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Antimicrobial susceptibilities of canine *Clostridium difficile* and *Clostridium perfringens* isolates to commonly utilized antimicrobial drugs

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Abstract

Clostridium difficile and *Clostridium perfringens* are anaerobic, Gram-positive bacilli that are common causes of enteritis and enterotoxemias in both domestic animals and humans. Both organisms have been associated with acute and chronic large and small bowel diarrhea, and acute hemorrhagic diarrheal syndrome in the dog. The objective of this study was to determine the in vitro antimicrobial susceptibilities of canine *C. difficile* and *C. perfringens* isolates in an effort to optimize antimicrobial therapy for dogs with clostridial-associated diarrhea. The minimum inhibitory concentrations (MIC) of antibiotics recommended for treating *C. difficile* (metronidazole, vancomycin) and *C. perfringens*-associated diarrhea in the dog (ampicillin, erythromycin, metronidazole, tetracycline, tylosin) were determined for 70 canine fecal *C. difficile* isolates and 131 *C. perfringens* isolates. All *C. difficile* isolates tested had an MIC of ≤ 1 for both metronidazole and vancomycin. Ninety-five percent (124/131) of *C. perfringens* isolates tested had an MIC for ampicillin of $\leq 0.125\,\mu g/ml$. Two *C. perfringens* isolates had an MIC of $\geq 256\,\mu g/ml$ for both erythromycin and tylosin. A third *C. perfringens* isolate had an MIC of $32\,\mu g/ml$ for metronidazole. Based on the results of this study, ampicillin, erythromycin, metronidazole, and tylosin appear to be effective antibiotics for the treatment of *C. perfringens*-associated diarrhea, although resistant strains do exist. However, because there is limited information regarding breakpoints for veterinary anaerobes, and because intestinal concentrations are not known, in vitro results should be interpreted with caution.
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1. Introduction

Clostridium difficile and *Clostridium perfringens* are spore-forming, anaerobic, Gram-positive bacilli that are common causes of enteritis and enterotoxemias in domestic animals and humans (Niilo, 1980; Jones et al., 1987; Kelly et al., 1994; Songer, 1996; Meer et al., 1997; Kelly and LaMont, 1998). Both organisms have been implicated as causes of canine acute and chronic large and small bowel diarrhea, as well as an acute hemorrhagic diarrheal syndrome (Berry and Levett, 1986; Kruth et al., 1989; Twedt, 1993; Meer et al., 1997; Sasaki et al., 1999; Weese et al., 2001a; Weese et al., 2001b; Cave et al., 2002; Marks et al., 2002). *C. perfringens* enterotoxin (CPE) is detected in 28–34% of diarrheic dogs and 5–14% of nondiarrheic dogs, while *C. difficile* toxin A or B is detected in 13–21% of diarrheic dogs and 2–7% of nondiarrheic dogs (Weese et al., 2001b; Marks et al., 2002). Diagnosis of *C. difficile* or *C. perfringens*-associated diarrhea in the dog is typically made based on detection of toxins in fecal specimens in conjunction with clinical signs, and antimicrobial administration is typically reserved for cases of severe diarrhea or for dogs with systemic manifestations of disease. Antibiotics recommended for the treatment of *C. difficile*-associated diarrhea in dogs include metronidazole, and to a lesser extent, vancomycin, whereas antibiotics recommended for the treatment of *C. perfringens*-associated diarrhea include beta-lactams (ampicillin and amoxicillin), macrolides (erythromycin and tylosin), metronidazole, and tetracyclines (Biberstein and Hirsh, 1999; Greene, 1998). However, the recommendations for treating clostridial-associated diarrhea in dogs have mostly been extrapolated from the human literature, as there is limited information concerning the in vitro or in vivo susceptibilities of these canine fecal isolates to these antibiotics. This information is particularly important in the face of increasing reports of resistance among anaerobic bacteria isolated from both humans and animals. Several studies have documented multiply antibiotic-resistant strains of *C. perfringens* (Dornbusch et al., 1975; Rood et al., 1978; Dutta and Devriese, 1981), and a recent study conducted at the University of California Davis Veterinary Medical Teaching Hospital (VMTH) in horses with *C. difficile*-associated diarrhea reported that 19% of the *C. difficile* isolates were resistant to metronidazole, one of the most commonly administered antibiotics used to kill this organism (Jang et al., 1997). Because exposure to antibiotics at concentrations close to or below the MIC for a particular organism is one factor involved in the selection of resistant bacterial strains (Schtentag et al., 2001), administration of antibiotics to treat the diseases associated with these organisms may in fact promote resistant strains if the fecal concentrations fall below the MIC values for each antibiotic. The objectives of this study were to determine the in vitro antimicrobial susceptibilities of canine *C. difficile* isolates to two antibiotics, and to determine the in vitro susceptibilities of canine *C. perfringens* isolates to five commonly utilized antibiotics to provide data on which to optimize antimicrobial therapy for dogs with clostridial-associated diarrhea.

2. Materials and methods

2.1. Bacterial isolates

Seventy *C. difficile* isolates, each obtained from an individual dog (59 diarrheic dogs, 11 nondiarrheic dogs), and 131 *C. perfringens* isolates, each obtained from an individual

dog (72 diarrheic dogs, 59 nondiarrheic dogs), were evaluated. All isolates were derived from diarrheic and nondiarrheic dogs that presented to the University of California Davis Veterinary Medical Teaching Hospital between 1995 and 2001. Isolates were frozen at $-80\,^\circ$C in 20% skim milk until analyzed. Frozen isolates were thawed, plated onto reduced 5% sheep blood agar (SBA) plates, and incubated at $37\,^\circ$C in an anaerobic chamber (Bactron IV, Sheldon Manufacturing Inc., Cornelius, OR). *C. perfringens* isolates were identified based on gram-stain morphology, a double zone of hemolysis on SBA, and lecithinase production on McClung's egg yolk agar (EYA). *C. difficile* isolates were identified on the basis of gram-stain, colony morphology and color on cycloserine–cefoxitin–fructose agar (CCFA), fluorescence, odor, and detection of L-proline aminopeptidase activity utilizing a commercially available kit (PRO Kit, Remel, Lenexa, KS).

2.2. Susceptibility testing

Minimum inhibitory concentrations (MICs) for metronidazole and vancomycin were determined for all *C. difficile* isolates ($n = 70$) and MICs for ampicillin, erythromycin, metronidazole, tetracycline, and tylosin were determined for all *C. perfringens* isolates ($n = 131$). All MIC values were determined using the National Committee for Clinical Laboratory Standards Reference Agar Dilution (NCCLS, 2001). Brucella plates supplemented with hemin, Vitamin K_1 and laked sheep blood, were prepared on the day of testing for each antibiotic at the following antibiotic concentrations: 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 μg/ml agar. Twenty-four hour cultures of each isolate were used to inoculate reduced brucella broth to match the turbidity of a 0.5 McFarland standard. Approximately 1 ml of the inoculated broth was then transferred to a well of a Steer's replicator seed block and plates were inoculated from the lowest concentration to the highest. Control plates containing no antibiotic were inoculated before and after the antibiotic plates as well as between each antibiotic series. Inoculated plates were incubated anaerobically at $37\,^\circ$C and MICs were read at 24 and 48 h. For quality control, *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Bacteroides fragilis* (ATCC 25285), *Bacteroides thetaiotamicron* (ATCC 29741), and *Eggerthella lenta* (ATCC 43055) were included on each plate. MIC values for each quality control organism were within reported ranges.

2.3. Statistical analysis

Each dog's medical record was evaluated to determine the antibiotic history in the 6 months prior to collection of isolates. The Mann–Whitney test was used to compare the MIC values obtained for each isolate with antibiotic exposure within 6 months of fecal collection for each dog. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Clostridium difficile

All *C. difficile* isolates were susceptible to metronidazole and vancomycin at $\leq 1\,\mu$g/ml. The MIC_{50} and MIC_{90} for metronidazole was 0.25 and 0.5 μg/ml, respectively. Both the

Table 1
MIC distribution of ampicillin, erythromycin, metronidazole, tetracycline, and tylosin for 131 canine *Clostridium perfringens* isolates

| | Number of *C. perfringens* isolates with MIC values (μg/ml) | | | | | | | | | | | | | |
	<0.125	0.25	0.5	1	2	4	8	16	32	64	128	>256	MIC$_{50}$	MIC$_{90}$
Ampicillin	124	4	0	1	2	0	0	0	0	0	0	0	0.125	0.125
Erythromycin	0	0	0	51	34	38	6	0	0	0	0	2	2	4
Metronidazole	3	3	21	76	19	8	0	0	0	1	0	0	1	2
Tetracycline	9	0	1	1	5	37	50	15	11	2	0	0	8	16
Tylosin	8	25	39	57	0	0	0	0	1	0	0	1	0.5	1

MIC$_{50}$ and MIC$_{90}$ for vancomycin were 1 μg/ml. Antibiotic histories were available for 63 of 70 dogs from which *C. difficile* was isolated. None of the dogs had received vancomycin 6 months prior to fecal collection, and 9/63 dogs (14%) had received metronidazole within 6 months of fecal collection.

3.2. Clostridium perfringens

The MIC values of each antibiotic for *C. perfringens* are summarized in Table 1. Ninety-five percent (124/131) of *C. perfringens* isolates tested had an MIC of ≤0.125 μg/ml for ampicillin. The MIC$_{90}$ for erythromycin and tylosin was 4 and 1 μg/ml, respectively. One *C. perfringens* isolate had an MIC of ≥256 μg/ml for both erythromycin and tylosin, 2 μg/ml for ampicillin, and 32 μg/ml for tetracycline. A second isolate had an MIC of >256 μg/ml for erythromycin and 32 μg/ml for tylosin. The MIC$_{90}$ for metronidazole was 2 μg/ml. One isolate had an MIC of 64 μg/ml for metronidazole. The MIC$_{90}$ for tetracycline was 16 μg/ml. Antibiotic treatment histories of 126 of the 131 dogs from which *C. perfringens* was isolated, were evaluated for determination of antibiotic administration in the 6 months prior to fecal collection. Ten dogs (8%) had received beta-lactam antibiotics within 6 months of fecal collection (five received amoxicillin/clavulanic acid, two received amoxicillin, and three received ampicillin). The isolates obtained from these dogs were all susceptible to ampicillin at 0.125 μg/ml. Three dogs had received tylosin prior to fecal collection and one dog had received erythromycin. One of the two *C. perfringens* isolates with a high MIC for both erythromycin and tylosin (≥256 μg/ml) was obtained from a dog that had received tylosin 2 months previously. The second *C. perfringens* isolate with a high MIC for the macrolides was obtained from a diarrheic dog with no history of macrolide antibiotic administration. Thirteen of the 126 dogs (10%) whose antibiotic histories could be determined had received metronidazole within 6 months of fecal collection. The isolates obtained from these dogs all had MICs of ≤2 μg/ml for metronidazole. Two of 126 dogs (1.6%) had received doxycycline within 6 months of fecal collection. The tetracycline MICs for these two isolates were 0.125 and 8 μg/ml. Analysis of antibiotic exposure and in vitro susceptibilities for each antibiotic revealed no association between antibiotic exposure and an increasing MIC.

4. Discussion

Because there is a paucity of information regarding breakpoints for anaerobic veterinary isolates to commonly utilized antibiotics, interpretation of in vitro MIC values is difficult. However, some inferences can be made. The two *C. perfringens* isolates that had an MIC for erythromycin of >256 μg/ml can be referred to as resistant based on previous reported MICs of erythromycin-resistant *C. perfringens* strains (Dornbusch et al., 1975; Rood et al., 1978). One of the two dogs from which a *C. perfringens* isolate with a high MIC for both erythromycin and tylosin (MIC > 256 μg/ml) was obtained, had received tylosin for a 1 month period at a very low dose (5 mg/kg twice daily), with treatment ending approximately 2 months before the fecal analysis was performed. In addition, the isolate obtained from this dog had an MIC of 2 μg/ml for ampicillin, and an MIC of 32 μg/ml for tetracycline. Only one *C. perfringens* isolate, obtained from a dog with nosocomial diarrhea, had an MIC supportive of a resistant isolate (MIC of 64 μg/ml). Metronidazole resistance among human and animal clinical *C. perfringens* isolates is rare, and to the authors' knowledge, this is the first report of a metronidazole-resistant clinical *C. perfringens* isolate obtained from a dog. This same isolate also had a high MIC (64 μg/ml) for tetracycline. Furthermore, the dog had no history of antibiotic exposure within 6 months of fecal analysis. Although the majority of clostridial isolates evaluated in this study were susceptible to the antibiotics tested, the high percentage of *C. perfringens* isolates (21%) with an MIC of ≥16 μg/ml for tetracycline (the breakpoint for resistance for human anaerobes and veterinary isolates (NCCLS, 2001; NCCLS, 1999)), and the discovery of multiply-resistant *C. perfringens* strains emphasizes the importance of surveying antimicrobial susceptibility profiles of common bacterial pathogens. Based on the results of this study, tetracycline is a poor antimicrobial choice for the treatment of *C. perfringens*-associated diarrhea in the dog, due to the reduced susceptibility among canine *C. perfringens* isolates. Furthermore, it has recently been shown that exposure to tetracycline concentrations below the MIC may actually induce conjugative transfer of tetracycline resistance plasmids from *Bacteroides* species, and it has been suggested that gene transfer can occur not only within the *Bacteroides* genus but also between *Bacteroides* spp. and Gram-positive bacteria (Shoemaker et al., 2001). Administration of tetracycline, especially at a low dose, for treatment of *C. perfringens*-associated diarrhea could not only select for resistant strains, but might potentially stimulate the transfer of tetracycline resistance.

Because of the paucity of information pertaining to clostridial MIC breakpoints for specific antibiotics in dogs, determination of intrinsic resistance ascertained by genetic analysis should provide additional clarification of these putative resistant isolates. These studies are currently underway to determine the presence of established tetracycline and macrolide resistance genes. In addition, fecal concentrations of ampicillin, metronidazole, and tylosin are currently being determined utilizing an HPLC method. Determination of MIC values for *C. perfringens* and *C. difficile* used in conjunction with both molecular resistance determinants and fecal antibiotic concentrations should provide insight as to whether bactericidal intracolonic concentrations are being reached in the lumen of the bowel, and may provide further information for antibiotic dosing.

5. Conclusion

The results of this study emphasize the importance of careful selection of antimicrobials used to treat dogs with clostridial-associated diarrhea. For the treatment of *C. perfringens*-associated diarrhea, ampicillin, metronidazole, and tylosin appeared to be the most effective antibiotics, although it should be noted that resistant strains do exist. Metronidazole appears to be an appropriate antibiotic for the treatment of canine *C. difficile*-associated diarrhea, due to the fact that all isolates were susceptible to $\leq 1\ \mu g/ml$ of antibiotic.

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