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Efficacy of Ceftriaxone, Cefepime, Doxycycline, Ciprofloxacin, and Combination Therapy for *Vibrio vulnificus* Foodborne Septicemia

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ABSTRACT Foodborne *Vibrio vulnificus* infections are associated with higher rates of sepsis and mortality than wound infections; however, antibiotic efficacy studies have not been performed in foodborne infection models. The efficacies of ceftriaxone, cefepime, doxycycline, ciprofloxacin, and combination therapy were assessed in *V. vulnificus* intestinal infection in mice in order to model foodborne infections. In accordance with prior studies of cefotaxime, cefepime was synergistic with doxycycline and ciprofloxacin *in vitro*; combination therapy significantly decreased bacterial growth, by $\geq 2 \log_{10}$ units, from that with antibiotic monotherapy ($P < 0.01$). *In vivo*, survival rates in the ceftriaxone (50%), doxycycline (79%), and ciprofloxacin (80%) groups were significantly higher than those in the control group (0%) ($P < 0.0001$). Survival was significantly higher with ceftriaxone-doxycycline (91%) or ceftriaxone-ciprofloxacin (100%) therapy than with ceftriaxone (50%) ($P \leq 0.05$). Survival with cefepime-doxycycline (96%) or cefepime-ciprofloxacin (90%) therapy was significantly higher than that with cefepime alone (20%) ($P < 0.001$). There was no difference in survival between the combination therapy groups. Thus, we conclude that combination therapy was the most effective treatment for foodborne *V. vulnificus* septicemia. In a septic patient with a recent ingestion of raw seafood, cefepime in combination with doxycycline or ciprofloxacin should be initiated for coverage of resistant Gram-negative organisms and *V. vulnificus* pending a microbiological diagnosis. Once a diagnosis of foodborne *V. vulnificus* septicemia is established, treatment can safely transition to ceftriaxone in combination with doxycycline or ciprofloxacin.

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Vibrio vulnificus is a Gram-negative pathogen found in marine and estuarine environments worldwide (1). The two most common clinical syndromes caused by *V. vulnificus* are foodborne disease and wound infections; foodborne disease results from the ingestion of raw seafood, particularly oysters, and wound infections occur after skin lesions are exposed to contaminated seawater (2, 3). According to U.S. surveillance data, patients with foodborne illness have higher rates of septicemia (87% versus 55%) and death (61% versus 17%) than those with wound infections (2, 3). Since *V. vulnificus* disseminates from the gastrointestinal tract to the bloodstream, patients with foodborne infections can progress rapidly to septicemia, disseminated intravascular coagulation, and multiorgan failure within days of consuming seafood (4, 5). Of concern, recent epidemiological studies have reported an increase in the incidence of *V. vulnificus* infections worldwide. According to FoodNet, an active surveillance network of

foodborne diseases in the United States, the annual incidence of *V. vulnificus* per 100,000 population increased from 0.01 to 0.05 between 1996 and 2010 (6). Furthermore, warming ocean patterns have coincided with the emergence of *V. vulnificus* infections along the northern U.S. Atlantic coast and Baltic Sea (7, 8).

Given the severity of foodborne *V. vulnificus* infections, effective empirical treatments and timely diagnosis are essential for halting the progression of disease and improving clinical outcomes (9). Based on *in vitro* and *in vivo* studies, the Centers for Disease Control and Prevention (CDC) recommend a fluoroquinolone or a third-generation cephalosporin in combination with a tetracycline for the treatment of *V. vulnificus* infections (9–12). A time-kill study found that cefotaxime and minocycline were synergistic *in vitro*, since subinhibitory concentrations of cefotaxime with minocycline reduced *V. vulnificus* growth by 6 orders of magnitude from that with either antibiotic alone (10). These results translated *in vivo*: cefotaxime-and-minocycline combination therapy resulted in survival rates significantly higher than those with minocycline monotherapy in a wound infection model in mice (11). Synergy was attributed to the fact that the antibiotics target different processes essential for *V. vulnificus* growth: cefotaxime targets cell wall growth, while minocycline inhibits protein synthesis (11). Fluoroquinolones were found to be equivalent in efficacy to combination therapy with cefotaxime and minocycline in wound infection models (12). The activity of fluoroquinolones against *V. vulnificus* may be related to their ability to decrease the production of the *V. vulnificus* multifunctional autoprocessing repeats-in-toxin (MARTX$_{Vv}$) toxin, the primary virulence factor associated with mortality in mice (13). Because all prior antibiotic efficacy studies were performed in wound infection models, there is a significant gap in our knowledge about the optimal antimicrobial therapy for foodborne *V. vulnificus* infections. Since foodborne infections are associated with worse clinical outcomes than wound infections, studies are needed to assess the efficacy of antibiotics in an intestinal *V. vulnificus* infection model.

While CDC treatment guidelines are applicable once a *V. vulnificus* diagnosis is confirmed, fluoroquinolones, third-generation cephalosporins, and tetracyclines are rarely administered in the initial management of sepsis pending a microbiological diagnosis. For initial treatment, the Surviving Sepsis Campaign recommends the initiation of extended-spectrum β-lactam antibiotics, including cefepime and piperacillin-tazobactam, for coverage of resistant Gram-negative organisms (14). Although sepsis is the most common initial presentation of patients with foodborne *V. vulnificus* infections, there are no studies on the efficacy of extended-spectrum β-lactam antibiotics for *V. vulnificus* foodborne septicemia. While one is awaiting a microbiological diagnosis for a septic patient with a recent history of raw seafood consumption, it is essential for the initial empirical antibiotic regimen to provide effective treatment for a possible foodborne *V. vulnificus* infection, since the illness can progress to death within days.

In this study, a mouse model of intestinal infection now routinely used in bacterial pathogenesis studies (15–18) was employed to assess antibiotic efficacy for the treatment of foodborne *V. vulnificus* infection. The antibiotics recommended by the CDC based on prior wound infection studies were tested, including ceftriaxone, doxycycline, ciprofloxacin, and combination therapy. We also assessed the efficacy of an extended-spectrum β-lactam antibiotic routinely administered in the empirical treatment of sepsis: cefepime. Cefepime was tested because prior antibiotic susceptibility studies of clinical and environmental *V. vulnificus* isolates have found lower levels of resistance to cefepime (3%) than to penicillins (10% to 100%) (19–22). In addition, it was hypothesized that cefepime, a fourth-generation cephalosporin, would perform as effectively as other cephalosporins (the first-generation cephalosporin cefazolin and the third-generation cephalosporin cefotaxime) in combination with tetracyclines and fluoroquinolones *in vitro* and *in vivo* against *V. vulnificus* (10, 11, 13, 23, 24). In an analysis of U.S. surveillance data, we found that 38% of patients with *V. vulnificus* bacteremia received an extended-spectrum β-lactam antibiotic, highlighting the need to assess the efficacy of these antimicrobials against foodborne *V. vulnificus* infections. In agreement with prior studies on cefotaxime, cefepime was synergistic with doxycycline and
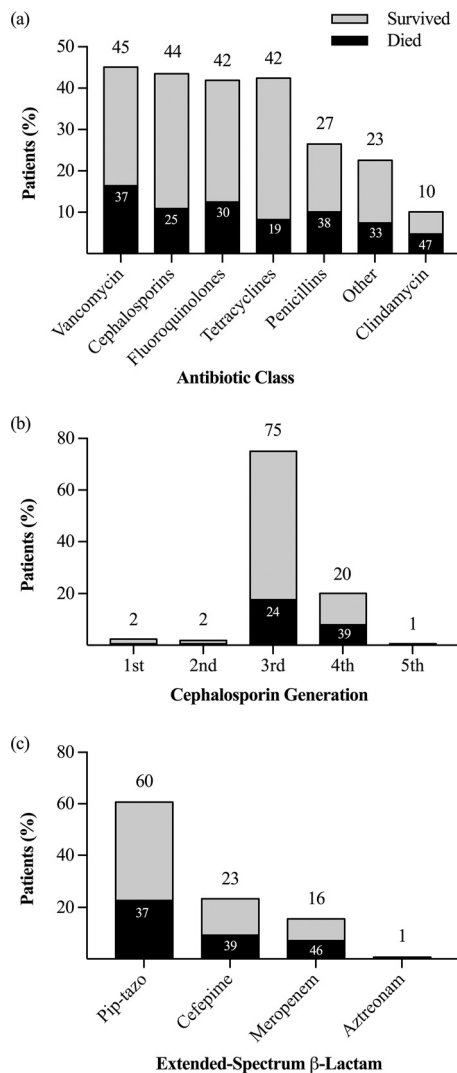
FIG 1 Mortality rates by antibiotic class (a), cephalosporin generation (b), and extended-spectrum β-lactam (c). (a) Frequency of patients with *V. vulnificus* bacteremia who received antibiotics of each class (*n* = 377). (b) Frequency of each generation of cephalosporins among patients on cephalosporins (*n* = 164). (c) Frequency of each antibiotic among patients on an extended-spectrum β-lactam antibiotic (*n* = 142). Pip-tazo, piperacillin-tazobactam.

ciprofloxacin *in vitro* against *V. vulnificus*. The ceftriaxone-doxycycline, ceftriaxone-ciprofloxacin, cefepime-doxycycline, and cefepime-ciprofloxacin combination therapies were the most effective antibiotic regimens for intestinal *V. vulnificus* infections in mice.

RESULTS

Thirty-eight percent of patients with *V. vulnificus* bacteremia in the United States received an extended-spectrum β-lactam antibiotic. Between 2007 and 2013, 500 cases of *V. vulnificus* bacteremia were reported to the Cholera and Other Vibrios Illness Surveillance (COVIS), and information on antimicrobial therapy was available for 377 (75%) of these. The most commonly prescribed antibiotics were vancomycin (45%), cephalosporins (44%), fluoroquinolones (42%), tetracyclines (42%), and penicillins (27%) (Fig. 1a). For the 164 (44%) patients treated with a cephalosporin, those most frequently administered were third-generation cephalosporins (ceftriaxone, ceftazidime, cefotaxime) (75%) and cefepime (20%) (Fig. 1b). A total of 142 (38%) patients were treated with an extended-spectrum β-lactam with coverage of resistant Gram-negative organisms, including piperacillin-tazobactam (60%), cefepime (23%),
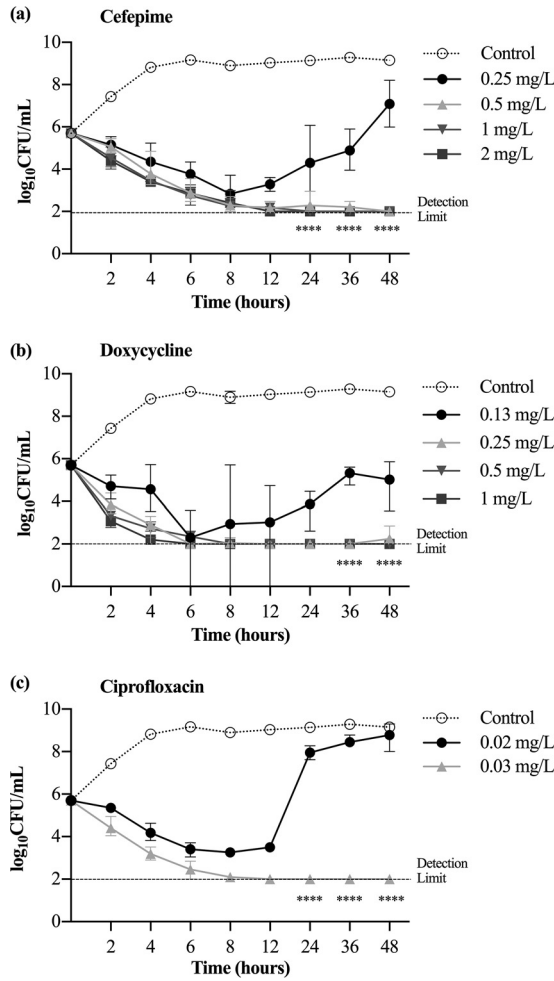
FIG 2 Inhibition of growth curves at various concentrations of cefepime (a), doxycycline (b), and ciprofloxacin (c). The concentration of antibiotic with minimal bacterial inhibition was 0.25 mg/liter for cefepime, 0.13 mg/liter for doxycycline, and 0.02 mg/liter for ciprofloxacin. Bacterial growth is represented as means $\pm$ standard deviations. The antibiotic concentration with minimal growth inhibition had a level of bacterial growth significantly higher, by ≥ 2 $\log_{10}$ units, than the detection limit (****, $P <$ 0.0001).

and meropenem (16%) (Fig. 1c). Approximately one-third of patients with *V. vulnificus* bacteremia (118 patients) died despite receiving antibiotic therapy. The antibiotics with the highest associated mortality rates included clindamycin (47%), meropenem (46%), cefepime (39%), piperacillin-tazobactam (37%), and vancomycin (37%).

Cefepime was synergistic with doxycycline and ciprofloxacin *in vitro* against *V. vulnificus*. For *V. vulnificus* M06-24/O, the ceftriaxone MIC was 0.06 mg/liter; the cefepime MIC, 0.25 mg/liter; the doxycycline MIC, 0.13 mg/liter; and the ciprofloxacin MIC, 0.03 mg/liter. The antibiotic concentration with minimal bacterial inhibition was 0.06 mg/liter for ceftriaxone, 0.25 mg/liter for cefepime, 0.13 mg/liter for doxycycline, and 0.02 mg/liter for ciprofloxacin; bacterial growth at these antibiotic concentrations was >2 $\log_{10}$ CFU/ml higher than the detection limit at 36 and 48 h ($P < 0.0001$) (Fig. 2). *V. vulnificus* developed antibiotic resistance after suspension in 0.06 mg/liter ceftriaxone and 0.25 mg/liter cefepime (the concentration with minimal bacterial inhibition) for 48 h, as evidenced by the fact that bacterial growth was 2 $\log_{10}$ CFU/ml greater than that of the control on the respective antibiotic agar at the MIC and three times the MIC ($P < 0.001$) (Fig. 3).

The combination of cefepime at 0.25 mg/liter and doxycycline at 0.13 mg/liter significantly decreased bacterial growth by >2 $\log_{10}$ CFU/ml from that with the
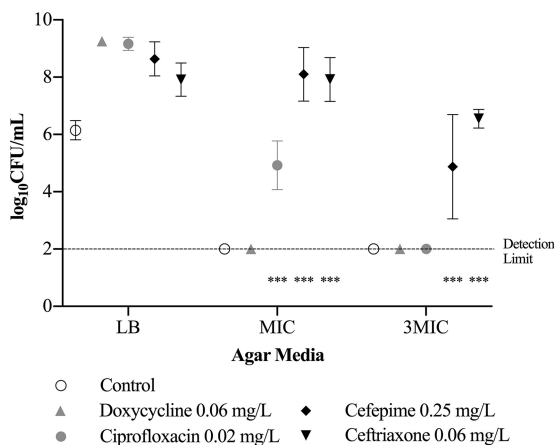
FIG 3 Antibiotic resistance in *V. vulnificus* after suspension in antibiotic concentrations with minimal bacterial inhibition for 48 h. Bacterial growth is represented as means $\pm$ standard deviations on LB agar and the respective antibiotic agar at the MIC and at three times the MIC (3MIC). Asterisks indicate antibiotic groups that had bacterial growth significantly higher, by 2 $\log_{10}$ units, than the control (***, $P <$ 0.001).

antibiotics alone at 24 h ($P < 0.01$), 36 h, and 48 h ($P < 0.0001$) (Fig. 4a). The combination of cefepime at 0.25 mg/liter and ciprofloxacin at 0.03 mg/liter significantly decreased bacterial growth by >2 $\log_{10}$ CFU/ml from that with the antibiotics alone at 24, 36, and 48 h ($P < 0.0001$) (Fig. 4b).

Survival rates in the ceftriaxone, doxycycline, and ciprofloxacin groups were significantly higher than those in the control group. All mice in the intestinal infection control group ($n = 23$) died within 14 h. In the groups receiving antibiotics, the survival rate was 80% with ciprofloxacin ($n = 20$), 79% with doxycycline ($n = 28$), 50% with ceftriaxone ($n = 20$), 20% with cefepime ($n = 10$), and 0% with high-dose cefepime ($n = 12$) (Fig. 5). Survival was significantly higher in the ceftriaxone, doxycycline, and ciprofloxacin groups than in the control group ($P < 0.0001$). When antibiotic groups were compared, there was no significant difference in survival between the ciprofloxacin and doxycycline groups ($P = 0.82$) or between the cefepime and high-dose cefepime groups ($P = 0.99$). Survival was significantly higher in the ceftriaxone group than in the cefepime group ($P \leq 0.05$).

Ceftriaxone in combination with doxycycline or ciprofloxacin improved survival over that with ceftriaxone monotherapy for intestinal *V. vulnificus* infection. The survival rate in the ceftriaxone-doxycycline group ($n = 11$) was 91%, and that in the ceftriaxone-ciprofloxacin group ($n = 11$) was 100% (Fig. 5a). Relative to that for the ceftriaxone group, survival was significantly improved in the ceftriaxone-doxycycline ($P \leq 0.05$) and ceftriaxone-ciprofloxacin ($P < 0.01$) groups. The survival curves did not differ significantly between the doxycycline and ceftriaxone-doxycycline groups ($P = 0.35$), the ciprofloxacin and ceftriaxone-ciprofloxacin groups ($P = 0.12$), and the ceftriaxone-doxycycline and ceftriaxone-ciprofloxacin groups ($P = 0.32$).

Cefepime in combination with doxycycline or ciprofloxacin improved survival over that with cefepime monotherapy for intestinal *V. vulnificus* infection. The survival rate in the cefepime-doxycycline group ($n = 23$) was 96%, and that in the cefepime-ciprofloxacin group ($n = 10$) was 90% (Fig. 5b). Survival was significantly improved in the cefepime-doxycycline ($P < 0.0001$) and cefepime-ciprofloxacin ($P < 0.001$) groups over that in the group receiving cefepime alone. Survival rates did not differ significantly between the ciprofloxacin and cefepime-ciprofloxacin groups ($P = 0.48$), the doxycycline and cefepime-doxycycline groups ($P = 0.07$), and the cefepime-doxycycline and cefepime-ciprofloxacin groups ($P = 0.56$).

There was no significant difference in survival rates between combination therapy groups. The survival rate in the ceftriaxone-doxycycline group did not differ significantly from those in the cefepime-doxycycline ($P = 0.61$) and cefepime-
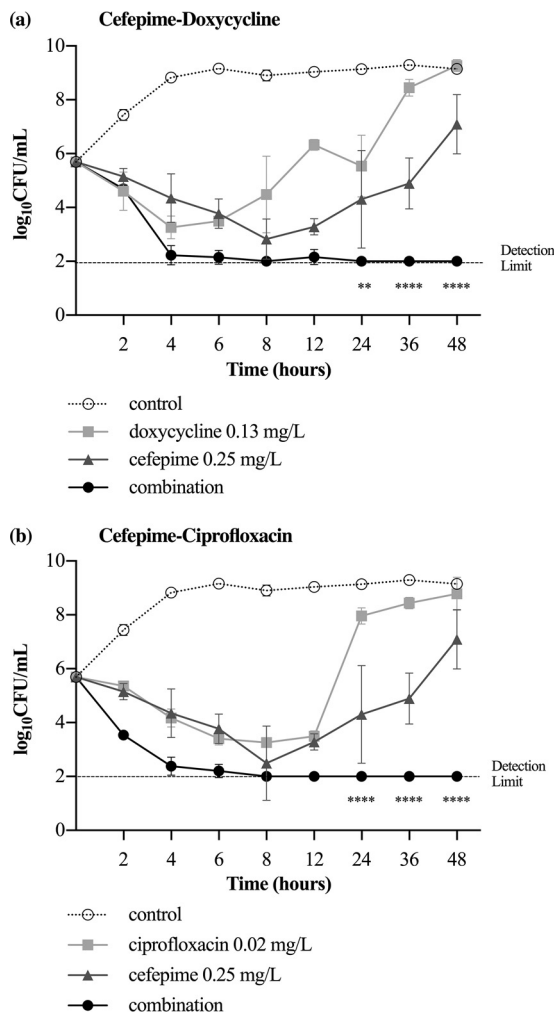
FIG 4 Inhibition of growth curves for *V. vulnificus* after incubation with cefepime, doxycycline, or a combination (a) and cefepime, ciprofloxacin, or a combination (b). Bacterial growth is represented as means ± standard deviations. The antibiotic combination significantly decreased bacterial growth, by ≥2 $\log_{10}$ CFU/ml, from that with the individual antibiotics (**, $P < 0.01$; ****, $P < 0.0001$).

ciprofloxacin ($P = 0.92$) groups. The survival rate in the ceftriaxone-ciprofloxacin group did not differ significantly from those in the cefepime-doxycycline ($P = 0.49$) and cefepime-ciprofloxacin ($P = 0.29$) groups.

There was no emergence of antibiotic resistance to ceftriaxone, cefepime, or high-dose cefepime *in vivo*. Because the ceftriaxone, cefepime, and high-dose cefepime groups had the highest mortality rates, the *in vivo* experiments for these treatment groups were repeated in order to assess the emergence of antibiotic resistance. At 8 h after infection (6 h after treatment), the liver and intestines were excised in order to be assessed for antibiotic-resistant *V. vulnificus*. There was no bacterial growth from the livers and intestines of the antibiotic-treated mice (Fig. 6). Each antibiotic-treated group had significantly less bacterial growth, by 2 $\log_{10}$ CFU/ml, in the intestines ($P < 0.0001$) and the liver ($P < 0.05$) than the control group. There was no bacterial growth from the excised organs of the antibiotic-treated mice on the respective antibiotic agar at the MIC and three times the MIC.

Despite differences in efficacy *in vivo*, cefepime, ceftriaxone, ciprofloxacin, and doxycycline had the same effect on *V. vulnificus* cytotoxicity. There was no significant difference in *V. vulnificus* cytotoxicity between the control group and the ceftriaxone, cefepime, ciprofloxacin, and doxycycline groups at 60, 120, and 180 min (Fig. 7).
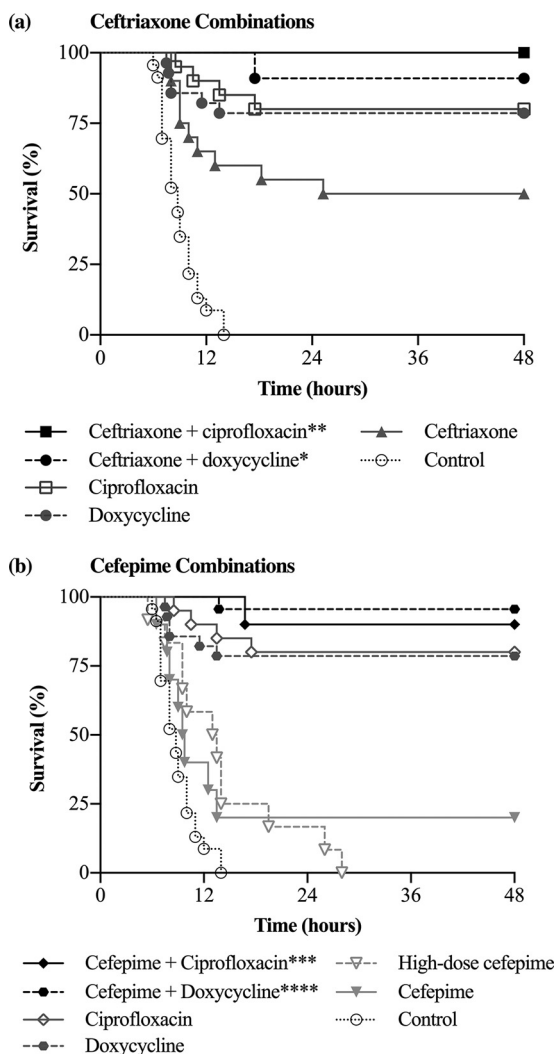
FIG 5 (a) Survival rates of mice treated with ceftriaxone alone or ceftriaxone combination therapy after *V. vulnificus* intestinal infection. Survival rates for the ceftriaxone-doxycycline (*, $P \leq 0.05$) and ceftriaxone-ciprofloxacin (**, $P < 0.01$) groups were significantly higher than those for the ceftriaxone group. (b) Survival rates of mice treated with cefepime alone or cefepime combination therapy. Survival in the cefepime-doxycycline (****, $P < 0.0001$) and cefepime-ciprofloxacin (***, $P < 0.001$) groups was significantly higher than that in the cefepime group.

DISCUSSION

Timely administration of effective antimicrobial therapy is required to halt the progression of foodborne *V. vulnificus* infection to septicemia and death. Indeed, studies have reported that human survival is strongly correlated with the immediate administration of appropriate antimicrobial therapy (9). Our study found that ciprofloxacin and doxycycline were the most effective antimicrobial agents for intestinal *V. vulnificus* infections in mice. The efficacy of fluoroquinolones against *V. vulnificus* wound infections was established previously (12), and this study confirms their utility for foodborne *V. vulnificus* infections. However, it was unexpected for doxycycline monotherapy to provide a survival benefit in a model of foodborne *V. vulnificus* infection, since it was found to be ineffective in a wound infection model. In a study by Chuang et al., the mortality rate was 100% among mice treated with minocycline after a subcutaneous injection of *V. vulnificus* (11). The enhanced efficacy of doxycycline for intestinal infections may be related to its ability to achieve high concentrations in the small intestine and the liver (25). Because doxycycline is excreted in the bile to the feces, drug concentrations are 10 to 25 times higher in the bile than in the serum (25).
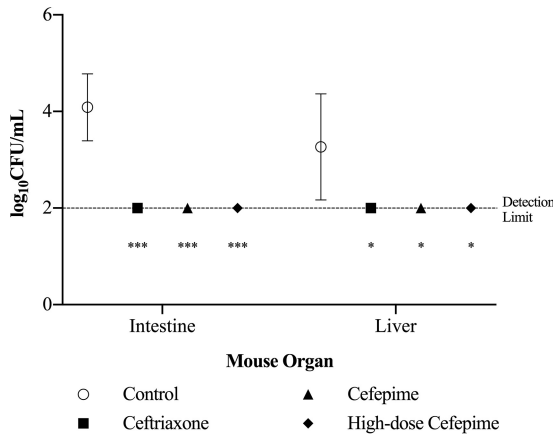
FIG 6 *V. vulnificus* isolated from organs 8 h after mice were inoculated intragastrically (or 6 h after the initiation of antimicrobial therapy). Bacterial growth is represented as means ± standard deviations. No bacterial growth was detected in the intestines and livers of antibiotic-treated mice. Asterisks indicate antibiotic groups that had significantly less bacterial growth than the control (***, by 2 $\log_{10}$ CFU/ml [$P < 0.0001$]; *, by $\log_{10}$ CFU/ml [$P < 0.05$]).

In our analysis of COVIS surveillance data, about one-third of patients with *V. vulnificus* bacteremia received an extended-spectrum β-lactam with coverage of resistant Gram-negative organisms, highlighting the need to assess the efficacy of these antibiotics for foodborne infections. Meropenem, cefepime, and piperacillin-tazobactam were among the antibiotics associated with the highest mortality rates; disease severity was likely a confounding factor, since patients with the most severe infections are usually treated with broad-spectrum antibiotics. The extended-spectrum β-lactam cefepime was tested because the rates of cefepime resistance among clinical and environmental isolates are lower than the rates of resistance to penicillins (20, 21). Although cefepime had good activity against *V. vulnificus in vitro*, cefepime at standard and high doses did not provide a survival benefit in the intestinal infection model. Similarly, ceftriaxone effectively decreased *V. vulnificus* growth *in vitro* but provided a survival benefit to only 50% of mice *in vivo*. These findings were consistent with those of prior studies on the efficacy of other cephalosporins against *V. vulnificus* wound infections in mice (11, 13, 24, 26). After subcutaneous injection of *V. vulnificus*, the
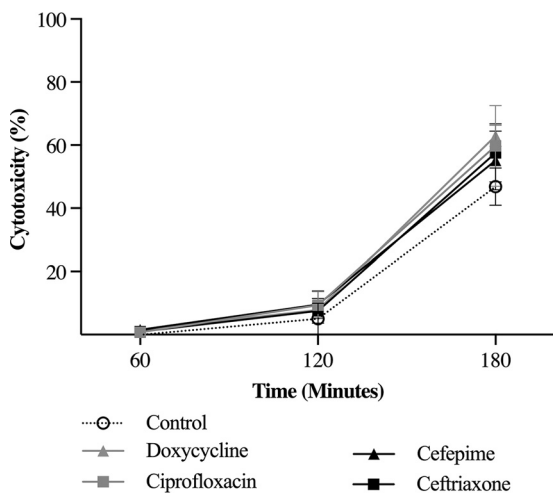

FIG 7 Effects of subinhibitory levels of antibiotics on *V. vulnificus* cytotoxicity. HeLa cells were suspended in antibiotics at a concentration of $^{1}/_{4}\times$ MIC and were inoculated with *V. vulnificus* at a multiplicity of infection of 2. There was no significant difference in cytotoxicity between the antibiotic groups and the control at 60, 120, and 180 min.

mortality rate was 100% for cefazolin- or cefotaxime-treated mice (11, 24, 26). Cefotaxime administered at five times the standard dose did not improve survival in the *V. vulnificus* wound infection model (mortality, 92%) (11). COVIS surveillance data also suggest that cephalosporins are not effective against *V. vulnificus* infections (9). In an analysis of 1,599 patients with *V. vulnificus* septicemia, gastroenteritis, and wound infection reported to the CDC, Wong et al. found that mortality was significantly higher among patients treated with cephalosporin monotherapy (all generations) (37%) than among those receiving fluoroquinolone monotherapy (17%) (9).

The emergence of antibiotic resistance *in vivo* did not explain the increased mortality among mice treated with cephalosporins. Although *V. vulnificus* developed resistance to ceftriaxone and cefepime *in vitro* when suspended in the concentration with minimal bacterial inhibition, both antibiotics effectively eliminated *V. vulnificus* from the intestines and the liver by 6 h after the initiation of treatment. Furthermore, development of *V. vulnificus* resistance to cefepime followed by outgrowth *in vivo* was unlikely, since serum cefepime concentrations were at least 40 times higher than the MIC (0.25 mg/liter). For the cefepime and high-dose cefepime groups, the antibiotic dosing was based on a pharmacokinetic study of cefepime in mice conducted by Maglio et al. (27). According to the study, the cefepime and high-dose cefepime groups received doses to achieve serum cefepime concentrations of 10 to 100 mg/liter and 50 to 500 mg/liter, respectively, over the 48-h treatment period (27). As confirmed in our cefepime time-kill study, a cefepime concentration of 0.5 mg/liter was sufficient to suppress *V. vulnificus* growth over the 48-h period during which the mice were observed (Fig. 2a).

To explain the differences in survival, it was hypothesized that ciprofloxacin and doxycycline inhibited *rtxA1* transcription and MARTX$_{Vv}$ toxin production to a greater degree than ceftriaxone and cefepime. Ciprofloxacin and doxycycline both inhibit processes essential for protein synthesis: ciprofloxacin inhibits DNA gyrase, an enzyme that prevents the supercoiling of DNA during replication and transcription (28), and doxycycline binds to the 30S ribosomal subunit and blocks the binding of aminoacyl-tRNA to mRNA (29). A prior study by Jang et al. found that ciprofloxacin significantly decreased *rtxA1* transcription and *V. vulnificus* cytotoxicity from those with cefotaxime (13). In contrast, our study found that the antibiotics did not differ in *V. vulnificus* cytotoxicity, suggesting that the antibiotics had no influence on MARTX$_{Vv}$ toxin production. Jang et al. may have overestimated the role of ciprofloxacin in *V. vulnificus* cytotoxicity, since there was a modest but significant difference of 20% in cytotoxicity between antibiotics. As seen in Fig. 7, cytotoxicity increased over time in our study, and despite variations at the midpoint, all antibiotics had 60% cytotoxicity by 180 min.

Differences in antibiotic efficacy may be related to how rapidly the antibiotics are able to halt the progression of a *V. vulnificus* infection. Because ciprofloxacin and doxycycline target processes essential for bacterial survival, these antibiotics can quickly eliminate the initial bacterial inoculum and any subsequent bacterial replicates (28, 29). Targeting the inoculum and subsequent replicates may halt the activation of a systemic inflammatory response. In contrast, because cephalosporins inhibit the final stage of cell wall synthesis, bacterial replicates are targeted, but the initial inoculum can activate an uncontrolled pathogenic systemic inflammatory response (30). In our *in vivo* experiments, although ceftriaxone and cefepime effectively eliminated *V. vulnificus* from the gastrointestinal organs by 6 h after treatment, we hypothesize that an uncontrolled systemic inflammatory response had already been activated, leading to high mortality rates. Indeed, a recent study has shown that it is the cytokine storm linked to uncontrolled proinflammatory responses that leads to the death of mice from *V. vulnificus* infections (31).

Despite differences in survival between the individual antibiotics, the efficacy of both cephalosporins for intestinal *V. vulnificus* infections increased with the addition of doxycycline or ciprofloxacin. The ceftriaxone-doxycycline, ceftriaxone-ciprofloxacin, cefepime-doxycycline, and cefepime-ciprofloxacin groups had the highest survival rates among the treatment groups. The improved efficacy of combination therapy in wound

and foodborne *V. vulnificus* infections can be attributed to the synergistic interaction of the antibiotics, as demonstrated *in vitro*. Although survival did not differ statistically between the combination therapy, doxycycline, and ciprofloxacin groups, the additional survival benefit with combination therapy may be clinically meaningful in the setting of an infection with high rates of septicemia and death.

In contrast to prior antibiotic efficacy studies, there were no differences in survival between the combination therapy groups. In a wound infection model, Jang et al. found that survival in the cefotaxime-ciprofloxacin group (75%) was significantly higher than that in the cefotaxime-minocycline group (33%) (13). The differences in survival were attributed to the ability of ciprofloxacin to decrease MARTX$_{Vv}$ transcription and the resulting *V. vulnificus* cytotoxicity (13). Our study found that there was no difference in *V. vulnificus* cytotoxicity between ciprofloxacin and the control over a 180-min period. For intestinal *V. vulnificus* infections, doxycycline may be as effective as ciprofloxacin in combination therapy, since both drugs penetrate gastrointestinal tissues at high concentrations (25, 32).

Overall, our study found that the most effective treatments for *V. vulnificus* foodborne septicemia include ceftriaxone-doxycycline, ceftriaxone-ciprofloxacin, cefepime-doxycycline, and cefepime-ciprofloxacin. For patients presenting in septic shock with a recent history of raw seafood consumption, we recommend initiating cefepime with doxycycline or ciprofloxacin for appropriate coverage of *V. vulnificus* and Gram-negative resistant organisms while awaiting microbiological cultures. Once a diagnosis of *V. vulnificus* is confirmed, the antibiotics can be safely deescalated to ceftriaxone with doxycycline or ciprofloxacin.

MATERIALS AND METHODS

Antibiotic use for *V. vulnificus* bacteremia in the United States. COVIS is a national passive surveillance system established by the CDC and the Food and Drug Administration (FDA) to monitor the incidence of *Vibrio* infections in the United States (6, 33). Health care providers submit a report that includes patient demographic, clinical, and epidemiologic information (6, 33). Deidentified COVIS data from 2007 through 2013 were obtained in order to assess the use of antibiotics for *V. vulnificus* bacteremia in the United States. A maximum of three antibiotics were reported, but start and end dates were not released, so as to protect patient confidentiality. Without prescribing dates, it was not known whether antibiotics were administered concurrently or consecutively, so data were recorded as the patient simply having received at least one dose of the reported antibiotics over the term of treatment. The mortality rate was determined for each antibiotic class, cephalosporin generation, and extended-spectrum β-lactam.

In vitro **antibiotic synergy testing.** *V. vulnificus* M06-24/O, a strain representative of U.S. clinical isolates, was used throughout the study. *In vitro* susceptibilities of *V. vulnificus* M06-24/O were determined for cefepime hydrochloride (U.S. Pharmacopeia Reference Standard, Rockville, MD), ceftriaxone sodium, doxycycline hyclate, and ciprofloxacin (Sigma-Aldrich, St. Louis, MO) using the broth microdilution method described in the Clinical and Laboratory Standards Institute (CLSI) guidelines (34). In a round-bottom 96-well plate, serial 2-fold antibiotic dilutions were performed in cation-adjusted Mueller-Hinton broth (CAMHB). Bacteria were inoculated into each well to achieve a final bacterial concentration of 5×10^5 CFU/ml. The inoculated plates were incubated at 30°C in an ambient air incubator. The MIC was the lowest antibiotic concentration that inhibited visible bacterial growth at 16 h. MIC results were confirmed using a SpectraMax M3 multimode microplate reader (Molecular Devices, Sunnyvale, CA); visible growth was defined as an optical density (OD) greater than 0.1 at 600 nm. *Escherichia coli* ATCC 25922 was used as the positive control.

In vitro synergism was established previously with time-kill assays for the third-generation cephalosporin cefotaxime in combination with minocycline and ciprofloxacin against *V. vulnificus* (10, 23). To maintain consistency with the prior *V. vulnificus* antibiotic efficacy studies, time-kill assays were performed as described previously in order to assess if cefepime was synergistic with doxycycline or ciprofloxacin *in vitro* (10, 23). For cefepime, doxycycline, and ciprofloxacin, volumetric dilutions were performed using CAMHB in 17- by 100-mm test tubes to make antibiotic concentrations of $1/4\times$ MIC, $1/2\times$ MIC, MIC, $2\times$ MIC, and $4\times$ MIC. M06-24/O was inoculated in each test tube to achieve a bacterial concentration of 5×10^5 CFU/ml. The broth cultures were incubated at 30°C and 250 rpm. For each antibiotic concentration, bacterial counts were measured at 2, 4, 6, 8, 12, 24, 36, and 48 h. The antibiotic concentration with minimal growth inhibition—the highest concentration with bacterial growth >2 log$_{10}$ CFU/ml higher than the detection limit at 48 h—was identified. The detection limit was 1×10^2 CFU/ml, based on the dilution method used to count bacterial colonies.

The emergence of antibiotic resistance was determined for *V. vulnificus* suspended in the antibiotic concentrations with minimal growth inhibition for 48 h. Broth cultures from the time-kill assays at 48 h were plated onto Luria-Bertani (LB) agar and the respective antibiotic agar at MIC and three times the

MIC. Antibiotic resistance was defined as bacterial growth $>2 \log_{10}$ CFU/ml higher than that of the control on the $3\times$ MIC antibiotic agar.

The cefepime-doxycycline and cefepime-ciprofloxacin combinations were tested using the antibiotic concentrations with minimal growth inhibition. Bacterial growth was measured over 48 h. The antibiotic combination was synergistic if bacterial growth was $2 \log_{10}$ lower than the starting inoculum and the growth with each antibiotic alone (10, 23).

i.g. *V. vulnificus* infection of mice. Mouse infections were conducted under protocols approved by the Northwestern University Institutional Animal Care and Use Committee. Each treatment group included 10 to 28 female CD-1 IGS mice (Charles River, Wilmington, MA) aged 32 to 38 days. The 10 treatment groups comprised control (0.9% sodium chloride), ceftriaxone, cefepime, high-dose cefepime, doxycycline, ciprofloxacin, ceftriaxone-doxycycline, ceftriaxone-ciprofloxacin, cefepime-doxycycline, and cefepime-ciprofloxacin groups. The cefepime and high-dose cefepime groups were tested to establish the optimal dose for *V. vulnificus* treatment. Mice were infected intragastrically (i.g.) as reported previously (17). Mice were anesthetized with an intraperitoneal injection of 10 μg/ml ketamine and 10 μg/ml xylazine suspended in 100 μl of phosphate buffer solution (PBS). By use of a feeding needle, mice were inoculated intragastrically with 50 μl of 8.5% sodium bicarbonate to neutralize the stomach, followed by 2×10^6 CFU of M06-24/O (10 times the 50% lethal dose [LD$_{50}$]). To confirm the bacterial dose administered, the inoculum was plated onto LB agar and was incubated at 30°C for 16 h (17).

Two hours after the intragastric bacterial inoculation, intraperitoneal antibiotics were administered for 48 h. Antibiotic dosing was based on prior mouse studies using *V. vulnificus* wound infection models: ceftriaxone (Hikma Farmaceutica, Terrugem, Portugal) at 50 mg/kg of body weight every 12 h (11), doxycycline (Frenius Kabi USA, Lake Zurich, IL) at 6 mg/kg every 24 h (11), and ciprofloxacin (Claris Injectibles Ltd., Gujarat, India) at a 16-mg/kg loading dose followed by 8 mg/kg every 12 h (12). Based on a study of cefepime pharmacokinetics in mice (27), the cefepime (Hospira, Inc., Lake Forest, IL) group received 80 mg/kg every 6 h (peak concentration, 175.7 mg/liter; rate of elimination, 0.64 h^{-1}) and the high-dose cefepime group received 300 mg/kg every 6 h (peak concentration, 510.5 mg/liter; rate of elimination, 0.46 h^{-1}). In another study of cefepime pharmacokinetics in mice using an alternative assay for measuring drug plasma levels, cefepime administered subcutaneously at a notably lower dose of 50 mg/kg had a half-life of 1.07 h and a bioavailability of 77% (35). Mice were observed every 2 h for the first 24 h and then every 6 h for the next 24 h for the primary endpoint, death.

The emergence of antibiotic resistance *in vivo* was determined for the antibiotics with the highest mortality rates: ceftriaxone, cefepime, and high-dose cefepime. In each treatment group, three mice were anesthetized, inoculated with *V. vulnificus* intragastrically, and treated with the respective antibiotics 2 h after infection. For the recovery of *V. vulnificus* from the gastrointestinal organs, the mice were euthanized 8 h after infection (6 h after antibiotic treatment). This time point was chosen for two reasons: (i) death in the cefepime and high-dose cefepime treatment groups started around hour 8 of infection, and (ii) serum cefepime concentrations were at trough levels, since it is dosed every 6 h. The liver and intestines were excised and were homogenized in PBS as described previously (17). The CFU count per organ was calculated by plating serially diluted homogenates onto CHROMagar *Vibrio* (CHROMagar, Springfield, NJ), a medium that isolates and detects *Vibrio* species based on differential colony colors (green-blue to turquoise-blue for *V. vulnificus*). The homogenates were plated on antibiotic agar at the MIC and three times the MIC to be assessed for the emergence of antibiotic resistance *in vivo*.

Effect of antibiotics on *V. vulnificus* cytotoxicity. Cytotoxicity assays were performed as described previously to assess the effect of the antibiotics on *V. vulnificus* MARTX$_{Vv}$ production (13). HeLa cervical epithelial cells (ATCC CLL-2) were seeded in six-well culture plates and were incubated at 37°C under 5% CO$_2$ for 16 h to reach 2×10^5 cells per well. The medium was changed to Dulbecco's modified Eagle's medium (DMEM) with an antibiotic concentration of $^1/_4\times$ MIC. Subinhibitory antibiotic concentrations were administered to assess the impact of the antibiotics on *V. vulnificus* MARTX$_{Vv}$ production. The treatment groups included cefepime, ceftriaxone, ciprofloxacin, and doxycycline. The positive control was DMEM alone. M06-24/O was washed with PBS and was inoculated at a multiplicity of infection (MOI) of 2 (4×10^5 CFU per well). Lactate dehydrogenase (LDH) release, a marker of cytotoxicity, was measured from the culture supernatant at 60, 120, and 180 min using the CytoTox96 nonradioactive cytotoxicity assay (Promega, Madison, WI) according to the manufacturer's protocol. Results were read on a SpectraMax M3 multimode microplate reader (Molecular Devices, Sunnyvale, CA) at 490 nm. The culture supernatant at 180 min was plated on LB agar and was incubated at 30°C for 16 h to ensure that the antibiotics were administered at subinhibitory concentrations.

Statistical analysis. Statistical analyses were performed using GraphPad Prism, version 7.00 for Mac OS X (GraphPad Software, La Jolla, CA). The time-kill and cytotoxicity assays were analyzed using two-way ANOVA (analysis of variance) and Tukey's multiple-comparison test. Survival analyses were conducted using the log rank test. Significance was set at a P value of ≤ 0.05.

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