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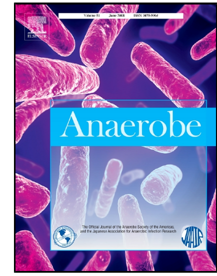

Antimicrobial resistance in large clostridial toxin-negative, binary toxin-positive *Clostridium difficile* ribotypes.

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1 **Antimicrobial resistance in large clostridial toxin-negative, binary toxin-**
2 **positive *Clostridium difficile* ribotypes.**
3

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18 **Running Title**: Antimicrobial susceptibilities of A$^-$B$^-$CDT$^+$ *C. difficile*
19

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26 **Abstract**

27 Antimicrobial resistance (AMR) is commonly found in *Clostridium difficile* strains and plays
28 a major role in strain evolution. We have previously reported the isolation of large clostridial
29 toxin-negative, binary toxin-producing ($A^-B^-CDT^+$) *C. difficile* strains from colonised (in
30 some instances diarrhoeic) food animals, as well as from patients with diarrhoea. To further
31 characterise these strains, we investigated the phenotypic and genotypic AMR profiles of a
32 diverse collection of $A^-B^-CDT^+$ *C. difficile* strains. The *in vitro* activities of 10 antimicrobial
33 agents were determined for 148 $A^-B^-CDT^+$ *C. difficile* strains using an agar dilution
34 methodology. Whole-genome sequencing and *in silico* genotyping was performed on 53
35 isolates to identify AMR genes. All strains were susceptible to vancomycin, metronidazole
36 and fidaxomicin, antimicrobials currently considered first-line treatments for *C. difficile*
37 infection (CDI). Differences in antimicrobial phenotypes between PCR ribotypes (RTs) were
38 observed but were minimal. Phenotypic resistance was observed in 13 isolates to tetracycline
39 (TetR, MIC=16 mg/L), moxifloxacin (MxfR, MIC=16 mg/L), erythromycin (EryR, MIC
40 ≥128 mg/L) and clindamycin (CliR, MIC=8 mg/L). The MxfR strain (RT033) possessed
41 mutations in *gyrA/B*, while the TetR (RT033) strain contained a *tetM* gene carried on the
42 conjugative transposon Tn*6190*. All EryR and CliR strains (RT033, QX521) were negative
43 for the erythromycin ribosomal methylase gene *ermB*, suggesting a possible alternative
44 mechanism of resistance. This work describes the presence of multiple AMR genes in A^-B^-
45 CDT^+ *C. difficile* strains and provides the first comprehensive analysis of the AMR repertoire
46 in these lineages isolated from human, animal, food and environmental sources.

47 **Introduction**

48 *Clostridium difficile* is a Gram-positive obligately anaerobic bacillus that can persist under
49 aerobic conditions in a non-vegetative spore form. *C. difficile* infection (CDI) is the leading
50 cause of antimicrobial-associated diarrhoea in most developed countries with high rates of
51 healthcare-related infections reported, especially in European and North American hospitals
52 (1). The prevalence of CDI has increased in parallel with the greater use of antimicrobials,
53 most nptably clindamycin, cephalosporins and fluoroquinolones (2-4). Antimicrobial
54 treatment destroys the commensal gut bacteria that contribute to the inhibition of *C. difficile*
55 spore outgrowth, creating an imbalance (5). A continual dysbiosis is exploited by *C. difficile*
56 resulting in CDI recurrences that have become a substantial strain on health care systems in
57 regions with high incidences of CDI (6).

58 In the early 2000s, a new generation quinolone (fluoroquinolone) resistant strain of *C.*
59 *difficile,* RT027/NAP1/B1, caused outbreaks of CDI in Europe and North America leading to
60 the implementation of antimicrobial stewardship policies in hospital settings in these regions
61 (7). Today, although infections due to *C. difficile* RT027 have decreased, fluoroquinolone
62 resistance continues to promote the spread of other *C. difficile* RTs. In particular,
63 moxifloxacin resistance is commonly found in *C. difficile* RTs, an indication of the need for
64 frequent review and audit of antimicrobial stewardship policies (8).

65 Oral vancomycin and metronidazole are the preferred therapeutic options for mild or
66 moderate CDI while a combination of oral metronidazole and intravenous vancomycin is
67 recommended for severe disease (9). Both antimicrobials have been linked to disease
68 recurrences due to the spore-forming nature of *C. difficile,* which is resistant to these
69 treatments (9). Fidaxomicin, a narrow-spectrum, sporicidal macrolide that is highly effective
70 against *C. difficile*, is occasionally used as a first-line therapy due to its microbiota preserving

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71 property that greatly reduces the probability of recurrent CDI (10, 11). Despite it's proven
72 effectiveness, the cost of fidaxomicin is substantially higher than other therapies and, coupled
73 with the substantial healthcare costs of CDI, it is not affordable in many parts of the world
74 (12). In the US, a region highly impacted with CDI, a case of recurrent CDI costs up to
75 $18,000 (12).

76 Currently, there is global widespread use of antimicrobials in both hospital and community
77 settings. Approximately 5 out of 6 individuals in the US receive a course of antibiotics
78 annually (13). As a result, increased antimicrobial resistance (AMR) and reduced
79 susceptibilities to multiple antimicrobials has become common (1, 14). On the other hand, the
80 use of alternate CDI therapies such as faecal microbiota transplantation and microbial
81 ecosystem therapeutics is becoming popular due to excellent recovery rates for recurrent
82 infections and the lack of dependency on antimicrobials (15). However, these carry the risk
83 of acquiring AMR genes from donors (1, 15). These concerns emphasize the importance of
84 AMR surveillance in both large clostridial toxin-positive (toxigenic) and large clostridial
85 toxin-negative *C. difficile* strains.

86 Antimicrobial susceptibility patterns of toxigenic *C. difficile* strains have been determined
87 periodically while large clostridial toxin-negative *C. difficile* strains have been ignored. Large
88 clostridial toxin-negative *C. difficile* strains lack the main virulence factors (toxins A and B),
89 however, they may encode a third binary toxin (CDT), the significance of which is not well
90 understood despite it being associated with more severe disease (16,17). CDT shares 80%-
91 85% homology with iota toxin (ι-toxin) produced by *C. perfringens* type E and also possesses
92 an ADP-ribosyltransferase activity that modifies actin in the host cells leading to its de-
93 polymerization and inability to form filaments, eventually resulting in destruction of the cell
94 cytoskeleton (17). *In vitro* experiments have confirmed toxicity of CDT and its crucial role
95 in adherence and colonisation (17). Recently, *C. difficile* strains producing only CDT (A^-B^-

96 CDT^{+}) have been isolated from diarrhoeic individuals with recurrent CDI symptoms
97 suggesting the possibility of CDI in the absence of toxigenic *C. difficile* strains (18).

98 Although the role of CDT in infection is unclear, we postulated that $A^{-}B^{-}CDT^{+}$ *C. difficile*
99 strains may harbour other non-toxin virulent factors, including antimicrobial resistance, that
100 contribute to their ability proliferate and cause symptoms in infected patients. The purpose of
101 this study was to determine the antimicrobial susceptibilities of a collection of $A^{-}B^{-}CDT^{+}$ *C.*
102 *difficile* strains to a range of antimicrobial agents. In addition, a selection of the strains was
103 whole-genome sequenced to corroborate the phenotypic results.

104 **Materials and methods**

105 **Bacterial isolates**

106 *C. difficile* isolates were selected based upon genetic uniqueness using previous molecular
107 analysis of PCR ribotypes (RTs), toxin gene profiles and multilocus sequence types (MLST,
108 STs). The strains belonged to ten RTs (033, 238, 239, 288, 585, 586, QX143, QX360,
109 QX444, QX521) and were collected from diverse sources (human faeces, *n*=28; foal, *n*=1;
110 calves, *n*=52; pigs, *n*=40; food, *n*=1; effluent, *n*=26). Table 1 illustrates the various RTs,
111 sequence types (STs) and general characteristics of the isolates analysed.

112 **Bacterial culture**

113 *C. difficile* isolates previously frozen at -80°C using brain heart infusion broth (supplemented
114 with 15% glycerol) were revived on blood agar (BA) plates. BA plates were incubated
115 anaerobically (A35 Anaerobic Workstation, Don Whitley Scientific, Shipley, West Yorkshire
116 BD17 7SE, United Kingdom) for 48 h to obtain pure cultures. *C. difficile* colonies were
117 confirmed by their chartreuse fluorescence under ultraviolet light.

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118 **Susceptibility testing**

119 The minimum inhibitory concentrations (MICs) of pure *C. difficile* isolates were determined
120 using a CLSI-recommended agar dilution method as previously described (19). A total of 148
121 $A^-B^-CDT^+$ *C. difficile* isolates were tested against 10 antimicrobials consisting of current CDI
122 therapies (vancomycin, metronidazole, fidaxomicin and rifaximin), antimicrobials associated
123 with high resistance and risk of CDI development (moxifloxacin, erythromycin, clindamycin)
124 and broad-spectrum antimicrobials frequently that may lead to CDI (meropenem,
125 amoxicillin/clavulanate and tetracycline). The MICs were interpreted using CLSI and
126 EUCAST guidelines where available (20, 21). For fidaxomicin and rifaximin, a European
127 Medical Agency proposed breakpoint of 1.0 mg/L (report WC500119707,
128 http://www.ema.europa.eu/) and recommended breakpoint of ≥32 mg/L (22) were used,
129 respectively.

130 **DNA sequencing, genome assembly and data analysis**

131 Whole-genome sequencing (WGS) of 53 *C. difficile* isolates representative of the 148 A^-B^-
132 CDT^+ isolates was performed using methods described by Knight *et al.* (23). Bacterial DNA
133 libraries were generated using standard Nextera XT protocols (Illumina® Inc., San Diego,
134 CA, USA) and paired-end (PE) sequencing was performed on the Illumina® Miseq Platform.
135 Quality control and bioinformatic processing of raw reads were performed as described by
136 Knight *et al.* (24). AMR genes and STs were detected *in silico* using the ARG-ANNOT and
137 PubMLST databases, respectively, compiled in the short-read typing algorithm SRST2 v0.1.8
138 (23-25). Draft genomes were assembled and annotated as previously described (23). Manual
139 investigation of acquired and intrinsic resistance loci and their underlying genomic context
140 was performed using a custom sequence library comprising mobile genetic elements
141 previously identified in *C. difficile* and other related Firmicutes, as previously described (23).

142 **Results**

143 All isolates were susceptible to fidaxomicin, rifaximin, vancomycin, metronidazole,
144 amoxicillin/clavulanate and meropenem (Table 2). Phenotypic resistance was observed in
145 9.3% (3/28) of human isolates, 38.5% (10/26) of effluent isolates and 0% of cattle (0/53) and
146 pig (0/40) isolates. A total of 13/148 *C. difficile* isolates from humans (*n*=3) and effluent
147 (*n*=10) exhibited phenotypic resistance to tetracycline (TetR, MIC=16mg/L), moxifloxacin
148 (MxfR, MIC=16mg/L), erythromycin (EryR, MIC ≥128mg/L) and clindamycin (CliR,
149 MIC=8mg/L). All cattle and pig *C. difficile* isolates were susceptible to all antimicrobial
150 agents tested. In total, 10 different RTs were analysed and the resistant isolates belonged to
151 two RTs only, RTs 033 (n=11, 84.6%) and QX521 (n=2, 15.4%).

152 Non-synonymous mutations in the DNA gyrase GyrA/B were detected in the MxfR isolate
153 (RT033) with distinct allele types (GyrA [Lys413Asn], GyrB [Gln160His, Ser366Val,
154 Ser416Ala, Asp426Asn]). The Asp426Asn and Ser416Ala mutations in GyrB correlated with
155 fluoroquinolone resistance and the other mutations were non-synonymous mutations that fell
156 outside the quinolone resistance-determining regions (QRDR) of GyrA and B. The TetR
157 strain, also belonging to RT033, contained a *tetM* gene (encoding a ribosomal protective
158 protein) carried on a conjugative transposon Tn*6190*, originally discovered in the M120 strain
159 of RT078 (accession NC_017174) isolated from an Irish diabetic patient (Table 3). No EryR
160 or CliR strain contained methylase *erm* genes, suggesting a possible alternative mechanism of
161 resistance in these strains.

162 Eight RT033 isolates also possessed aminoglycoside resistance genes (*aph3-III* and *sat4A)*
163 and harboured a 7269bp fragment of a multidrug resistance gene cassette from the ruminant
164 facultative anaerobe *Erysipelothrix rhusiopathiae* (99% nucleotide seq ID to KP339868.1).
165 Interestingly, this cassette also had a third (syntenic) aminoglycoside gene (*ant6-Ia)*, which

166 was not picked up by SRST2 analysis but identified on manual curation of the assembled
167 genome. Further manual curation of the $A^-B^-CDT^+$ *C. difficile* genomes detected genes
168 encoding a *β*-lactamase inducing penicillin-binding protein (*blaR)* and a multidrug resistance
169 transporter protein *(cme)*, loci that have been reported previously in other *C. difficile* lineages
170 (Table 3).

171 **Discussion**

172 This work illustrates antimicrobial phenotypic resistance and the presence of multiple AMR
173 genes in $A^-B^-CDT^+$ *C. difficile* RTs isolated from human, animal and environmental (effluent)
174 sources. Our collection of *C. difficile* RT033 strains exhibited resistance to more
175 antimicrobials of different classes than any other $A^-B^-CDT^+$ *C. difficile* RT tested. This is
176 noteworthy because this RT, despite being thought of as not clinically relevant, has been
177 associated with human infections in Australia, Europe and North America (18, 26-28). We
178 hypothesize that the presence of multiple AMR genes in this RT may be a factor driving the
179 increased incidence of RT033 human and animal infections.

180 *C. difficile* RT033, also classified as toxinotype XI, is common in food animals, especially
181 piglets and veal calves (29). It belongs to ST11 and MLST clade 5, a clade known to cause
182 significant mortality that contains the so-called "hypervirulent" RT078 strain (22).
183 Symptomatic human cases of RT033 infection described in the literature include single cases
184 from Australia, Italy and North America, and four cases from France (18, 26-28). We
185 recently reported the discovery of a *vanB2*-like vancomycin resistance operon from an
186 RT033 *C. difficile* strain isolated from an Australian veal calf at slaughter (31). Although
187 phenotypically inactive, possibly due to fragmentation in the *vanRB* gene, the origin of this
188 element in vancomycin-resistant *Enterococcus* species illustrates the possibility that a fully
189 vancomycin-resistant strain of *C. difficile* may emerge. None of our RT033 *C. difficile*

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190 isolates contained a *vanB2* operon and they were all susceptible to vancomycin (MIC=1-2
191 mg/L, Table 2). However, they showed similar phenotypic resistance characteristics to
192 clinically relevant toxigenic *C. difficile* strains.

193 Since the initial association between CDI and antimicrobial therapy was confirmed, many
194 toxigenic *C. difficile* strains have been reported as resistant to clindamycin and erythromycin,
195 often related to the rRNA adenine *N-6*-methyltransferase encoded by the *ermB* gene (32,33).
196 Approximately 17 mobile elements have been linked to macrolide-lincosamide-streptogramin
197 B (MLS_B) resistance in *C. difficile* but Tn*5398* is the most commonly identified *ermB*-
198 containing element found in CliR and EryR *C. difficile* strains (1). Notably, this non-
199 conjugative element contains two copies of *ermB* genes (1). Some of our $A^-B^-CDT^+$ *C.*
200 *difficile* RTs (033, *n=8* and QX521, *n=2)* displayed an MLS_B phenotype yet did not harbour
201 any of the known methylase subclasses (*ermB*, *ermC* or *ermTR*). This discordance has been
202 observed in *C. difficile* previously, and publications have suggested that mutations in L4/L22
203 riboproteins and 23s rRNA could explain the MLS_B resistance (1). Analysis of the sequenced
204 genomes showed that both the L4/L22 riboprotein genes and 23s rRNA genes in this
205 population were full-length and wildtype with no variations identified that were found
206 exclusively in MLS_B^+ strains. However, analysis of the multiple 23s rRNA alleles present in
207 a typical *C. difficile* genome was not possible with the Illumina short-read sequencing
208 approach used in this study.

209 Fluoroquinolone resistance (FQR) in *C. difficile* has been continually documented since the
210 outbreaks caused by two independently evolved FQR lineages of *C. difficile* RT027/BI/NAP1
211 in Canada, USA and Europe between 2002 and 2006 (1, 23). Although the incidence of *C.*
212 *difficile* RT027 infections has markedly reduced in some countries, FQR in other *C. difficile*
213 RTs continues to emerge, most notably in ST11 and RT017 lineages (23). Mutations within

214 the defined QRDRs of DNA gyrase subunits GyrA and/or GyrB generally confer resistance
215 to FQs, however, non-QRDR polymorphisms resulting in FQR have been observed (33). We
216 identified both QRDR and non-QRDR mutations in *gyrA/B*. These mutations were identified
217 in an isolate (RT033) that was phenotypically resistant to moxifloxacin (MIC=16mg/L). The
218 isolate originated from a patient in France who was considered to have CDI and had only A^-
219 B^-CDT^+ *C. difficile* RT033 isolated from stool specimens. The patient fully recovered after
220 treatment with oral metronidazole, however, this case exemplifies acquisition and possible
221 proliferation of the FQR genotypes within $A^-B^-CDT^+$ *C. difficile* strains (18).

222 With regard to tetracycline, resistance in *C. difficile* is thought to be less common and varies
223 between countries and RTs (34). *C. difficile* tetracycline resistance genes are commonly
224 carried on Tn*916* and Tn*5397*-like mobile elements, however, mobile elements that carry
225 TetR genes from other bacterial species have been identified in *C. difficile* e.g. *tetA/B* (23).
226 The TetR strain in our $A^-B^-CDT^+$ *C. difficile* collection, also an RT033 strain, contained
227 a *tetM* gene carried on a conjugative transposon Tn*6190*, originally discovered in *C. difficile*
228 RT078 strain M120 and, to date, only reported in *C. difficile* ST11 lineages RT126 and
229 RT078 (35). Tn*6190* is 97% homologous to Tn*916* and considered to circulate in pigs (36).
230 Our TetR isolate originated from a patient with idiopathic diarrhoea suggesting possible
231 zoonotic transmission, although a higher-resolution typing approach such as core genome
232 SNP analysis would be needed to confirm this (35).

233 In Australia and The Netherlands, bi-directional transmission (zoonotic and anthroponotic) of
234 *C. difficile* has been demonstrated that may be facilitating dissemination of AMR genes (23,
235 37). However, in this study, we observe the possible multi-directional transmission of AMR
236 genes from human, animal and effluent sources. Ten of 13 resistant isolates (76.9%) came
237 from an environmental source (effluent from a piggery) and indicated phenotypic resistance

238 to erythromycin (≥128mg/L). These isolates belonged to RTs 033 and QX521 (novel
239 ribotype). We did not isolate QX521 from any other source, however, *C. difficile* RT033 was
240 detected from all the sources (human, animal, food and effluent) and at least one RT033
241 isolate from each source (besides food) contained AMR genes (Table 3). Additionally, the
242 RT033 isolates from human and effluent sources exhibited multi-drug resistant (MDR)
243 phenotypes (resistance to two or more antimicrobials) to moxifloxacin, clindamycin,
244 erythromycin and tetracycline. These results emphasize the importance of a 'One Health'
245 approach to combating AMR in *C. difficile* (38).

246 While considerable effort is being made in directing antimicrobial stewardship, there is
247 increasing concern about the development of resistance to clinically consequential
248 antimicrobials. In this study, we successfully demonstrated that $A^-B^-CDT^+$ *C. difficile* strains
249 from diverse sources are reservoirs of AMR genes that have also been identified in clinically
250 relevant toxigenic *C. difficile* strains.

251 **Conclusion**

252 AMR is a One Health issue that highlights the importance of the association between human
253 health, animal health and the environment. While the role of $A^-B^-CDT^+$ *C. difficile* strains in
254 idiopathic diarrhoea is still unclear, these strains remain common in food animals and could
255 potentially transmit AMR genes. In the future, we will further investigate the evolution and
256 transmission of these strains using high-resolution core genome phylogenetics. However, the
257 present study provides a basis for this with a comprehensive analysis of AMR profiles of
258 various $A^-B^-CDT^+$ *C. difficile* strains isolated from humans, animals, food and environmental
259 sources.

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270

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385 **Table 1.** Distribution of PCR ribotypes and sequence types (STs) of the various $A^{-}B^{-}CDT^{+}$ *C. difficile* isolates (*n*=148) included in the study.

	***C. difficile* MLST genes**												
RIBOTYPE	***adk***	***atpA***	***dxr***	***glyA***	***recA***	***sodA***	***tpi***	**ST**	**CLADE**	**SOURCE**	**COUNTRY**	**SYMPTOMATIC/ ASYMPTOMATIC**	**RIBOTYPE PATTERNS**
RT 238	5	8	5	26	15	29	8	169	5	Pigs	Australia, *n*=23	NI[1]	
QX 143	5	8	5	28	15	28	59	386	5	Human	Australia, *n*=1	Symptomatic	
RT 585	5	15	5	27	15	29	20	164	5	Human	Australia, *n*=4	Symptomatic	
										Foal	Australia, *n*=1	Symptomatic	
RT 239	10	8	19	11	15	29	22	168	5	Human	Australia, *n*=2	Symptomatic	
RT 033	5	8	8	11	9	11	8	11	5	Human	Australia, *n*=11	Symptomatic	
										Human	France, *n*=6	Symptomatic	
										Pigs	Australia, *n*=17	NI	
										Food	Australia, *n*=1	NA[2]	
										Effluent	Australia, *n*=10	NA	
										Calves	Australia, *n*=24	Asymptomatic	
RT 586	5	8	5	27	15	29	22	167	5	Human	Australia, *n*=1	Symptomatic	
RT 288	5	8	5	11	9	11	8	11	5	Calves	Australia, *n*=28	NI	
										Human	Australia, *n*=1	Symptomatic	
QX 444	5	8	5	26	15	29	8	169	5	Human	Australia, *n*=1	Symptomatic	

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QX 521	5	8	5	27	15	28	8	280	5	Effluent	Australia, *n*=16	NA	
QX 629	5	8	5	27	15	29	8	315	5	Human	Australia, *n*=1	Symptomatic	

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388 All isolates belonged to the evolutionary divergent lineage clade 5 and were distributed within eight STs. MLST- Multi-Locus Sequence Type.

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Table 2: Susceptibility of $A^-B^-CDT^+$ *C. difficile* strains to 10 antimicrobial agents.

Antimicrobial Agent (Clinical Breakpoints) (S/I/R)	Sources										Total (*n*=148)		%S/I/R
	Human (*n*=28)		Food (*n*=1)		Cattle (*n*=54)		Pigs (*n*=39)		Effluent (*n*=26)				
	MIC Range	$MIC_{50/90}$ (mg/L)	MIC Range	$MIC_{50/90}$ (mg/L)	MIC Range	$MIC_{50/90}$ (mg/L)	MIC Range	$MIC_{50/90}$ (mg/L)	MIC Range	$MIC_{50/90}$ (mg/L)	MIC Range	$MIC_{50/90}$ (mg/L)	
[a]VAN	1 - 2	1/1	1	1/1	1	1/1	1 - 2	1/1	1	1/1	1 - 2	1/1	100/-/0
[a]MTZ	0.25 – 0.5	0.5/0.5	0.5	0.5/0.5	0.12 – 0.5	0.5/0.5	0.25 - 1	0.25/0.5	0.12 – 0.5	0.25/0.5	0.12 - 1	0.5/1	100/-/0
[b]FDX	0.004 – 0.12	0.03/0.06	0.015	0.015/0.015	0.004 – 0.06	0.015/0.06	0.004 – 0.06	0.03/0.03	0.008 – 0.12	0.03/0.06	0.004 – 0.12	0.03/0.12	100/-/0
[c]MXF	1 - 16	1/1	1	1/1	1 - 2	1/1	1 - 2	1/2	1 - 4	1/2	1 - 2	1/1	98.6/0.7/0.7
[c]CLI	0.12 - 8	0.5/4	0.5	0.5/0.5	0.12 - 4	1/4	0.03 - 4	0.5/1	0.03 - 8	1/4	0.03-8	0.5/4	92.6/6.7/0.7
[c]ERY	0.12 - 128	1/1	0.5	0.5/0.5	0.25 - 4	0.5/0.5	0.5 - 4	1/1	0.25 - 128	8/128	0.12 - 128	1/4	-/-/6.7
[c]MER	1 - 4	2/2	2	2/2	2	2/2	2 - 4	2/4	2 - 4	2/2	2 - 4	2/2	100/0/0
[c]AUG	0.25 - 1	0.5/1	0.5	0.5/0.5	0.25 – 0.5	0.5/0.5	0.25 - 2	0.5/0.5	0.25 – 0.5	0.5/0.5	0.25 -2	0.5/1	100/0/0
[d]RFX	0.004 - 2	0.008/0.008	0.008	0.008/0.008	0.004	0.004/0.004	0.004 - 1	0.008/0.008	0.004 – 0.015	0.008/0.008	0.004 - 2	0.004/0.015	100/-/0
[c]TET	0.03 – 0.25	0.12/0.25	0.12	0.12/0.12	0.06 – 0.12	0.12/0.12	0.06 - 8	0.12/2	0.06 – 0.12	0.06/0.12	0.06 - 8	0.12/2	98.0/1.3/0.7

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390 S- susceptible, I-intermediate, R- resistant. Breakpoints (minimum inhibitory concentration [mg/L]; S, I, R) for each antibiotic were as follows: VAN-
391 Vancomycin (≤2/-/>2), MET- Metranidazole (≤2/-/>2), FDX- Fidaxomicin (-/-/≥1), MXF- Moxifloxacin (≤2/4/≥8), CLI- Clindamycin (≤2/4/≥8), ERY-
392 Erythromycin (-/-/>8), MER-Meropenem (≤4/8/≥16), AUG- Amoxicillin/clavulanate (≤4/8/≥16), RFX- Rifaximin (-/-/≥32), TET- Tetracyline (≤4/8/≥16).
393 [a]EUCAST breakpoints (21). [b]Resistance (≥1.0 mg/L) as described by European Medical Agency (report WC500119707, http://www.ema.europa.eu/).
394 [c]Breakpoints as recommended by CLSI (20). [d]Resistance (≥32 mg/L) as described by O'Connor *et al* (22).

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Table 3. AMR genes detected from raw sequence reads of A^{-}B^{-}CDT^{+} *C. difficile* strains, *n*=53.

Phenotype	Gene(s)	Ribotype	Toxin profile	Source
Aminoglycoside resistance a	aph3-III-sat4A-ant6-Ia	RT 033	A-B-CDT+	Human, n=1, pigs, n=3 and effluent, n=4
	aph3-III-sat4A-npmA- ant6-Ia	RT 033	A-B-CDT+	Pig, n=1
β-lactam resistance b	blaR cme	RT 033	A-B-CDT+	Human, n=16, calves, n=6, pigs, n=3, effluent, n=4 and food, n=1
		RT 238	A-B-CDT+	Pigs, n=2 and calf, n=1
		RT 239	A-B-CDT+	Human, n=2
		RT 288	A-B-CDT+	Human, n=1 and calf, n=3
		RT 585	A-B-CDT+	Human, n=4 and foal, n=1
		RT 586	A-B-CDT+	Human, n=1
		QX 143	A-B-CDT+	Human, n=1
		QX 444	A-B-CDT+	Human, n=1
		QX 521	A-B-CDT+	Effluent, n=5
		QX 629	A-B-CDT+	Human, n=1
Fluoroquinolone resistance	gyrA (Lys413Asn) gyrB (Gln160His, Ser366Val, Ser416Ala, Asp426Asn)	RT 033	A-B-CDT+	Human, n=1
Glycopeptide resistance	van B2 operon	RT 033	A-B-CDT+	Calf, n=1
Tetracycline resistance	TetM	RT 033	A-B-CDT+	Human, n=1

397 [a]All genomes positive for aminoglycoside resistance genes *aph3-III* and *sat4A* harboured a
398 7269bp fragment of a resistance gene cassette from the ruminant facultative anaerobe
399 *Erysipelothrix rhusiopathiae* (99% seq ID to KP339868.1). [b]Results obtained by manual
400 curation of all A^{-}B^{-}CDT^{+} *C. difficile* genomes.

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Highlights

- Antimicrobial resistance (AMR) is common in *C. difficile*.
- Susceptibility testing generally focuses on toxigenic *C. difficile* strains.
- CDI due to non-toxigenic CDT producing strains ($A^-B^-CDT^+$) is under-reported.
- AMR genes were identified in $A^-B^-CDT^+$ *C. difficile* strains from various sources.
- These findings emphasize the importance of a One Health approach in combating AMR.

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