CLOSTRIDIUM DIFFICILE ISOLATED FROM ANIMALS: SUSCEPTIBILITY TO 30 ANTIMICROBIAL AGENTS USING BROTH MICRODILUTION METHOD

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Introduction

The antibiotic resistant pattern could differ in different hosts and while human isolates are regularly surveilled for antimicrobial susceptibility, the information on animal *C. difficile* strains is more deficient and has been not often sistematically determined (1,2,3).

We chose 31 *C. difficile* isolates from pigs, calves, dogs and a horse to examine their antimicrobial susceptibility to 30 antimicrobial agents. The method used was broth microdilution on commercially available plates for monitoring resistance of anaerobic and Gram-positive bacteria.

Materials and methods

Bacterial isolates

31 fecal isolates of *C. difficile* from pigs (21), calves (5), dogs (4) and a horse were collected and identified previously (4). The bacteria were recovered from frozen storage (-70°C). To ensure good growth and purity the isolates were subcultured by at least two serial transfers on 5% sheep blood agar prior to testing.

Broth microdilution

The method was performed on commercially available 96-well broth microdilution plates for monitoring resistance of anaerobic and Gram-positive bacteria, Sensititre Anaerobe Plate ANO2B format and Sensititre Gram-Positive Plate GPALL1F format, (Trek Diagnostic Systems, Ltd., UK). The reference method for susceptibility testing of anaerobic bacteria is agar dilution as recommended by the CLSI (5), broth microdilution is currently limited to testing of *Bacteroides fragilis* group and selected antibiotics.

The antimicrobial agents tested were ampicillin, ampicillin/sulbactam, amoxicillin/clavulanic acid, cefotetan, cefoxitin, chloramphenicol, ciprofloxacin clindamycin, daptomycin, erythromycin, gentamicin, imipenem, levofloxacin, linezolid, meropenem, metronidazole, mezlocillin, moxifloxacin, nitrofurantoin, oxacillin, penicillin, piperacillin, piperacillin/tazobactam, quinupristin/dalfopristin, rifampin, streptomycin, tetracycline, tigecycline, trimethoprim sulfamethoxazole and vancomycin.

The colonies were selected from 48-hour anaerobic culture, the inoculation was done in aerobic atmosphere, but the organisms were not exposed to air for more than 15 minutes.

The procedure was done as recommended by the producer, using reduced cation adjusted Mueller-Hinton Broth and supplemented Brucella Broth (Trek Diagnostic Systems, Ltd., UK) to make an inoculum containing 1×10^5 - 1×10^6 cfu/ml. The plates were incubated at 37°C for 48h in anaerobic conditions (GENbox anaerobic jar and GENbox Anaer generators, BioMerieux, France), up to three in a stack.

The MIC endpoints were determined where no growth or the most significant reduction of growth was observed.

Quality Control

The following control strains were included: *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, *Staphylococcus aureus* ATCC 29213 (aerobic control for Gram-positive plate). *C. difficile* ATCC 8864 was included for the internal control of the procedure.

The tests for ten isolates were performed in duplicate. The inoculum suspension was checked for purity by subculturing onto 5% sheep blood agar plates and incubated anaerobically and aerobically. All plates include control wells.

Results

Broth microdilution

Certain antimicrobial agents (chloramphenicol, tetracycline, ampicillin, penicillin, clindamycin) were included in both panels, for testing anaerobes and Gram-positive organisms. The MICs did not differ for more than one two-fold dilution for any of the antimicrobial agents. The results of anaerobe panel were chosen for presentation of the results for these agents. Also, duplicates did not differ for more than one two-fold dilution for any of ten tested strains and we chose the first duplicate for presentation of the results.

The results for MIC determination, MIC_{50} and MIC_{90} are given in Table 1.

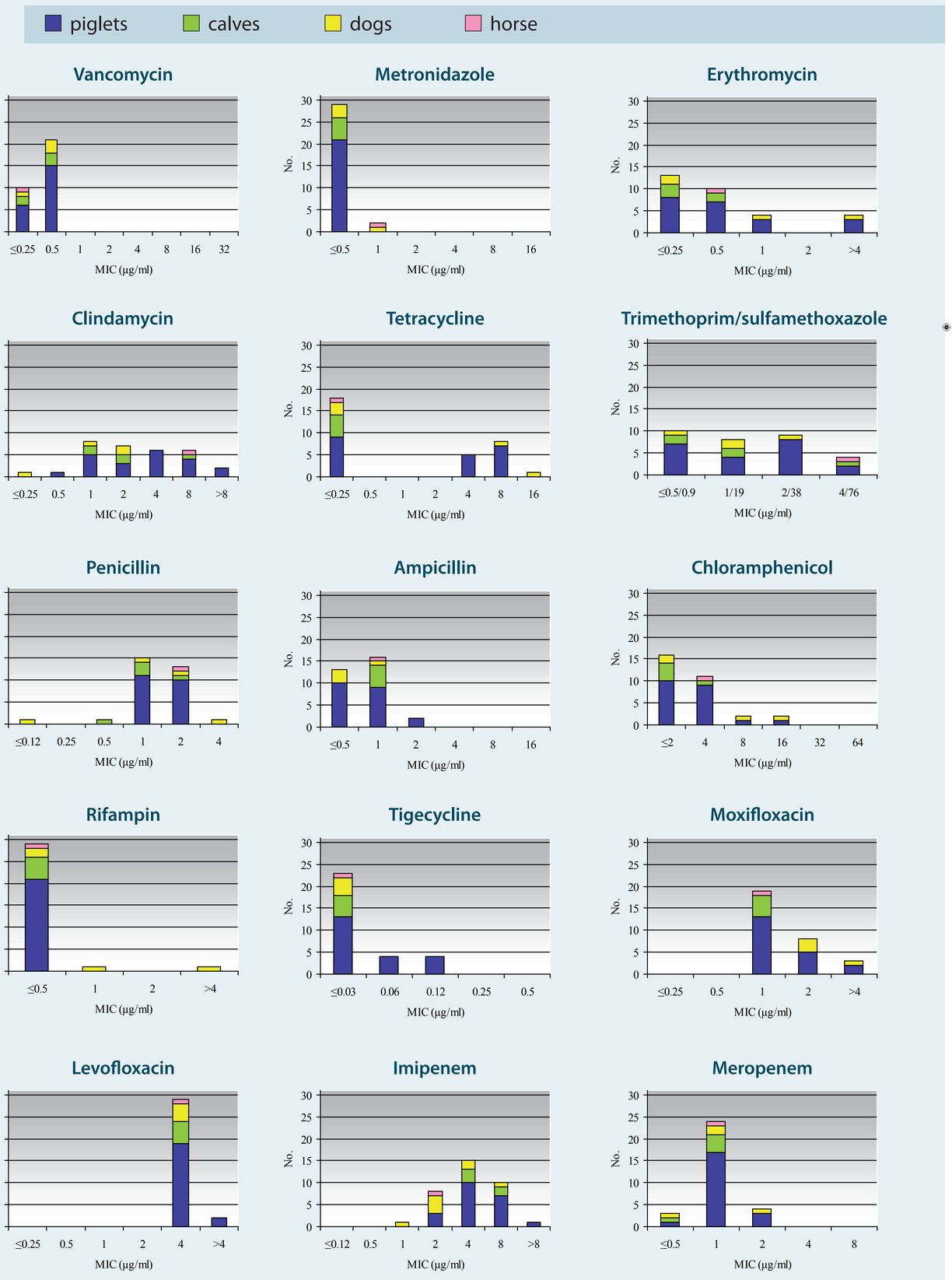
TABLE 1. Minimum inhibitory concentrations for animal isolates of C. difficile by broth microdilution

Antimicrobial agent	MIC (µg/ml)		
	MIC range	MIC_{50}*	MIC_{90}*
Chloramphenicol	≤2 - 16	≤2	4
Daptomycin	≤0.5 - >4	1	4
Gentamicin	>16	>16	>16
Linezolid	≤1-4	≤1	2
Rifampin	≤0.5->4	≤0.5	≤0.5
Trimethoprim/sulfamethoxazole	≤0.5/0.9-4/76	1/19	2/38
Quinupristin/dalfopristin	≤0.5->4	≤0.5	1
Tetracycline	≤0.25-16	≤0.25	8
Erythromycin	≤0.25->4	0.5	1
Oxacillin	≤0.25->4	≤0.25	4
Ampicillin	≤0.5-2	1	1
Penicillin	≤0.12-4	1	2
Vancomycin	≤0.25-0.5	0.5	0.5
Levofloxacin	4->4	4	4
Tigecycline	≤0.03-0.12	≤0.03	0.06
Moxifloxacin	1->4	1	2
Clindamycin	≤0.25->8	2	8
Streptomycin	≤1000	≤1000	≤1000
Ciprofloxacin	>2	>2	>2
Nitrofurantoin	≤32	≤32	≤32
Ampicillin/sulbactam	≤0.5/0.25-1/0.5	≤0.5/0.25	1/0.5
Amoxicillin/clavulanic acid	≤0.5/0.25-1/0.5	≤0.5/0.25	≤0.5/0.25
Cefotetan	≤4-16	16	16
Imipenem	1->8	4	8
Meropenem	≤0.5-2	1	1
Cefoxitin	>32	>32	>32
Metronidazole	≤0.5-1	≤0.5	≤0.5
Piperacillin	≤4-8	1	1
Mezlocillin	≤4	≤4	≤4
Piperacillin/tazobactam	1/4-8/4	4/4	8/4

** MIC_{50} and MIC_{90} are minimum inhibitory concentrations at which 50% and 90% of the isolates, respectively, were inhibited.*

Distribution of MIC for specific antimicrobial agents is showed in Figure 1.

FIGURE 1. Distribution of the MICs from various animal species for selected antimicrobial agents: vancomycin, metronidazole, erythromycin, clindamycin, tetracycline, trimethoprim/sulfamethoxazole, penicillin, ampicillin, chloramphenicol, rifampin, tigecyclin, moxifloxacin, levofloxacin, imipenem and meropenem



Discussion

From the viewpoint of antibiotic therapy the emerging resistance to metronidazole, and vancomycin is the main concern, as the agents are the drugs of choice for human treatment. Resistance to other antibiotics is also important as it enables the growth in the presence of increased antibiotic levels that disrupt the normal gut flora.

All tested isolates were inhibited by concentrations that did not exceed 0.5 µg/ml for vancomycin and 1 µg/ml for metronidazole.

Some studies found correlation with resistance to chloramphenicol and erythromycin, clindamycin and tetracycline, the pattern found almost exclusively in serogroup C (6). In our study, decreased susceptibility to chloramphenicol (16 µg/ml) was observed in two isolates and one of them also had decreased susceptibility for clindamycin (2 µg/ml), tetracycline (8 µg/ml), rifampin (>4 µg/ml) and moxifloxacin (>4 µg/ml). The strain was isolated from a dog and belongs to toxinotype 0 (unpublished data), which is commonly found in serogroup C. MICs for clindamycin, erythromycin and trimethoprim/sulfamethoxazole were distributed within whole tested range, which is consistent to findings of a wide range of susceptibility in those antimicrobial agents. MICs for tetracycline showed bimodal distribution: 58% of isolates had MICs ≤0.25 µg/mL and 42% of isolates had MICs 4-8 µg/mL. Rifampin showed high level activity against all *C. difficile* isolates but one, with MIC_{90} ≤0.5 µg/ml.

The broth microdilution method in this study showed as reproducible and reliable, MIC endpoints were generally clearly determined. However, in the case of some antimicrobial agents, the dilution range was too narrow to make conclusions on MIC values or to identify resistant strains.

REFERENCES: **1.** Baverud V, Gunnarsson A, Karlsson M, Franklin A. 2004. Antimicrobial susceptibility of equine and environmental isolates of *Clostridium difficile.* Microb. Drug Resist. 10: 57-63. **2.** Post KW, Songer GJ. 2004. Antimicrobial Susceptibility of *Clostridium difficile* isolated from neonatal pigs with enteritis. Anaerobe 10: 47-50. **3.** Marks SL, Kather E. 2003. Antimicrobial susceptibilities of canine *Clostridium difficile* and *Clostridium perfringens* isolates to commonly utilized antimicrobial drugs. Vet Microbiol 94: 39–45. **4.** Avbersek J, Janezic S, Pate M, Rupnik M, Zidaric V, Logar K, Vengust M, Zemljic M, Pirs T, Ocepek M. 2009. Diversity of Clostridium difficile in pigs and other animals in Slovenia.Anaerobe 15(6):252-5. **5.** Clinical and Laboratory Standards Institute. 2007. Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard—7th edition. Clinical and Laboratory, Standards Institute, Wayne, PA. **6.** Barbut F, Decre D, Burghoffer B, Lessage D, Delisle F, Lalande V, Delmee M, Avesani V, Sano N, Coudert C, Petit JC.1999. Antimicrobial susceptibilities and serogroups of clinical strains of Clostridium difficile isolated in France 1991 and 1997. Antimicrob. Agents Chemother. 43:2607-11.