had novel sequence types. When applied to the validation set, the derived prediction rules demonstrated an overall positive predictive value of 98% and negative predictive value of 99% compared to standard phenotypic methods. Most errors were falsely resistant predictions for tetracycline and doxycycline. Further analysis of genotypic-phenotypic discrepancies revealed potentially novel *pbp5* and *tet*(M) alleles that provide insight into ampicillin and tetracycline class resistance mechanisms. The prediction rules demonstrated generalizability when tested on an external data set. In conclusion, known AMR genes and mutations can predict *E. faecium* phenotypic susceptibility with high accuracy for most routinely tested antibiotics, providing opportunities for advancing molecular diagnostics.

KEYWORDS antibiotic resistance, antimicrobial resistance, *Enterococcus*, whole-genome sequencing, genomics, prediction, VRE, microbiology, antimicrobial agents, genome analysis, vancomycin resistance

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Over the last 4 decades, *Enterococcus faecium* has transformed from a harmless gastrointestinal colonizer into a serious pathogen causing antibiotic-resistant clinical disease and hospital-acquired infections (1–4). The multidrug resistant, hospital-associated strains of *E. faecium* mostly belong to the clonal complex 17 (CC17) lineage and

are a leading cause of nosocomial urinary tract infections, bacteremia, endocarditis, and wound infections (5). The optimal antibiotic treatment for *E. faecium* infections is a cell wall-active agent, such as ampicillin or vancomycin, which in serious infections may be used in combination with an aminoglycoside like gentamicin for bactericidal synergism (6). Unfortunately, rising rates of resistance have rendered β-lactams, vancomycin, and aminoglycosides increasingly inactive against *E. faecium*. Consequently, standard empirical antimicrobial therapy regimens containing vancomycin for Gram-positive coverage fail to treat more than 75% of patients with *E. faecium* bloodstream infections in the United States, resulting in higher mortality rates and health care costs and highlighting the need to improve our diagnostic and therapeutic capabilities for *E. faecium* (7, 8).

Mechanisms of *E. faecium* resistance to the most commonly used antimicrobial agents are well described (2). Briefly, vancomycin resistance is conferred by the vancomycin resistance (Van) operon, which disrupts the D-Ala–D-Ala vancomycin binding site on the peptidoglycan cell wall (9–11). In contrast, ampicillin resistance primarily occurs by a more complicated set of alterations in the low-affinity penicillin-binding protein 5 (PBP5) (12). For serious ampicillin- and vancomycin-resistant *E. faecium* infections, the treatment options are very limited, with only one FDA-approved drug—linezolid—and off-label use of other drugs, such as daptomycin, tigecycline, quinupristin-dalfopristin, and oritavancin. The Clinical and Laboratory Standards Institute (CLSI) also provides interpretative criteria for fluoroquinolones (ciprofloxacin and levofloxacin), tetracyclines (tetracycline and doxycycline), and nitrofurantoin, which may be used in less serious infections.

Whole-genome sequencing (WGS) provides a powerful tool to discover more about *E. faecium* antimicrobial resistance (AMR) mechanisms and transmission patterns (13, 14). WGS data have primarily been used to predict AMR by applying a rules-based approach, where the presence of one or more known AMR genes or single nucleotide polymorphisms (SNPs) is used to determine resistance to an antibiotic. Alternatively, model-based approaches, which use statistical methods to train a classifier that often determines predictive genetic loci without prior knowledge, have also been used successfully to predict AMR (15, 16). For well-studied organisms and antibiotics for which there is ample understanding of the genotypic-phenotypic relationship, a rules-based approach has the advantage of being easily interpretable and has successfully been used for *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* (17–19), with limited results for *E. faecium* (19–21). Therefore, there remains a need to systematically test the efficacy of a rules-based approach for a broad range of clinically relevant antibiotics in *E. faecium*.

In this study, we developed and tested AMR prediction rules for *E. faecium* based on a collection of clinical isolates from a large U.S. academic health center clinical microbiology laboratory. The isolate set was temporally divided into derivation and validation sets. As expected for this organism, 92% of isolates were resistant to more than one drug class, highlighting the high degree of resistance in circulating clinical isolates and the importance of determining antimicrobial susceptibility to guide clinical management. After deriving the genotypic prediction rules using the derivation set, the rules were applied to the validation set and discrepancies were analyzed. The final set of AMR prediction rules was applied to an external data set of previously characterized *E. faecium* clinical isolates collected from Germany to confirm the accuracy of the prediction rules from a geographically distinct source.

RESULTS

Characterization of isolate sources, sequence type diversity, and AMR profiles. We first examined the population structure of our isolate collection. Using multilocus sequence typing (MLST), the universal typing method that designates sequence types (STs) based on allelic assignments of seven species-specific housekeeping genes (22), we found that over 88% of isolates belonged to the high-risk, hospital-associated

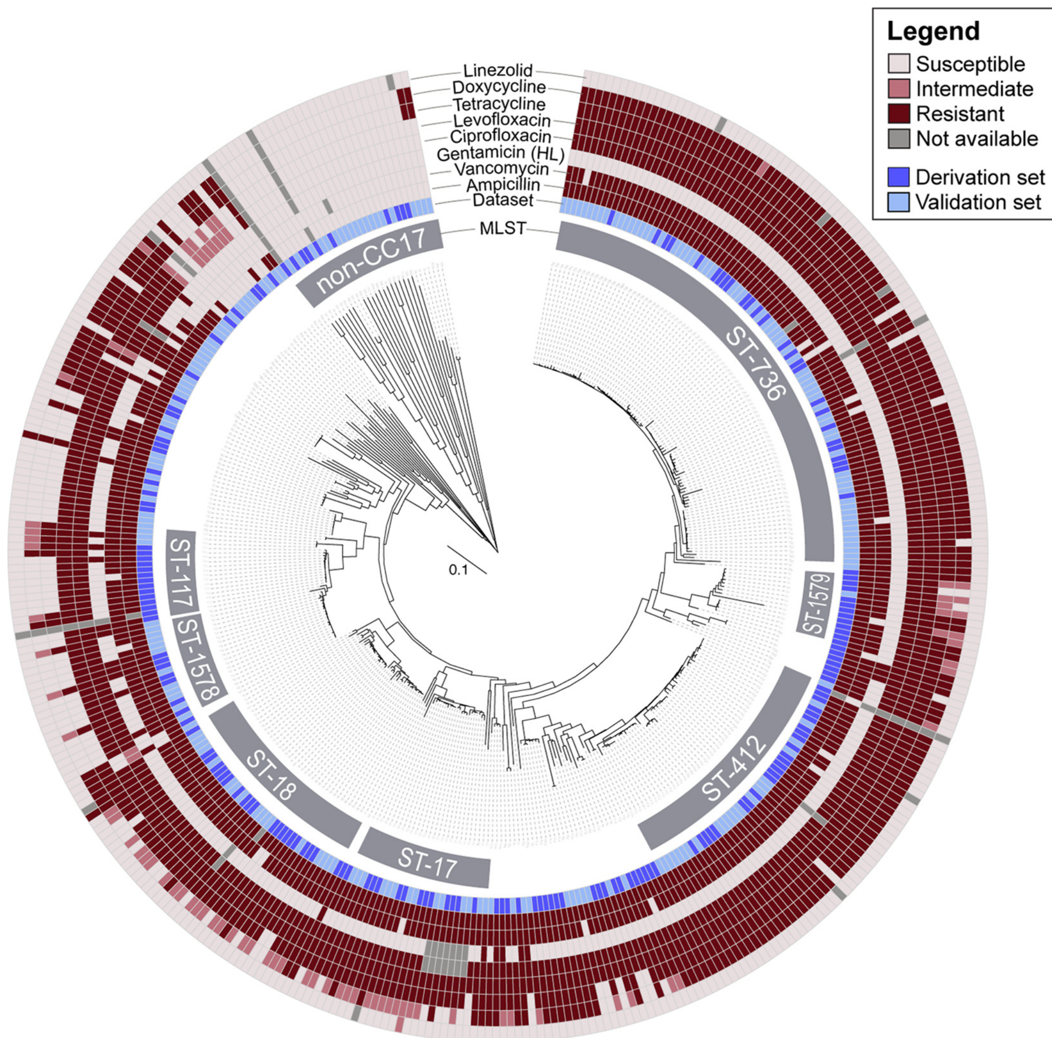

FIG 1 Core genome MLST-based neighbor joining tree demonstrating the relationship between phylogeny and antimicrobial resistance in the derivation and validation sets. Phenotypic susceptibility results are displayed for ampicillin, vancomycin, high-level (HL) gentamicin, ciprofloxacin, levofloxacin, tetracycline, doxycycline, and linezolid.

clonal complex 17. The most common STs were ST-736 (23%), ST-18 (13%), ST-412 (13%), ST-17 (7%), and ST-117 (5%) (Fig. 1). We identified numerous novel sequence types, including two locally common STs belonging to CC17, now designated ST-1578 and ST-1579 (Fig. 1; see also Table S1 in the supplemental material). Compared to all 1,138 clinical *E. faecium* isolates in the international PubMLST database, our single hospital data set captured most subgroup founders in CC17 and CC94 (Fig. S1), indicating that the diversity of this collection provides a comprehensive set of isolates to test a generalizable genotypic AMR prediction approach (23).

Using the higher-resolution core genome MLST (cgMLST) system, we examined the relationship between phylogeny and AMR (Fig. 1). Seven percent of isolates were resistant to all three of the key antimicrobials used in the treatment of *E. faecium* infections—vancomycin, ampicillin, and gentamicin—and belonged to a range of STs within CC17, though most commonly ST-80 and ST-1578. The most common STs, ST-736 and ST-412, had uniform susceptibility to gentamicin and linezolid. Isolates that were not in CC17 tended to be susceptible to all tested antibiotics. While there is clearly a correlation between population structure and antimicrobial resistance, these

findings indicate that the population structure can only partially inform the AMR predictions and that further characterization is needed.

Developing a rules-based approach to predicting AMR. We analyzed the AMR gene content of the derivation set to develop a simple rules-based approach based upon published genes to predict *E. faecium* resistance. Ampicillin resistance in *E. faecium* is associated with mutations in the penicillin-binding protein 5 (*pbp5*) gene, where dominant susceptibility (*pbp5*-S) and resistance (*pbp5*-R) alleles have been described (24). PBP5-R and PBP5-S differ by 21 amino acids, which decrease the protein's ampicillin affinity and result in an increase in the MIC from ≤2 to ≥16 μg/mL, with hybrid alleles exhibiting a range of MICs in between (24). Despite the complex variability in *pbp5*, most resistance alleles have a mutation in codon 485, which results in a methionine-to-alanine or -threonine substitution and independently increases the ampicillin MIC at least 4-fold (25). In the derivation set, the Met485 substitution was found in every ampicillin-resistant isolate and appeared to be a simple predictor of the presence of an ampicillin resistance *pbp5* allele and phenotypic ampicillin resistance.

To predict vancomycin resistance, we used the same approach as FDA-cleared molecular vancomycin-resistant enterococcus (VRE) assays by detecting the *vanA* or *vanB* gene as a surrogate for the presence of the vancomycin resistance operon (9, 26). We found *vanA* in 97% of resistant isolates and *vanB* in the remaining 3%. We did not detect other vancomycin resistance genes (*vanC*, *vanD*, *vanE*, *vanF*, *vanG*, *vanL*, *vanM*, or *vanN*) in any isolates (Table S2).

High-level gentamicin (HLG) resistance was associated with the presence of an intact *aac(6′)-Ie-aph(2″)-Ia* gene, which encodes a bifunctional aminoglycoside-modifying enzyme that decreases the binding affinity of gentamicin for the ribosome and is the most common described mechanism of HLG resistance in *E. faecium* (2, 27). Other aminoglycoside-modifying genes, such as *aph(3′)-Ia*, *aph(3′)-IIIa*, *aac(6′)-Ii*, and *ant(6′)-Ia*, were commonly found but were not associated with high-level gentamicin resistance (Table S3) (28).

Ciprofloxacin and levofloxacin resistances are associated with classic mutations in *gyrA* (Ser84) and *parC* (Ser82). Both of these mutations were found exclusively in all fluoroquinolone-resistant isolates and not found in any fluoroquinolone-susceptible isolates of the derivation set. However, it was not possible to distinguish derivation set isolates that were fully susceptible to fluoroquinolones from strains that had intermediate resistance according to the CLSI breakpoints, even when additional genes such as *gyrB* and *parB* were incorporated. This is consistent with the ciprofloxacin epidemiologic cutoff value (ECV) of 8 μg/mL being above the CLSI breakpoint for resistance (4 μg/mL) and the levofloxacin ECV of 4 μg/mL coinciding with the intermediate CLSI interpretative category (29). Given that the wild-type fluoroquinolone MIC distributions include both the susceptible and intermediate CLSI interpretive categories, intermediate isolates were categorized with susceptible organisms for the purpose of genotypic prediction.

Numerous tetracycline resistance genes have been reported for enterococci, but resistance to tetracycline among the derivation set isolates appeared to be associated with the presence of either the *tet*(L) (drug efflux), *tet*(M) (ribosomal protection), or *tet* (S) (ribosomal protection) gene, with a sensitivity of 99% and specificity of 87%. Doxycycline resistance had the strongest correlation with the presence of *tet*(M) alone, but there were 14 isolates that nominally had *tet*(M) yet were phenotypically susceptible. Alignment to "*tet*(U)" was found frequently, within 84% of ST-17 isolates and 32% of ST-18 isolates, and in both tetracycline-susceptible and -resistant isolates. Some isolates demonstrated a very high depth of coverage of the *tet*(U) gene, up to 10 times higher than the rest of the genome. The lack of correlation with phenotypic resistance and the disproportionately high alignment rates are supportive of a previous report that *tet*(U) is a misannotation of a portion of the rolling-circle replication initiator (*rep*) gene found in plasmids rather than a tetracycline resistance determinant (30).

Finally, we were not able to initially develop a rules-based method for linezolid

based on our derivation set due to a paucity of linezolid-resistant isolates. However, others have shown that linezolid resistance in *E. faecium* is most commonly due to a G2576T mutation in at least three of six copies of the 23S rRNA gene (2, 31), which is the rule that we incorporated. Other linezolid resistance genes, such as *poxtA*, *optrA*, and the *cfr*-like genes, have been described but were absent or were present only in linezolid-susceptible isolates in our data set (Table S4).

Determining the accuracy of genotypic prediction rules. We next evaluated the accuracy of the rules-based genotypic predictions on the Boston validation set. After resolving genotyping or phenotyping errors through repeat testing, the genotypic-phenotypic categorical agreement was generally excellent, with an overall positive predictive value of 98% and negative predictive value of 99%. All drugs achieved a percent categorical agreement above 89.9%, which is the threshold that the FDA considers to be acceptable performance for antimicrobial susceptibility testing (AST) devices compared to a CLSI reference method (Table 1) (32). The very major error (VME) rate, also known as the false-negative rate, was 1.4% or lower for all drugs (Table 1). The major error (ME) rate, or the false-positive rate, was below 3% for ampicillin, vancomycin, HL gentamicin, ciprofloxacin, and levofloxacin (Table 1). The tetracycline and doxycycline ME rates were above the FDA-accepted threshold of 3% (14% and 27%, respectively) due to the presence of *tet* genes in phenotypically susceptible isolates.

We found G2576T mutations in three validation set isolates, which included the only two linezolid-resistant isolates. The two linezolid-resistant isolates each had a linezolid MIC of 8 μg/mL and G2576T mutations in 46% of reads, corresponding to three out of six gene copies. The third isolate had the G2576T mutation in only 16% of reads, or one out of six gene copies, and was phenotypically susceptible to linezolid, with an MIC of 2 μg/mL. Thus, the presence of the G2576T mutation in multiple copies of the 23S rRNA gene appeared to correlate well with phenotypic resistance to linezolid in our data set. Overall, the first iteration of the genotypic prediction rules achieved excellent performance for ampicillin, vancomycin, HL gentamicin, ciprofloxacin, levofloxacin, and linezolid.

Investigating the cause of genotypic-phenotypic discrepancies in the validation set. To further improve the accuracy of the genotypic prediction rules, we examined the isolates for which the predicted and observed AMR phenotypes did not match. The only isolate with a vancomycin discrepancy was phenotypically susceptible to vancomycin (MIC of 1 μg/mL) yet contained the *vanA* gene. Further analysis of the vancomycin resistance (Van) operon revealed that the isolate also contained the *vanX*, *vanY*, and *vanH* components of the Van operon with very high coverage but lacked the *vanS* (sensor) or *vanR* (regulator) genes that form a two-component regulatory system that is necessary for the expression of the *vanHAX* gene cluster (33) (Table S5). While this was the only isolate out of 302 *vanA*-containing isolates in our collection that lacked *vanRS*, other *E. faecium* isolates with a "silent" *vanA* gene via the same mechanism have been described in the literature as vancomycin-variable enterococci (VVE) because they can gain vancomycin resistance during treatment and thereby lead to treatment failure (34–36). To prospectively identify these VVE isolates, the vancomycin genotypic prediction rule can be modified to identify not only *vanA/B* but also the remaining genes of the Van operon (*vanX*, *vanY*, *vanH*, *vanR*, and *vanS)* and flag the presence of *vanHAX* without *vanRS*. This example highlights the potential for whole-genome-sequencing-based approaches to identify rich and potentially clinically valuable information beyond what is routinely available today.

Two isolates, belonging to ST-56 and ST-640, were falsely predicted to have ampicillin susceptibility based on a wild-type methionine at position 485 yet were confirmed by multiple phenotypic testing methods to be resistant. Subsequent analysis demonstrated that these isolates contained potentially novel *pbp5* alleles encoding an aspartic acid insertion after position 466 (Asp466′ [Table 2]). Resistance occurring in these Met485, Asp466′ isolates support an independent contribution of 466′ insertions to the reduced binding of ampicillin to the active site of PBP5, as suggested by crystal structure studies (25). Updating the rules-based prediction of ampicillin resistance to

TABLE 1 Accuracy of genotypic prediction rules V.1 when applied to the Boston validation set[a]

	V.1 rules applied to the Boston validation set							
Antimicrobial	**Total no. of isolates**	**Phenotypic susceptibility rate, %**	**Genotype used for resistance prediction (V.1)**	**Categorical agreement, %**	**Very major error rate (FN), % (95% CI)**	**Major error rate (FP), % (95% CI)**	**PPV, %**	**NPV, %**
Ampicillin	203	12	Mutation of *pbp5* 485M	99	1.1 (0.13, 4.0)[b]	0 (0, 13)	100	92.6
Vancomycin	204	21	Presence of *vanA* or *vanB*	99	0 (0, 2.3)	2.3 (0.06, 12)[c]	99.4	100
Gentamicin, high level	198	94	Presence of *aac(6′)-Ie-aph(2″)-Ia*	100	0 (0, 28)	0 (0, 1.9)	100	100
Ciprofloxacin[d]	198	15	Mutation of *gyrA* (84S) or *parC* (82S)	100	0 (0, 2.2)	0 (0, 11)	100	100
Levofloxacin[d]	205	15	Mutation of *gyrA* (84S) or *parC* (82S)	100	0 (0, 2.1)	0 (0, 11)	100	100
Tetracycline	205	24	Presence of *tet*(L), *tet*(M), or *tet*(S)	97	0 (0, 2.4)	14 (5.8, 27)	95.7	100
Doxycycline	205	29	Presence of *tet*(M)	91	1.4 (0.16, 4.9)	27 (16, 40)	90.0	95.7
Linezolid[d]	200	99	Mutation of 23S rRNA G2576T in at least 3 alleles	100	0 (0, 84)	0 (0, 1.8)	100	100

[a]FN, false negative; FP, false positive; PPV, positive predictive value; NPV, negative predictive value.
[b]Both isolates that were phenotypically resistant to ampicillin harbored wild-type *pbp5* Met485 but had an aspartic acid inserted after position 466 (Asp466′).
[c]A single vancomycin-variable enterococcus, which appeared phenotypically susceptible but could gain vancomycin resistance during treatment, was conservatively classified as a major error.
[d]Antibiotic for which intermediate isolates were considered with susceptible isolates.

TABLE 2 PBP5 alleles found in the validation set isolates[a]

MLST type (# of isolates in validation set)	AMP MIC, µg/mL	PBP5-S/R type	Amino acid position within PBP5: 24	27	34	66	68	85	100	144	172	177	204	216	324	466′	485	496	499	525	586	629	667
PBP5-S consensus sequence	≤2		V	S	R	G	A	E	E	K	T	L	D	A	T	-	M	N	A	E	V	E	P
ST-253 (2), ST-361 (2), ST-583 (1), ST-648 (1), ST-854 (1), ST-1192 (1)	≤2	S_{21}/R_0																					
ST-2057 (1)	≤2	S_{20}/R_1																					S
ST-862 (1)	≤2	S_{19}/R_2								Q					A								
ST-623 (1)	≤2	S_{18}/R_3		G										V	A								
ST-329 (2)	≤2	S_{16}/R_5	A	G	Q					Q					A								
ST-74 (1), ST 94 (2), ST-2059 (1)	≤2	S_{16}/R_5	A	G						Q					A			K					
ST-29 (1), ST-54 (1), ST-2058 (1)	≤2	S_8/R_{13}	A	G	Q	E			Q	Q	A	I		S	A			K	I	D			
ST-22 (1), ST-32 (1), ST-533 (1)	≤2	S_4/R_{17}	A	G	Q	E	T	D	Q	Q	A	I	G	S	A			K	T	D			S
ST-56 (1)	**≥32**	S_2/R_{19}	A	G	Q	E	T	D	Q	Q	A	I	G	S	A	**D**		K	T	D		V	S
ST-640 (1)	**16**	S_1/R_{20}	A	G	Q	E	T	D	Q	Q	A	I	G	S	A	**D**		K	T	D	L	V	S
ST-38 (1)	16	S_2/R_{19}	A	G	Q	E	T	D	Q	Q	A	I	G	S	A		T	K	T	D		V	S
ST-18 (2), ST-262 (1), ST-294 (1)	≥32	S_1/R_{20}	A	G	Q	E	T	D	Q	Q	A	I	G	S	A		A	K	T	D	L	V	S
ST-203 (1)	≥16	S_1/R_{20}	A	G	Q	E	T	D	Q	Q	A	I	G	S	A	S	A	K	T	D		V	S
ST-17 (11), ST-18 (21), ST-78 (3), ST-80 (5), ST-117 (3), ST-203 (7), ST-266 (1), ST-412 (19), ST-734 (2), ST-736 (60), ST-750 (2), ST-761 (3), ST-1283 (1), ST-1391 (1), ST-1471 (1), ST-1516 (1), ST-1578 (11)	≥32	S_1/R_{20}	A	G	Q	E	T	D	Q	Q	A	I	G	S	A	S	A	K	T	D		V	S
PBP5-R consensus sequence	≥32		A	G	Q	E	T	D	Q	Q	A	I	G	S	A	S / D	A / T	K	T	D	L	V	S

[a]PBP5-S/R types were assigned using the nomenclature of Pietta et al. (24). Blank amino acid positions indicate an identical amino acid as the PBP5-S consensus sequence. AMP, ampicillin. Data for the specific positions discussed in the text are highlighted in yellow. MICs of ≤8 μg/mL, corresponding to the susceptible category, are in green, and MICs of ≥16 μg/mL, corresponding to the resistant category, are in red.

include either a mutation at 485M or the presence of 466′S/D would correctly classify these unusual organisms and flag them for confirmatory laboratory testing.

The tetracyclines had strikingly high false-positive rates of 14% for tetracycline and 27% for doxycycline. We hypothesized that distinct allelic forms of *tet*(L) and *tet*(M) were responsible for the phenotypic variability. We found that the presence of either the wild-type *tet*(L) allele or the *tet*(L) allele with a 34-bp deletion at the N terminus and an SNP at position 61 was highly correlated with both tetracycline and doxycycline resistance. There was no apparent correlation between the *tet*(L) allelic forms and tetracycline phenotypic susceptibility (Fig. 2), but the *tet*(L) allele with a 34-bp deletion, which likely requires translation initiation from an alternative upstream start codon (37), was found uniformly in ST-736, ST-412, and ST-203 isolates. Conversely, *tet*(M) had far more sequence variability, with up to 85 SNPs and 187-bp deletions (Fig. 2). A cluster of isolates, mostly ST-17, ST-18, and ST-117, lacked *tet*(L) but harbored *tet*(M) alleles with SNPs or deletions predominantly in domain V of TetM, and they could be distinguished from other *tet*(M)-containing isolates based on multiple SNPs, such as L528F. Most isolates with *tet*(M) L528F were resistant to tetracycline but intermediate to doxycycline, but 5 of the 39 isolates were highly susceptible to both tetracycline and doxycycline despite some having identical *tet*(M) protein sequences as isolates that tested not susceptible to both drugs. Further work is needed to understand the contribution of this L528F-containing *tet*(M) allele to doxycycline susceptibility, but detection of *tet* (M) L528F could flag isolates with indeterminant doxycycline susceptibilities to avoid misclassifications.

Applying updated genotypic prediction rules to an external test data set. To assess the generalizability of the AMR prediction rules, we assessed the revised rules' classification accuracy on an external test data set of 50 clinical *E. faecium* isolates

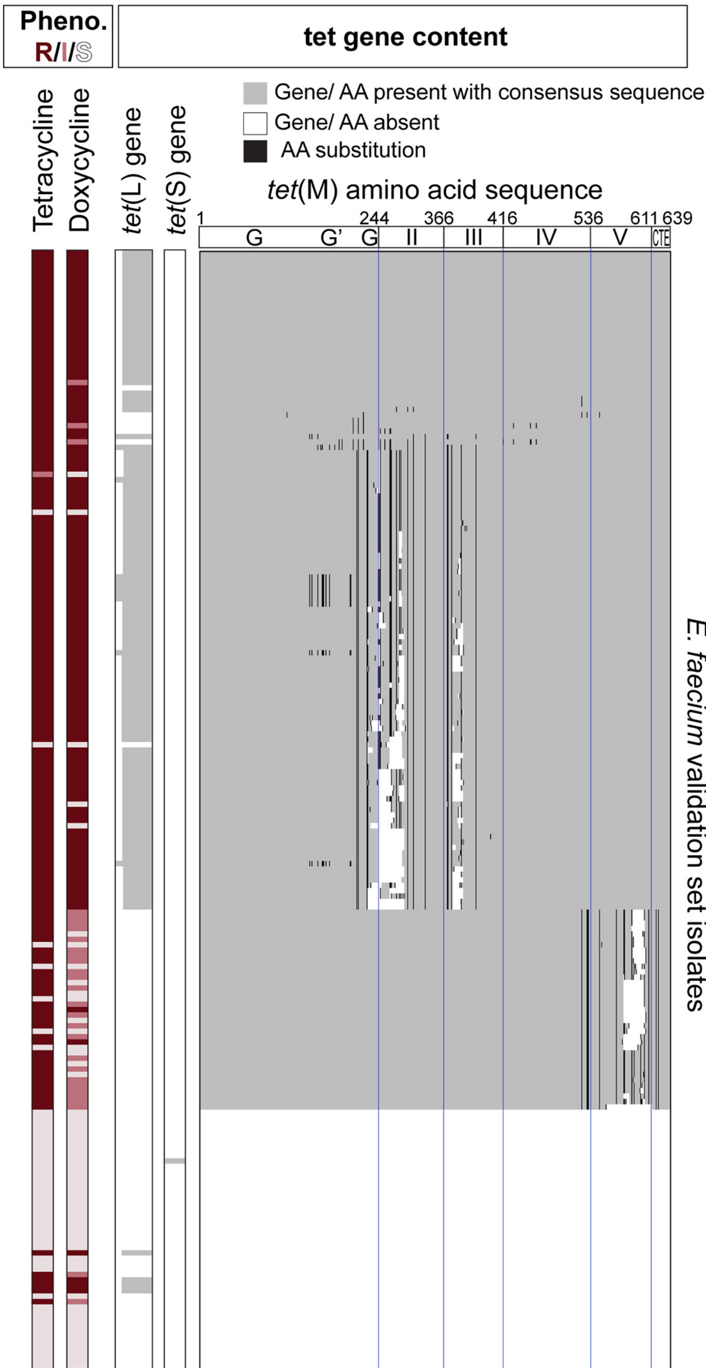

FIG 2 Phenotypic-genotypic correlations between tetracycline and doxycycline susceptibility testing results and *tet* gene content. Rows represent individual *E. faecium* validation set isolates. Columns represent phenotypic susceptibility testing results or *tet* gene content. The allelic form of *tet*(M) containing deletions and SNPs in the fifth TetM domain comprised the majority of isolates with phenotypic-genotypic discrepancies, and this allelic form could be distinguished from others based on *tet*(M) L528F. R, resistant; I, intermediate; S, susceptible; AA, amino acid.

collected from Germany that had been previously published and characterized (19). All isolates belonged to CC17, with 38% ST-117, 14% ST-80, 12% ST-262, and 8% ST-203. The genotypic predictions and phenotypes had 100% categorical agreement for ampicillin, vancomycin, and ciprofloxacin (Table 3). Gentamicin susceptibility testing results were reported for only 13 isolates, for all of which the gentamicin MIC was at or above

TABLE 3 Accuracy of genotypic prediction rules V.2 when applied to the German test set

Antimicrobial	Total no. of isolates	Phenotypic susceptibility rate, %	Genotype used for prediction (V.2)	Categorical agreement, %	Very major error rate (FN), % (95% CI)	Major error rate (FP), % (95% CI)	PPV, %	NPV, %
Ampicillin	50	0	Mutation of *pbp5* 485M or presence of 466′S/D	100	0 (0, 7)	NA[a]	100	NA
Vancomycin	50	20	Presence of *vanA* or *vanB* Flag isolates with *vanHAX* but without *vanRS* as potential VVE	100	0 (0, 8.8)	0 (0, 31)	100	100
Gentamicin, high level[b]	13	30	Presence of *aac(6′)-Ie-aph(2″)-Ia*	69	10 (0.25, 44)	100 (29, 100)	75	0
Ciprofloxacin[c]	50	0	Mutation of *gyrA* (84S) or *parC* (82S)	100	0 (0, 7)	NA	100	NA
Levofloxacin[c]	NA		Mutation of *gyrA* (84S) or *parC* (82S)					
Tetracycline	50	56	Presence of *tet*(L), *tet*(M), or *tet*(S)	94	0 (0, 17)	3.6 (0, 18)	95	90
Doxycycline	NA		Presence of *tet*(M) flag isolates with *tet*(M) L528F as indeterminant					
Linezolid[c]	50	96	Mutation of 23S rRNA G2576T in at least 3 alleles	100	0 (0, 84)	0 (0, 7.4)	100	100

[a]NA, not available.

[b]Gentamicin was only tested on a subset of isolates using an alternative phenotypic method from that used in the derivation and validation sets. For all three isolates that were phenotypically susceptible, the gentamicin MIC was 128 μg/mL, which is 1 dilution below the EUCAST breakpoint, and the isolate contained the *aac(6′)-Ie-aph(2″)-Ia* gene.

[c]Antibiotic for which intermediate isolates were considered with susceptible isolates.

128 μg/mL. The genotypic prediction rule only had 69% categorical agreement due to one false negative from an isolate that reportedly contained the *aph(2″)-Ih* gene and three false positives from isolates that all contained the *aac(6′)-Ie-aph(2″)-Ia* gene yet had gentamicin MICs of 128 μg/mL, which is 1 dilution below the EUCAST breakpoint (19). These discrepancies may be due to methodological differences in incubation times, broth composition, and antibiotic concentrations used between the two data sets or associated with the phylogenetic background of the isolates, which were all ST-262. Tetracycline had 94% categorical agreement, with two minor errors and one major error in a *tet*(M)-containing isolate with a tetracycline MIC within 1 dilution of the breakpoint. Phenotypic data for doxycycline and levofloxacin were not available. Finally, linezolid had 100% categorical agreement, where both linezolid-resistant (MIC 32 μg/mL) isolates had at least three alleles with the G2576T mutation. Six isolates tested as linezolid intermediate, but only one of those isolates had the G2576T mutation, and it was found in two alleles. Overall, the prediction rules appeared to generalize very well when applied to an isolate set from a different continent.

DISCUSSION

E. faecium is one of the most common and difficult-to-treat multidrug-resistant pathogens, yet systematic, genome-wide surveys of its antibiotic resistance mechanisms in clinical isolates have not been performed. In this study, we collected, sequenced, and analyzed a large and diverse set of clinical *E. faecium* isolates from Massachusetts General Hospital (MGH) and found a surprisingly small set of AMR genes and SNPs that we incorporated into simple antibiotic resistance prediction rules. When applied to a validation set that was prospectively collected from the same hospital, our rules predicted phenotypic susceptibility testing results with an average categorical agreement of 98% across eight commonly used antibiotics. When applied to a geographically distant test set, the genotypic-phenotypic agreement was 97% across six antibiotics analyzed. In addition to supporting rule generalizability, the diverse sample set enabled the detection of rare alleles, such as the ampicillin-resistant Asp466′ and Met485 *pbp5* alleles, enhanced the generalizability of the prediction rules, and may elucidate antibiotic resistance mechanisms. Our data suggest that genomic methods are potentially useful for guiding *E. faecium* antibiotic selection.

We were able to resolve all genotype-phenotype discrepancies in the validation set except for tetracycline and doxycycline, which had false-positive rates of 14% and 27%, respectively, when the presence of *tet*(L), *tet*(M), or *tet*(S) was used for resistance prediction. The majority of false-positive genotypic predictions occurred with one allelic form of *tet*(M) that contained deletions and SNPs in the fifth TetM domain, which makes critical contacts with 23S rRNA (38). In the short term, L528F or other SNPs in the fifth TetM domain may be used as a proxy to flag isolates with "indeterminant" genotypic predictions. Future studies may examine the mechanism for the observed variability, such as expression differences due to variation in the *tet*(M) promoter sequence, and assess how the *tet*(L) and *tet*(M) allele variation impacts resistance to other important tetracycline-derived antibiotics like minocycline and tigecycline. Finally, while *tet*(S) was included in the prediction rule, its contribution to tetracycline resistance is unclear because it was found as the sole *tet* gene, in its wild-type form, in only three isolates: two tetracycline- and doxycycline-resistant derivation set isolates but also one tetracycline- and doxycycline-susceptible validation set isolate.

Our study also had some notable limitations. The phenotypic susceptibility testing results for the majority of isolates were generated by the Vitek 2 automated instrument, rather than the broth microdilution reference method, though previous studies have demonstrated 99 to 100% essential agreement between the two methods with the drugs included in our prediction rules (39, 40). In addition, our genotypic prediction rules included most, but not all, antibiotics with CLSI breakpoints for *E. faecium*. Daptomycin has obvious clinical importance in the treatment of vancomycin-resistant *E. faecium* infections, but resistant isolates remain very rare in most hospitals, the

phenotypic gold standard is prone to error (41), the genotypic resistance mechanism is incompletely characterized, and daptomycin susceptibility results are available for only a subset of isolates because of selective testing of *E. faecium* in our laboratory. Likewise, enterococci are not routinely tested for quinupristin-dalfopristin, rifampin, teicoplanin, and minocycline susceptibility at our institution, leading to a dearth of derivation data. More generally, we specifically determined phenotypic categories using CLSI breakpoints rather than the ECVs for maximal translatability to clinical practice, though ECV-based predictions may have led to higher agreement due to their better correlation with wild-type and non-wild-type categories (42). Like for most other genotype-based predictions, we did not explicitly predict the intermediate category, though doing so may have had the most notable performance improvement on the doxycycline predictions. While these simple rules appeared to generalize well when applied to a test set of German clinical *E. faecium* isolates, further testing with geographically diverse isolates is warranted, as geographic variations in resistance mechanisms have been described (43). In particular, additional acquired resistance genes that are not included in our rules, such as *optrA* and *poxtA* for linezolid, may need to be included in genotypic prediction strategies when applied in settings where those antibiotics are used more commonly and selective pressures are stronger (44). Finally, these genotypic prediction rules may need to be updated in the future to reflect emerging mechanisms of AMR.

While WGS is not yet routinely implemented in most clinical microbiology labs due to several factors, including the lack of FDA-cleared *in vitro* diagnostics and cost, our findings can be used to improve existing *E. faecium* diagnostics and inform the development and interpretation of future testing strategies. Rapid molecular VRE detection methods used in many clinical laboratories currently only predict vancomycin resistance but could be modified to predict resistance to other highly relevant antibiotics, like ampicillin. Implementing our genotypic prediction rules that are based on essential sites like *pbp5* 485M and 466′ rather than the complete set of mutations that may exist in a given gene may simplify assay design and improve generalizability compared to other rules-based prediction methods (19). The development and validation of prediction rules like ours rely heavily on existing literature but can be used to supplement more agnostic, unsupervised approaches and provide interpretability for clinicians (16). Finally, our findings also underscore the value of genomic surveillance in enhancing our understanding of the molecular epidemiology of AMR and improving patient care through the development of more rapid methods to determine AMR.

MATERIALS AND METHODS

Microbiologic species identification and susceptibility testing. For all isolates, species identification was performed using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Vitek MS; bioMérieux). Initial susceptibility testing was performed using the AST-GP75 card on the bioMérieux Vitek 2 instrument for ampicillin, high-level gentamicin, ciprofloxacin, levofloxacin, linezolid, vancomycin, tetracycline, and doxycycline (validated within our laboratory). Confirmatory testing was performed by broth microdilution (Sensititre Gram-positive GPALL3F AST plate; Thermo Fisher Scientific), CLSI reference disk diffusion (45), and gradient diffusion (Etest; bioMérieux) when needed. Categorical interpretations were assigned using CLSI document M100 (30th edition) breakpoints (46). Intermediate isolates were considered with susceptible isolates for ciprofloxacin, levofloxacin, and linezolid and with resistant isolates for vancomycin, tetracycline, and doxycycline, based on the epidemiologic cutoff value (ECV).

Clinical isolate collection. The derivation and validation sets were collected from the Massachusetts General Hospital (MGH) Microbiology Laboratory. The derivation set included a random sampling of *E. faecium* isolates collected from January 2016 to December 2017. The validation set was collected from January 2018 to September 2019 with an attempt to enrich for organisms with unusual phenotypic resistance to linezolid, high-level gentamicin, or daptomycin, though no daptomycin-resistant isolates were available. Duplicate isolates collected from the same patient (e.g., same isolation site over multiple days or multiple isolation sites on the same day) were removed from the data sets. The derivation set included 177 isolates and the validation set included 205 isolates. Further details regarding isolate collection are available in the Supplemental Methods. All isolates were collected under a research protocol reviewed by the MGH internal review board (IRB; protocol 2017P000376). The test set (phenotypic AST and whole-genome sequencing data) was obtained from a previous study (19), in which a convenience set of *E. faecium* isolates from Germany was tested via broth microdilution, which we reinterpreted using CLSI clinical breakpoints.

DNA extraction and sequencing. Purified DNA was extracted from isolates as previously described (47), with minor modifications (see the Supplemental Methods). Library preparation was performed with the Nextera DNA library preparation kit using a protocol adapted from that of Baym et al. (48). Paired-end, 150-bp sequencing was performed on Illumina platforms (MiSeq, HiSeq, and NextSeq). Sequencing reads were quality filtered using Trimmomatic (49), with a threshold quality score of 20 for leading, trimming, and sliding-window trimming and a minimum read length of 36. Eight isolates with under 20× genome coverage were excluded from the analysis.

Species confirmation and MLST. Species identification was confirmed using Kraken v1.0 (http://ccb.jhu.edu/software/kraken/). Multilocus sequencing typing (MLST) was performed on whole-genome sequences using SRST2 (50) with an *E. faecium* allele table retrieved from PubMLST containing 1,518 sequence types (STs). GoeBURST analysis was performed at the single-locus variant level with visualization of the first two groups: hospital-associated CC17 and clonal complex 94 (CC94). Members of a clonal complex share at least four alleles with the central sequence type (ST-17 to CC17 and ST-94 to CC94). Core genome MLST (cgMLST) (51) assignment and generation of a neighbor joining tree (ignoring missing values) were performed with Ridom SeqSphere+ v.8.0.0 software on contigs from *de novo* assembly using SPAdes v.3.15.2 (default parameters with the isolate flag). Phylogenetic tree visualization was performed with ggtree v.2.2.1 (52) in RStudio v.1.3.959.

Resistance gene and SNP identification. Resistance genes were detected using SRST2 v.0.2.0 (with a minimum coverage cutoff of 90%, maximum divergence cutoff of 10%, minimum depth of 5×, and maximum of 10 mismatches per read with the option to report all consensus sequences and pileups) with the ARG-ANNOT v.3 (53) database supplemented with *gyrA* (GenBank accession no. NC_017960.1 and NCBI:protein accession no. WP_002288365.1), *parC* (GenBank accession no. NC_017960.1 and NCBI:protein accession no. WP_002296998.1), *pbp5-S* (GenBank accession no. GG670325.1 and NCBI:protein accession no. EEV61481), and 23S rRNA (GenBank accession no. CP046077.1) sequences. Additional *van* genes, aminoglycoside-modifying genes, and linezolid resistance genes were detected using CARD v.3.0.8, as noted in Tables S2, S3, and S4, respectively. Consensus sequences generated by SRST2 for *pbp5*, *gyrA*, *parC*, and *tet*(M) were imported to AliView (54) for single nucleotide polymorphism (SNP) identification. The 23S rRNA G2576T allele frequency was determined using the base calls mapping to position 2576 within the pileups generated by SRST2. Specifically, the allele fraction was the ratio of the number of reads with 2576T to the total number of aligned reads covering that position. Allele fractions were visually confirmed in Integrated Genomics Viewer. The complete resistance gene and SNP information for each isolate was collated in Table S5. Diagnostic accuracy and confidence intervals were determined with the epiR v.1.0-15 R package.

Implementation of genotypic predictions. Antimicrobial resistance predictions were made by implementing the rules (Supplemental Methods) with a custom R script using the genotypic information described above as the input.

Genotypic-phenotypic discrepancy resolution. Fifteen validation set isolates with genotypic-phenotypic discrepancies were revived and underwent repeat phenotyping, repeat genotyping, or both. Repeat testing resolved discrepancies in four isolates and confirmed the original results in the remaining isolates. Two samples were found to contain two or more colony morphotypes of *E. faecium*, which were individually isolated, phenotyped, and sequenced.

Ethics approval and consent to participate. This study was approved by the Mass General Brigham Institutional Review Board with a waiver of written informed consent.

Data availability. The raw sequencing reads have been deposited to the NCBI BioProject database under accession number PRJNA771404.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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