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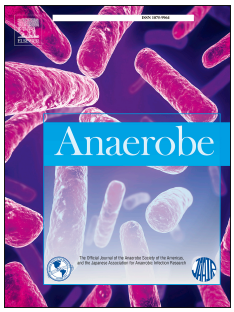

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Identification and antimicrobial susceptibility of obligate anaerobic bacteria from clinical samples of animal origin

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Highlights

- Anaerobic bacteria from animal infections were identified by gas chromatography
- Susceptibility profiles to eight antimicrobials were determined
- A high level of resistance to cephalosporins, fluoroquinolones and amoxicillin was detected
- The *erm*, *tet*, *cepA*, *cfxA* and *cfiA* resistance genes were infrequently detected

ABSTRACT

The etiology of veterinary infectious diseases has been the focus of considerable research, yet relatively little is known about the causative agents of anaerobic infections. Susceptibility studies have documented the emergence of antimicrobial resistance and indicate distinct differences in resistance patterns related to veterinary hospitals, geographic regions, and antibiotic-prescribing regimens. The aim of the present study was to identify the obligate anaerobic bacteria from veterinary clinical samples and to determinate the *in vitro* susceptibility to eight antimicrobials and their resistance-associated genes. 81 clinical specimens obtained from food-producing animals, pets and wild animals were examined to determine the relative prevalence of obligate anaerobic bacteria, and the species represented. *Bacteroides* spp, *Prevotella* spp and *Clostridium* spp represented approximately 80% of all anaerobic isolates. Resistance to metronidazole, clindamycin, tetracycline and fluoroquinolones was found in strains isolated from food-producing animals. Ciprofloxacin, enrofloxacin and cephalotin showed the highest resistance in all isolates. In 17%, 4% and 14% of tetracycline-resistant isolates, the resistance genes *tetL*, *tetM* and *tetW* were respectively amplified by PCR whereas in 4% of clindamycin-resistant strains the *ermG* gene was detected. 26% of the isolates were positive for *cepA*, while only 6% harbored the *cfxA* (resistance-conferring genes to beta-lactams). In this study, the obligate anaerobic bacteria from Costa Rica showed a high degree of resistance to most antimicrobials tested. Nevertheless, in the majority of cases this resistance was not related to the resistance acquired genes usually described in anaerobes. It is important to address and regulate the use of antimicrobials in the agricultural industry and the empirical therapy in anaerobic bacterial infections in veterinary medicine, especially since antibiotics and resistant bacteria can persist in the environment.

Keywords: Antimicrobial resistance, resistance-associated genes, veterinary anaerobes.

1. Introduction

Anaerobic bacteria are an important part of the indigenous microbiota of animals and humans. They are mainly found in the urinary tract, digestive and upper respiratory systems, oral cavity and skin [1]. Nevertheless, they can be considered opportunistic pathogens as they are responsible for numerous endogenous infections in susceptible hosts [2].

The most common diseases in animals associated to anaerobic bacteria are abscesses, myositis, osteomyelitis, myonecrosis, abdominal infections, periodontal disease, enteritis, mastitis (in livestock and pigs) and foot-rot (in sheep and goats) [1,3,4]. Species of the genera *Bacteroides* , *Prevotella* and *Porphyromonas* are the most frequent Gram-negative rods isolated from veterinary sources [5] and *C. difficile*, *C. perfringens*, *C. chauvoei*, *C. septicum*, *C. noyvi* and *C. sordelli* are the main pathogenic clostridia isolated [6].

One of the main limitations in veterinary medicine is that only a few clinical laboratories isolate and work with anaerobic bacteria. Hence, there is little information available in regards to anaerobic infections, and antimicrobial treatment in these cases is mostly empirical [7]. Antimicrobial use in food-producing animals and treatment of bacterial infections in veterinary medicine [8] has widely contributed to the increase of antimicrobial resistance in the indigenous microbiota [9]. Furthermore, contact between humans and animals has been suggested as a possible mechanism for horizontal gene transfer between microbiotas [10,11].

The aim of the present study was to identify obligate anaerobic bacteria from veterinary clinical samples and to determinate the *in vitro* susceptibility to eight antimicrobials and their resistance-associated genes.

2. Materials and methods

2.1 Bacterial identification

From June, 2013 to May 2014, eighty one anaerobic isolates from clinical samples were sent by the Laboratory of Bacteriology of the School of Veterinary Medicine, National University of Costa Rica to the Research Laboratory on Anaerobic Bacteriology, University of Costa Rica for identification. Strains were isolated from animal samples previously diagnosed with anaerobic bacterial infections. The samples included abscess materials (metatarsals, soft tissues, ear and prostate), tissue samples (lung, liver, tongue, brain, spleen and kidney) and fluid samples (pleura, joint, blood, endometrium, udder and pericardium). Identification of the isolated bacteria was confirmed by RapID 32A system (bioMérieux, Lyon, France) and membrane fatty acid profile with a 6850 GC equipment according to manufacturer guidelines (Agilent Technologies, Santa Clara, CA, USA), which was then analyzed using the Sherlock MIDI (Agilent Technologies) software. All isolates were further classified into three groups: food-producing animals (horses, pigs, sheep, cattle, duck and buffalo; n=56), pets (dogs and rabbits; n=19) and wild animals (snakes and coati; n=6).

2.2 Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of antimicrobials commonly used in veterinary medicine (metronidazole, ciprofloxacin, clindamycin, amoxicillin, tetracycline, enrofloxacin, cephalotin and trimethopim-sulfamethaxazole) were determined by E-test (bioMérieux) [12] and *Bacteroides fragilis* ATCC® 25825 was used for quality control..

Susceptibility was determined according to the CLSI breakpoints [13] for human isolates as there are no breakpoints for anaerobic bacteria of veterinary source. The ciprofloxacin and enrofloxacin results were interpreted using the breakpoint for moxifloxacin (4 µg/ml) and for cephalotin the third-generation cephalosporin breakpoint was used (32 µg/ml)

[13].MIC_{50} and MIC_{90} were determined with the respective percentile values. MIC_{50} and MIC_{90} values were defined as the lowest concentration of the antimicrobial at which 50% and 90% of the strains were inhibited, respectively.

2.3 Detection of antimicrobial resistance genes

DNA from each clinical isolate was obtained from overnight cultures in brain heart infusion broth (BHI; Oxoid, Hampshire, United Kingdom) with vitamin K (1 µg/ml) and hemin (5 µg/ml) (Sigma), using the DNeasy Blood & Tissue Kit (Qiagen Inc., Hilden, Germany). Fragments of *ermA, ermB, ermG, ermF* (clindamycin resistance), *tetQ, tetM, tetW, tetO, tetL* (tetracycline resistance), *cepA, cfxA* and *cfiA* (beta-lactam) were amplified by PCR according to Chung *et al*. [14], Shoemaker *et al*. [15] and Boente *et al*. [16]. *Escherichia coli* V1438 (*ermA), B. fragilis* BF-12256 (*ermF*), *E. coli* V1910 (*ermB)*, *B. thetaiotaomicron* BT-4009 (*ermG* and *tetQ)*, *Enterococcus faecalis Tn916+, (tetM, tetW, tetO, tetL), B. fragilis* 22285 (*cepA), B. fragilis* AA10 (*cfiA)* and *B. fragilis* Es36.2 (*cfxA)* were used as positive controls for the corresponding genes. *B. fragilis* ATCC® 25825 was used as a negative control for all genes, except for *cepA*.

3. Results

In this study, 81 anaerobic bacterial strains were isolated from diverse veterinary clinical samples. 46% (n=37) of the isolates were identified as *Bacteroides* spp, followed by *Prevotella* spp (18%) and *Clostridium* spp (15%) (Table 1 and Figure S1). *Bacteroides* spp was also the main genus isolated in the three distinct groups (food-producing animals, pets and wild animals) and *B. fragilis* was the most common species (Table 1). Antimicrobial susceptibility tests showed that in general, a small number of strains were resistant to metronidazole (5%), whereas most of the strains were resistant to ciprofloxacin

(63%). Comparisons between Gram-positive and Gram-negative anaerobes bared differences among these, as Gram-negative bacteria were resistant to ciprofloxacin (80%) and cephalotin (66%); while most of the Gram-positive isolates were resistant to tetracycline or enrofloxacin (50%). Resistance and MIC_{90} values were different as well, especially for tetracycline, ciprofloxacin, amoxicillin, and cephalotin (Table 2). In addition, MIC_{50} and MIC_{90} values for enrofloxacin differed in both Gram-positive and Gram-negative bacteria (2-3 µg/ml vs. 32 µg/ml) (Table 2).

Bacteroides spp resistance to metronidazole (5%), tetracycline (3%) and enrofloxacin (46%) was lower than the overall resistance of the Gram-negative group; but this genus was more resistant to ciprofloxacin (46%), amoxicillin (73%) and cephalotin (89%). Nonetheless, the MIC_{50} and MIC_{90} values were the same for *Bacteroides* spp and the Gram negative-group.

Antimicrobial susceptibility among the three groups varied as well. Antimicrobial resistance was higher in strains isolated from food-producing animals and 84% of the strains resistant to tetracycline were isolated from this group. Even though metronidazole resistance was low, three out of four resistant strains were isolated from food-producing animals. Approximately 30-35% of the strains isolated from pets were resistant to almost all antibiotics, except to metronidazole and only 12% were resistant to tetracycline. On the other hand, most of the strains isolated from wild animals were not resistant to any of the antibiotics of this study. However, a small percent of the strains (12%) was resistant to ciprofloxacin or cephalotin (Figure S2).

Despite the fact that 20% of the isolates were resistant to clindamycin, only 4% of these harbor the *ermG* gene and the *ermF*, *ermA* and *ermB* genes were not detected. In the case of tetracycline, the resistance-conferring genes *tetL*, *tetM* and *tetW* were found in 17%, 4% and 14% of the strains, respectively. The resistance-conferring gene to beta-lactam *cfiA* was not detected in any of the isolated bacteria and only 6% harbored the *cfxA*

gene, while 26% of the isolates were positive for *cepA*. Interestingly, most of the strains with at least one of these resistance genes belonged to the *B. fragilis* group (62%). Moreover, resistance-conferring genes were found in 85% of the bacteria isolated from food-producing animals and in 65% of the isolates obtained from pets. Meanwhile, there was no amplification for any of these genes in the samples of wild animals.

4. Discussion

The etiological agents found in this study are similar to those described in different geographic areas. Reports from Almeida et al. [3], Stephan et al. [17], and Cahalan et al. [18] indicate that *Bacteroides* spp is the most prevalent genus isolated from different groups of animals. In our study, *Bacteroides* spp were isolated from normally sterile sites including lung, liver, kidney, and brain tissue samples, abscess material from soft tissue infections, and fluids such as blood and endometrial fluid. Furthermore, in these and other reports, *Prevotella* spp has been the second most common strain, mainly isolated from various pathologies in head and neck. *Clostridium* spp have also been implicated in numerous infections in different groups of animals [19–21]. This was true in our case as well, with C. *perfringens* as the most common *Clostridium* species isolated.
Additionally, our results highlight the association between antimicrobial resistance and etiological agent, as antimicrobial susceptibility was different between Gram-positive and Gram-negative anaerobes. Resistance to beta-lactams (e.g. amoxicillin and cephalotin) and fluoroquinolones (ciprofloxacin) was high in *Bacteroides* spp and the Gram-negative group, as has been described before [22–25]. On the contrary, resistance to the antimicrobials tested was lower in Gram-positive strains. Thus, a preliminary identification of the etiological anaerobic agent is hepful prior to the administration of an empirical treatment in veterinary medicine.

Antimicrobial resistance was also higher in strains isolated from food producing animals, which could be a consequence of the antibiotic prophylaxis in this group of animals [26]. In addition, studies in Costa Rica have revealed that antimicrobial concentrations in food given to breeding animals are higher than the values allowed and indicated in food labels [27]. The high levels of antimicrobial resistance in strains isolated from food-producing animals and the low resistance in wild animals could therefore be associated to these activities. Even though antibiotic prophylaxis is advantageous for production [28], resistant strains may be favored. Resistant strains in animals may then pass on to humans [24,25], and as a consequence of the close contact between humans and animals, horizontal gene transfer might occur. Nonetheless, in this study there is not enough evidence to support this fact as few resistance genes were detected.

Tetracycline is widely used due to its low cost. Schwarz & Chaslus-Dancla [26] have reported that tetracycline is the number one antibiotic use in food-producing animals in the United States and Switzerland. A strong correlation between tetracycline use and its antimicrobial resistance has been found [29]. In this study, tetracycline resistance was higher in food-producing animals. Tetracycline resistance was higher in Gram-positive bacteria than in Gram-negative anaerobes (50% and 24%). However, resistance among genera and species varies [31]. Resistance-conferring genes for tetracycline found in this study (*tetL*, *tetM* and *tetW*) have been previously described in both Gram-positive and Gram-negative anaerobic bacteria [26,31]. In our case, most of the strains that harbored *tetM* belonged to the *Clostridium* spp as reported by Spigaglia et al. [32].

Metronidazole resistance in strains isolated form clinical samples is uncommon [33], although it is widely used [34]. In this study, only four strains (*Peptostreptococcus anerobius* and *Prevotella zoogleoformans* isolated from food-producing animals) were resistant to metronidazole and the MIC_{90} value was 0.5 µg/ml. Overall, these results suggest that metronidazole can still be used as empirical treatment for anaerobic related

infections in animals. Nevertheless, empirical use of metronidazole should be discouraged, particularly in large animals. It should only be used for specific infections.

Similar to data previously reported [35], resistance to clindamycin was found in 14% of the Gram-positive and 22% of the Gram-negative isolates. Still, clindamycin is one of the primary treatments in anaerobic infections in animals and humans. Macrolides are widely used in cattle [36] and probably in other animals as well, which may contribute to the decrease of clindamycin susceptibility. In our case, resistance to clindamycin was not associated to the usual resistant determinants like *erm* genes. *ermG* was the only gene found in three strains *(B. fragilis* and *Prevotella denticola*). In previous studies in Costa Rica done with strains isolated from human samples, clindamycin resistance was not associated to *erm* genes [37].

As reported before, resistance to amoxicillin and cephalotin was high in Gram-negative strains (54%, MIC_{90} 128 µg/ml and 66%, MIC_{90} 256 µg/ml) [16,35]. A strong association between beta-lactam use in animals and its antimicrobial resistance has been found [29]. Beta-lactam resistance is primarily due to beta-lactamase action or modification of PBP [25]. In the *Bacteroides* spp three main groups of genes have been described: *cepA* (endogenous cephalosporinase), *cfxA* (class A cephalosporinase) and *cfiA* (beta-lactamase) [24]. In this work *cepA* and *cfxA* were found mostly in *Bacteroides* spp, as has been reported in strains isolated from human samples in Costa Rica [22] and elsewhere [39]. The *cfiA* gene, which confers resistance to carbapenems, was not found.

Studies have suggested that *in vitro* ciprofloxacin is more effective against Gram-negative than Gram-positive anaerobic bacteria [40], but ciprofloxacin is not effective in vivo against anaerobes. In our case, 80% of the Gram-negative anaerobes were resistant to ciprofloxacin and the MIC_{90} was considerably higher in this group. However, ciprofloxacin is not usually recommended for treating anaerobic infections as it was found in very early studies to have poor activity [41], thus the use of this antibiotic has been reduced. Our

results suggest that ciprofloxacin should not be considered as a therapeutic option in clinical veterinary in our country, especially in infections caused by Gram-negative anaerobes. In addition, half of the Gram-positive strains were highly resistant to enrofloxacin. This antibiotic was developed exclusively for clinical veterinary use in Gram-negative aerobic bacterial infections, thus, this antibiotic should not be recommended for anaerobic bacterial infections.

5. Conclusions

The results of this study support the importance of identifying the anaerobic etiological agents involve in veterinary infections and their antimicrobial susceptibilities, as well as their resistance-conferring genes. It is important to address and regulate the use of antimicrobials in the agricultural industry and the empirical therapy in anaerobic bacterial infections in veterinary medicine. Especially since antibiotics and resistant bacteria can persist in the environment, guidelines must be established. If this situation remains unattended, bacterial horizontal gene transfer between human and animals could result in a serious public health issue.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure S1 Bacterial genera identified from veterinary clinical samples (n=81).
*It corresponds to an isolate of each of the following genus: *Eggerthella, Eubacterium, Propionibacterium, Ruminococcus* and *Porphyromonas* spp.

Figure S2 Antibiotic resistance in anaerobic bacteria isolated from veterinary samples based on human-animal contact (food-producing animals n= 53, pets n=21 and wild animals n=7)

Table 1 Identification of anaerobic species from clinical veterinary samples

Food producing animals (n=55)	
Host	**Bacterial identification**
Cattle	*Bacteroides fragilis* (n=10) *Clostridium perfringens* (n=5), *C. mangenotii* (n=2), *C. cadaveris* (n=2) *Prevotella heparinolytica* (n=2), *P. veroralis* (n=2), *P. zoogleoformans, P. oralis, P. denticola* *Fusobacterium nucleatum* (n=2), *F. necrophorum* *Jonhsonella ignava* (n=2) *Peptostreptococcus anaerobius* *Propionibacterium propionicus*
Horse	*Bacteroides fragilis* (n= 6), *B. ovatus* *Peptostreptococcus anaerobius* *Eggerthella lenta, Eubacterium hadrum* *Prevotella oris, P. zoogleoformans,*
Pig	*Bacteroides ovatus* (n=2), *B. fragilis* *Clostridium perfringens* *Fusobacterium necrophorum* *Peptostreptococcus anaerobius*
Sheep	*Bacteroides ovatus* *Prevotella zoogleoformans*
Duck	*Bacteroides ovatus,* *Johnsonella ignava*
Buffalo	*Clostridium bifermentans*
Pets (n=21)	
Dog	*Bacteroides fragilis* (n=4), *B. ovatus, B. thetaiotaomicron* *C. perfringens* *Prevotella heparinolyticus* (n=2) *P. tannerae, P. buccalis,* *P. catoniae* *Ruminococcus* spp.
Rabbit	*Bacteroides fragilis* (n=2), *B. ovatus* (n=2), *B. thetaiotaomicron* *Fusobacterium varium*
Wild animals (n= 6)	
Coati	*Bacteroides fragilis* (n=3) *Fusobacterium naviforme*
Snake	*Bacteroides fragilis* (n=2)

Table 2 Resistance, MIC_{50} and MIC_{90} to eight antimicrobials obtained by E-test in anaerobic veterinary isolates

Antimicrobial	Strains		
	Total (n=81)	**Gram Positive (n=22)**	**Gram Negative (n=59)**
Metronidazole			
% Resistance	5	5	5
MIC_{50}	0.02	0.09	0.13
MIC_{90}	0.5	0.5	0.38
Tetracycline			
% Resistance	31	50	24
MIC_{50}	1.5	3	1
MIC_{90}	48	12	48
Clindamycin			
% Resistance	20	14	22
MIC_{50}	0.5	0.06	0.75
MIC_{90}	16	3	0.16
Ciprofloxacin			
% Resistance	63	18	80
MIC_{50}	4	0.13	12
MIC_{90}	32	0.25	32
Amoxicillin			
% Resistance	44	18	54
MIC_{50}	0.75	0.06	12
MIC_{90}	128	0.25	128
Enrofloxacin			
% Resistance	52	50	53
MIC_{50}	2	2	3
MIC_{90}	32	32	32
Cephalotin			
% Resistance	51	9	66
MIC_{50}	8	1	96
MIC_{90}	256	4	256
Trimethoprim-sulfametoxazole	*	*	*
% Resistance	32	32	8
MIC_{50}	32	32	32
MIC_{90}			

*The breakpoint for trimethoprim-sulfa resistance has not been established in anaerobic bacteria.

Table 3 Resistance conferring genes in veterinary isolates

Detected genes	Number of strains	Gram-positive strains	Gram-negative strains
ermG	3		*Bacteroides fragilis, Prevotella denticola*
tetL	14	*Peptostreptococcus anaerobius, Johnsonella ignava, E.gerthella lenta, Propioniobacterium propionicus, Clostridium cadaveris, C. mangenotii*	*B. ovatus, B. fragiis*
tetM	3	*C. cadaveris, C. mangenotii*	*B. ovatus*
tetW	11	*P. anaerobius, J. ignava, E. lenta, C. cadaveris*	*B. ovatus, B. fragilis, P. zoogleoformans*
cepA	22	*E. lenta*	*B. fragilis , B. ovatus, P. veroralis, P. zoogleoforman, P. oris*
cfxA	5	*P. propionicus*	*B. fragilis*

Antimicrobial resistance and identification of obligate anaerobic bacteria isolated from clinical veterinary samples

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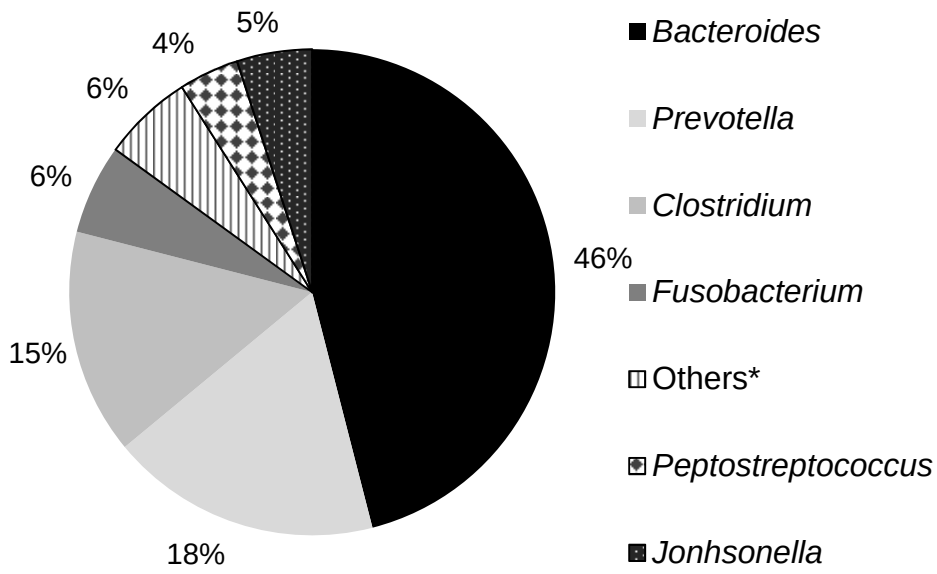

Figure S1

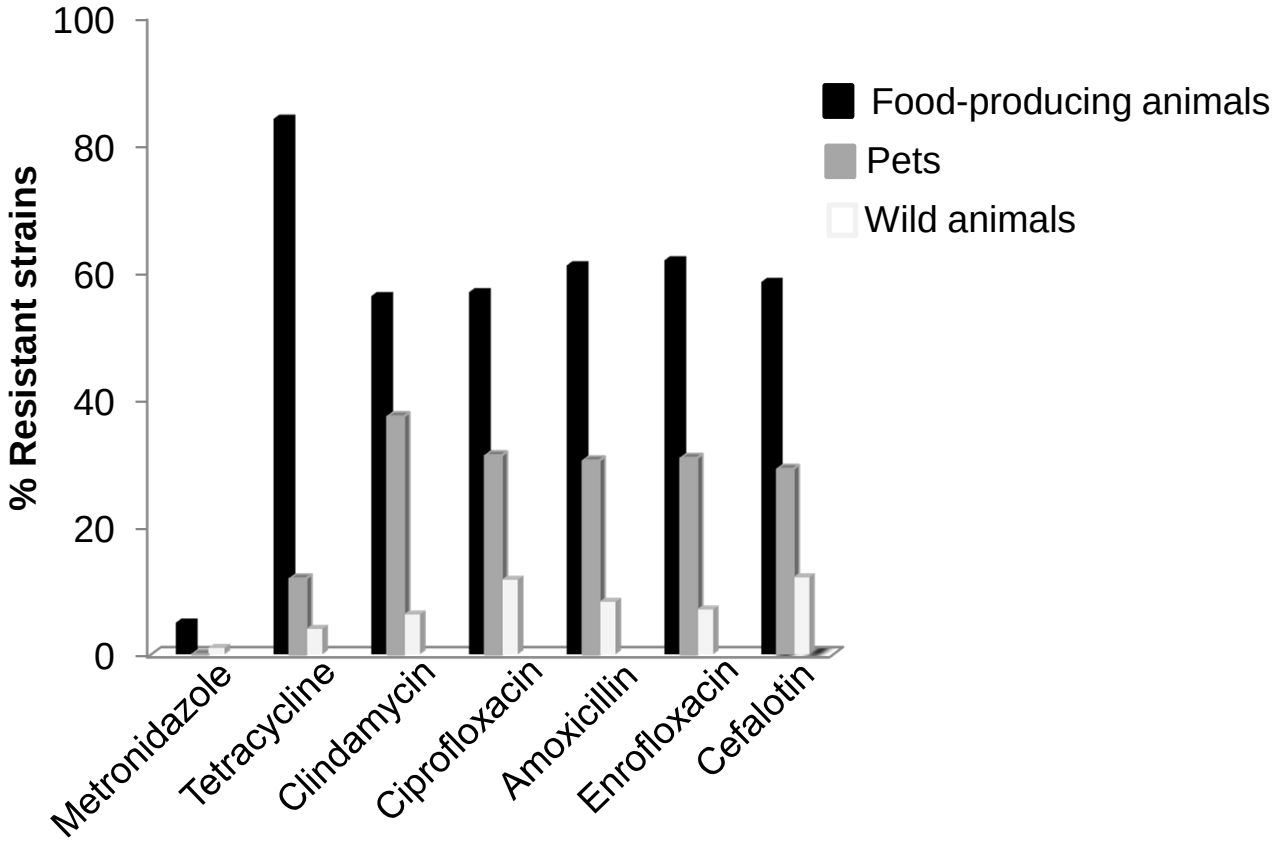
100
80
60
40
20
0
% Resistant strains
Food-producing animals
Pets
Wild animals
Metronidazole
Tetracycline
Clindamycin
Ciprofloxacin
Amoxicillin
Enrofloxacin
Cefalotin

Figure S2

Identification and antimicrobial susceptibility of obligate anaerobic bacteria from clinical samples of animal origin

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Highlights

- Anaerobic bacteria from animal infections were identified by gas chromatography
- Susceptibility profiles to eight antimicrobials were determined
- A high level of resistance to cephalosporins, fluoroquinolones and amoxicillin was detected
- The *erm*, *tet*, *cepA*, *cfxA* and *cfiA* resistance genes were infrequently detected