J Antimicrob Chemother 2016; **71**: 2213–2217
doi:10.1093/jac/dkw124 Advance Access publication 29 April 2016

Journal of Antimicrobial Chemotherapy

Clostridium difficile clade 5 in Australia: antimicrobial susceptibility profiling of PCR ribotypes of human and animal origin

Daniel R. Knight[1] and Thomas V. Riley[1,2]*

[1]*Microbiology & Immunology, School of Pathology and Laboratory Medicine, The University of Western Australia, Nedlands, Western Australia, Australia;* [2]*Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands 6009, Western Australia, Australia*

*Corresponding author. Tel: +61-8-6383-4355; E-mail: thomas.riley@uwa.edu.au

Received 15 December 2015; returned 10 February 2016; revised 29 February 2016; accepted 15 March 2016

Objectives: Increasing reports of genetic overlap between animal and human sources of *Clostridium difficile* necessitate an understanding of antimicrobial susceptibility and resistance in these populations. In this study, we sought to investigate the *in vitro* activities of 13 antimicrobials against a unique collection of clade 5 *C. difficile* isolates of Australian animal and human origin.

Methods: The collection comprised 171 *C. difficile* strains of human ($n=91$) and animal ($n=80$) origin in Australia in the last decade. The collection encompassed seven different clade 5 PCR ribotypes (RTs; 033, 078, 126, 127, 237, 281 and 288), seven STs and three toxin gene profiles. MICs were determined by agar incorporation methodology.

Results: All isolates were fully susceptible, with little variation between strain populations, to vancomycin, metronidazole, fidaxomicin, rifaximin, amoxicillin/clavulanate, meropenem and piperacillin/tazobactam. Resistance to one or more of tetracycline, moxifloxacin, clindamycin or erythromycin was seen in 26.9% (46 of 171) of isolates, predominantly RTs 126 and 078 of human origin. Fifty per cent of this group were MDR. Human and animal isolates belonging to RTs 033, 237, 281 and 288 presented very similar and generally susceptible phenotypic profiles.

Conclusions: Similar pan-susceptible phenotypic profiles seen for a large proportion of human and animal isolates of RTs 033, 237, 281 and 288 support the hypothesis of a common source. Conversely, human strain populations of RTs 126 and 127 show high proportions of antimicrobial resistance and limited phenotypic overlap with their animal counterparts.

Introduction

Clostridium difficile presents a significant burden to public health systems worldwide.[1] Exposure of the gut microbiota to antimicrobial therapy, particularly clindamycin, cephalosporins and aminopenicillins, is the major risk factor for developing *C. difficile* infection (CDI) in humans and non-human animals.[2] Australia has in recent years seen a substantial increase in the rate of CDI, particularly the proportion of cases attributed to community acquisition (CA-CDI).[3] In both the northern hemisphere and in Australia, livestock have been identified as potential reservoirs for CDI, with increasing reports of significant genetic overlap between *C. difficile* isolates of animal and human origin.[4] Outside Australia, PCR ribotype (RT) 078 is well-established in porcine and bovine populations and greatly contributes to the burden of CA-CDI.[5] We have previously shown that Australian livestock are reservoirs for a heterogeneous population of *C. difficile* RTs belonging predominantly to the same lineage as RT 078, MLST clade 5.[6–8] Many of these clade 5 RTs have been isolated from humans with CDI in Australia and are of emerging One Health importance (www.onehealthinitiative.com/).[9]

In this study, to define further the extent of overlap, a unique collection of clade 5 RTs originating from human and non-human sources was investigated for phenotypic susceptibility and resistance to 13 antimicrobial agents.

Materials and methods

Clade 5 strain collection

The collection comprised 171 *C. difficile* strains isolated from human (cases of CDI, $n=91$) and animal (porcine, bovine, equine and macropodine, $n=80$) origin in Australia in the last decade (see Table S1, available as Supplementary data at *JAC* Online). Previous molecular analysis for RT, toxin gene profile and MLST ST characterized the strains as belonging to the evolutionarily divergent lineage MLST clade 5, encompassing seven

different RTs (033, 078, 126, 127, 237, 281 and 288), seven STs (11, 167, 169, 174, 258, 315 and 316) and three toxin gene profiles (*tcdA*$^+$/*tcdB*$^+$/*cdtAB*$^+$, *tcdA*$^-$/*tcdB*$^+$/*cdtAB*$^+$ and *tcdA*$^-$/*tcdB*$^-$/*cdtAB*$^+$) (see Table S1).

MIC determination using agar incorporation

MICs of fidaxomicin, vancomycin, metronidazole, rifaximin, clindamycin, erythromycin, amoxicillin/clavulanate, ceftriaxone, meropenem, moxifloxacin, tetracycline, piperacillin/tazobactam and trimethoprim were determined by agar incorporation methodology as previously described.[10] Where available, clinical breakpoints for antimicrobial agents are shown in Table 1 and are based on recommendations of EUCAST and CLSI.[11,12]

Results and discussion

All isolates were fully susceptible, with little variation between strain populations, to vancomycin, metronidazole, rifaximin, amoxicillin/clavulanate, meropenem and piperacillin/tazobactam (Table 1). These data are consistent with our recent *C. difficile* national surveillance study[10] and generally agree with *C. difficile* surveillance studies conducted in the northern hemisphere.[13–15] However, we did not observe any reduced susceptibility to meropenem or metronidazole (Table 1), which has often been reported in some European populations.[14–16]

Fidaxomicin showed potent *in vitro* activity against all isolates, irrespective of host origin or strain type (MIC range 0.004–0.25 mg/L), inhibiting 97.1% (166 of 171) of isolates at 0.12 mg/L and all isolates at 0.25 mg/L (Table 1). The *in vitro* activity of fidaxomicin (MIC_{50}/MIC_{90} 0.03/0.12 mg/L) was superior to recommended first-line treatment agents for CDI [metronidazole (MIC_{50}/MIC_{90} 0.5/1 mg/L) and vancomycin (MIC_{50}/MIC_{90} 1/1 mg/L)] (Table 1) and supports the *in vivo* efficacy demonstrated in clinical trials.[2] Currently there are no clinical breakpoints for fidaxomicin; however, the MICs presented herein are several orders of magnitude lower than the concentrations of fidaxomicin that are achieved in the gut lumen and stool.[2] Importantly, no vancomycin or metronidazole resistance was observed. Fidaxomicin, vancomycin and metronidazole were all less active than rifaximin (MIC_{50}/MIC_{90} 0.004/0.008 mg/L) (Table 1). MICs of both trimethoprim and ceftriaxone ranged from 8 to 64 mg/L for all isolates (Table 1).

Overall, the human and animal isolates showed little variation and no significant difference in MICs of vancomycin, metronidazole, fidaxomicin, rifaximin, amoxicillin/clavulanate, meropenem, piperacillin/tazobactam, ceftriaxone or trimethoprim (Table 1). Approximately 27% (46 of 171) of isolates showed resistance to either tetracycline, moxifloxacin, clindamycin or erythromycin with significant differences observed between human and animal populations [human resistant isolates (44 of 91) and animal resistant isolates (2 of 80) (Fisher's exact $P \leq 0.05$)]. ST 11 RTs dominated this resistant group; RTs 126 (50%), 078 (23.9%), 127 (21.7%) and 033 (2.2%) (Figure 1).

Multidrug resistance, defined as resistance to three or more of tetracycline, moxifloxacin, clindamycin or erythromycin, was observed in 50% (23 of 46) of resistant isolates, all of human origin and only within ST 11 (RTs 078 and 126).

Fluoroquinolone resistance in European populations is as high as 40%, and is associated with hospital outbreaks and with transcontinental dissemination of epidemic lineage RT 027.[13,17,18] In this current work, resistance to moxifloxacin was observed in 25% (4 of 16) of RT 078 and 39.3% (11 of 28) of RT 126 strains originating from humans, but was notably absent from the animal strain population (Figure 1). These data contrast with our recent baseline surveillance where only 3.4% (15 of 440) were moxifloxacin resistant, although limited numbers of clade 5 RTs were examined in that study.[10]

Clindamycin exposure has been documented as a specific risk factor for CDI and clindamycin-resistant *C. difficile* are common in Europe, Asia and Australia.[10,13,17,19] Clindamycin-resistant isolates of *C. difficile* are usually also resistant to macrolide antimicrobials such as erythromycin. Overall, combined clindamycin and erythromycin resistance in human clade 5 strains was 50%, comparable to the findings of a recent pan-European study.[13] Clindamycin resistance was observed exclusively in the human strain population in: 31.3% (5 of 16) of RT 078; 35.7% (10 of 28) of RT 126; and 6.5% (2 of 31) of RT 127. Erythromycin resistance was observed in 56.3% (9 of 16) of RT 078, 82.1% (23 of 28) of RT 126 and 6.5% (2 of 31) of RT 127 of human origin, and 4.5% (1 of 22) of animal RT 127 strains.

Although tetracycline is considered low risk for CDI induction, tetracycline-resistant strains of *C. difficile* are clinically important as they represent genetic reservoirs for a range of resistance genes encoded on mobile genetic elements, capable of moving between bacterial species within the human and animal gut.[18] Reports from Europe show tetracycline resistance varies widely between country and RT.[13] Resistance to tetracycline was observed in 56.3% (9 of 16) of RT 078, 82.1% (23 of 28) of RT 126, 22.6% (7 of 31) of RT 127 and 10% (1 of 10) of RT 033 strains originating from humans. For the animal strain population, tetracycline resistance was seen in a single isolate of RT 237 only (Figure 1).

This work presents the first phenotypic evaluation of Australian animal and human clade 5 *C. difficile* strains, and is the first to include non-078 RTs from animal origin. The vast majority of isolates belonging to RTs 033, 237, 281 and 288 originating from both humans and animals shared similar, pan-susceptible phenotypic profiles. These data further contribute to the hypothesis that human and animal clade 5 strains in Australia have a common origin. Interestingly, these RTs all have uncommon toxin profiles; RTs 033 and 288 are both large clostridial toxin negative (A^-B^-) and binary toxin positive (CDT^+), whilst RTs 237 and 281 are negative for *tcdA* ($A^-B^+CDT^+$).

Based on antimicrobial phenotype, the majority of strains of human RT 126 and to a lesser extent human RT 127 appear quite different from their animal counterparts and this may suggest the absence of a common source. However, acquired resistance to antimicrobials in *C. difficile* is conferred by a wide range of mobile genetic elements including *Tn*5397 (tetR) and *Tn*5398 (eryR).[18] These mobile genetic elements form part of the highly dynamic accessory genome and their acquisition and loss from the bacterium occurs readily under forces of selection such as antimicrobial exposure.[18]

It is possible that acquisition of resistance may have occurred within the healthcare system and was driven by differences in antimicrobial selection pressure in piggery and hospital environments. For example, ciprofloxacin is among the 20 most commonly prescribed antimicrobial agents in Australian hospitals accounting for ~2.8% of all prescriptions in 2013 and 2014,[20] but fluoroquinolones are banned from use in food animals of any kind in Australia.[21] These data could account for the

Table 1. Susceptibility and summary MIC data for 13 antimicrobials against 171 clade 5 *C. difficile* isolates, by strain origin

Agent	Host	*N*	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Clinical breakpoints			S, *n* (%)	I, *n* (%)	R, *n* (%)
						S	I	R			
VAN[a]	human	91	0.5–2	1	1	≤2	—	>2	91 (100)	0 (0)	0 (0)
	animal	80	0.5–2	1	1				80 (100)	0 (0)	0 (0)
	total	171	0.5–2	1	1				171 (100)	0 (0)	0 (0)
MTZ[b]	human	91	0.12–1	0.5	0.5	≤2	—	>2	91 (100)	0 (0)	0 (0)
	animal	80	0.12–1	0.5	1				80 (100)	0 (0)	0 (0)
	total	171	0.12–1	0.5	1				171 (100)	0 (0)	0 (0)
FDX	human	91	0.004–0.25	0.03	0.12	—	—	—	—	—	—
	animal	80	0.008–0.25	0.03	0.06				—	—	—
	total	171	0.004–0.25	0.03	0.12				—	—	—
RFX[c]	human	91	0.002–0.008	0.004	0.008	—	—	≥32	—	—	—
	animal	80	0.001–0.015	0.004	0.008				—	—	—
	total	171	0.001–0.015	0.004	0.008				—	—	—
CLI[b]	human	91	0.12 to >32	1	>32	≤2	4	>8	70 (76.9)	4 (4.4)	17 (18.7)
	animal	80	0.12–4	1	4				70 (87.5)	10 (12.5)	0 (0)
	total	171	0.12 to >32	1	4				140 (81.9)	14 (8.2)	17 (9.9)
ERY[b]	human	91	0.12 to >256	1	>256	—	—	>8	—	—	34 (37.4)
	animal	80	0.12 to >256	0.5	1				—	—	1 (1.3)
	total	171	0.12 to >256	1	>256				—	—	35 (20.5)
AMC[b]	human	91	0.12–0.5	0.25	0.25	≤4	8	≥16	91 (100)	0 (0)	0 (0)
	animal	80	0.12–0.5	0.25	0.5				80 (100)	0 (0)	0 (0)
	total	171	0.12–0.5	0.25	0.5				171 (100)	0 (0)	0 (0)
CRO[b]	human	91	8–64	16	16	≤16	32	≥64	87 (95.6)	2 (2.2)	2 (2.2)
	animal	80	8–32	16	32				66 (82.5)	14 (17.5)	0 (0)
	total	171	8–64	16	32				153 (89.5)	16 (9.4)	2 (1.2)
MEM[b]	human	91	0.5–2	2	2	≤4	8	≥16	91 (100)	0 (0)	0 (0)
	animal	80	0.5–2	2	2				80 (100)	0 (0)	0 (0)
	total	171	0.5–2	2	2				171 (100)	0 (0)	0 (0)
MXF[b]	human	91	0.5–16	1	16	≤2	4	≥8	75 (82.4)	1 (1.1)	15 (16.5)
	animal	80	0.5–1	1	1				80 (100)	0 (0)	0 (0)
	total	171	0.5–16	1	2				155 (90.6)	1 (0.6)	15 (8.8)
TET[b]	human	91	0.06–32	0.25	32	≤4	8	≥16	51 (56)	1 (1.1)	39 (42.9)
	animal	80	0.06–16	0.12	0.25				79 (98.8)	0 (0)	1 (1.3)
	total	174	0.06–32	0.12	16				130 (76)	1 (0.6)	40 (23.4)
TZP[b]	human	91	0.5–8	4	8	≤32	64	≥128	91 (100)	0 (0)	0 (0)
	animal	80	1–8	4	8				80 (100)	0 (0)	0 (0)
	total	171	0.5–8	4	8				171 (100)	0 (0)	0 (0)
TMP	human	91	8–64	64	64	—	—	—	—	—	—
	animal	80	16–64	64	64				—	—	—
	total	171	8–64	64	64				—	—	—

VAN, vancomycin; MTZ, metronidazole; FDX, fidaxomicin; RFX, rifaximin; CLI, clindamycin; ERY, erythromycin; AMC, amoxicillin/clavulanate; CRO, ceftriaxone; MEM, meropenem; MXF, moxifloxacin; TET, tetracycline; TZP, piperacillin/tazobactam; TMP, trimethoprim; S, susceptible; I, intermediate; R, resistant.

No breakpoints are currently available for fidaxomicin or trimethoprim.

[a]Breakpoints are those recommended by EUCAST[11] and are based on epidemiological cut-off values that distinguish 'WT' isolates from those with reduced susceptibility.

[b]Breakpoints are those recommended for anaerobes by CLSI.[12]

[c]Resistance (≥32 mg/L) is as described by O'Connor *et al.*[22]

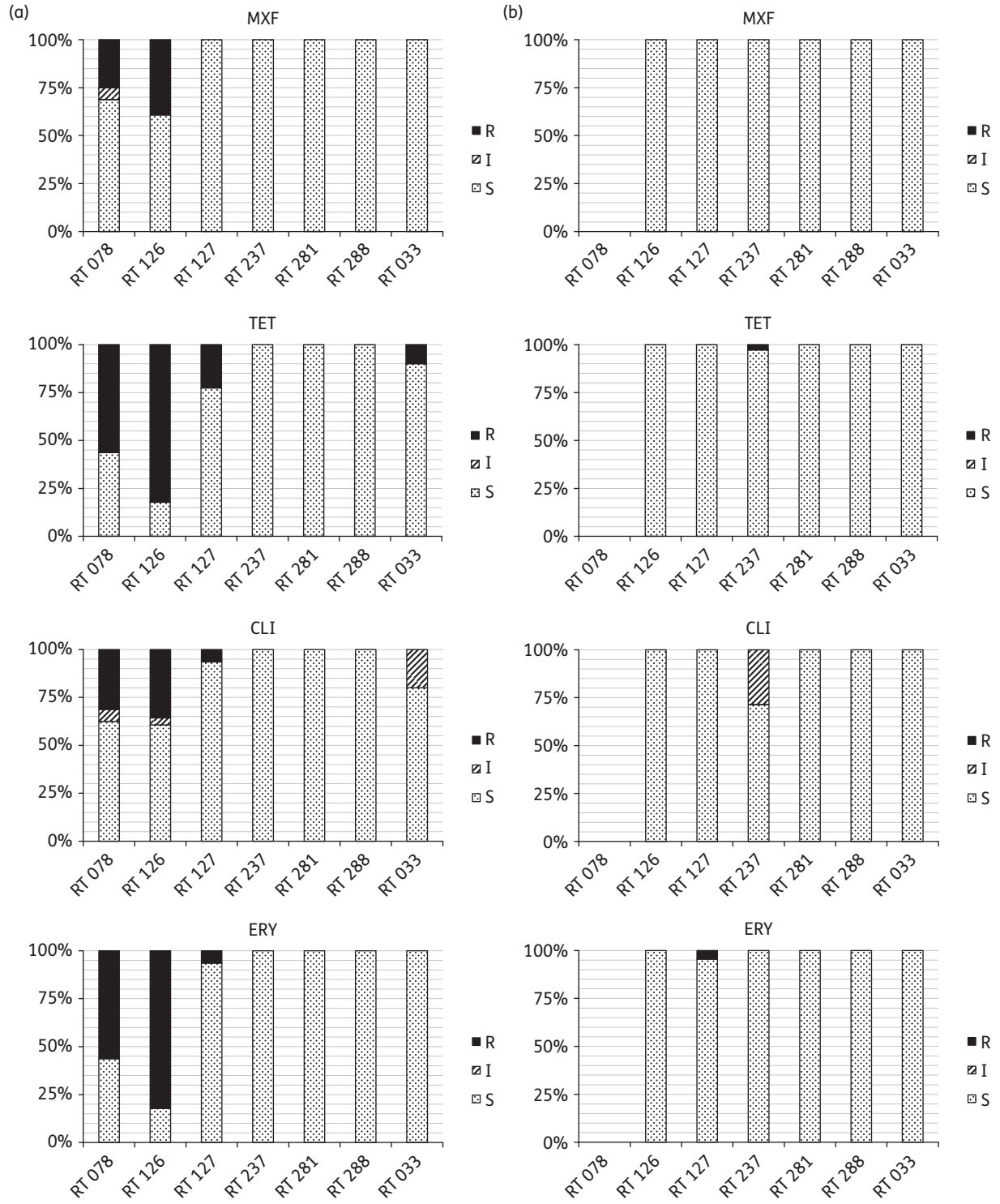

Figure 1. Percentage susceptibility data for moxifloxacin, tetracycline, clindamycin and erythromycin, grouped by host and RT. (a) Humans. (b) Animals. Breakpoints are those recommended for anaerobes by CLSI.[12] MXF, moxifloxacin; TET, tetracycline; CLI, clindamycin; ERY, erythromycin; S, susceptible; I, intermediate; R, resistant.

discordant fluoroquinolone-resistant phenotypes of RT 126 seen in this study [animal isolates (0%) and human isolates (39.3%)].

Human strains of RT 126 and RT 127 share a very similar and generally antimicrobial-resistant phenotype to RT 078. This RT is well established in porcine and bovine populations in Europe

and North America, but is notably absent from livestock populations in Australia.[6–8] The number of CDI cases attributable to RT 078 in Australia is low[3,10] and the limited number of cases is likely a result of importation from overseas where this RT is among the most common in human carriers or on food.[13,14,17] Given the phenotypic overlap, it is possible that strains of RT 126, and to a lesser extent RT 127, are also imported strains. This would be an interesting avenue for further investigation. Ultimately, the fine-scale resolution offered by WGS and core genome phylogenetics will resolve the true ancestral relationship of this strain collection. This is currently underway and will elucidate the underlying genetic mechanisms conferring the resistance seen in this study.

In conclusion, the pan-susceptible phenotypic profiles seen for the large proportion of human and animal isolates of RTs 033, 237, 281 and 288 suggest genetic overlap and further support the hypothesis of a common source. Conversely, human populations of RTs 126 and RT 127 show high proportions of antimicrobial resistance and limited genetic overlap with their animal counterparts.

Acknowledgements

We are grateful to Mr John Boehm and colleagues at PathWest Media (Mt Claremont, Western Australia, Australia) for preparation of the testing media.

Funding

This study was supported by internal funding. D. R. K is funded by an Australian Postgraduate Award conferred by The University of Western Australia.

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Wilcox MH. Progress with a difficult disease. *Lancet Infect Dis* 2012; **12**: 256–7.

2 Baines SD, Wilcox MH. Antimicrobial resistance and reduced susceptibility in *Clostridium difficile*: potential consequences for induction, treatment, and recurrence of *C. difficile* infection. *Antibiotics* 2015; **4**: 267–98.

3 Slimings C, Armstrong P, Beckingham WD *et al*. Increasing incidence of *Clostridium difficile* infection, Australia, 2011–2012. *Med J Aust* 2014; **200**: 272–6.

4 Hensgens MP, Keessen EC, Squire MM *et al*. *Clostridium difficile* infection in the community: a zoonotic disease? *Clin Microbiol Infect* 2012; **18**: 635–45.

5 Jhung MA, Thompson AD, Killgore GE *et al*. Toxinotype V *Clostridium difficile* in humans and food animals. *Emerg Infect Dis* 2008; **14**: 1039–45.

6 Knight DR, Thean S, Putsathit P *et al*. Cross-sectional study reveals high prevalence of *Clostridium difficile* non-PCR ribotype 078 strains in Australian veal calves at slaughter. *Appl Environ Microbiol* 2013; **79**: 2630–5.

7 Knight DR, Squire MM, Riley TV. *Clostridium difficile* in Australian neonatal pigs; nationwide surveillance study shows high prevalence and heterogeneity of PCR ribotypes. *Appl Environ Microbiol* 2014; **81**: 119–23.

8 Knight DR, Putsathit P, Elliott B *et al*. Contamination of Australian newborn calf carcasses at slaughter with *Clostridium difficile*. *Clin Microbiol Infect* 2016; **22**: 266.e1–7.

9 Squire MM, Knight DR, Riley TV. Community-acquired *Clostridium difficile* infection and Australian food animals. *Microbiol Aust* 2015; 10.1071/MA15040.

10 Knight DR, Giglio S, Huntington PG *et al*. Surveillance for antimicrobial resistance in Australian isolates of *Clostridium difficile*, 2013–14. *J Antimicrob Chemother* 2015; **70**: 2992–9.

11 EUCAST. *Breakpoint Tables for Interpretation of MICs and Zone Diameters, Version 5.0, 2015*.

12 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-third Informational Supplement M100-S23*. CLSI, Wayne, PA, USA, 2013.

13 Freeman J, Vernon J, Morris K *et al*. Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. *Clin Microbiol Infect* 2015; **21**: 248.e9–16.

14 Keessen EC, Hensgens MPM, Spigaglia P *et al*. Antimicrobial susceptibility profiles of human and piglet *Clostridium difficile* PCR-ribotype 078. *Antimicrob Resist Infect Control* 2013; **2**: 14.

15 Lachowicz D, Pituch H, Obuch-Woszczatynski P. Antimicrobial susceptibility patterns of *Clostridium difficile* strains belonging to different polymerase chain reaction ribotypes isolated in Poland in 2012. *Anaerobe* 2014; **31**: 37–41.

16 Pirš T, Avberšek J, Zdovc I *et al*. Antimicrobial susceptibility of animal and human isolates of *Clostridium difficile* by broth microdilution. *J Med Microbiol* 2013; **62**: 1478–85.

17 Spigaglia P, Barbanti F, Mastrantonio P. Multidrug resistance in European *Clostridium difficile* clinical isolates. *J Antimicrob Chemother* 2011; **66**: 2227–34.

18 Knight DR, Elliott B, Chang BJ. Diversity and evolution in the genome of *Clostridium difficile*. *Clin Microbiol Rev* 2015; **28**: 721–41.

19 Lee JH, Lee Y, Lee K *et al*. The changes of PCR ribotype and antimicrobial resistance of *Clostridium difficile* in a tertiary care hospital over 10 years. *J Med Microbiol* 2014; **63**: 819–23.

20 Australian Commission on Safety and Quality in Health Care. *Antimicrobial Prescribing Practice in Australian Hospitals: Results of the 2014 National Antimicrobial Prescribing Survey*. Sydney, NSW, Australia: ACSQHC, 2015. http://www.safetyandquality.gov.au/wp-content/uploads/2015/07/Antimicrobial-prescribing-practice-in-Aust-hospitals-NAPS-2014-Results.pdf.

21 Jordan D, Chin JJC, Fahy VA *et al*. Antimicrobial use in the Australian pig industry: results of a national survey. *Aust Vet J 2009:* **87**: 222–9.

22 O'Connor JR, Galang MA, Sambol SP *et al*. Rifampin and rifaximin resistance in clinical isolates of *Clostridium difficile*. *Antimicrob Agents Chemother* 2008; **52**: 2813–7.