Gut Microbiota Predict Enterococcus Expansion but Not Vancomycin-Resistant Enterococcus Acquisition

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ABSTRACT Vancomycin-resistant Enterococcus (VRE) is a leading cause of hospital-acquired infections and continues to spread despite widespread implementation of pathogen-targeted control guidelines. Commensal gut microbiota provide colonization resistance to VRE, but the role of gut microbiota in VRE acquisition in at-risk patients is unknown. To address this gap in our understanding, we performed a case-control study of gut microbiota in hospitalized patients who did (cases) and did not (controls) acquire VRE. We matched case subjects to control subjects by known risk factors and “time at risk,” defined as the time elapsed between admission until positive VRE screen. We characterized gut bacterial communities using 16S rRNA gene amplicon sequencing of rectal swab specimens. We analyzed 236 samples from 59 matched case-control pairs. At baseline, case and control subjects did not differ in gut microbiota when measured by community diversity ($P = 0.33$) or composition ($P = 0.30$). After hospitalization, gut communities of cases and controls differed only in the abundance of the Enterococcus-containing operational taxonomic unit (OTU), with the gut microbiota of case subjects having more of this OTU than time-matched control subjects ($P = 0.01$). Otherwise, case and control communities after the time at risk did not differ in diversity ($P = 0.33$) or community structure ($P = 0.12$). Among patients who became VRE colonized, those having the Blautia-containing OTU on admission had lower Enterococcus relative abundance once colonized ($P = 0.004$). Our results demonstrate that the 16S profile of the gut microbiome does not predict VRE acquisition in hospitalized patients, likely due to rapid and profound microbiota change. The gut microbiome does not predict VRE acquisition, but it may be associated with Enterococcus expansion, suggesting that these should be considered two distinct processes.

IMPORTANCE The Centers for Disease Control and Prevention estimates that VRE causes an estimated 54,000 infections and 539 million dollars in attributable healthcare costs annually. Despite improvements in hand washing, environmental cleaning, and antibiotic use, VRE is still prevalent in many hospitals. There is a pressing need to better understand the processes by which patients acquire VRE. Multiple lines of evidence suggest that intestinal microbiota may help some patients resist VRE acquisition. In this large case-control study, we compared the 16S profile of intestinal microbiota on admission in patients that did and did not subsequently acquire VRE. The 16S profile did not predict subsequent VRE acquisition, in part due to rapid and dramatic change in the gut microbiome following hospitalization. However, Blautia spp. present on admission predicted decreased Enterococcus abundance after VRE acquisition, and Lactobacillus spp. present on admission predicted Enterococcus dom-

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inance after VRE acquisition. Thus, VRE acquisition and domination may be distinct processes.

**KEYWORDS** microbiome, vancomycin-resistant *Enterococcus*, colonization resistance, hospital-acquired infection

Vancomycin-resistant *Enterococcus* (VRE) species are highly antibiotic-resistant bacteria, are a leading cause of health care-associated infections, and are classified as a serious public health threat by the Centers for Disease Control and Prevention (1, 2). Colonization with VRE precedes infection (3, 4), and molecular epidemiologic analyses show patient-to-patient hospital transmission is the primary means of spread (5). Preventing transmission between hospitalized patients is a significant challenge, and despite the widespread application of pathogen-targeted control measures (6), VRE remains prevalent in many hospitals (1, 2).

Both indirect human evidence and animal experimentation demonstrate that gut microbiota prevent VRE colonization when a patient is exposed, a phenomenon termed "colonization resistance" (7–9). Colonization resistance may entail competition for resources, secretion of bactericidal factors (10, 11), and indirect stimulation of host immune defense mechanisms that target VRE (12, 13). Though colonization resistance plays a crucial role in suppressing VRE expansion and preventing VRE infection (14, 15), to date, no study has evaluated whether variation in intestinal microbiota can explain variation in VRE acquisition among at-risk patients.

To address this gap in our understanding of VRE transmission, we investigated whether the gut microbiome of at-risk patients predicts VRE colonization in a hospitalized patient population. We hypothesized that if the gut microbiome can confer colonization resistance for VRE acquisition, variation in baseline microbiota would explain variation in patient susceptibility to VRE acquisition. To test this hypothesis, we designed a case-control study using 16S rRNA gene amplicon sequencing of rectal swabs acquired from hospitalized patients.

**RESULTS**

**Study population and medication exposures.** We studied gut microbiome communities in 236 rectal swab samples from 59 matched pairs of case and control subjects (Table 1). Cases and controls did not differ in demographics (age, sex, ethnicity) or in the relative frequency of common comorbidities (e.g., immunosuppression, malignancy, or gastrointestinal disease). Antibiotic use was widespread among all subjects and was nearly equal across groups (Table 2). Vancomycin, cefepime, metronidazole, and piperacillin-tazobactam were the most commonly used antibiotics in the study population. Cases and controls did not differ significantly in their exposure to any specific antibiotics prior to initial sampling. More cases received proton pump inhibitors prior to initial sampling ($P = 0.04$). During time at risk (between initial and subsequent sampling), case and controls did not differ in their exposure to antibiotics or proton pump inhibitors.

**Admission gut microbiota do not predict VRE acquisition.** We first compared baseline microbiota across patients who did (cases) and did not (controls) subsequently acquire VRE. Baseline gut communities of cases and controls did not differ in their community composition, determined either via permutation testing ($P = 0.30$ by permutational multivariate analysis of variance [PERMANOVA]) or via visualization (principal-component analysis; Fig. 1, left). Similarly, baseline gut communities of cases and controls did not differ in their species diversity as measured by the Shannon diversity index (mean of 2.72 ± 0.90 for controls, mean of 2.71 ± 0.76 for cases, $P = 0.96$ for all matched case-control pairs) (Fig. 1, right). We noted that *Enterococcus* (OTU0004) was among the top 10 operational taxonomic units (OTUs) identified in both cases and controls at the time of admission. Despite the high abundance of the genus *Enterococcus* on admission, none of these swabs had vancomycin-resistant *Enterococcus* at the time of admission when evaluated with VRESelect chromogenic medium. *Enterococcus*...
(OTU0002) colonization did not imply VRE colonization, as OTU0004 captured both resistant and sensitive strains. We concluded that the gut microbiota, as represented by the 16S profile of these samples of hospitalized patients, do not predict subsequent VRE acquisition. At the time of VRE detection, the gut communities of cases and controls differ only in the abundance of Enterococcus. We next compared gut communities across matched cases and controls after time at risk: after cases had been colonized and

**TABLE 1** Demographics and comorbidities of matched cohorts

| Demographic or clinical characteristic | No. of individuals (proportion) with characteristic or value specified | Controls (n = 59) | Cases (n = 59) | P value |
|---------------------------------------|------------------------------------------------------------------------|-----------------|---------------|---------|
| Demographics                          |                                                                        |                 |               |         |
| Age (mean ± SE)                       |                                                                        | 57.19 ± 1.62    | 60.2 ± 1.95   | 0.23    |
| Female                                |                                                                        | 23 (0.39)       | 22 (0.38)     | 0.56    |
| Nonwhite race                         |                                                                        | 9 (0.15)        | 9 (0.15)      | 0.28    |
| Diagnoses and comorbidities           |                                                                        |                 |               |         |
| C. difficile infection                 |                                                                        | 4 (0.07)        | 11 (0.18)     | 0.07    |
| Leukemia                              |                                                                        | 21 (0.36)       | 17 (0.29)     | 0.38    |
| Lymphoma                              |                                                                        | 12 (0.21)       | 10 (0.17)     | 0.49    |
| Bone marrow transplant                |                                                                        | 15 (0.25)       | 14 (0.24)     | 0.64    |
| Solid organ malignancy                |                                                                        | 35 (0.6)        | 40 (0.67)     | 0.33    |
| Metastatic malignancy                 |                                                                        | 29 (0.49)       | 35 (0.59)     | 0.10    |
| Diabetes                              |                                                                        | 27 (0.46)       | 23 (0.39)     | 0.59    |
| Coronary artery disease               |                                                                        | 6 (0.11)        | 10 (0.17)     | 0.72    |
| Congestive heart failure              |                                                                        | 19 (0.32)       | 18 (0.3)      | 0.60    |
| COPD                                  |                                                                        | 21 (0.35)       | 36 (0.61)     | 0.02    |
| Peripheral vascular disease           |                                                                        | 6 (0.1)         | 2 (0.03)      | 0.31    |
| End-stage renal disease               |                                                                        | 18 (0.31)       | 26 (0.44)     | 0.07    |
| Connective tissue disease             |                                                                        | 1 (0.01)        | 4 (0.06)      | 0.35    |
| Peptic ulcer disease                  |                                                                        | 9 (0.15)        | 6 (0.11)      | 0.50    |
| Cirrhosis                             |                                                                        | 2 (0.04)        | 9 (0.15)      | 0.06    |
| Cerebrovascular disease               |                                                                        | 12 (0.21)       | 17 (0.29)     | 0.54    |
| Hemiplegia                            |                                                                        | 4 (0.06)        | 12 (0.2)      | 0.07    |
| Dementia                              |                                                                        | 1 (0.01)        | 3 (0.05)      | 0.34    |
| Charlson score (mean ± SE)            |                                                                        | 3.71 ± 0.22     | 4.45 ± 0.25   | 0.05    |

*Cases and controls were matched by “time at risk” and unit or ward.
*C. difficile, Clostridium difficile; COPD, chronic obstructive pulmonary disease.

**TABLE 2** Medication exposure of matched cohorts

| Sampling time and medication          | Prevalence of exposurea | P value | Duration of exposureb | P value |
|---------------------------------------|-------------------------|---------|-----------------------|---------|
| Prior to admission swab               | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases |
| Antibiotics                           |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |
| Any antibiotics                       | 29 (0.49) | 40 (0.68) | 0.05    | 1.72 ± 0.62 | 2.32 ± 0.47 | 0.44   |
| Vancomycin                            | 14 (0.24) | 21 (0.36) | 0.17    | 0.52 ± 0.3  | 0.36 ± 0.06 | 0.61   |
| Metronidazole                         | 8 (0.14)  | 14 (0.24) | 0.17    | 0.14 ± 0.04 | 0.4 ± 0.16 | 0.16   |
| Piperacillin-tazobactam               | 8 (0.14)  | 12 (0.2)  | 0.29    | 0.14 ± 0.04 | 0.21 ± 0.06 | 0.25   |
| Cefepime                              | 7 (0.12)  | 11 (0.19) | 0.32    | 0.4 ± 0.3   | 0.32 ± 0.12 | 0.81   |
| Proton pump inhibitors                | 9 (0.15)  | 19 (0.32) | 0.04    | 0.2 ± 0.07  | 0.61 ± 0.19 | 0.07   |
| Between admission and *time at risk* swab |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |
| Antibiotics                           |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |          |       |
| Any antibiotics                       | 56 (0.95) | 52 (0.88) | 0.18    | 21.66 ± 4.98 | 22.36 ± 3.66 | 0.82   |
| Vancomycin                            | 37 (0.63) | 39 (0.66) | 0.66    | 3.55 ± 0.82  | 3.33 ± 0.82 | 0.73   |
| Metronidazole                         | 20 (0.34) | 24 (0.41) | 0.43    | 2.01 ± 0.72  | 2.29 ± 0.6  | 0.75   |
| Piperacillin-tazobactam               | 26 (0.44) | 25 (0.42) | 0.83    | 3.66 ± 1.13  | 2.8 ± 0.64  | 0.42   |
| Cefepime                              | 21 (0.36) | 24 (0.41) | 0.56    | 2.92 ± 1.01  | 3.1 ± 0.82  | 0.85   |
| Proton pump inhibitors                | 33 (0.56) | 39 (0.66) | 0.23    | 5.15 ± 1.48  | 6.75 ± 1.32 | 0.13   |

*aPrevalence values are reported as number of case or control individuals (proportion).
bDuration values are reported as numbers of days of therapy ± standard deviation (SD).
time-matched controls had not. After time at risk, gut microbiota did differ across cases and controls ($P < 0.001$ by PERMANOVA), though Shannon diversity index did not (mean of 2.38 ± 0.11 for controls, mean of 2.22 ± 0.12 for cases, $P = 0.33$ for all matched case-control pairs). The difference in gut microbiota was driven by the increased relative abundance of a single OTU, the Enterococcus-classified taxonomic group (OTU0004), which was greater in cases than controls ($P = 0.01$ via mvabund, $P < 0.001$ via random forest) (Fig. 2). When this Enterococcus OTU was excluded from the analysis, we found no significant difference in communities across cases and controls ($P = 0.12$ by PERMANOVA) (Fig. 3). We thus concluded that at the time of VRE acquisition, the gut microbiota of VRE-infected and uninfected patients differ only in the relative abundance of Enterococcus and do not consistently differ in their non-Enterococcus microbiota.

**Gut microbiota change rapidly and profoundly in hospitalized patients.** Given the lack of differentiation of gut communities across cases and controls at admission and at the time of VRE colonization, we then asked whether the temporal change in gut microbiota could distinguish the two groups. We did this by calculating the relative dissimilarity of admission and index (time-at-risk) communities for each subject using Jaccard distance, a metric of dissimilarity between gut microbial communities measured on a scale of 0 (complete similarity) to 1 (complete dissimilarity) (Fig. 4). The gut communities of both groups underwent a rapid, profound change in composition. Within several days of admission, gut communities of both cases and controls bore little similarity to the communities detected at the time of admission. The size of the change in gut communities did not differ across cohorts ($0.87 ± 0.02$ for cases, $0.86 ± 0.02$ controls, $P = 0.84$). Cases and controls also had similar decreases in Shannon diversity ($−0.48 ± 0.10$ for cases, $−0.40 ± 0.08$ for controls, $P = 0.93$ for all matched case-control pairs). We found that Jaccard distance was significantly correlated with time (Spearman’s rank correlation coefficient $ρ = 0.32$, $P = 0.0006$) and determined that a negative exponential model best fit the data, with gut microbiota approaching complete dissimilarity at an exponential rate of $0.47 \times e^{-0.47t+32}$ (t representing the time between swabs). We found no significant difference in the rate of change between the two groups. We noted that the predicted mean Jaccard distance for two rectal swab
samples taken on the same day ($t = 0$) was $0.79 \pm 0.058$, implying a substantial amount of variation in community structure within the same day of admission.

**Gut microbiota on admission are associated with Enterococcus expansion.** Finding no difference in the community composition, diversity, or temporal rate of change across patients who did (cases) and did not (controls) acquire VRE during their hospitalization, we asked whether gut microbiota on admission could predict the relative abundance of *Enterococcus* in VRE-colonized patients. We built a random forest
A random forest regression model was used to identify taxa present on admission that were predictive of final *Enterococcus* relative abundance. In cases, only *Blautia* and *Lactobacillus* were significant after correcting for multiple testing and feature importance bias (Fig. 5; see Tables S1 and S2 and Fig. S2 in the supplemental material). In cases, *Blautia* spp. (OTU 0092) on admission was predictive of decreased *Enterococcus* (−10.3% relative-abundance-adjusted P [relative abundance P] = 0.004 by Mann-Whitney U test), and *Lactobacillus* spp. (OTU 0026) was predictive with an increased abundance of *Enterococcus* after the time at risk (12.5% relative abundance P = 0.007 by Mann-Whitney U test). A random forest regression model applied to the control population identified the same *Lactobacillus* and *Blautia* taxa as predictive of *Enterococcus* abundance after the time at risk (−3.7% relative abundance P = 2.4 × 10^{-8} and +3.6% relative abundance for *Lactobacillus* P = 0.003 by Mann-Whitney U test). In controls, *Lactobacillus* and *Blautia* were not the only predictive taxa, as *Phascolarctobacterium*, *Prevotella*, and *Bifidobacterium* were also predictive of decreased *Enterococcus* abundance. While more taxa were predictive of final *Enterococcus* abundance in controls, the effect size of these taxa was smaller, as controls had a lower abundance of *Enterococcus* after the time at risk (Table S2). Thus, we found that the presence of specific anaerobes previously implicated in *Enterococcus* colonization resistance (8, 15, 16) is predictive of decreased *Enterococcus* abundance in both VRE-colonized and uncolonized patients. These findings suggest that VRE acquisition and *Enterococcus* expansion are two distinct processes with different risk factors and pathophysiology.

**DISCUSSION**

In this study, gut microbiota did not predict VRE acquisition in hospitalized patients. Secondary analysis identified individual members of the gut microbiota that do predict
Enterococcus abundance at the time of VRE acquisition, implying that acquisition and expansion of VRE may be distinct processes. The community composition, diversity, and temporal rate of change did not differ across patients who did (cases) and did not (controls) acquire VRE during their hospitalization. As expected based on the study design, gut communities of cases had a greater abundance of Enterococcus than controls after the time at risk.

Gut communities of all subjects demonstrated a rapid and dramatic change during hospitalization that was time dependent. In this population, antibiotic use was prevalent (Table 2), gut microbial communities were remarkably dynamic (Fig. 5), and admission gut microbiota provided very little information about microbiota after the time at risk. Our model of Jaccard distance over time estimated a mean Jaccard distance of 0.79 between two rectal swabs taken on the same day of admission, implying that only 21% of gut microbiota remain constant with resampling within 24 h. Given the significant correlation between Jaccard distance and time, some of this change is likely due to the disruptive pressures that face gut microbiota upon hospitalization (i.e., antibiotics). However, a large portion of this change may represent stochasticity and noise introduced by variation in sample collection and storage. Other studies have found that gut microbiota change dramatically during hospitalization (17–19), but to our knowledge, ours is the largest study to examine this change, the only study to look at the rate of change, and the only study show that most of the change occurs very early (within 72 h). These results have important implications for the clinical use of gut microbiota for therapy, prediction, and risk stratification. Given the rapid change of gut communities upon hospitalization, a single static 16S analysis of gut microbiota may miss subtle dynamics important for VRE acquisition and is subject to a large amount of noise that may obscure a true biologically meaningful association. Future study of the gut microbiota in VRE acquisition may need to move beyond traditional 16S analysis, which can be time-consuming and miss important species-level information (20, 21). Real-time metagenomics and rapid, ultrasensitive quantification technologies hold promise as tools with better resolution to evaluate these processes (22, 23).
Despite the dramatic change in community structure, we did find some evidence of colonization resistance, as admission microbiota were predictive of Enterococcus abundance at the time of VRE detection. VRE-colonized subjects with Blautia had less Enterococcus expansion, consistent with prior studies (8, 15, 16). We hypothesize that there may be a distinction between VRE acquisition and VRE expansion. In conjunction with earlier studies (8, 16, 24), our findings suggest that commensal anaerobes may play a significant role in suppressing VRE once colonized. In this context, our results further support the possibility of microbiome manipulation to reduce VRE burden even in patients already colonized to prevent progression to VRE infection in the individual patient (15, 24) or transmission into the surrounding environment and other hospitalized patients (7, 8, 16).

We noted that more cases received proton pump inhibitor (PPI) therapy prior to initial sampling than controls (Table 2), consistent with prior studies showing that PPI use is a risk factor for VRE colonization (25, 26). Despite this difference in treatment, we found no meaningful difference in the community structure of cases and controls on admission (Fig. 1). This may imply that the increased risk of VRE colonization from PPI therapy is not mediated through changes in gut microbiota, but by elimination of the gut acid barrier to ingested bacteria (27). We believe these findings are hypothesis generating for future studies of the role of PPI therapy in VRE acquisition.

In this retrospective case-control study, we controlled for multiple confounders with our time- and unit-matched design. We used machine learning algorithms robust to multicollinearity and overfitting, and applied permutation heuristics to correct for feature importance bias and decrease our false discovery rate. This study reveals an opportunity for future studies to delineate key differences in pathophysiology between VRE acquisition and domination.

In summary, VRE acquisition and expansion may be two distinct processes, and efforts to manipulate the microbiome to prevent the spread of VRE may be more beneficial in reducing VRE domination in colonized patients than in preventing VRE...
acquisition in uncolonized patients. Future studies of the role of the gut microbiota in VRE acquisition may need to move beyond single time point 16S analyses and address the role of temporal dynamics and stochasticity of gut microbiota in the acquisition and expansion of VRE.

MATERIALS AND METHODS

Study setting and design. We previously conducted a retrospective case-control study of clinical risk factors for VRE acquisition among patients who did (cases) and did not (controls) acquire VRE during their admissions at the University of Michigan Healthcare System from January 2013 until June 2016 (25). We studied gut microbiome communities in 236 rectal swab samples from 59 matched pairs of case and control subjects from patients admitted to the University of Michigan Hospital in 2016. Sixty-four out of 118 subjects in this study (54%) were a part of our previous clinical risk factor analysis. The remaining subjects were admitted from June to December 2016 (outside the time frame of the previous study by 6 months). The University of Michigan Healthcare system consists of ~1,000 inpatient beds and serves as a tertiary referral hospital for southeastern Michigan. The institutional review board at the University of Michigan approved the study before its initiation.

VRE case definition. The infection control practice throughout the study period was to perform routine surveillance for VRE on eight adult units, including intensive care units, the hematology and oncology ward, and the bone marrow transplant ward. All patients were routinely screened on admission and weekly thereafter with rectal swabs that were tested by Bio-Rad VRESelect chromogenic medium to detect VRE. Cases were defined as subjects with an initial negative swab followed by a positive swab when evaluated by this selective culture. We further identified the “time at risk” for each case patient, here defined as the time elapsed between admission and positive VRE screen. We matched each case subject to a control subject with an initial negative swab followed by repeat negative swab within the same time at risk (±5%). An additional matching factor was the unit from which the first positive VRE was recovered, for cases or the matched swab after the time at risk for controls.

Bacterial DNA isolation. Genomic DNA was extracted from rectal swabs resuspended in 360 µL ATB buffer (Qiagen DNaseasy blood and tissue kit) and homogenized in fecal DNA bead tubes using a modified protocol previously demonstrated to isolate bacterial DNA (28, 29). Sterile laboratory water and AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) used in DNA isolation were collected and analyzed as potential sources of contamination. ZymoBIOMICS microbial community DNA standard (Zymo Research catalog no. D6306) was sequenced for error analysis.

16S rRNA gene sequencing. The V4 region of the 16S rRNA gene was amplified using published primers and the dual-indexing sequencing strategy developed previously (28). Sequencing was performed using the Illumina MiSeq platform (San Diego, CA) and a MiSeq reagent kit V2 (500 cycles) according to the manufacturer’s instructions with modifications found in the standard operating procedure of the laboratory of Patrick Schloss (28, 30). Accuprime high-fidelity Taq was used in place of Accuprim Pfx SuperMix (31). Primary PCR cycling conditions were 95°C for 2 min, followed by 20 cycles of touchdown PCR (1 cycle consisting of 95°C for 20 s, 60°C for 20 s and decreasing 0.3 degrees each cycle, 72°C for 5 min), and then 20 cycles of standard PCR (1 cycle consisting of 95°C for 20 s, 55°C for 15 s, and 72°C for 5 min), and finished with 72°C for 10 min.

Statistical analyses. Sequence data were processed and analyzed using the software mothur v.1.33.0 (32) according to the standard operating procedure for MiSeq sequence data using a minimum sequence length of 250 bp (28, 33). To summarize, the SILVA rRNA database (34) was used as a reference for sequence alignment and taxonomic classification. K-mer searching with 8-mers was used to assign raw sequences to their closest matching template in the reference database, and pairwise alignment was performed with the Needleman-Wunsch algorithm (35) and NAST algorithms (36). A k-mer-based naïve Bayesian classifier (37) was used to assign sequences to their correct taxonomy with a bootstrap confidence score threshold of 80. Pairwise distances between aligned sequences were calculated by the method employed by Sogin et al. (38), where pairwise distance equals mismatches, including indels, divided by sequence length. A distance matrix was passed to the OptiCLUST clustering algorithm (39) to cluster sequences into “operational taxonomic units” (OTUs) by maximizing the Matthews correlation coefficient with a dissimilarity threshold of 3% (40).

After clustering and classification of raw sequencing data, we evaluated differences in community structure with permutation multivariate analysis of variance (PERMANOVA) in the vegan package (v 2.0-4) (41) in R (v 3.6.4) (42). We performed resampling of multiple generalized linear models with the mvabund (43) package in R to look for individual OTU differences between communities. We set a significance threshold of 0.01 after adjusting for multiple comparisons using a stepdown resampling procedure to reduce the type I error rate (44). We confirmed individual OTU differences with random forest classification and regression models built with the ranger package in R (v 0.11.2) (45). We used the caret (v 6.0-84) (46) package in R for cross-validation and to optimize the hyperparameters of the number of decision trees in the model and the number of features considered by each tree when splitting a node. We corrected for feature importance bias in random forest models with a permutation importance (PIMP) heuristic developed by Altmann et al. (47). All OTUs were included in diversity and abundance analyses. We performed bivariate analysis with conditional logistic regression using the survival (v 3.1-8) package in R (48, 49). Levels of final Enterococcus abundance were compared with the nonparametric Mann-Whitney U test. We used the vegan package in R to calculate both the average species diversity in an individual rectal swab (Shannon diversity) and the change in community structure between the initial swab and second swab for each subject (Jaccard distance). We used Spearman’s rank
correlation coefficient to determine whether Jaccard distance was significantly correlated with the time between swabs, and we used nonlinear least-squares regression to fit a model of Jaccard distance over time for cases and controls.

**Adequacy of sequencing.** We performed 16S rRNA gene amplicon sequencing on 236 rectal swab specimens and 15 negative-control specimens, which identified 1,188 unique operational taxonomic units (genus-level bacterial taxa) at a dissimilarity threshold of 3%. After bioinformatics processing, the mean number of reads per sample was 71,484 ± 2,684. No specimens were excluded from the analysis. Rectal swab specimens had clear differences in community structure compared to control specimens, which was confirmed as statistically significant using multiple methods of hypothesis testing (mvabund and PERMANOVA [adonis], \( P < 0.01 \) for both) (see Fig. S1 in the supplemental material). Sequences generated from negative-control specimens were dominated by a single *Pseudomonas*-classified OTU (OTU001). This OTU was included in all reported analyses, though the exclusion of this OTU did not affect any of the reported results.

**Data availability.** Sequences are available via the NCBI Sequence Read Archive (accession number PRJNA633879). OTU tables, taxonomy classification tables, and metadata tables are available at https://github.com/rishichanderraj/Microbiota_Predictors_VRE_Acquisition. We have excluded protected health information (PHI) attached with this metadata. Potential collaborators are welcome to contact our group with reasonable requests that guarantee patient safety and privacy.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, EPS file, 1.6 MB.
**FIG S2**, EPS file, 1.4 MB.
**TABLE S1**, DOCX file, 0.01 MB.
**TABLE S2**, DOCX file, 0.01 MB.

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We declare that we have no conflicts of interest to disclose.

**REFERENCES**

1. Centers for Disease Control and Prevention. 2019. Antibiotic resistance threats in the United States, 2019. Antibiotic Resistance Coordination and Strategy Unit, Division of Healthcare Quality Promotion, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA.

2. Weiner LM, Webb AK, Limbago B, Dudek MA, Patel J, Kallen AJ, Edwards JR, Sievert DM, 2016. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011–2014. Infect Control Hosp Epidemiol 37:1228 –1301. https://doi.org/10.1017/ice.2016.174.

3. Zirakzadeh A, Gastineau DA, Mandrekar JN, Burke JP, Johnston PB, Patel R. 2008. Vancomycin-resistant enterococcal colonization appears associated with increased mortality among allogeneic hematopoietic stem cell transplant recipients. Bone Marrow Transplant 41:385–392. https://doi.org/10.1038/sj.bmt.17075912.

4. Zirakzadeh A, Patel R. 2006. Vancomycin-resistant enterococci: colonization, infection, detection, and treatment. Mayo Clin Proc 81:529–536. https://doi.org/10.4065/81.4.529.

5. Bonten MJ, Hayden MK, Nathan C, van Voorhis J, Matushek M, Slaughter K, Peled JU, Hendrickson RC, Taur Y, van den Brink MRM, Pamer EG. 2017. Cooperating commensals restore colonization resistance to vancomycin-resistant Enterococcus faecium. Cell Host Microbe 21:592–602.e4. https://doi.org/10.1016/j.chom.2017.04.002.

6. Buffie CG, Pamer EG. 2013. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol 13:790–801. https://doi.org/10.1038/nri3535.

7. Kim SG, Becattini S, Moore YU, Littmann ER, Seok R, Gjonbalaj M, Eaton V, Fontana E, Amoretti L, Wright R, Caballero S, Wang ZMX, Jung HJ, Morjaria SM, Leiner IM, Qin W, Ramos RJF, Cross JR, Narushima S, Honda K, Peled JU, Hendrickson RC, Taur Y, van den Brink MR, Pamer EG. 2019. Microbiota-derived lantibiotic restores resistance against vancomycin-resistant Enterococcus. Nature 572:665–669. https://doi.org/10.1038/s41586-019-1501-z.

8. Cash KL, Whitham CV, Behrendt CL, Hooper LV. 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science 313:1126 –1130. https://doi.org/10.1126/science.1127119.

9. Bhalla A, Pultz NJ, Ray AJ, Hoyen CK, Eckstein EC, Donkey CJ. 2003. Antanaerobic antibiotic therapy promotes overgrowth of antibiotic-resistant, Gram-negative bacilli and vancomycin-resistant enterococci in the stool of colonized patients. Infect Control Hosp Epidemiol 24:646–649. https://doi.org/10.1086/502267.

10. Taur Y, Xavier JB, Lipuma L, Ubeda C, Goldberg J, Gobourne A, Lee YJ, Dubin KA, Socci ND, Viale A, Perales M-A, Jenq RR, van den Brink MR,
Pamer EG. 2012. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin Infect Dis 55:905–914. https://doi.org/10.1093/cid/cis580.

16. Donskey CJ, Chowdhry TK, Hecker MT, Hoyen CK, Hanrahan JA, Hujer AM, Hutton-Thomas RA, Whalen CC, Bonomo RA, Rice LB. 2000. Effect of antibiotic therapy on the density of vancomycin-resistant enterococci in the stool of colonized patients. N Engl J Med 343:1925–1932. https://doi.org/10.1056/NEJM200012283426004.

17. McDonald D, Ackermann K, Kahlова L, Baird C, Heyland D, Kozar R, Lemieux M, Derenski K, King J, Vis-Kampen C, Knight R, Wischmeyer PE. 2016. Extreme dysbiosis of the microbiome in critical illness. mSphere 1:199–215. https://doi.org/10.1128/mSphere.00199-16.

18. Ravi A, Halstead FD, Bamford A, Casey A, Thomson NM, van Schaik W, Snelsen C, Goulden R, Foster-Nyarak E, Savva GM, Whitehouse T, Pallen MJ, Oppenheim BA. 2019. Loss of microbial diversity and pathogen domination of the gut microbiota in critically ill patients. Microb Genom 5:e000293. https://doi.org/10.1099/mgen.0.000293.

19. Livanos AE, Snider EJ, Whitteet S, Chong DH, Abrams JA, Freedberg DE. 2018. Rapid gastrointestinal loss of clostrocoidal clusters IV and XIVA in the ICU associates with an expansion of gut pathogens. PLoS One 13:e0200322. https://doi.org/10.1371/journal.pone.0200322.

22. Pendleton KM, Erb-Downward JR, Bao Y, Branton WR, Falkowski NR, Newton DW, Huffnagle GB, Dickson RP. 2017. Rapid pathogen identification in bacterial pneumonia using real-time metagenomics. Am J Respir Crit Care Med 195:1610–1612. https://doi.org/10.1164/rccm.201703-0537LE.

23. Chanderjari R, Dickson RP. 2018. Rethinking pneumonia: a paradigm shift with practical utility. Proc Natl Acad Sci U S A 115:13148–13154. https://doi.org/10.1073/pnas.1819024116.

24. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. https://doi.org/10.1128/AEM.01541-09.

25. Schloss PD. 2019. MiSeq SOP:mothur. https://mthor.org/wiki/miseq_sop/.

26. Schloss PD, Hostacka RE, Cloud D, Reddy JP, Williams BP, Maisonneuve H, Zimmerman L, Rangel-Mino R, King J, et al. 2016. Extreme dysbiosis of the microbiome in critical illness. mSphere 1:199–215. https://doi.org/10.1128/mSphere.00199-16.

27. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. New Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267. https://doi.org/10.1128/AEM.01562-07.

28. Vreeland S, Burtner J, Murphy M, Reddy JP, Williams BP, Maisonneuve H, Zimmerman L, Rangel-Mino R, King J, et al. 2016. Extreme dysbiosis of the microbiome in critical illness. mSphere 1:199–215. https://doi.org/10.1128/mSphere.00199-16.

29. Wang Y, Naumann U, Wright ST, Warton DI. 2012. mvabund R package version 2.0-2. https://cran.r-project.org/web/packages/mvabund/mvabund.pdf.

30. Wang Y, Naumann U, Wright ST, Warton DI. 2012. mvabund R package version 2.0-2. https://cran.r-project.org/web/packages/mvabund/mvabund.pdf.

31. Korbe CJ, Mattick JS. 2008. Touchdown PCR for increased specificity and sensitivity in PCR amplification. Nat Protoc 3:1452–1456. https://doi.org/10.1038/nprot.2008.133.

32. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. https://doi.org/10.1128/AEM.01541-09.