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MBX-500 Is Effective for Treatment of *Clostridium difficile* Infection in Gnotobiotic Piglets

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The novel antibiotic MBX-500, dosed at 100, 200, or 400 mg/kg twice daily for 7 days, was evaluated for the treatment of *Clostridium difficile* infection (CDI) in the gnotobiotic pig model. MBX-500 increased survival at all doses and at high doses improved clinical signs and reduced lesion severity, similar to vancomycin. Our results show that MBX-500 is an effective antibiotic for the treatment of diarrhea associated with CDI and prevents severe systemic disease.

Clostridium difficile is a Gram-positive, spore-forming, anaerobic bacterium and the leading infective cause of nosocomial, antibiotic-associated diarrhea in the United States. *C. difficile* infection (CDI) results in consequences ranging from asymptomatic infection to mild or chronic diarrhea to life-threatening disease. The incidence of CDI has markedly increased since 2000, and the increasing morbidity and mortality, as well as the economic burden, have renewed research efforts on effective treatment and prevention strategies (1, 2).

CDI often occurs following the administration of broad-spectrum antibiotics that alter the normal gastrointestinal flora, making colonization by *C. difficile* possible. The current treatment of *C. difficile* includes primarily metronidazole and vancomycin, which have been the two mainstays of antibiotic treatment for decades (3), and fidaxomicin, which was approved in 2011 (4), although many other antimicrobial and nonantimicrobial treatments are under investigation. Treatment failure or recurrence of CDI in patients treated with current drugs (3, 5) has led to the investigation of novel antibiotic therapies for CDI, including an antibiotic developed by Microbiotix, Inc., that is an anilinouracil DNA polymerase inhibitor linked to a fluoroquinolone (6). This drug, named MBX-500, has demonstrated *in vitro* efficacy against and selectivity for *C. difficile*, with a bacterial spectrum of action narrower than that of metronidazole or vancomycin and increased survival and reduced disease severity in the hamster and mouse models (6). Here, we investigated the efficacy of the drug in the gnotobiotic piglet model of CDI, which more closely mimics human CDI than rodent models do (7).

Gnotobiotic piglets were delivered via Cesarean section and maintained in sterile isolators as we have previously described (7). All piglets were inoculated with 1×10^6 spores of *C. difficile* 027/NAP1/BI strain UK6 (8) by oral gavage at 5 days of age. A total of 31 piglets were divided as follows: 8 untreated controls, 9 treated with 100 mg/kg MBX-500, 4 treated with 200 mg/kg MBX-500, 6 treated with 400 mg/kg MBX-500, and 4 treated with 20 mg/kg vancomycin to serve as treatment controls. All piglet groups inoculated with pathogenic strain UK6 were compared to two control piglets inoculated with 10^8 vegetative cells of nontoxigenic *C. difficile* strain CD37, which does not cause pathology (7). Spores were prepared by inoculating UK6 vegetative cells into brain heart infusion medium, incubating them anaerobically for 10 to 14 days, washing them, and suspending them in water as we have previously described (7). Vancomycin and MBX-500 were prepared for oral administration by sonication or homogenization plus sonication with sterile phosphate-buffered saline. Antibiotics were administered twice per day for 7 days, starting at the onset of

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TABLE 1 Clinical outcomes of control and antibiotic-treated piglets

Treatment (no. of piglets)	Severity of:		Posttreatment *C. difficile* count (CFU/g)	No. with fatal systemic disease/total[b]
	Diarrhea posttreatment	GI lesions[a]		
None[c] (8)	Moderate to severe	Moderate to severe	10^4–10^7	6/8
MBX-500, 100 mg/kg (9)	Moderate to severe	Moderate to severe	10^4–10^9	1/9
MBX-500, 200 mg/kg (4)	Moderate to severe	Moderate to severe	10^3–10^{10}	1/4
MBX-500, 400 mg/kg (6)	4 resolved, 2 mild	None to mild	0–10^5	0/6
Vancomycin, 20 mg/kg (4)	3 resolved, 1 mild	None to mild	0	0/4
None, CD37 inoculation (2)	None	None	10^{12}	0/2

[a] Gastrointestinal lesions were noted during necropsy and on histopathologic examination of tissues. Lesion severity was based on the extent of dilatation, mesocolonic edema, hemorrhage, mucosal erosions and ulcerations, pseudomembrane formation, and degree of neutrophilic inflammation.
[b] Determination of severe systemic illness was based on the presence of respiratory distress, lethargy, and dehydration and the development of pleural or abdominal effusion.
[c] Control.

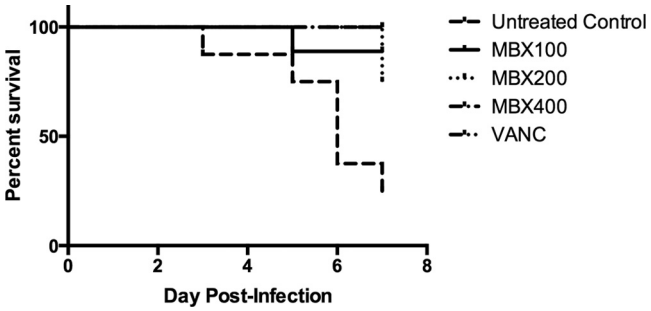

FIG 1 Kaplan-Meier survival curves of control and antibiotic-treated piglets. Piglets developing severe CDI became moribund between days 3 and 7 postinfection. The survival rate of piglets treated with vancomycin (VANC) or 400 mg/kg MBX-500 (MBX400) was 100%, compared with 89% for those treated with 100 mg/kg MBX-500, 75% for those treated with 200 mg/kg MBX-500, and 25% for untreated infected controls.

diarrhea, which occurred 48 h after inoculation with *C. difficile*. All animals were cared for according to Institutional Animal Care and Use Committee guidelines.

Daily fecal samples were spread on taurocholate-cefoxitin-cycloserine-fructose agar selective medium and incubated anaerobically to assess *C. difficile* shedding. *C. difficile* in the colon contents was also quantified at the time of necropsy. We used the ultrasensitive immunocytotoxicity assay developed by our laboratory (9) to measure TcdA and TcdB in serum, body fluid, and fecal samples. The concentrations of the cytokines interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12, tumor necrosis factor alpha (TNF-α), TGF-β, and gamma interferon in the large intestinal contents collected at the time of necropsy were determined with porcine cytokine quantification kits (Invitrogen and Research & Diagnostics Systems, Inc.).

As expected, all of the untreated, UK6-infected control piglets developed moderate-to-severe clinical signs of CDI, including watery/mucoid diarrhea, lethargy, anorexia, and weakness, with severe systemic disease occurring in six of the eight piglets in this group (Table 1). In the groups receiving either 100 or 200 mg/kg MBX-500, the piglets continued to have moderate-to-severe diarrhea after the initiation of treatment but other clinical signs of illness, such as anorexia, lethargy, and weakness, were less severe than in untreated controls, and systemic disease occurred in only 2 of the 13 piglets in these groups (Table 1). Piglets receiving 400 mg/kg MBX-500 had mild (2/6) or resolved (4/6) diarrhea after treatment, and none developed severe clinical disease, similar to those treated with vancomycin. The survival plot in Fig. 1 shows the times at which any piglet in each group became moribund, ranging from day 3 to day 7 after inoculation. Piglets inoculated with *C. difficile* strain CD37 did not develop any clinical signs of illness but were colonized and had *C. difficile* present in their feces beginning at 48 h after inoculation. Culture for *C. difficile* demonstrated that 400 mg/kg MBX-500 completely cleared the infection in four of six treated piglets, but lower doses did not clear *C. difficile* in any of the piglets (Table 1). TcdA and TcdB were present in the fecal and intestinal samples of piglets with positive *C. difficile* cultures but were not identified in the samples from piglets treated with 400 mg/kg MBX-500 or vancomycin, which had complete clearance of infection (data not shown).

Untreated, UK6-infected control piglets had typical moderate-to-severe lesions of CDI affecting the large intestine, including mesocolonic edema, luminal dilation, serosal hyperemia, mucosal erosions and ulcerations, and pseudomembrane formation (Fig. 2). Piglets treated with either 100 or 200 mg/kg MBX-500 also developed moderate-to-severe lesions of the large intestine. Piglets treated with 400 mg/kg MBX-500 had either no apparent lesions or mild lesions on gross or microscopic examination (Fig. 2), similar to those treated with vancomycin. Piglets inoculated with CD37 did not develop any gross or microscopic lesions (Fig. 2).

For cytokine concentration analysis, the Kruskal-Wallis test was performed to compare the mean intestinal cytokine concentrations in the UK6-infected control piglets and those treated with

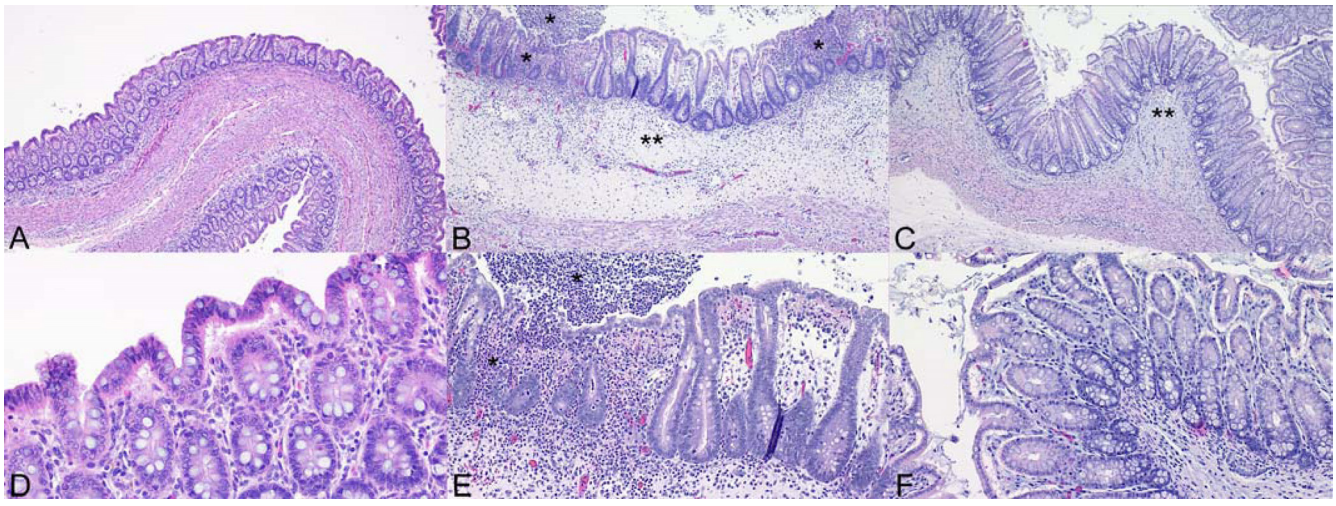

FIG 2 Histopathologic lesions in MBX-500-treated and untreated control piglets. *, examples of neutrophils, fibrin, and cellular debris in the lumen and lamina propria; **, examples of submucosal edema. (A) Colon of a *C. difficile* strain CD37 (nonpathogenic)-infected control piglet showing normal microscopic architecture (×4 magnification). (B) Colon of a *C. difficile* strain UK6-infected control piglet with severe fibrinonecrotic suppurative colitis, epithelial ulceration, and submucosal edema (×4 magnification). (C) Colon of an MBX-500 (400 mg/kg)-treated piglet showing intact mucosa with mild submucosal edema compared to that of infected controls (×4 magnification). (D) Normal colonic mucosa of a *C. difficile* CD37-infected control piglet (×20 magnification). (E) Neutrophilic infiltration, epithelial ulceration, and pseudomembrane formation in the colon of an untreated, *C. difficile* UK6-infected control piglet (×20 magnification). (F) Normal intact mucosa of an MBX-500 (400 mg/kg)-treated piglet (×20 magnification).

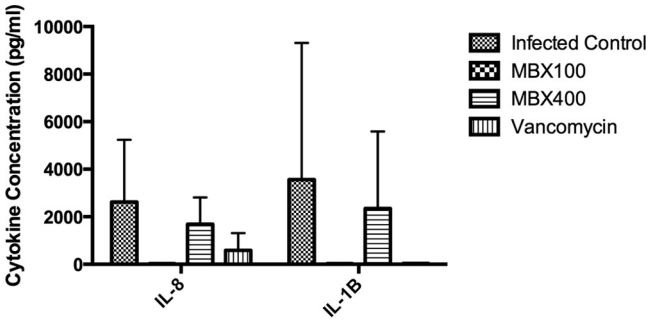

FIG 3 Intestinal cytokine concentrations in infected control and antibiotic-treated piglets. Cytokine concentrations in intestinal contents were measured via enzyme-linked immunosorbent assay. Kruskal-Wallis analysis demonstrated significantly greater mean IL-8 and IL-1β levels in control piglets than in those treated with antibiotics ($P < 0.05$).

100 or 400 mg/kg MBX-500 or vancomycin. Our previous experiments have indicated that CDI is associated with increases in proinflammatory cytokines that play a role in neutrophil migration (7, 10). Here we observed significant differences in IL-8 ($P = 0.015$) and IL-1β ($P = 0.026$), as shown in Fig. 3, with control piglets having the greatest average concentrations of these inflammatory cytokines and treated piglets having lower concentrations.

Our data show that MBX-500 is an effective compound against *C. difficile* in experimental infection of gnotobiotic piglets. Treatment reduced clinical signs of diarrhea and systemic disease; reduced or eliminated bacteria; and reduced or eliminated *C. difficile* toxins in a dose-dependent manner. With low doses (100 or 200 mg/kg twice daily), survival was increased but there were moderate effects on clinical infection severity. At a higher dose (400 mg/kg twice daily), MBX-500 was highly effective, allowing 100% survival of treated animals, resolved or mild diarrhea after treatment, and clearance of *C. difficile* in over 65% (4/6) of the treated animals. Overall, these results provide evidence that this novel antibiotic, at the highest dose evaluated, has a clinical efficacy in the gnotobiotic pig model similar to that of vancomycin.

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