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Antimicrobial susceptibility of animal and human isolates of *Clostridium difficile* by broth microdilution

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A total of 188 human (n=92) and animal (n=96) isolates of *Clostridium difficile* of different PCR ribotypes were screened for susceptibility to 30 antimicrobials using broth microdilution. When comparing the prevalence of antimicrobial resistance, the isolates of animal origin were significantly more often resistant to oxacillin, gentamicin and trimethoprim/sulfamethoxazole (P<0.01). The most significant difference between the animal and human populations (P=0.0006) was found in the level of imipenem resistance, with a prevalence of 53.3 % in isolates of human origin and 28.1 % in isolates of animal origin. Overall, the results show similar MICs for the majority of tested antimicrobials for isolates from human and animal sources, which were collected from the same geographical region and in the same time interval. This supports the hypothesis that *C. difficile* could be transmissible between human and animal hosts. Resistant isolates have been found in all animal species tested, including food and companion animals, and also among non-toxigenic isolates. The isolates of the most prevalent PCR ribotype 014/020 had low resistance rates for moxifloxacin, erythromycin, rifampicin and daptomycin, but a high resistance rate for imipenem. Multiresistant strains were found in animals and humans, belonging to PCR ribotypes 012, 017, 027, 045, 046, 078 and 150, and also to non-toxigenic strains of PCR ribotypes 010 and SLO 080.

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INTRODUCTION

Clostridium difficile has been well established as a cause of human intestinal disease and, until recently, has been mostly seen as a hospital-acquired infection in elderly patients following antibiotic treatment in hospitals (Bartlett, 1992; Rupnik *et al.*, 2009). In animals, *C. difficile* has been described as an important cause of enteritis or, more often, as a commensal in various animal species, including pigs, poultry, cattle, horses and dogs (Båverud *et al.*, 2003; Songer & Anderson, 2006; Rodriguez-Palacios *et al.*, 2006; Keel *et al.*, 2007; Hammitt *et al.*, 2008; Pirs *et al.*, 2008; Zidaric *et al.*, 2008; Avbersek *et al.*, 2009; Alvarez-Perez *et al.*, 2009). The overlap of types in humans, animals, the environment and food suggests transmission from/to various hosts and animals as a reservoir for human infections (Gould & Limbago, 2010; Weese, 2010; Koene *et al.*, 2012; Janezic *et al.*, 2012).

Antimicrobial therapy has a key role in the development of *C. difficile* infection (CDI). Clindamycin, cephalosporins and, more recently, fluoroquinolones have been linked to a high risk of CDI (Kuijper *et al.*, 2008). Although the great majority of isolates are still susceptible to the drugs of choice for human treatment, metronidazole and vancomycin, the resistance to other antimicrobials is also important as it enables the growth in the presence of increased antibiotic levels that disrupt the indigenous intestinal microbiota. The resistance to other antimicrobials varies widely in different countries (Huang *et al.*, 2009). While human isolates are regularly surveyed for antimicrobial susceptibility, there is less information

Abbreviations: CDI, *Clostridium difficile* infection; CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing.

available on resistance in animal *C. difficile* isolates. Also, this information has not often been systematically compared to human isolates from the same geographical regions (Marks & Kather, 2003; Båverud *et al.*, 2004; Post & Songer, 2004; Bakker *et al.*, 2010; Janezic *et al.*, 2012; Fry *et al.*, 2012; Keessen *et al.*, 2013).

The aim of our study was to determine and compare the antimicrobial susceptibility of human and animal isolates from the same geographical region and the same time interval to a variety of antimicrobials. The method used was broth microdilution, which is a convenient method for simultaneous determination of susceptibilities to several antimicrobials in a single isolate.

METHODS

Broth microdilution. The method was performed on commercially available 96-well broth microdilution plates for monitoring resistance of anaerobic and Gram-positive bacteria: the Sensititre Anaerobe plate ANO2B format and the Sensititre Gram-Positive plate GPALL1F format (Trek Diagnostic Systems). Thirty antimicrobial agents were tested, including 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, rifampicin, streptomycin, tetracycline, tigecycline, trimethoprim/sulfamethoxazole and vancomycin.

The colonies were selected from 48 h anaerobic culture on 5 % sheep blood agar (Columbia blood agar base; Oxoid). The suspensions and inoculation of microdilution plates were carried out in an aerobic atmosphere, but the organisms were not exposed to air for more than 30 min.

The procedure was carried out using reduced cation-adjusted Mueller–Hinton broth for initial suspension and supplemented Brucella broth (Trek Diagnostic Systems) to make an inoculum containing 1×10^5–1×10^6 c.f.u. ml^{-1} in final suspension, which was transferred to a broth microdilution plate following the manufacturer's instructions. The plates were incubated at 35 °C for 48 h in anaerobic conditions (GENbox anaerobic jar and GENbox Anaer generators; bioMérieux).

The MIC end points were determined where no growth was observed, or, in cases where growth was observed in the last tested dilution, the results were interpreted as at or above the next twofold dilution. Certain antimicrobial agents (chloramphenicol, tetracycline, ampicillin, penicillin, clindamycin) were included in both panels, for testing anaerobes and Gram-positive organisms. The results of the panel with a wider range were chosen for presentation of the results for these agents. Breakpoints were defined according to the Clinical and Laboratory Standards Institute (CLSI) (M11-A8, 2012, and M-100-S23, 2013) recommendations (CLSI, 2012, 2013) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, and are listed in Table 3. The resistance rates in two populations were compared with Fisher's exact test, $P<0.01$ was considered significant.

Bacterial isolates. A total of 188 isolates of *C. difficile* from Slovenia were selected from our collection: 92 human strains were isolated from patients with clinical manifestation of CDI in 2008–2011, and 96 animal isolates, mostly from animals without clinical signs of intestinal disorder, which were isolated in 2005–2011. The animal isolates included six isolates from a poultry environment (water, litter and soil). The isolates have been characterized previously (Avbersek *et al.*, 2009; M. Rupnik, A. Andlovic & T. Lejko-Zupanc, unpublished data). They belonged to 44 different PCR ribotypes. The origins of the isolates are presented in Table 1. 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.

Quality control. Control strains *C. difficile* ATCC 700057, *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741 were included for the internal control of the procedure. The tests for ten isolates were performed in duplicate.

RESULTS AND DISCUSSION

The results for the MIC_{50} and MIC_{90} for all 30 tested antimicrobials against the 188 human and animal isolates are summarized in Table 2. The proportions of resistant isolates of human and animal origin for all tested antimicrobials are listed in Table 3. The most prevalent PCR ribotypes in Slovenia (014/020, 002, 001/072, 012 and others) are shared between humans and animals (Janezic *et al.*, 2012). In our study, the following common PCR ribotypes were tested: 014/020, 002, 001/072, 029, 150, SLO 080 (internal nomenclature, non-toxigenic strain) and 045. Table 4 shows the percentage of resistant isolates for the five most prevalent PCR ribotypes that are shared between animals and humans for selected antimicrobials.

From the viewpoint of antibiotic therapy for humans, the emerging resistance to metronidazole and vancomycin is the main concern and all strains tested in this study were susceptible to both antimicrobials, with low MIC_{90} values of 0.5 and $\leqslant$0.5 μg ml^{-1}, respectively. Three isolates (human PCR ribotype 078 and two pig isolates of PCR ribotype 045) had metronidazole MIC values of 2 μg ml^{-1}, which is just at the recently published EUCAST breakpoint (EUCAST, 2013) based on epidemiological cut-off values, which distinguish wild-type isolates from those with reduced susceptibility.

Fluoroquinolone resistance in Europe has been connected with hospital outbreaks and is common among the prevalent PCR ribotypes (Spigaglia *et al.*, 2008; Solomon *et al.*, 2011). In studies of clinical isolates and animal isolates in Europe, resistance to moxifloxacin was associated with mutations in the quinolone resistance-determining region *gyr* genes, with the majority of the resistant strains showing amino acid substitution in GyrA (Spigaglia *et al.*, 2008, 2010; Keessen *et al.*, 2013). The fluoroquinolones have been associated with CDI in particular in outbreaks caused by the PCR ribotype 027 strain and the acquisition of resistance has been assumed as one of the reasons for its dissemination (Muto *et al.*, 2005; Kuijper *et al.*, 2008; Spigaglia *et al.*, 2008). As expected, the results for ciprofloxacin show a high rate of resistance in humans and animals (100 and 97.9 %, respectively). All strains with decreased susceptibility to moxifloxacin showed MIC values for levofloxacin of $\geqslant$4 μg ml^{-1}, but this has also

Table 1. Origin of *C. difficile* tested in the study

Host	No. of strains tested	No. of different PCR ribotypes tested	PCR ribotypes*	
			Common	Specific for host
Human	92	38	002, 001, 012, 014/020, 029, 046, 056, 064, SLO 116	017, 027, 078, 126, 003, 015, 023, 005, 087, 011/049, SLO 064, SLO 083, SLO 009, SLO 020, SLO 036, SLO 010, SLO 011, SLO 023, SLO 025, SLO 027, SLO 033, SLO 046, SLO 048, SLO 063, SLO 070, SLO 110, SLO 120, SLO 127, SLO 134
Poultry	39	13	014/020, 002, 001, 010, 045, 046, SLO 116	018, 070, SLO 020, SLO 131, SLO 160, SLO 080
Pig	42	4	010, 029, 045	150
Ruminant	9	7	014/020, 002, 033, 056, 010	SLO 061, SLO 151
Dog	5	3	014/020, 010, 012	–
Horse	1	1	033	–

*Isolates were designated by standard Cardiff nomenclature or internal nomenclature.

Table 2. MICs for human and animal isolates of *C. difficile* by broth microdilution

Antimicrobial agent	MIC (μg ml^{-1})						
	MIC range	MIC_{50}*			MIC_{90}*		
		All	Human	Animal	All	Human	Animal
Metronidazole	⩽0.5–2	⩽0.5	⩽0.5	⩽0.5	1	1	⩽0.5
Vancomycin	⩽0.25–1	0.5	0.5	0.5	0.5	0.5	0.5
Daptomycin	⩽0.5–>4	1	1	1	4	2	4
Rifampicin	⩽0.5–>4	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5	⩽0.5
Linezolid	⩽1–2	⩽1	⩽1	⩽1	2	2	2
Oxacillin	⩽0.25–>4	⩽0.25	⩽0.25	⩽0.25	>4	2	>4
Ampicillin	⩽0.5–4	1	1	1	1	2	2
Ampicillin/sulbactam	⩽0.5/0.25–2/1	1/0.5	1/0.5	1/0.5	1/0.5	1/0.5	1/0.5
Penicillin	0.5–8	1	1	1	4	4	4
Amoxicillin/clavulanic acid	⩽0.5/0.25–2/1	1/0.5	⩽0.5/0.25	⩽0.5/0.25	1/0.5	1/0.5	1/0.5
Piperacillin	⩽4–16	8	8	8	8	8	8
Piperacillin/tazobactam	2/4–16/4	4/4	8/4	4/4	8/4	8/4	8/4
Mezlocillin	⩽4–8	⩽4	⩽4	⩽4	8	8	⩽4
Cefotetan	8–64	16	16	16	16	32	16
Cefoxitin	>32	>32	>32	>32	>32	>32	>32
Gentamicin	>16	>16	>16	>16	>16	>16	>16
Streptomycin	⩽1000–>1000	⩽1000	⩽1000	⩽1000	⩽1000	>1000	>1000
Trimethoprim/sulfamethoxazole	⩽0.5/9.5–>4/76	2/38	2/38	2/38	>4/76	4/76	>4/76
Quinupristin/dalfopristin	⩽0.5–>4	⩽0.5	⩽0.5	⩽0.5	1	1	1
Tetracycline	⩽0.25–>16	⩽0.25	⩽0.25	⩽0.25	16	16	8
Tigecycline	⩽0.03–0.25	⩽0.03	⩽0.03	⩽0.03	0.06	0.06	0.12
Erythromycin	⩽0.25–>4	0.5	1	0.5	>4	>4	1
Clindamycin	⩽0.25–>8	4	4	4	>8	>8	8
Chloramphenicol	⩽2–16	4	4	4	4	4	4
Moxifloxacin	1–>4	2	1	2	2	4	2
Ciprofloxacin	>2	>2	>2	>2	>2	>2	>2
Levofloxacin	4–>4	4	4	4	>4	>4	>4
Nitrofurantoin	⩽32	⩽32	⩽32	⩽32	⩽32	⩽32	⩽32
Imipenem	2–>8	8	>8	8	>8	>8	>8
Meropenem	1–8	2	2	1	2	2	2

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

Table 3. The proportions of resistant isolates of human and animal origin for all tested antimicrobials

Antimicrobial	Breakpoint (μg ml^{-1})*	Resistance (%)	
		Human (n=92)	Animal (n=96)
Ampicillin	⩾2	22.8	20.8
Penicillin	⩾2	40.2	38.5
Amoxicillin/clavulanic acid	⩾16/8	0	0
Oxacillin	⩾4	4.3	17.7
Ampicillin/sulbactam	⩾32/16	0	0
Piperacillin	⩾128	0	0
Mezlocillin	⩾128	0	0
Piperacillin/tazobactam	⩾128/2	0	0
Cefotetan	⩾64	1.1	0
Cefoxitin	⩾64	98.9	97.9
Gentamicin	⩾16†	78.3	91.7
Streptomycin	⩾1000†	20.7	29.2
Ciprofloxacin	⩾4†	100	97.9
Levofloxacin	⩾4†	98.9	100
Moxifloxacin	>4‡	11.9	4.2
Metronidazole	⩾2‡	0	0
Vancomycin	⩾2‡	0	0
Tetracycline	⩾16	11.9	9.4
Tigecycline	>4‡	1.1	2.1
Daptomycin	>4‡	2.2	2.1
Rifampicin	>4†	2.2	1.0
Trimethoprim/sulfamethoxazole	>4/76†	10.9	26.0
Erythromycin	⩾8†	13.0	8.3
Clindamycin	⩾8	42.4	38.5
Imipenem	⩾16	53.3	28.1
Meropenem	⩾16	0	1.0
Chloramphenicol	⩾32	0	0
Linezolid	⩾8†	0	0
Quinupristin/dalfopristin	⩾4†	0	2.1

*Breakpoints were defined according to the CLSI (M11-A8, 2012, and M-100-S23, 2013) recommendations for anaerobes (CLSI, 2012, 2013).
†Breakpoints according to the CLSI M-100-S23 (interpretative values for *Staphylococcus aureus*, streptomycin for *Enterococcus*) (CLSI, 2013).
‡Breakpoints according to EUCAST guidelines based on the epidemiological cut-off value for the 'wild-type' population.

been confirmed in other strains susceptible to moxifloxacin, with the MIC_{90} for levofloxacin being >4 μg ml^{-1}. For moxifloxacin, the resistance rates were 4.2 % (4/96) in animal isolates and 12 % (11/92) in human isolates, which seems low compared to published results in Europe, where a 37.5 % level of resistant strains was observed (Barbut *et al.*, 2007). However, it is shown in the same European report that PCR ribotypes 014/020 and 002 had low moxifloxacin resistance rates (6.9 and 0 %, respectively). This is comparable with our results for these two PCR ribotypes, which are the most prevalent in Slovenia: the resistance rates for PCR ribotype 014/020 were 8.3 % (1/12) for isolates of human origin and 0 % (0/11) for isolates of animal origin. None of the isolates of PCR ribotype 002 was resistant to moxifloxacin. In neighbouring Italy, Spigaglia *et al.* (2010) reported that the number of fluoroquinolone-resistant *C. difficile* strains increased from 10 % in 1985–2001 to 56 % in 2002–2008. One of the reasons for such a difference in resistance rates might also be a large inter-country variation in the consumption of quinolones. The reports for the two countries correlate with lower resistance in our strains, as the human quinolone consumption is lower in Slovenia than in Italy (ECDC, 2013). The reports on antimicrobial consumption in veterinary medicine are scarce, but a recent report on sales of antibiotic in Slovenia showed that veterinary use of antimicrobials is moderate, and the most used antimicrobial classes are penicillins, followed by tetracyclines and sulfonamides (EMA, 2012).

Resistance to MLS_B (macrolide–lincosamide–streptogramin B) antibiotics in *C. difficile* is most commonly due to the *erm*(B) gene encoding rRNA methylase carried by conjugative transposon Tn*5398*, although there is a great heterogeneity in genetic arrangements of resistance determinants (Spigaglia *et al.*, 2011). An increased number of *erm*(B) negative resistant strains has been described, where the resistance mechanisms still have to be identified (Ackermann *et al.*, 2003; Kuijper *et al.*, 2008; Spigaglia *et al.*, 2011).

Table 4. Resistance to selected antimicrobials for the five most common PCR ribotypes of *C. difficile* shared by humans and animals

CLI, clindamycin; ERY, erythromycin; IMI, imipenem; MOX, moxifloxacin; OXA, oxacillin; RIF, rifampicin; STR, streptomycin; TET, tetracycline.

PCR ribotype*	Source and no. of isolates	Resistance (%)									
		TET	CLI	ERY	MOX	RIF	OXA	STR	IMI	MOX+RIF	ERY+CLI+ TET
014/020	Humans (n=12)	0	41.7	0	8.3	0	0	25	50	0	0
	Animals (n=11)	0	36.4	0	0	0	0	0	27.3	0	0
002	Humans (n=11)	0	9.1	0	0	0	0	9	45.5	0	0
	Animals (n=7)	0	85.7	0	0	0	0	14.3	85.7	0	0
029	Humans (n=4)	0	25	0	0	0	0	25	25	0	0
	Animals (n=2)	0	0	0	0	0	0	0	0	0	0
046	Humans (n=1)	100	100	100	0	0	0	100	100	0	100
	Animals (n=2)	100	50	0	0	0	0	0	100	0	0
012	Humans (n=3)	33.3	100	100	0	0	0	33.3	0	0	33.3
	Animals (n=1)	100	0	100	100	100	0	0	0	100	0

*PCR ribotype 001/072, which is among the most prevalent ribotypes in Slovenia, is not included as there was no resistance found.

In our study, MIC values for clindamycin and erythromycin, members of the MLS_B group of antibiotics, were distributed within the whole tested range ($\leqslant$0.25– >8 μg ml^{-1} and $\leqslant$0.25 – >4 μg ml^{-1}, respectively). This is consistent with findings of a wide range of susceptibility to those antimicrobial agents. However, 38.5 % (37/96) of animal strains and 42.4 % (39/92) of human strains had reduced susceptibility to clindamycin, 69.7 % (53/76) of these strains displayed a MIC of 8 μg ml^{-1}, which is just at the breakpoint limit. Among the strains interpreted as having reduced susceptibility, 69.7 % (53/76) of these strains displayed an MIC of 8 μg ml^{-1}, which is just at the breakpoint limit. Reduced clindamycin susceptibility was observed among several PCR ribotypes, among them PCR ribotype 014/020, 017, 078, 012 and 126. Interestingly, reduced clindamycin susceptibility has been observed in a high proportion of animal isolates of PCR ribotype 002 (85.7 %, 6/7), as opposed to human isolates of the same PCR ribotype, where the proportion was 9.1 % (1/11). Other reports on animal strains also describe great variation in susceptibilities to clindamycin and erythromycin, and the same for rifampicin, chloramphenicol and tetracycline (Båverud *et al.*, 2004; Post & Songer, 2004; Janezic *et al.*, 2012). Among strains with reduced susceptibility to clindamycin, 70.3 % (26/37) of animal isolates and 56.4 % (22/39) of human isolates showed reduced susceptibility only to clindamycin. A total of 8.1 % (3/37) of animal isolates and 5.1 % (2/39) of human isolates had reduced susceptibility only to erythromycin, and 21.6 % (8/37) of animal isolates and 38.5 % (15/39) of human isolates had reduced susceptibility to both antimicrobials. An unusual reduced clindamycin susceptibility and erythromycin sensitive type of pattern has been described before in *erm*(B) negative isolates with low-level clindamycin resistance (Spigaglia *et al.*, 2010), and also with high-level resistance (Solomon *et al.*, 2011). However, considering the limitation of the method, which did not allow us to recognize high-level resistance to both antimicrobials, these strains should be tested further.

Increased MICs for rifampicin were observed only in three strains, two human isolates of PCR ribotype 017 and one dog isolate of PCR ribotype 012. They were all resistant to multiple antimicrobials, including moxifloxacin, which is a usual co-resistance, based on coexisting amino acid substitutions in Gyr A and RpoB, the β subunit of RNA polymerase (Spigaglia *et al.*, 2011). The rifamycin group of antibiotics has been recently tested for use in relapses of CDI, but the reports of an increased number of resistant isolates cause concern and suggest moderate use (Curry *et al.*, 2009; Norén *et al.*, 2010).

In general, tetracycline displayed low MICs ($\leqslant$0.25 μg ml^{-1}), i.e. in 65.6 % animal isolates and 83.7 % human isolates. The resistance rate was 11.9 % in human strains and 9.4 % in animal strains. The resistant strains belonged

to various PCR ribotypes, but with the highest proportions of resistance among PCR ribotypes 046, 012, 017 and 078, which is consistent with other reports on the predominance of tetracycline resistance in these PCR ribotypes (Barbut *et al.*, 2007; Huang *et al.*, 2009; Bakker *et al.*, 2010). For the related antibiotic tigecycline, 1.1 and 2.1 % resistant strains were found in human and animal isolates, respectively.

Regarding aminoglycosides, resistance to streptomycin was often found in pig isolates of PCR ribotypes 045 and 150, and in human isolates of PCR ribotypes 078, 014/020, 017 and 027. The proportion of streptomycin- and oxacillin-resistant strains was high in PCR ribotype 045 [57.1 % (12/21) and 76.2 % (16/21), respectively], which is the most prevalent PCR ribotype on pig farms in Slovenia (Avbersek *et al.*, 2009). This could be associated with the use of antimicrobials in intensive pig farming. On the majority of the farms with resistant PCR ribotype 045, the application of antimicrobials to piglets was usual, either for prophylaxis or treatment. The commonly used antimicrobials were predominantly amoxicillin/clavulanic acid, which was regularly given to 1-day-old piglets, gentamicin and trimethoprim/sulfadoxine. The resistance to gentamicin was significantly higher in animals, and all but two streptomycin-resistant isolates were also resistant to gentamicin.

However, tested poultry isolates did not show distinctively decreased susceptibility even if there was a regular use of antimicrobials for flock treatment (amoxicillin, enrofloxacin and oxytetracycline for broilers, and oxytetracycline and amoxicillin/clavulanic acid for breeding flocks). In contrast to the pig isolates, all isolates but two (PCR ribotypes 001 and 002) were susceptible to streptomycin, and all were susceptible to oxacillin. MICs for tetracycline showed bimodal distribution: the great majority of the isolates had a low MIC ($\leqslant 0.25$ μg ml^{-1}) and 10.3 % (4/39) of isolates were resistant (belonging to PCR ribotypes 046 and non-toxigenic SLO 080).

Several multiresistant strains were found in this study, in both human and animal isolates belonging to PCR ribotypes 012, 017, 027, 045, 046, 078 and 150, and also to non-toxigenic strains of PCR ribotypes 010 and SLO 080. Combined reduced susceptibility/resistance to clindamycin, erythromycin, rifampicin and moxifloxacin was found in two human isolates of PCR ribotype 017; one of them was additionally resistant to tetracycline. One human isolate of PCR ribotype 078 was resistant to tetracycline, daptomycin, moxifloxacin, streptomycin and oxacillin.

There were several animal isolates with decreased susceptibility to multiple antibiotics. A dog isolate of PCR ribotype 012 was resistant to penicillin, ampicillin, tetracycline, moxifloxacin, gentamicin, streptomycin and rifampicin. The dog had been treated for a prolonged period of time due to chronic enteric disorders and had received various antimicrobials, including amoxicillin, gentamicin and enrofloxacin, before being submitted to bacteriological examination. It may be mentioned that another multiresistant bacterium, extended-spectrum β-lactamase-producing *Escherichia coli*, was isolated from the same dog. Although no direct transmission *C. difficile* from companion animals to humans has been described to date, there are several studies that indicate the risk for zoonotic transmission (Lefebvre *et al.*, 2006; Weese *et al.*, 2010).

Additionally, non-toxigenic strains isolated from animals had reduced susceptibility/resistance to multiple antibiotics: PCR ribotype 010 to clindamycin, erythromycin and streptomycin, and SLO 080 (internal nomenclature) to clindamycin, oxacillin and streptomycin. Non-toxigenic strains could serve as a source of resistance determinants for pathogenic strains.

In several studies it has been described that most prevalent strains regardless of PCR ribotype displayed higher resistance (Huang *et al.*, 2010; Taori *et al.*, 2010; Solomon *et al.*, 2011), but the most prevalent PCR ribotype 014/020 in our study overall displayed low resistance, including to erythromycin (0 %), rifampicin (0 %), daptomycin (0 %) and moxifloxacin (8.3 % in human isolates and 0 % in animal isolates). The clindamycin resistance rate was 41.7 % (5/12) in human isolates and 36.4 % (4/11) in animal isolates, and the imipenem resistance rate was 50 % (6/12) in human isolates and 27.3 % (3/11) in animal isolates.

When comparing the prevalence of antimicrobial resistance of animal and human isolates, some significant differences were found. There was a significantly higher rate of resistance to oxacillin, gentamicin and trimethoprim/sulfamethoxazole in isolates of animal origin ($P<0.01$). The most significant difference between animal and human populations ($P=0.0006$) was found in the level of imipenem resistance, with a prevalence of 53.3 % in isolates of human origin and 28.1 % in isolates of animal origin. This could be explained by different antibiotic pressure, as carbapenems are not used in veterinary medicine. Despite these differences and the different use of antimicrobials in animals and humans, the overall resistance patterns seemed to be more PCR ribotype related than species related. However, the selection of PCR ribotypes was diverse ($n=44$) and the number of tested isolates within one PCR ribotype too low to make conclusions on strain transmission between the two populations.

Overall, the results show similar MICs for the majority of tested antimicrobials for isolates from human and animal sources, which were collected from the same geographical region and in the same time interval. This supports the hypothesis that *C. difficile* could be transmissible between human and animal hosts. Resistant isolates have been found in all animal species tested, including food and companion animals, and also among non-toxigenic isolates. This raises concerns about transmission to humans, either directly or indirectly via food from animal origins or the environment, and also about the transmission of resistant determinants from non-toxigenic to pathogenic strains.

The broth microdilution method is currently limited to the testing of the *B. fragilis* group according to the CLSI (2012), but it proved convenient for monitoring purposes. Ten isolates were tested in duplicate. MICs for the duplicates did not differ for more than one twofold dilution for any of the ten tested strains. In general, MIC breakpoints were easily determined. There is currently no commercially available selection of broth microdilution platforms for testing *C. difficile*. Therefore, the limitation of our study was too narrow dilution ranges in the case of some antimicrobial agents, which did not allow us to identify highly resistant strains. However, the method shows a potential for extensive screening for resistant strains and obtaining results for a wide variety of antimicrobials.

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REFERENCES

Ackermann, G., Degner, A., Cohen, S. H., Silva, J., Jr & Rodloff, A. C. (2003). Prevalence and association of macrolide-lincosamide-streptogramin B (MLS_B) resistance with resistance to moxifloxacin in *Clostridium difficile*. *J Antimicrob Chemother* **51**, 599–603.

Alvarez-Perez, S., Blanco, J. L., Bouza, E., Alba, P., Gibert, X., Maldonado, J. & Garcia, M. E. (2009). Prevalence of *Clostridium difficile* in diarrhoeic and non-diarrhoeic piglets. *Vet Microbiol* **137**, 302–305.

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**, 252–255.

Bakker, D., Corver, J., Harmanus, C., Goorhuis, A., Keessen, E. C., Fawley, W. N., Wilcox, M. H. & Kuijper, E. J. (2010). Relatedness of human and animal *Clostridium difficile* PCR ribotype 078 isolates determined on the basis of multilocus variable-number tandem-repeat analysis and tetracycline resistance. *J Clin Microbiol* **48**, 3744–3749.

Barbut, F., Mastrantonio, P., Delmée, M., Brazier, J., Kuijper, E. & Poxton, I. on behalf of the European Study Group on *Clostridium difficile* (ESGCD) (2007). Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. *Clin Microbiol Infect* **13**, 1048–1057.

Bartlett, J. G. (1992). Antibiotic-associated diarrhea. *Clin Infect Dis* **15**, 573–581.

Båverud, V., Gustafsson, A., Franklin, A., Aspán, A. & Gunnarsson, A. (2003). *Clostridium difficile:* prevalence in horses and environment, and antimicrobial susceptibility. *Equine Vet J* **35**, 465–471.

Båverud, V., Gunnarsson, A., Karlsson, M. & Franklin, A. (2004). Antimicrobial susceptibility of equine and environmental isolates of *Clostridium difficile*. *Microb Drug Resist* **10**, 57–63.

CLSI (2012). *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*; Approved Standard, M11-A8, 8th edn. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI (2013). *Performance Standards for Antimicrobial Susceptibility Testing*; 23rd Informational Supplement M100-S23. Wayne, PA: Clinical and Laboratory Standards Institute.

Curry, S. R., Marsh, J. W., Shutt, K. A., Muto, C. A., O'Leary, M. M., Saul, M. I., Pasculle, A. W. & Harrison, L. H. (2009). High frequency of rifampin resistance identified in an epidemic *Clostridium difficile* clone from a large teaching hospital. *Clin Infect Dis* **48**, 425–429.

ECDC (2013). *Surveillance of Antimicrobial Consumption in Europe, 2010*. Stockholm: European Centre for Disease Prevention and Control.

EMA (2012). *Sales of Veterinary Antimicrobial Agents in 19 EU/EEA Countries in 2010*; EMA/88728/2012. London: European Medicines Agency.

EUCAST (2013). *Breakpoint Tables for Interpretation of MICs and Zone Diameters*; version 3.0, 2013. http://www.eucast.org/clinical_breakpoints (accessed January 2013).Basel; European Committee on Antimicrobial Susceptibility Testing.

Fry, P. R., Thakur, S., Abley, M. & Gebreyes, W. A. (2012). Antimicrobial resistance, toxinotype, and genotypic profiling of *Clostridium difficile* isolates of swine origin. *J Clin Microbiol* **50**, 2366–2372.

Gould, L. H. & Limbago, B. (2010). *Clostridium difficile* in food and domestic animals: a new foodborne pathogen? *Clin Infect Dis* **51**, 577–582.

Hammitt, M. C., Bueschel, D. M., Keel, M. K., Glock, R. D., Cuneo, P., DeYoung, D. W., Reggiardo, C., Trinh, H. T. & Songer, J. G. (2008). A possible role for *Clostridium difficile* in the etiology of calf enteritis. *Vet Microbiol* **127**, 343–352.

Huang, H., Weintraub, A., Fang, H. & Nord, C. E. (2009). Antimicrobial resistance in *Clostridium difficile*. *Int J Antimicrob Agents* **34**, 516–522.

Huang, H., Weintraub, A., Fang, H., Wu, S., Zhang, Y. & Nord, C. E. (2010). Antimicrobial susceptibility and heteroresistance in Chinese *Clostridium difficile* strains. *Anaerobe* **16**, 633–635.

Janezic, S., Ocepek, M., Zidaric, V. & Rupnik, M. (2012). *Clostridium difficile* genotypes other than ribotype 078 that are prevalent among human, animal and environmental isolates. *BMC Microbiol* **12**, 48.

Keel, K., Brazier, J. S., Post, K. W., Weese, S. & Songer, J. G. (2007). Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. *J Clin Microbiol* **45**, 1963–1964.

Keessen, E. C., Hensgens, M. P. M., Spigaglia, P., Barbanti, F., Sanders, I. M. J. G., Kuijper, E. J. & Lipman, L. J. A. (2013). Antimicrobial susceptibility profiles of human and piglet *Clostridium difficile* PCR-ribotype 078. *Antimicrob Resist Infect Control* **2**, 14.

Koene, M. G. J., Mevius, D., Wagenaar, J. A., Harmanus, C., Hensgens, M. P. M., Meetsma, A. M., Putirulan, F. F., van Bergen, M. A. P. & Kuijper, E. J. (2012). *Clostridium difficile* in Dutch animals: their presence, characteristics and similarities with human isolates. *Clin Microbiol Infect* **18**, 778–784.

Kuijper, E. J., Barbut, F., Brazier, J. S., Kleinkauf, N., Eckmanns, T., Lambert, M. L., Drudy, D., Fitzpatrick, F., Wiuff, C. & other authors (2008). Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. *Euro Surveill* **13**, 18942.

Lefebvre, S. L., Arroyo, L. G. & Weese, J. S. (2006). Epidemic *Clostridium difficile* strain in hospital visitation dog. *Emerg Infect Dis* **12**, 1036–1037.

Marks, S. L. & Kather, E. J. (2003). Antimicrobial susceptibilities of canine *Clostridium difficile* and *Clostridium perfringens* isolates to commonly utilized antimicrobial drugs. *Vet Microbiol* **94**, 39–45.

Muto, C. A., Pokrywka, M., Shutt, K., Mendelsohn, A. B., Nouri, K., Posey, K., Roberts, T., Croyle, K., Krystofiak, S. & other authors (2005). A large outbreak of *Clostridium difficile*-associated disease with an unexpected proportion of deaths and colectomies at a

teaching hospital following increased fluoroquinolone use. *Infect Control Hosp Epidemiol* **26**, 273–280.

Norén, T., Alriksson, I., Akerlund, T., Burman, L. G. & Unemo, M. (2010). In vitro susceptibility to 17 antimicrobials of clinical *Clostridium difficile* isolates collected in 1993-2007 in Sweden. *Clin Microbiol Infect* **16**, 1104–1110.

Pirs, T., Ocepek, M. & Rupnik, M. (2008). Isolation of *Clostridium difficile* from food animals in Slovenia. *J Med Microbiol* **57**, 790–792.

Post, K. W. & Songer, J. G. (2004). Antimicrobial susceptibility of *Clostridium difficile* isolated from neonatal pigs with enteritis. *Anaerobe* **10**, 47–50.

Rodriguez-Palacios, A., Stämpfli, H. R., Duffield, T., Peregrine, A. S., Trotz-Williams, L. A., Arroyo, L. G., Brazier, J. S. & Weese, J. S. (2006). *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis* **12**, 1730–1736.

Rupnik, M., Wilcox, M. H. & Gerding, D. N. (2009). *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol* **7**, 526–536.

Solomon, K., Fanning, S., McDermott, S., Murray, S., Scott, L., Martin, A., Skally, M., Burns, K., Kuijper, E. & other authors (2011). PCR ribotype prevalence and molecular basis of macrolide-lincosamide-streptogramin B (MLS_B) and fluoroquinolone resistance in Irish clinical *Clostridium difficile* isolates. *J Antimicrob Chemother* **66**, 1976–1982.

Songer, J. G. & Anderson, M. A. (2006). *Clostridium difficile*: an important pathogen of food animals. *Anaerobe* **12**, 1–4.

Spigaglia, P., Barbanti, F., Mastrantonio, P., Brazier, J. S., Barbut, F., Delmée, M., Kuijper, E. & Poxton, I. R. on behalf of the European Study Group on *Clostridium difficile* (ESGCD) (2008). Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. *J Med Microbiol* **57**, 784–789.

Spigaglia, P., Barbanti, F., Dionisi, A. M. & Mastrantonio, P. (2010). *Clostridium difficile* isolates resistant to fluoroquinolones in Italy: emergence of PCR ribotype 018. *J Clin Microbiol* **48**, 2892–2896.

Spigaglia, P., Barbanti, F. & Mastrantonio, P. on behalf of the European Study Group on *Clostridium difficile* (ESGCD) (2011). Multidrug resistance in European *Clostridium difficile* clinical isolates. *J Antimicrob Chemother* **66**, 2227–2234.

Taori, S. K., Hall, V. & Poxton, I. R. (2010). Changes in antibiotic susceptibility and ribotypes in *Clostridium difficile* isolates from southern Scotland, 1979-2004. *J Med Microbiol* **59**, 338–344.

Weese, J. S. (2010). *Clostridium difficile* in food–innocent bystander or serious threat? *Clin Microbiol Infect* **16**, 3–10.

Weese, J. S., Finley, R., Reid-Smith, R. R., Janecko, N. & Rousseau, J. (2010). Evaluation of *Clostridium difficile* in dogs and the household environment. *Epidemiol Infect* **138**, 1100–1104.

Zidaric, V., Zemljic, M., Janezic, S., Kocuvan, A. & Rupnik, M. (2008). High diversity of *Clostridium difficile* genotypes isolated from a single poultry farm producing replacement laying hens. *Anaerobe* **14**, 325–327.