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

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Running Title: Antimicrobial susceptibilities of A-B-CDT\textsuperscript{+} *C. difficile*

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Word count (abstract): 246
Abstract

Antimicrobial resistance (AMR) is commonly found in *Clostridium difficile* strains and plays a major role in strain evolution. We have previously reported the isolation of large clostridial toxin-negative, binary toxin-producing (A⁻B⁻CDT⁺) *C. difficile* strains from colonised (in some instances diarrhoeic) food animals, as well as from patients with diarrhoea. To further characterise these strains, we investigated the phenotypic and genotypic AMR profiles of a diverse collection of A⁻B⁻CDT⁺ *C. difficile* strains. The *in vitro* activities of 10 antimicrobial agents were determined for 148 A⁻B⁻CDT⁺ *C. difficile* strains using an agar dilution methodology. Whole-genome sequencing and *in silico* genotyping was performed on 53 isolates to identify AMR genes. All strains were susceptible to vancomycin, metronidazole and fidaxomicin, antimicrobials currently considered first-line treatments for *C. difficile* infection (CDI). Differences in antimicrobial phenotypes between PCR ribotypes (RTs) were observed but were minimal. Phenotypic resistance was observed in 13 isolates to tetracycline (TetR, MIC=16 mg/L), moxifloxacin (MxfR, MIC=16 mg/L), erythromycin (EryR, MIC ≥128 mg/L) and clindamycin (CliR, MIC=8 mg/L). The MxfR strain (RT033) possessed mutations in *gyrA/B*, while the TetR (RT033) strain contained a *tetM* gene carried on the conjugative transposon Tn6190. All EryR and CliR strains (RT033, QX521) were negative for the erythromycin ribosomal methylase gene *ermB*, suggesting a possible alternative mechanism of resistance. This work describes the presence of multiple AMR genes in A⁻B⁻CDT⁺ *C. difficile* strains and provides the first comprehensive analysis of the AMR repertoire in these lineages isolated from human, animal, food and environmental sources.
Introduction

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

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

Oral vancomycin and metronidazole are the preferred therapeutic options for mild or moderate CDI while a combination of oral metronidazole and intravenous vancomycin is recommended for severe disease (9). Both antimicrobials have been linked to disease recurrences due to the spore-forming nature of _C. difficile_, which is resistant to these treatments (9). Fidaxomicin, a narrow-spectrum, sporicidal macrolide that is highly effective against _C. difficile_, is occasionally used as a first-line therapy due to its microbiota preserving
property that greatly reduces the probability of recurrent CDI (10, 11). Despite its proven
effectiveness, the cost of fidaxomicin is substantially higher than other therapies and, coupled
with the substantial healthcare costs of CDI, it is not affordable in many parts of the world
(12). In the US, a region highly impacted with CDI, a case of recurrent CDI costs up to
$18,000 (12).

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

Antimicrobial susceptibility patterns of toxigenic C. difficile strains have been determined
periodically while large clostridial toxin-negative C. difficile strains have been ignored. Large
clostridial toxin-negative C. difficile strains lack the main virulence factors (toxins A and B),
however, they may encode a third binary toxin (CDT), the significance of which is not well
understood despite it being associated with more severe disease (16,17). CDT shares 80%-85%
homology with iota toxin (i-toxin) produced by C. perfringens type E and also possesses
an ADP-ribosyltransferase activity that modifies actin in the host cells leading to its de-
polymerization and inability to form filaments, eventually resulting in destruction of the cell
cytoskeleton (17). In vitro experiments have confirmed toxicity of CDT and its crucial role
in adherence and colonisation (17). Recently, C. difficile strains producing only CDT (A-B-
CTD+ have been isolated from diarrhoeic individuals with recurrent CDI symptoms suggesting the possibility of CDI in the absence of toxigenic *C. difficile* strains (18).

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

**Materials and methods**

**Bacterial isolates**

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

**Bacterial culture**

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

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

DNA sequencing, genome assembly and data analysis

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

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

Non-synonymous mutations in the DNA gyrase GyrA/B were detected in the MxfR isolate (RT033) with distinct allele types (GyrA [Lys413Asn], GyrB [Gln160His, Ser366Val, Ser416Ala, Asp426Asn]). The Asp426Asn and Ser416Ala mutations in GyrB correlated with fluoroquinolone resistance and the other mutations were non-synonymous mutations that fell outside the quinolone resistance-determining regions (QRDR) of GyrA and B. The TetR strain, also belonging to RT033, contained a tetM gene (encoding a ribosomal protective protein) carried on a conjugative transposon Tn6190, originally discovered in the M120 strain of RT078 (accession NC_017174) isolated from an Irish diabetic patient (Table 3). No EryR or CliR strain contained methylase erm genes, suggesting a possible alternative mechanism of resistance in these strains.

Eight RT033 isolates also possessed aminoglycoside resistance genes (aph3-III and sat4A) and harbour a 7269bp fragment of a multidrug resistance gene cassette from the ruminant facultative anaerobe Erysipelothrix rhusiopathiae (99% nucleotide seq ID to KP339868.1). Interestingly, this cassette also had a third (syntenic) aminoglycoside gene (ant6-la), which
was not picked up by SRST2 analysis but identified on manual curation of the assembled genome. Further manual curation of the A·B·CDT+ *C. difficile* genomes detected genes encoding a β-lactamase inducing penicillin-binding protein (*blaR*) and a multidrug resistance transporter protein (*cme*), loci that have been reported previously in other *C. difficile* lineages (Table 3).

**Discussion**

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

*C. difficile* RT033, also classified as toxinotype XI, is common in food animals, especially piglets and veal calves (29). It belongs to ST11 and MLST clade 5, a clade known to cause significant mortality that contains the so-called “hypervirulent” RT078 strain (22). Symptomatic human cases of RT033 infection described in the literature include single cases from Australia, Italy and North America, and four cases from France (18, 26-28). We recently reported the discovery of a *vanB2*-like vancomycin resistance operon from an RT033 *C. difficile* strain isolated from an Australian veal calf at slaughter (31). Although phenotypically inactive, possibly due to fragmentation in the *vanRB* gene, the origin of this element in vancomycin-resistant *Enterococcus* species illustrates the possibility that a fully vancomycin-resistant strain of *C. difficile* may emerge. None of our RT033 *C. difficile*
isolates contained a vanB2 operon and they were all susceptible to vancomycin (MIC=1-2 mg/L, Table 2). However, they showed similar phenotypic resistance characteristics to clinically relevant toxigenic C. difficile strains.

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

Fluoroquinolone resistance (FQR) in C. difficile has been continually documented since the outbreaks caused by two independently evolved FQR lineages of C. difficile RT027/BI/NAP1 in Canada, USA and Europe between 2002 and 2006 (1, 23). Although the incidence of C. difficile RT027 infections has markedly reduced in some countries, FQR in other C. difficile RTs continues to emerge, most notably in ST11 and RT017 lineages (23). Mutations within
the defined QRDRs of DNA gyrase subunits GyrA and/or GyrB generally confer resistance
to FQs, however, non-QRDR polymorphisms resulting in FQR have been observed (33). We
identified both QRDR and non-QRDR mutations in gyrA/B. These mutations were identified
in an isolate (RT033) that was phenotypically resistant to moxifloxacin (MIC=16mg/L). The
isolate originated from a patient in France who was considered to have CDI and had only A- B' CDT+ C. difficile RT033 isolated from stool specimens. The patient fully recovered after
treatment with oral metronidazole, however, this case exemplifies acquisition and possible
proliferation of the FQR genotypes within A' B' CDT+ C. difficile strains (18).

With regard to tetracycline, resistance in C. difficile is thought to be less common and varies
between countries and RTs (34). C. difficile tetracycline resistance genes are commonly
carried on Tn916 and Tn5397-like mobile elements, however, mobile elements that carry
TetR genes from other bacterial species have been identified in C. difficile e.g. tetA/B (23).
The TetR strain in our A' B' CDT+ C. difficile collection, also an RT033 strain, contained
a tetM gene carried on a conjugative transposon Tn6190, originally discovered in C. difficile
RT078 strain M120 and, to date, only reported in C. difficile ST11 lineages RT126 and
RT078 (35). Tn6190 is 97% homologous to Tn916 and considered to circulate in pigs (36).
Our TetR isolate originated from a patient with idiopathic diarrhoea suggesting possible
zoonotic transmission, although a higher-resolution typing approach such as core genome
SNP analysis would be needed to confirm this (35).

In Australia and The Netherlands, bi-directional transmission (zoonotic and anthropogenic) of
C. difficile has been demonstrated that may be facilitating dissemination of AMR genes (23,
37). However, in this study, we observe the possible multi-directional transmission of AMR
genes from human, animal and effluent sources. Ten of 13 resistant isolates (76.9%) came
from an environmental source (effluent from a piggery) and indicated phenotypic resistance
to erythromycin (≥128mg/L). These isolates belonged to RTs 033 and QX521 (novel ribotype). We did not isolate QX521 from any other source, however, *C. difficile* RT033 was detected from all the sources (human, animal, food and effluent) and at least one RT033 isolate from each source (besides food) contained AMR genes (Table 3). Additionally, the RT033 isolates from human and effluent sources exhibited multi-drug resistant (MDR) phenotypes (resistance to two or more antimicrobials) to moxifloxacin, clindamycin, erythromycin and tetracycline. These results emphasize the importance of a ‘One Health’ approach to combating AMR in *C. difficile* (38).

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

**Conclusion**

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

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We thank Dr. Frédéric Barbut (National Reference Laboratory for \textit{C. difficile}, Hôpital Saint-Antoine, Paris, France) for providing additional A-B-CDT\(^+\) \textit{C. difficile} strains for this study and the PathWest Laboratory Medicine Media Section for providing the bacteriological media for the study.

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**Transparency declarations**

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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.

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<th>atpA</th>
<th>dxr</th>
<th>glyA</th>
<th>recA</th>
<th>sodA</th>
<th>tpi</th>
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<th>CLADE</th>
<th>SOURCE</th>
<th>COUNTRY</th>
<th>SYMPTOMATIC/ASYMPTOMATIC</th>
<th>RIBOTYPE PATTERNS</th>
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<td>5</td>
<td>26</td>
<td>15</td>
<td>29</td>
<td>8</td>
<td>169</td>
<td>5</td>
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<td>5</td>
<td>Calves</td>
<td>Australia, <em>n</em>=28</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Human</td>
<td>Australia, <em>n</em>=1</td>
<td>Symptomatic</td>
<td></td>
</tr>
<tr>
<td>QX 444</td>
<td>5</td>
<td>8</td>
<td>5</td>
<td>26</td>
<td>15</td>
<td>29</td>
<td>8</td>
<td>169</td>
<td>5</td>
<td>Human</td>
<td>Australia, <em>n</em>=1</td>
<td>Symptomatic</td>
<td></td>
</tr>
</tbody>
</table>
All isolates belonged to the evolutionary divergent lineage clade 5 and were distributed within eight STs. MLST- Multi-Locus Sequence Type.

**Table 2:** Susceptibility of A-B-CDT+ C. difficile strains to 10 antimicrobial agents.
S- susceptible, I-intermediate, R- resistant. Breakpoints (minimum inhibitory concentration [mg/L]; S, I, R) for each antibiotic were as follows: VAN-
Vancomycin (≤2/-/≥2), MET- Metranidazole (≤2/-/≥2), FDX- Fidaxomicin (≤2/-/≥1), MXF- Moxifloxacin (≤2/4/≥8), CLI- Clindamycin (≤2/4/≥8), ERY-

\(^a\)EUCAST breakpoints (21). \(^b\)Resistance (≥1.0 mg/L) as described by European Medical Agency (report WC500119707, http://www.ema.europa.eu/).

\(^c\)Breakpoints as recommended by CLSI (20). \(^d\)Resistance (≥32 mg/L) as described by O’Connor et al (22).
Table 3. AMR genes detected from raw sequence reads of A·B·CDT+ *C. difficile* strains, *n=53*.

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

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