The Journal of Veterinary Medical Science

NOTE

Bacteriology

Antimicrobial susceptibility patterns of anaerobic bacteria identified from clinical specimens of diseased dogs and cats

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J. Vet. Med. Sci.
82(9): 1316–1320, 2020
doi: 10.1292/jvms.20-0294

Received: 18 May 2020
Accepted: 16 July 2020
Advanced Epub:
27 July 2020

ABSTRACT. We aimed to clarify antimicrobial susceptibility patterns of anaerobes from diseased companion animals. Bacterial identification was based on the Japanese 2012 guidelines for the testing of anaerobic bacteria. AST was performed using the broth microdilution method. The anaerobe-containing samples collected from 2014 to 2018 included blood (anaerobe recovery rate, 5.0%), bile (9.4%), joint fluids (0.6%), pleural effusions (42.6%), ascites (64.1%), cerebrospinal fluids (3.0%), and punctures (75.0%). The anaerobes identified included *Bacteroides* spp. (33.2%), *Peptostreptococcus* spp. (19.6%), *Prevotella* spp. (13.6%), *Propionibacterium* spp. (10.3%), *Clostridium* spp. (9.3%), and *Fusobacterium* spp. (7.5%). *Bacteroides fragilis* group isolates were resistant to penicillin G (100%), ampicillin (100%), cefmetazole (63.6%), ceftizoxime (90.0%), and clindamycin (40.0%). Our observations demonstrated antimicrobial susceptibility in anaerobes isolated from Japanese companion animals.

KEY WORDS: antimicrobial resistance, companion animals, identification, Japan, veterinary anaerobes

Anaerobic bacteria are increasingly identified as important obligate pathogens in animals and humans. These anaerobes are intrinsically present in the oral cavity, upper and lower respiratory tracts, digestive system, urinary tract, and skin [20]. Therefore, they can be considered as main pathogens in opportunistic or bacterial superinfections in susceptible hosts [13].

The predominant infections caused by anaerobic bacteria in companion animals (household dogs and cats) include periodontal disease [17, 26], pyothorax [2, 29], osteomyelitis [15, 24], soft-tissue infections [19], purulent lesions [16], and infections of the central nervous system [10]. The most frequent Gram-negative rods isolated from veterinary clinical settings include *Bacteroides*, *Prevotella*, and *Porphyromonas* [25].

Despite the prevalence of anaerobe-associated diseases, only a few veterinary clinical laboratories isolate anaerobic organisms in their facilities. Consequently, there is limited information available on anaerobic infectious diseases and the empirical antimicrobial treatment strategies [21, 23]. Ministry of Agriculture, Forestry and Fisheries of Japan [22] has reported that penicillins and the first/second-generation cephalosporins constituted 24.5% and 42.1% of the overall antimicrobials (converted weight in kilograms to bulk powder) used for companion animals in 2016. Empirical antimicrobial use has substantially contributed to increased antimicrobial resistance (AMR) in intrinsic bacteria such as *Staphylococcus intermedius* group and *Escherichia coli* [18]. Veterinarians should pay special attention to the emergence of AMR in anaerobes as well as aerobes [4]. Therefore, we aimed to clarify the *in vitro* susceptibility patterns of anaerobes to antimicrobial agents that can be administered in the Japanese veterinary clinical settings.

Genus/species-level identification and antimicrobial susceptibility testing (AST) were conducted using clinical specimens including the blood, bile, joint fluids, pleural effusions, ascites, cerebrospinal fluids, and punctures that veterinarians obtained from diseased companion animals from March 2014 to March 2018. The specimens were collected from 516 animal hospitals and clinics located in 39 prefectures in Japan (except for Akita, Yamagata, Yamanashi, Toyama, Ishikawa, Ehime, Tokushima, and Kouchi), with a total number of 1,742 specimens (23 collected in 2014, 476 in 2015, 558 in 2016, 530 in 2017, and 155 in 2018).

For the bacterial isolation, blood samples were cultured in anaerobic bottles using the Versa TREK system (Kohjin Bio Co.,

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(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

Ltd., Saitama, Japan). For the remaining specimens, semi-fluid medium for anaerobic bacteria (Kohjin Bio Co., Ltd.), anaerobe sheep blood agar medium (Kohjin Bio Co., Ltd.), and Bacteroides Bile Esculin (BBE) agar medium (Kohjin Bio Co., Ltd.) were used. Cultures were maintained under anaerobic conditions at 35°C for seven days in the AnaeroPack system (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

Bacterial identification was based on the Japanese 2012 guidelines for the testing of anaerobic bacteria [9]. The guidelines took into consideration the colony appearances on blood agar plates for anaerobes, Gram-positive/negative staining and morphology, bacterial growth in selective confirmative media (BBE medium for *Bacteroides fragilis* group, *Bacteroides* medium for other *Bacteroides* spp., and modified FM medium for *Fusobacterium* spp.), biochemical characteristics [using reverse CAMP test, catalase test, indole test, and metronidazole (MNZ) susceptibility test], and the identification using a manual kit (BD BBLCRYSTAL ANR Kit, Japan Becton, Dickinson and Co., Tokyo, Japan). When the colony appearances were suggestive of anaerobes, their growths in both aerobic and anaerobic conditions were verified and only those growing in anaerobic conditions alone were defined as anaerobes. Matrix-assisted laser desorption ionization-time of flight mass spectrometry [6, 27], which had been introduced in October 2016, was employed to confirm the identification results since April 2017.

Although *Clostridioides difficile*, *Cutibacterium acnes*, and *Eggerthella lenta* are now used as examples of the novel taxonomic species, *Clostridium difficile*, *Propionibacterium acnes*, and *Eubacterium lentum* were used as examples of the old taxonomic species in this study.

Genus/species-level identification manually conducted using the Japanese 2012 guidelines, was verified against genetic analysis using 16S rRNA sequencing. As the isolates collected from March 2014 to December 2017 were not stored, the 17 isolates collected and stored from January 2018 to March 2018 were genetically identified. DNA was extracted from the isolates by suspending the colonies in Tris-EDTA buffer and boiling the suspensions at 97°C for 10 min [11]. Isolates with ≥98.7% similarity to the 16S rRNA sequence of the corresponding reference strains were unambiguously identified by 16S-based IDs in the EzBioCloud database (https://www.ezbiocloud.net/) [11, 28].

The minimum inhibitory concentrations of various antimicrobial agents including penicillin G (PCG), ampicillin (ABPC), clavulanic acid-amoxicillin (CVA/AMPC), minocycline (MINO), chloramphenicol (CP), cefmetazole (CMZ), ceftizoxime (CZX), clindamycin (CLDM), imipenem (IPM), and meropenem (MEPM), were determined by the broth microdilution method using the Dry Plate Eiken DP43 and the Brucella broth supplemented with hemin (5 μg/m*l*), vitamin K1 (1 μg/m*l*), and 5% horse hemolysate (Eiken Chemical Co., Ltd., Tokyo, Japan). The breakpoints in the Clinical and Laboratory Standards Institute (CLSI) guidelines were applied in this study [8]. The tetracycline breakpoint was used for the susceptibility/resistance testing of MINO according to the CLSI guidelines.

AMR genotyping including *cepA* (cephalosporinases) [12], *cfxA* (broad-spectrum β-lactamase) [1], *cfiA* (metallo-β-lactamase) [12], *ermF* (ribosome methylase) [7], and *tetQ* (ribosome protection protein) [7] was assessed by polymerase chain reaction (PCR) amplifications among the 17 isolates that received genetic identification. All purified PCR products were subjected to direct sequencing to confirm the AMR gene sequences. We analyzed the relationship between the AMR genotypes and phenotypes.

The Ethics Committee of the Sanritsu Zelkova Veterinary Laboratory approved the study design (approval number SZ20180316) to ensure anonymity of the companion animals included in this study.

Of the 1,742 clinical specimens collected from diseased dogs and cats, 626 were tested positive for bacteria with an isolation rate of 35.9%. A total of 848 bacterial isolates consisted of 634 aerobic and 214 anaerobic isolates, with multiple aerobes and anaerobes isolated from the same specimens. The anaerobic isolation rates among the 175 anaerobe-containing samples were 5.0% for blood (52/1,034), 9.4% for bile (31/330), 0.6% for joint fluids (1/169), 42.6% for pleural effusions (46/108), 64.1% for ascites (41/64), 3.0% for cerebrospinal fluids (1/33), and 75.0% for punctures (3/4). At the genus/species level, the identified anaerobes belonged to *Bacteroides* spp. including *B. fragilis* group and *B. vulgatus* (*n*=71, 33.2%), *Peptostreptococcus* spp. specifically *P. anaerobius* (*n*=42, 19.6%), *Prevotella* spp. including *P. intermedia* and *P. denticola* (*n*=29, 13.6%), *Propionibacterium* spp. specifically *P. acnes* (*n*=22, 10.3%), *Clostridium* spp. including *C. perfringens* and *C. difficile* (*n*=20, 9.3%), *Fusobacterium* spp. specifically *F. nucleatum* (*n*=16, 7.5%), and others including *Actinomyces* spp., *Eubacterium* spp., *Porphyromonas* spp., *Lactobacillus* spp., *Bifidobacterium* spp., and *Bilophila* spp. (*n*=14, 6.5%).

Concordance of the genus (*Peptostreptococcus* sp. and *Fusobacterium* sp.) and species (*Bacteroides fragilis* group, *Propionibacterium acnes*, *Clostridium difficile*, *Bilophila wadsworthia*, and *Eubacterium lentum*) by manual identification was compared with species identification by genetic analysis in a limited number of isolates (*n*=17), and the results supported the validity of genus/species identification by manual identification (Supplementary Table 1). Demographics of the 17 isolates from eight dogs and six cats were as follows: mean age, 7.5 years; age range, 1–11 years; sex, twelve males and two females. The sources of these isolates included pleural effusions (*n*=5), ascites (*n*=5), bile (*n*=3), and blood (*n*=1).

AST patterns were assessed in the *Bacteroides fragilis* group (*n*=29), *Peptostreptococcus* (*n*=28), *Prevotella* (*n*=17), *Clostridium perfringens* (*n*=11), and *Fusobacterium* isolates (*n*=11). Figure 1 shows the antimicrobial activities of antibiotics against the *B. fragilis* group isolates. All the evaluated anaerobes were susceptible to MINO, IPM, and MEPM. *B. fragilis* group isolates were resistant to PCG (100%), ABPC (100%), CMZ (63.6%), CZX (90.0%), and CLDM (40.0%). In contrast, *Peptostreptococcus* isolates were susceptible to almost all tested antimicrobials. *Prevotella* isolates were partially resistant to PCG (14.3%), ABPC (12.5%), and CLDM (10.0%). *C. perfringens* isolates were partially resistant to CLDM (50.0%) and CP (14.3%). *Fusobacterium* isolates were partially resistant to PCG (20.0%), ABPC (20.0%), and CVA/AMPC (14.3%). The AST patterns were similar in samples collected in different years.

The AMR genotypes and phenotypes among a limited number of isolates (*n*=17) are shown in Table 1, in which all belonged to

the *B. fragilis* group.

The prevalence of invasive anaerobes obtained from the sterile sites of diseased companion animals was assessed. All clinical samples containing anaerobic bacteria belonged to the category A (clinical samples with which anaerobic culture should be performed) of the Japanese 2012 guidelines for the testing of anaerobic bacteria [9]: blood (category A-1), bile (A-3), joint fluids (A-1), pleural effusions (A-1), ascites (A-3), cerebrospinal fluids (A-1), and punctate (A-1/A-2/ A-3) (Supplementary Table 2). This supports the validity of the Japanese 2012 guidelines. Veterinarians are therefore suggested to conduct these recommended sampling methods when requesting anaerobic culture.

In general, the AST data showed that the penicillin family, the lincomycin family, and CP are the preferred antimicrobials to treat anaerobic infections in dogs and cats [3]. Drugs selected for the treatment of anaerobic infections in small animals include penicillins, cephalosporins, carbapenems, CP, CLDM, MNZ, and vancomycin [5]. Jang *et al.* [14] reported that 97 isolates from dogs and cats in the US were all susceptible to ABPC, CVA/AMPC, CP, CLDM, and most were susceptible to MNZ as described in 1997. Seventy-one percent and eighty-three percent of the *Bacteroides* isolates were susceptible to ABPC and CLDM, respectively, while 80% of the *Clostridium* isolates were susceptible to CLDM. In contrast, our study showed that all isolates were susceptible to MINO, IPM, and MEPM. The *B. fragilis* group isolates were resistant to PCG (100%), ABPC (100%), CMZ (63.6%), CZX (90.0%), and CLDM (40.0%). Some resistant isolates might produce class A chromosomal β-lactamases encoded by the *cepA* gene, although other isolates might be naturally resistant to penicillins and cephalosporins [4]. The *C. perfringens* isolates were resistant to CLDM (50.0%) and CP (14.3%). Thus, different AST patterns were observed in our study compared to those in

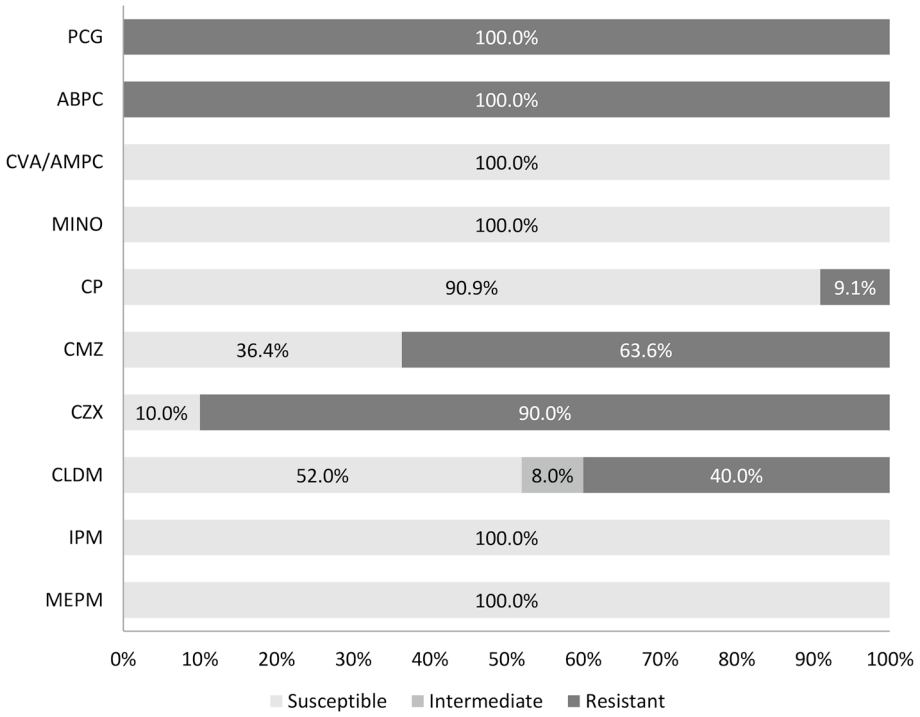

Fig. 1. Antimicrobial activities of antibiotics against *Bacteroides fragilis* group isolates (n=29) from diseased dogs and cats during March 2014–March 2018. PCG, penicillin G; ABPC, ampicillin; CVA/AMPC, clavulanic acid-amoxicillin; MINO, minocycline; CP, chloramphenicol; CMZ, cefmetazole; CZX, ceftizoxime; CLDM, clindamycin; IPM, imipenem; MEPM, meropenem.

Table 1. Antimicrobial resistance (AMR) genotypes and phenotypes among limited stored isolates (n=17)

AMR gene (encoding protein)	Number of isolates with AMR gene (%)	Identified anaerobe (number of isolates)	AMR phenotype in identified anaerobe (number of isolates)
cepA (cephalosporinases)	8 (47.1)	*Bacteroides fragilis* group (8)	Resistance to penicillin G (8), ampicillin (8), and ceftizoxime (8) in *B. fragilis* group
cfxA (broad spectrum β-lactamase)	0		
cfiA (metallo-β-lactamase)	0		
ermF (ribosome methylase)	3 (17.6)	*B. fragilis* group (3)	Resistance to clindamycin (3) in *B. fragilis* group
tetQ (ribosome protection protein)	2 (11.8)	*B. fragilis* group (2)	Resistance to minocycline (0) in *B. fragilis* group

AMR genotypes were *cepA* alone (n=5), *cepA*+*ermF*+*tetQ* (n=2), and *cepA*+*ermF* (n=1), all of which were detected from *B. fragilis* group.

other studies, likely due to different geographical locations and time periods. We should pay special attention to the changes of AST patterns of anaerobes because of the possibility of drastic changes in future.

In this study, all the AMR genotypes were detected in the *B. fragilis* group, suggesting genetic advances in AMR among this group. Interestingly, Boente *et al.* [4] reported that *cepA*, *cfiA*, *cfxA*, *tetQ*, and *ermF* were found in 69.2%, 17.3%, 9.6%, 50%, and 7.7% of *Bacteroides* isolates, respectively, indicating more advanced presence of AMR genes.

This study had some limitations. The demographic information about the enrolled animals was very limited. More details including underlying conditions, infectious diseases diagnosis, therapeutic approaches (surgical procedures, supportive and antimicrobial treatments), and outcomes (survival/death and related sequelae) should be further collected from Japanese veterinarians. The relationship between AST and antimicrobials administered in these animals should also be investigated.

To our knowledge, this study presents the first report on the AST of anaerobes isolated from diseased Japanese dogs and cats. Veterinarians would benefit from detailed AST data through the establishment of expanded research and clinical network in the future.

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