Effect of Antibiotic Treatment on Establishment and Elimination of Intestinal Colonization by KPC-Producing *Klebsiella pneumoniae* in Mice

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An understanding of the impact of antibiotics on the intestinal reservoir of KPC carbapenemase-producing *Klebsiella pneumoniae* (KPC-Kp) is important to prevent its emergence. We used a mouse model to examine the effect of antibiotic treatment on the establishment and elimination of intestinal colonization with KPC-Kp. Mice (10 per group) received subcutaneous antibiotics daily for 5 days. On day 3 of treatment, 10^9 CFU of KPC-Kp was given orogastrically, and concentrations of KPC-Kp in stool were monitored. Additional experiments assessed the effects of antibiotic treatment on concentrations of total anaerobes and *Bacteroides* spp. in stool and the efficacy of orogastric gentamicin and polymyxin E in suppressing KPC-Kp colonization. Of four antibiotics with minimal activity against the KPC-Kp test strain (MIC ≥ 16 μg/ml), those that suppressed total anaerobes and bacteroides (i.e., clindamycin and piperacillin-tazobactam) promoted colonization by KPC-Kp (P < 0.001), whereas agents that did not suppress total anaerobes or bacteroides (i.e., ciprofloxacin and ceftimox) did not (P = 0.835). Of two agents with moderate activity against the KPC-Kp test strain, ertapenem (MIC, 4 μg/ml) did not promote colonization by KPC-Kp, whereas tigecycline (MIC, 3 μg/ml) did (P < 0.001), despite not reducing levels of total anaerobes or bacteroides. Orogastic treatment with gentamicin and polymyxin E suppressed KPC-Kp to undetectable levels in the majority of mice. These data suggest that antibiotics that disturb the intestinal anaerobic microflora and lack significant activity against KPC-Kp promote colonization by this organism. The administration of nonabsorbed oral antibiotics may be an effective strategy to suppress colonization with KPC-Kp.

The emergence of *Klebsiella pneumoniae* strains with decreased susceptibility to carbapenems is a significant threat to hospitalized patients worldwide (19). Resistance to carbapenems in these organisms is most frequently mediated by *K. pneumoniae* carbapenemase (KPC), a class A β-lactamase that also confers resistance to broad-spectrum cephalosporins and commercially available β-lactam/β-lactamase inhibitor combinations (24). KPC-producing *K. pneumoniae* (KPC-Kp) strains often harbor resistance determinants against several other classes of antimicrobials, including aminoglycosides and fluoroquinolones, resulting in truly multidrug-resistant (MDR) organisms (12). Given the paucity of antimicrobial options available, infections caused by KPC-Kp pose a tremendous therapeutic challenge and are associated with poor clinical outcomes (20). Therefore, the prevention and control of KPC-Kp have become a priority (26).

A key factor in the epidemiology of many nosocomial pathogens is their ability to colonize the intestinal tract of humans. Antibiotics that select for resistant strains and eliminate competing flora, therefore nullifying “colonization resistance,” facilitate intestinal colonization (6). For example, for patients colonized with vancomycin-resistant enterococci (VRE), treatment with antianaerobic antibiotics that lack inhibitory activity against VRE results in the promotion of high-density colonization (7). By the same token, the existence of an intestinal reservoir of nosocomial pathogens also poses an opportunity for intervention, if established intestinal colonization can be eliminated.

Efforts to limit the transmission of KPC-Kp strains are focusing on basic and enhanced infection control measures (2). The role of the intestinal reservoir of KPC-Kp and its modulation by antibiotics remain largely unexplored. In this study we used a previously validated mouse model to examine the effect of antibiotics on the establishment and elimination of intestinal colonization with KPC-Kp (13).

(This work was previously presented at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 12 to 14 September 2009 [20a]).

MATERIALS AND METHODS

*Mice.* The experimental protocol was approved by the Cleveland Veterans Affairs Medical Center’s institutional animal care committee. Female CF1 mice (Harlan Sprague-Dawley, Indianapolis, IN) were used in all experiments. The mice were individually housed and weighed between 25 and 30 g.

*Strain.* We studied a thoroughly characterized strain of *K. pneumoniae, VA-367* (8, 9, 25). This clinical isolate is genetically related to the KPC-Kp strain circulating in the Eastern United States. Characterization of the resistance mechanisms in *K. pneumoniae* VA-367 with PCR and DNA sequence analysis revealed the presence of *bla*KPC-3, *bla*TEM-1, *bla*SHV-11, and *bla*SHV-12 as well as *qnrB19* and *aac(6’)/Ib*. Additionally, PCR and DNA sequencing revealed disruptions in the coding sequences of the following outer membrane protein genes: ompK35, ompK36, and ompK37. Table 1 summarizes the results of antibiotic susceptibility testing (AST) performed with the agar dilution method and interpreted according to current recommendations from the Clinical and Laboratory Standards Institute (CLSI) (4a). A modified Hodge test was performed, according to a

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TABLE 1. Antibiotic susceptibility and resistance profile of Klebsiella pneumoniae strain VA-367

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (μg/ml)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin-tazobactam</td>
<td>512</td>
<td>R</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>3</td>
<td>I</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>4</td>
<td>I (R)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≥32</td>
<td>R</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>16</td>
<td>R</td>
</tr>
<tr>
<td>Polymyxin E&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5</td>
<td>S</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2</td>
<td>S (R)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Meropenem</td>
<td>1</td>
<td>S (R)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> AST was done with an Etest (for all others, the agar dilution method was used).
<sup>b</sup> AST results interpreted according to FDA criteria (for all others, 2010 CLSI criteria were used [4a]).
<sup>c</sup> R, resistant; I, intermediate; S, susceptible.
<sup>d</sup> A modified Hodge test suggested the presence of carbapenemase.

The method described previously by Anderson et al. (1), with ertapenem, meropenem, and imipenem. Tigecycline and polymyxin E were evaluated by Etest susceptibility assays (AB bioMérieux, Solna, Sweden). Results for tigecycline were interpreted as suggested by the U.S. Food and Drug Administration (FDA) and according to CLSI recommendations (criteria for Pseudomonas) for polymyxin E.

Effect of subcutaneous antibiotics on establishment of colonization. An initial set of experiments was carried out to determine the effect of subcutaneous antibiotics on the establishment of colonization with KPC-Kp VA-367. Mice (10 per group) were assigned to the following treatment groups: clindamycin, piperacillin-tazobactam, tigecycline, ertapenem, ceftazidime, and ciprofloxacin, and normal saline (controls). All antibiotics were administered subcutaneously once each day for 8 days at doses based on the daily dose recommended for human adults (in mg per kg of body weight), as follows: clindamycin at 1.4 mg/day, piperacillin-tazobactam at 8 mg/day, tigecycline at 0.05 mg/day, ertapenem at 0.5 mg/day, cefepime at 2 mg/day, and ciprofloxacin at 0.25 mg/day. On the third day of the administration of the antibiotic, 10<sup>5</sup> CFU of KPC-Kp VA-367 diluted in 0.5 ml phosphate-buffered saline (PBS) was administered by orogastric gavage using a stainless-steel feeding tube (Perfektum; Popper & Sons, New Hyde Park, NY). Stool samples were collected 1, 4, 6, and 11 days after the administration of KPC-Kp in order to measure the concentration of carbapenem-resistant K. pneumoniae. Stool samples (∼100 mg diluted in 800 ml of PBS) were plated onto MacConkey agar with and without 0.5 μg/ml of imipenem, and the number of CFU per gram of stool was determined.

Effect of subcutaneous antibiotic treatment on indigenous intestinal microflora. Mice (n = 8 per group) received daily subcutaneous antibiotic treatment as described above. Stool samples were collected 5 days after the administration to assess the effects of the antibiotics on the stool microflora and to measure antibiotic levels in stool. To assess the effects on the microflora, fresh stool samples were diluted in prebuffered PBS and plated onto Enterococcus fluorescens agar, MacConkey agar, Brucella agar, and Bacteroides bile-soluble agar (Becton Dickinson) to measure concentrations of enterococci, total and facultative Gram-negative bacilli, total anaerobes, and Bacteroides spp., respectively. Cultures of total anaerobes and Bacteroides spp. were performed inside an anaerobic chamber (Coy Laboratories). The lower limits of detection were ∼4 log<sub>10</sub> CFU/g of stool for total anaerobes and ∼2 log<sub>10</sub> CFU/g of stool for bacteroides.

Bioassay for concentrations of antibiotics in stool. The concentrations of antibiotics in stool were determined by an agar diffusion assay with Escherichia coli (for piperacillin-tazobactam, tigecycline, ertapenem, ceftazidime, and ciprofloxacin) or Clostridium perfringens (for clindamycin) as the indicator strain (18).

The limit of detection was 1 μg/ml of feces.


Orogastric antibiotics and elimination of KPC-Kp intestinal colonization. Additional experiments were performed to examine whether the orogastric administration of antibiotics resulted in the elimination or persistence of colonization with KPC-Kp VA-367. Mice were treated with subcutaneous clindamycin to reduce the normal intestinal flora 1 day before receiving 10<sup>5</sup> CFU of KPC-Kp VA-367 by orogastric gavage, and the mice continued to receive subcutaneous clindamycin every other day for 7 days. Concurrently, for 7 days after orogastric gavage with KPC-Kp, mice received orogastric normal saline (control group), gentamicin at 6 times (0.9 mg/day) and 12 times (1.8 mg/day) the human-equivalent dose (in milligrams per kilogram of body weight), and polymyxin E at 6 times and 12 times the human-equivalent dose (1.02 mg/day and 2.04 mg/day, respectively). An additional dose of subcutaneous clindamycin was administered 20 days after the administration of KPC-Kp VA-367 to assess whether low levels of carbapenem-resistant K. pneumoniae were present that could be augmented by the elimination of the anaerobic microflora. Stool samples were collected at baseline and at 3, 6, 8, 11, 16, and 21 days after KPC-Kp VA-367 was given by gavage.

Statistical analysis. Data analyses were performed with the use of Stata software (version 6.0; Stata, College Station, TX). A one-way analysis of variance (ANOVA) was performed to compare the groups, with P values adjusted for multiple comparisons using the Scheffe correction. For the assessment of the effect of antibiotics on the elimination of intestinal microflora, a Student's t-test was used to compare mean densities during treatment with baseline densities.

RESULTS

Effect of subcutaneous antibiotics on establishment of colonization. Data for the effects of different antibiotic treatments on the establishment of intestinal colonization with KPC-Kp VA-367 are shown in Fig. 1. Clindamycin, piperacillin-tazobactam, and tigecycline promoted the overgrowth of carbapenem-resistant K. pneumoniae (P < 0.001 in comparison to saline controls). In contrast, ertapenem, ceftazidime, and ciprofloxacin

FIG. 1. Effect of subcutaneous antibiotic administration on the establishment of intestinal colonization with KPC-Kp in mice. Mice received 10<sup>5</sup> CFU of KPC-Kp strain VA-367 by orogastric gavage on day 0. Subcutaneous doses of antibiotics equal to human doses on a milligram-per-kilogram basis were administered from 2 days prior to 5 days after the administration of bacteria (n = 10 mice per antibiotic treatment group). Densities of carbapenem-resistant K. pneumoniae are shown for days 0, 1, 4, 6, and 11. If carbapenem-resistant organisms were not detected in the stool, the lower limit of detection (−2 log<sub>10</sub> CFU/g) was assigned. Bars represent standard errors.
did not promote overgrowth \((P = 0.35\) in comparison to saline controls). After the discontinuation of the antibiotics that promoted colonization (day 5), the concentrations of KPC-Kp VA-367 in stool decreased.

**Effect of subcutaneous antibiotic treatment on indigenous intestinal microflora.** Data for the effects of antibiotic treatment on the stool microflora are shown in Fig. 2. In comparison to saline controls, clindamycin, piperacillin-tazobactam, and ertapenem resulted in significant reductions in concentrations of total anaerobes and *Bacteroides* spp. \((P < 0.001)\), whereas tigecycline, cefepime, and ciprofloxacin did not \((P = 0.09)\). With the exception of clindamycin and cefepime, each of the antibiotics resulted in significant reductions in numbers of facultative Gram-negative bacilli \((P < 0.001)\). Piperacillin-tazobactam significantly reduced concentrations of enterococci in stool \((P < 0.001)\), whereas the other agents did not \((P = 0.23)\).

**Concentrations of antibiotics in stool.** The mean concentrations of the antibiotics in stool were 48.0 \(\mu g/g\) (range, 12 to 250 \(\mu g/g\)) for clindamycin, 46.5 \(\mu g/g\) (range, 0 to 125 \(\mu g/g\)) for piperacillin-tazobactam, 3.6 \(\mu g/g\) (range, 0 to 12 \(\mu g/g\)) for tigecycline, 12.5 \(\mu g/g\) (range, 0 to 64 \(\mu g/g\)) for ertapenem, 0 \(\mu g/g\) (not detected in any samples) for cefepime, and 126.5 \(\mu g/g\) (range, 64 to 612 \(\mu g/g\)) for ciprofloxacin.

**Orogastric antibiotics and elimination of KPC-Kp intestinal colonization.** The orogastric administration of polymyxin E and gentamicin resulted in the suppression of colonization with carbapenem-resistant *K. pneumoniae* (Fig. 3). All the regi-

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**FIG. 2.** Effect of antibiotic treatment on concentrations of total anaerobes and *Bacteroides* spp. Mice \((n = 8\) per group) received daily subcutaneous antibiotic treatment for 5 days. Stool samples were collected on day 5, diluted in prereduced phosphate-buffered saline, and plated onto selective media to measure concentrations of total anaerobes, *Bacteroides* spp., facultative Gram-negative bacilli, and enterococci. Cultures of total anaerobes and *Bacteroides* spp. were performed inside an anaerobic chamber. The lower limits of detection were \(-4\) log\(_{10}\) CFU/g of stool for total anaerobes and \(-2\) log\(_{10}\) CFU/g of stool for bacteroides. Error bars represent standard errors.

**FIG. 3.** Effect of antibiotics on elimination of intestinal colonization with KPC-Kp in mice. Mice received \(10^7\) CFU of KPC-Kp strain VA-367 by orogastric gavage on day 0. From days 1 through 7, control mice \((n = 4)\) received orogastric normal saline, and treatment groups received orogastric polymyxin E at \(6 \times (n = 6)\) or \(12 \times (n = 6)\) the human-equivalent dose (mg/kg) and gentamicin at \(6 \times (n = 5)\) or \(12 \times (n = 4)\) the human-equivalent dose. Subcutaneous clindamycin was administered to all groups on days \(-1, 3, 5, 7, \) and 20 of intestinal colonization with VA-367. The densities of carbapenem-resistant *K. pneumoniae* in stool samples from days 0, 3, 6, 8, 11, 16, and 21 are presented. If the pathogens were not detected in stool, the lower limit of detection \((-2.5\) log\(_{10}\) CFU/g) was assigned (shown as a line on the graph). Arrows indicate the subcutaneous injection of clindamycin.
mens, except for polymyxin E at 6 times the human dose, resulted in up to 5-log declines in the density of carbapenem-resistant K. pneumoniae in stool. After stopping treatment (day 7), rebound overgrowth was not seen for any of the treatment groups (including the controls). Rechallenge with subcutaneously clindamycin 20 days after the initial administration of bacteria resulted in relapse (approximately a 2-log increase) of colonization in all of the mice in the lower-dose polymyxin E group and in a subset of the mice in the control group (2/4 mice), the low-dose gentamicin group (2/5), and the high-dose gentamicin group (1/4). In contrast, mice treated with high-dose polymyxin E did not have carbapenem-resistant K. pneumoniae detectable in their stool samples on day 21 after rechallenge with clindamycin.

**DISCUSSION**

Our findings suggest that antibiotics that inhibit the anaerobic intestinal microflora and have limited activity against KPC-Kp may promote the establishment of intestinal colonization with that organism. This is epitomized by clindamycin, which resulted in the highest levels of intestinal colonization with KPC-Kp. Clindamycin achieves high levels in stool, causes a marked suppression of the anaerobic microflora of the colon, and has no direct bactericidal activity against the Enterobacteriaceae (15). Piperacillin-tazobactam also promoted colonization, consistent with the high concentrations achieved in stool, the inhibition of the anaerobic microflora, and its limited activity against the KPC-Kp test strain (MIC, 512 μg/ml). In contrast, we previously demonstrated that piperacillin-tazobactam inhibits the establishment of colonization by extended-spectrum-β-lactamase (ESBL)-producing K. pneumoniae strains that exhibit much lower MICs against this antibiotic (22). In human volunteers, piperacillin-tazobactam achieved levels in stool (range, 1.2 to 276 μg/g) that were similar to the levels in mice and inhibited the anaerobic intestinal microflora (17).

Tigecycline and ertapenem both have moderate *in vitro* activity against the KPC-Kp test strain (MICs, 3 and 4 μg/ml, respectively), and both may cause a significant disruption of the anaerobic microflora of the colon of healthy humans (18, 21). Notably, tigecycline did not inhibit total anaerobes or bacteroides in the current study; tigecycline also did not reduce numbers of total anaerobes or bacteroides in human volunteers but caused a marked suppression of bifidobacteria and lactobacilli (18). In the current study, tigecycline promoted a significant overgrowth of KPC-Kp, but ertapenem did not. This discrepancy may be related to the fact that ertapenem achieves higher levels in the intestinal tract than tigecycline (mean concentrations in the current study, 12.5 versus 3.6 μg/g, respectively; mean concentrations in stool samples of human volunteers on day 8 of treatment, 32.7 versus 5.6 μg/g, respectively) (18, 21), thereby providing sufficient levels of drug to inhibit the establishment of colonization. Of note, healthy human subjects treated with tigecycline developed a significant overgrowth of tigecycline-resistant Enterobacteriaceae (18).

In this model, two antibiotics with limited impacts on the anaerobic intestinal microflora (cefepime and ciprofloxacin) did not cause an overgrowth of KPC-Kp, even though K. pneumoniae VA-367 was highly resistant to these agents. These results are in concordance with data from previous studies using animal models which showed that antibiotics that do not disrupt the anaerobic microflora may be less likely to promote colonization with MDR Gram-negative bacteria (6, 23). In humans, stool concentrations of cefepime have been estimated to be extremely low, while concentrations of ciprofloxacin vary widely (from undetectable to 858 μg/g) (4, 28). These concentrations are similar to the levels that we detected in the stool of mice.

Our observations also suggest that the orogastric administration of antibiotics may play a role in the elimination of colonization with KPC-Kp. We found reductions of the density of carbapenem-resistant K. pneumoniae in the stool of mice treated with gentamicin and with polymyxin E. These results are especially promising because rebound was not seen after treatment with these antibiotics was stopped. Furthermore, recolonization occurred only for a minority of antibiotic-treated mice (3/13 mice), even after rechallenging with antianaerobic antibiotics, and did not occur at all when mice were treated with high-dose polymyxin E.

The conclusions derived from this model are limited by the intrinsic physiological differences between mice and humans that may result in disparate microbiological and pharmacological responses. The antibiotic dosages and dosing frequency that were used do not mimic systemic exposures in patients. However, we previously found that the administration of equivalent doses (mg/kg) to mice once or twice daily results in levels of drug in stool and effects on the intestinal microflora that are similar to those seen with healthy human volunteers (6, 22, 23). In the current study, the concentrations of antibiotics in stool samples of mice and the degree of alteration of the microflora are consistent with data from previous studies of human volunteers or patients (4, 17, 21). Furthermore, this model does not incorporate clinical factors that modify the risk of the acquisition and persistence of colonization, such as a simultaneous exposure to multiple classes of antibiotics, integrity of the skin and bowel, immune response, severity of illness, environmental contamination, and functional status. These same biases have to be considered in the interpretation of data from clinical studies assessing the effect of antibiotics on the establishment and elimination of colonization with carbapenem-resistant bacteria.

Among several case-control studies that examined the effect of antibiotics on the acquisition of carbapenem-resistant K. pneumoniae colonization in human subjects, only one focused on antianaerobic antibiotics. Such therapy, unlike therapy with carbapenems and fluoroquinolones, was not a risk factor for colonization with carbapenem-resistant K. pneumoniae (10, 11, 14, 16, 27). In contrast, a previously reported prospective study indicated that antianaerobic antibiotic therapy is associated with high-density stool colonization with strains of *Pseudomonas aeruginosa* and *Enterobacteriaceae* that are resistant to extended-spectrum cephalosporins and fluoroquinolones (3). Similar clinical studies are needed to demonstrate whether antianaerobic regimens also contribute to colonization with carbapenem-resistant organisms.

The elimination of colonization with MDR organisms in human subjects with selective oropharyngeal and/or digestive tract decontamination is both a highly studied and controversial topic. The combination of tobramycin and polymyxin E, in decontamination regimens that also include amphotericin B
and systemic ceftazidime, results in marked decreases in the incidence of colonization with Enterobacteriaceae (5). Such approaches have not been applied specifically against carbapenem-resistant organisms. The potential emergence of polymyxin-resistant bacteria as a result of decolonization is a critical consideration given that this class of drugs represents the treatment of last resort for carbapenem-resistant organisms.

In summary, our findings suggest that antibiotics that disrupt the intestinal anaerobic microflora but lack activity against KPC-Kp may promote intestinal colonization. Furthermore, the selective decontamination of the digestive tract with polymyxin E and/or aminoglycosides appears to be a promising approach to eliminate KPC-Kp from the gastrointestinal tract. The translation of these insights into the clinical arena depends on carefully designed studies with human subjects. This may lead to the development and refinement of antibiotic stewardship efforts and therapies to prevent and control carbapenem-resistant K. pneumoniae.

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