Gut Microbiota and Clinical Features Distinguish Colonization With *Klebsiella pneumoniae* Carbapenemase-Producing *Klebsiella pneumoniae* at the Time of Admission to a Long-Term Acute Care Hospital

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**Background.** Identification of gut microbiota features associated with antibiotic-resistant bacterial colonization may reveal new infection prevention targets.

**Methods.** We conducted a matched, case–control study of long-term acute care hospital (LTACH) patients to identify gut microbiota and clinical features associated with colonization by *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella pneumoniae* (KPC-Kp), an urgent antibiotic resistance threat. Fecal or rectal swab specimens were collected and tested for KPC-Kp; 16S rRNA gene-based sequencing was performed. Comparisons were made between cases and controls in calibration and validation subsamples using microbiota similarity indices, logistic regression, and unit-weighted predictive models.

**Results.** Case (n = 32) and control (n = 99) patients had distinct fecal microbiota communities, but neither microbiota diversity nor inherent clustering into community types distinguished case and control specimens. Comparison of differentially abundant operational taxonomic units (OTUs) revealed 1 OTU associated with case status in both calibration (n = 51) and validation (n = 80) subsamples that matched the canonical KPC-Kp strain ST258. Permutation analysis using the presence or absence of OTUs and hierarchical logistic regression identified 2 OTUs (belonging to genus *Desulfovibrio* and family *Ruminococcaceae*) associated with KPC-Kp colonization. Among clinical variables, the presence of a decubitus ulcer alone was independently and consistently associated with case status. Combining the presence of the OTUs *Desulfovibrio* and *Ruminococcaceae* with decubitus ulcer increased the likelihood of KPC-Kp colonization to >38% in a unit-weighted predictive model.

**Conclusions.** We identified microbiota and clinical features that distinguished KPC-Kp gut colonization in LTACH patients, a population particularly susceptible to KPC-Kp infection. These features may warrant further investigation as markers of risk for KPC-Kp colonization.

**Keywords.** carbapenem-resistant *Enterobacteriaceae*; *Klebsiella pneumoniae*; microbiota; long-term acute care hospitals; microbiome.

Health care–associated infections due to multidrug-resistant organisms (MDROs) pose an increasing public health threat. Carbapenemase-producing *Enterobacteriaceae* (CPE) are one of the most concerning MDROs because of their ability to spread rapidly across regions [1, 2] and to cause invasive infections associated with high mortality [3]. Although CPE control programs have been successful in some locales [4], globally the problem continues to worsen [5], necessitating more effective prevention strategies.

Colonization of the gastrointestinal tract by CPE usually precedes infection. Although a healthy, indigenous gut microbiota likely provides resistance to colonization by enteric MDROs such as CPE, exposure to antibiotics and other clinical factors can disrupt gut microbial community homeostasis and reduce “colonization resistance,” thus increasing CPE colonization risk [6]. Development of “microbiome disruption indices,” that is, measurements of microbiota characteristics that inversely associate with colonization resistance, has been proposed as an infection prevention strategy to improve identification of patients at risk for MDRO colonization, infection, or transmission [7]. A first step toward development of a microbiome disruption index for predicting CPE colonization is to determine whether the composition of the microbiota differs
in CPE-colonized and -noncolonized patients. To this end, we compared the fecal microbiota and clinical care exposures of patients with and without colonization by *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella pneumoniae* (KPC-Kp), the most common CPE in the United States, and sought features that distinguished the 2 groups.

**METHODS**

**Study Design and Enrollment Criteria**

We conducted a matched, case–control study in 1 long-term acute care hospital (LTACH) in Chicago, Illinois. Cases were defined as patients with KPC-Kp intestinal colonization identified by fecal or rectal swab screening within 3 days of admission to the LTACH and no evidence of colonization or infection with CPE other than KPC-Kp. Patients who had negative admission screening tests and no history of CPE colonization or infection were eligible to serve as controls and were matched 1:1 or 1:2 to cases by admission date (±14 days), depending on availability.

Patients were excluded from study participation if they had a history of acute bacterial or viral colitis (including *Clostridium difficile* infection) or active inflammatory bowel disease within 14 days before sample collection, or if they had a rectal tube, fecal incontinence device, or colostomy at the time of sample collection. These exclusion criteria were applied to reduce potential confounding effects of colonic inflammation and ensure that samples were representative of recently passed feces, respectively. Patients participated in the study only once.

The study protocol was reviewed and granted expedited approval by the institutional review board at Rush University Medical Center. Written informed consent was waived.

**Specimen Collection, Processing, and Screening for CPE**

Fecal or rectal swab specimens were collected from study participants, screened for KPC-Kp, and used for microbiota community analysis (Supplementary Table 1). In a previous investigation, we determined that fecal and rectal swab microbiota from the same subject were highly similar, thus justifying the combined analysis of those 2 specimen types in the current investigation [8]. Specimens were screened for *bla*-KPC by polymerase chain reaction (PCR) [9, 10] and plated simultaneously on MacConkey agar (Remel Inc, San Diego, CA); plates were incubated at 35°C in ambient air for up to 48 hours. Unique colony morphologies of suspected *K. pneumoniae* were identified to the species level, and antibiotic susceptibility was determined using the Microscan Walkaway Plus system (Beckman Coulter, Indianapolis, IN). Carbapenem-resistant *K. pneumoniae* isolates underwent a second round of confirmatory PCR testing for *bla*-KPC.

**16S rRNA Gene Sequencing**

To determine if the fecal microbial community of KPC-Kp(+) patients differed from that of KPC-Kp(-) patients, we purified DNA from fecal specimens or rectal swabs using the PowerMag Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) on the EpMotion 5075 (Eppendorf, Hamburg, Germany). The bacterial 16S rRNA genes were amplified by PCR using dual-index primers specific to the V4 region [11] from 1 μL of the sample DNA, as described previously [12]. Amplicons were prepared for sequencing and sequenced using the 500 cycle MiSeq Reagent Kit, v. 2 (Illumina, catalog No. MS-102–2003), on a MiSeq (Illumina, San Diego, CA) by the University of Michigan Microbial Systems Molecular Biology Laboratory as described previously [12].

**Sequence Analysis of Microbiota**

Sequence files were deposited in the NCBI’s Sequence Read Archive (SRA; Bioproject PRJNA428477, SAMN8292036-8292166). Sequences were processed and analyzed using mothur (v. 1.39.5) (Supplementary Methods) [13]. Briefly, sequences were aligned to the recreated SILVA SEED reference alignment (release 119) and trimmed to the same start and end positions [14]. Chimeras were removed using uchime [15]. Samples with <1000 sequences after processing were not included. For all microbiota analyses, sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity by the average neighbor method. Shannon diversity was calculated based on OTU composition. A distance metric that takes relative abundances of both shared and nonshared OTUs into account, θYC, was calculated between samples [16], and analysis of molecular variance (AMOVA) [17] was used to test for significant differences between the communities of KPC-Kp(+) and KPC-Kp(-) patients. Principal coordinates analysis (PCoA) was used to visualize the θYC distances between samples. Linear discriminant analysis (LDA) effect size (LDAseq) was used to determine if specific OTUs were differentially abundant in KPC-Kp(+) and KPC-Kp(-) specimens [18]. We also investigated the taxonomic composition by classifying sequences within microbiomes using the Ribosomal Database Project (RDP) training set (v. 10) [19]. Samples were grouped into community types using partitioning around medoids (PAM), calculated from the Jensen-Shannon divergence of the taxonomic composition, as used previously to group fecal microbiota profiles into enterotypes [20]. To investigate the sequences included in each OTU, bin.seqs was used. The unique OTU003 sequence was further analyzed using RDP’s online Sequence Match against 16S rRNA gene sequences for isolates (type and non-type), size ≥1200 nucleotides, and good quality (on December 20, 2017) [9] and compared with specific genomes by BLAST in PATRIC [21]. Stepwise sequence analysis is available at https://github.com/aseekatz/Rush_CX.study.

**Clinical Data**

Demographic and clinical data were extracted from hospital electronic medical records. Time-dependent exposures (eg, antibiotics) were measured from LTACH admission to the time...
of fecal or rectal swab specimen collection and coded as present or absent.

Development of Predictive Models

KPC-Kp colonization predictive indices were developed using a 2-stage approach. First, permutation analysis was used to reduce the initial set of 300 candidate OTU predictors to a number that was more manageable for constructing a robust, linear predictive model. Each OTU was recoded from a continuous quantity (relative abundance) to a dummy-coded variable based on presence or absence in a patient’s specimen. The entire patient sample was randomly assigned to 2 groups, and the random assignment was permuted 100 times. For each permutation of the random assignments, risk ratios for each OTU were calculated for both groups. An OTU was considered validated if the risk ratio in the 2 groups both exceeded an absolute value of 2.0 and predicted KPC-Kp colonization status in the same direction. The use of this permutation approach is equivalent to a k-fold cross-validation.

Second, the patient sample was divided into calibration and validation subsamples based on date of LTACH admission: The first 51 patients who participated in the study were included in the calibration subsample, and the next 80 patients who participated in the study were included in the validation subsample. The larger number in the validation subsample was designed to increase the power of the validation analyses.

Logistic regression was used to develop optimal clinical and microbiome predictive models in the calibration subsample. The dummy-coded OTUs and clinical variables that were found to be independent predictors of KPC-Kp status by logistic regression were then combined into simple unit-weighted models to create more robust predictions. Finally, the unit-weighted models were cross-validated in the validation subsample of patients to determine if they were generalizable across the entire sample. Analyses were conducted using SPSS software, version 19 (IBM SPSS, Chicago, IL), and R, version 2.13.1 (http://CRAN.R-project.org).

RESULTS

Characteristics of Study Participants

One hundred thirty-one patients (32 KPC-Kp(+) case patients and 99 KPC-Kp(-) control patients) were included in the study between June 19, 2014, and January 15, 2016 (Figure 1). On average, patients were elderly, and most (56%) were receiving antibiotics at the time of fecal or rectal swab specimen collection. In bivariate analyses, case patients were more likely than control patients to have poor functional status, comorbid medical conditions, decubitus ulcers, and medical device use (Table 1). In a multivariable logistic regression model, the sole clinical predictor of KPC-Kp colonization was presence of a decubitus ulcer (odds ratio [OR], 11.2; 95% confidence interval [CI], 1.3–95; P = .026).

The Fecal Microbiota Community, but not Microbiota Diversity, Distinguished Patients With or Without KPC-Kp Colonization

The overall diversity of the fecal microbiota as measured by the Shannon diversity index (H) did not differ between specimens from KPC-Kp(+) and KPC-Kp(-) patients (Figure 2A). However, diversity was significantly decreased in patients who received antibiotics between the time of admission and fecal sample collection, independent of KPC-Kp status (Wilcoxon test, P < .01) (Figure 2B). This difference was consistent across different antibiotic classes (Supplementary Figure 1).

Use of AMOVA on θYC distances to compare communities from KPC-Kp(+) and KPC-Kp(-) patients indicated that the microbial communities in patients from these 2 groups were distinct (P = .017), although this relatively subtle difference was not obvious by PCoA (Figure 3A). Next, we tried community clustering using the PAM algorithm based on Jensen-Shannon divergence (Figure 3B) [20, 22]. Likely due to high microbiota variability between subjects (Supplementary Figure 2), clustering was relatively weak, with a maximum silhouette score of 0.28 for 3 clusters, each defined by different dominant genera (Figure 3B, C). PAM cluster 1 was a high-diversity cluster with several Bacteroidetes and Firmicutes OTUs, PAM cluster 2 was defined by mainly Bacteroidetes OTUs, and PAM cluster 3 was characterized by high abundances of OTU001 (Enterococcus), OTU002 (Escherichia), and OTU003 (Enterobacteriaceae). Examination of the binned OTU sequences revealed that OTU003 contained a single unique sequence, matching various Enterobacteriaceae family members, including 16S rRNA gene sequences from the canonical KPC-Kp strain ST258 (Supplementary Methods). Significant overrepresentation of specimens from KPC-Kp(+) patients was not observed in any of the clusters (chi-square test).

We next sought to determine if specific features of the microbiota associated with KPC-Kp status. LEfSe evaluation revealed 12 OTUs that were differentially abundant in KPC-Kp(+) vs KPC-Kp(-) patients using the entire sample (n = 131 patients). To validate this observation, we ran LEfSe estimation independently on the calibration and validation subsamples. Different OTUs were observed to be differentially abundant in the calibration and validation subsets (Figure 4). The only OTU that cross-validated across the 2 subsamples was OTU003, which was also identified as differentially abundant in all samples, suggesting that a higher relative abundance of the OTU containing KPC-Kp was associated with KPC-Kp colonization (Figure 4).

In a final effort to identify features that distinguished the fecal microbiota of KPC-Kp(+) and KPC-Kp(-) patients, we applied a permutation analysis to investigate whether specific OTUs were differentially present or absent in fecal specimens from case and control patients. In the first stage of the analysis and based on cross-validated risk ratios of 2.0, the number of candidate differential OTUs was reduced from a filtered count of 302 (present in
at least 0.01% of the total data set and in at least 5% of all samples) to 30. A risk ratio of 2.0 was selected because, assuming a sample size of 30 in both the calibration and validation subsamples and a base likelihood of 18% OTU relative abundance (the median OTU relative abundance for the 302 OTUs considered in the entire patient sample), there was a statistically significant likelihood of achieving a risk ratio of 2.0 ($P = .014$). Identification of candidate OTUs was not ambiguous: all discarded OTUs had 0 cross-validations. Of the 30 candidate OTUs that were retained, the number of cross-validations varied from 15 to 87 out of 100 permutations, and all cross-validated agreements were in the same direction. Hierarchical logistic regression analysis was used to further reduce the number of OTUs from 30 to 2: (OTU109 [*Desulfovibrio*] and OTU179 [*Ruminococcaceae*]). The elimination process was based on Wald statistics in the logistic regression analysis. If there was a tie in the Wald statistics, the bivariate risk ratio was used as a tie-breaker.

**Unit-Weighted Models to Predict KPC-Kp Colonization at the Time of LTACH Admission**

Unit-weighted predictive functions (index values) were created using the 1 clinical and 2 microbiome variables that were found to be independent, statistically significant predictors of KPC-Kp status in logistic regression models, that is, decubitus ulcer, OTU109 (*Desulfovibrio*), and OTU179 (*Ruminococcaceae*). These 3 variables were dummy coded as “0” if absent and “1” if present and used to create simple clinical, microbiome, and combined index value models (Figure 5). The clinical index model and the microbiome index model were statistically significant predictors of KPC-Kp status in both the calibration and validation subsets (Figure 5A, B, D, E). The combined model, which included both clinical and microbiome predictors, performed better than either model alone (Figure 5C, F). The increase in model performance occurred for both the calibration and validation subsets, and the predictive function was a statistically significant predictor in both subsets (Figure 5C, F)

**DISCUSSION**

The appearance of MDROs such as KPC-Kp in healthcare settings poses a significant threat. Recently, prevention efforts have begun to target long-term care facilities such as LTACHs, which can serve as foci for the amplification and spread of KPC-Kp. 

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**Figure 1.** Patient participation. Patients were screened for participation between June 10, 2014, and January 17, 2016. The calibration subsample was collected between June 19, 2014, and February 6, 2015. The validation subsample was collected between March 21, 2015, and January 15, 2016. Case patients grew carbapenem-resistant, Klebsiella pneumoniae isolates from fecal or rectal swab samples that were verified to carry the *bla*-KPC gene by polymerase chain reaction (PCR). Control patients’ specimens were negative for *bla*-KPC and *bla*-NDM genes by screening PCR and culture-negative for carbapenem-resistant Enterobacteriaceae. Abbreviations: CPE, carbapenemase-producing Enterobacteriaceae; EIA, enzyme immunoassay; KPC-Kp, Klebsiella pneumoniae carbapenemase-producing Klebsiella pneumoniae.
and other MDROs [23–29]. Improved knowledge of factors that predispose to KPC-Kp carriage in LTACH populations could lead to new, more effective prevention strategies.

A growing body of evidence suggests that the indigenous microbiota of the gastrointestinal tract plays a critical role in determining susceptibility to MDRO carriage and infection [30–32]. The prime example of this is infection with *Clostridium difficile*, which is associated with alteration of the gut microbiota following antibiotic administration. Antibiotics have been shown to alter the structure and function of the gut microbiota in a manner that decreases colonization resistance against *C. difficile*. Restoration of the gut microbiota by means of fecal microbiota transplantation further demonstrates the critical role that the gut microbiome can play in *C. difficile* infection [33, 34].

Our current study indicates that patients who are colonized with KPC-Kp upon admission to an LTACH have a distinct gut microbiome compared with noncolonized patients. The overall bacterial community structure of fecal specimens from KPC-Kp(+) patients was dissimilar to that of fecal specimens from KPC-Kp(-) patients (Figure 3A), but overall diversity did not differ significantly between the 2 groups. Decreased

![Figure 2. Diversity of the fecal microbiota in patients admitted to 1 long-term acute care hospital.](https://academic.oup.com/ofid/article-abstract/5/8/ofy190/5062196/5062196)
microbiota diversity has been observed in patients with recurrent *C. difficile* infection, which is a rationale for treating this infection with microbiota transplantation [35, 36]. In the current study, the absence of association between KPC-Kp status and microbiota diversity may be explained in part by antibiotic exposure in >50% of both cases and controls at the time of specimen collection, which in and of itself is associated with decreased microbiota diversity [37–39]. Although we did not have access to patients’ antibiotic exposure histories before LTACH admission, it is likely that many patients would have received multiple prior antibiotic courses [23], which would also have been expected to reduce the diversity of their microbiota.

We utilized 2 approaches to seek specific members of patients’ gut microbiota that associated with KPC-Kp colonization. Analysis by LEfSe was used to detect significant differential abundance of microbial community members between different groups, followed by linear discriminant analysis to evaluate the effect size of those features. We refined our use of LEfSe by performing this analysis on samples from calibration and validation subsets of patients. Whereas multiple OTUs were identified as differentially abundant in KPC-Kp(+) patients compared with KPC-Kp(-) patients, only 1 OTU (OTU003) was identified as significant in both calibration and validation subsamples. This OTU003 is predicted to be the OTU that contains the common KPC-Kp strain ST258. Although this may seem to be a trivial result, it is important to recall that LEfSe analysis takes into account relative abundance of a given OTU when determining significance. In fact, our alternate approach for examining OTUs that was based on presence or absence of all OTUs did not identify OTU003 as being significantly associated with KPC-Kp colonization. Closer examination of these data suggests that virtually all patient specimens included sequences classified as OTU003. Therefore, it appears that KPC-Kp-colonized patients are distinguished from noncolonized patients by having KPC-Kp as a relatively abundant member of the community.

![Figure 3](https://academic.oup.com/ofid/article-abstract/5/8/ofy190/5062196/1533258)

**Figure 3.** Fecal microbiota structure and composition in long-term acute care hospital patients. A, Principal coordinates analysis (PCoA) based on the Yue & Clayton dissimilarity index suggested a difference in the microbiota structure of the fecal microbiota in *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella pneumoniae* (KPC-Kp) (+) and KPC-Kp(-) patients (analysis of molecular variance, \*P < .05). B, Biplot visualizing the operational taxonomic units (OTUs) driving the microbiota community structure in all patients, as represented by 3 community clusters calculated using partitioning around medoids (PAM) clustering (based on Jensen-Shannon divergence). C, Mean relative abundance of the 40 most abundant OTUs in each PAM cluster (n = number of samples; d = Shannon diversity).
In a second, independent analytic approach that sub-
set the study sample using permutation, the association 
of OTU109 (*Desulfovibrio*) and OTU179 (Ruminococcaceae) with 
KPC-Kp colonization was consistent across calibration and 
validation subsets (Figure 5.) Assessing the generalizability 
of microbiota analyses across different subsets of specimens 
can limit spurious associations identified with these complex, 
heterogeneous data sets where collinearity is likely to be a prob-
lem. Although we did not investigate the biological significance 
of the association between OTU109 (*Desulfovibrio*) or OTU179 
(Ruminococcaceae) and KPC-Kp colonization, the consistency 
of the association across all patient subsets suggests that these 
OTUs might be candidates for investigation in longitudinal 
alyses as microbiome indices of KPC-Kp acquisition risk. The
association between a clinical factor—presence of a decubitus ulcer—and KPC-Kp colonization is likely an indicator of poor functional status associated with KPC-Kp carriage. As in our previous work with *C. difficile* [22], models that included both clinical and microbiome variables improved the prediction of KPC-Kp status compared with either model alone.

The strengths of our study include its size and use of calibration and validation subsets to investigate the generalizability of associations between the microbiome and clinical features and KPC-Kp status. Our study also has limitations. We used a cross-sectional design, which prevents us from knowing whether microbiome features that were observed to be associated with KPC-Kp positivity were present before colonization. Our ability to detect KPC-Kp is not limiting, and there is little clinical utility to using microbiota analysis for detection of KPC-Kp. However, although 16S rRNA gene sequence analysis of full bacterial communities may not be a feasible clinical diagnostic tool, such analyses are the first step toward identifying potential biomarkers for future validation and development. Additionally, studying the fecal microbiota of KPC-Kp(-) patients longitudinally over time would provide an opportunity to investigate features of the microbiota associated with acquisition of KPC-Kp colonization. Such a longitudinal design could also be used to interrogate characteristics of the microbiota of KPC-Kp(+) patients that are associated with development of clinical infection. The potential value of this approach has been suggested by work that showed that the relative abundance of vancomycin-resistant enterococci or Enterobacteriaceae was associated with an increased risk of bacteremia [40].

In summary, we observed statistically significant and consistent differences in clinical characteristics and microbiome features of fecal specimens from KPC-Kp-colonized and -non-colonized LTACH patients. These results provide support for continuing investigation of the gut microbiota as a potential diagnostic tool to identify patients at risk of KPC-Kp acquisition, providing possible targets for intervention to prevent or eliminate KPC-Kp colonization and reduce the risk of invasive infection.

**Supplementary Data**

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader,
the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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