

A species-wide genetic atlas of antimicrobial resistance in *Clostridioides difficile*

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Abstract

Antimicrobial resistance (AMR) plays an important role in the pathogenesis and spread of *Clostridioides difficile* infection (CDI), the leading healthcare-related gastrointestinal infection in the world. An association between AMR and CDI outbreaks is well documented, however, data is limited to a few 'epidemic' strains in specific geographical regions. Here, through detailed analysis of 10330 publicly-available *C. difficile* genomes from strains isolated worldwide (spanning 270 multilocus sequence types (STs) across all known evolutionary clades), this study provides the first species-wide snapshot of AMR genomic epidemiology in *C. difficile*. Of the 10330 *C. difficile* genomes, 4532 (43.9%) in 89 STs across clades 1–5 carried at least one genotypic AMR determinant, with 901 genomes (8.7%) carrying AMR determinants for three or more antimicrobial classes (multidrug-resistant, MDR). No AMR genotype was identified in any strains belonging to the cryptic clades. *C. difficile* from Australia/New Zealand had the lowest AMR prevalence compared to strains from Asia, Europe and North America ($P < 0.0001$). Based on the phylogenetic clade, AMR prevalence was higher in clades 2 (84.3%), 4 (81.5%) and 5 (64.8%) compared to other clades (collectively 26.9%) ($P < 0.0001$). MDR prevalence was highest in clade 4 (61.6%) which was over three times higher than in clade 2, the clade with the second-highest MDR prevalence (18.3%). There was a strong association between specific AMR determinants and three major epidemic *C. difficile* STs: ST1 (clade 2) with fluoroquinolone resistance (mainly T82I substitution in GyrA) ($P < 0.0001$), ST11 (clade 5) with tetracycline resistance (various *tet*-family genes) ($P < 0.0001$) and ST37 (clade 4) with macrolide-lincosamide-streptogramin B (MLS_B) resistance (mainly *ermB*) ($P < 0.0001$) and MDR ($P < 0.0001$). A novel and previously overlooked *tetM*-positive transposon designated Tn6944 was identified, predominantly among clade 2 strains. This study provides a comprehensive review of AMR in the global *C. difficile* population which may aid in the early detection of drug-resistant *C. difficile* strains, and prevention of their dissemination worldwide.

DATA SUMMARY

This study utilises publicly available raw sequence reads available at the NCBI Sequence Read Archive (SRA) as of January 2020. The details of all genomes are available in the Supplementary Data (10.6084/m9.figshare.14623533).

INTRODUCTION

Antimicrobial resistance (AMR) is one of the biggest threats to modern medicine. Without focused interventions and collaborations across all government sectors, AMR could be responsible for

an estimated ten million deaths and the loss of up to US\$210 trillion of annual global income by 2050 [1]. The US Centers for Disease Control and Prevention (CDC) reported on AMR health threats in 2013 [2], with an update in 2019 [3], highlighting organisms with the highest AMR burden and threat [3].

Clostridioides (Clostridium) difficile infection (CDI) causes major gastrointestinal illness worldwide [4], responsible for as many as 14000 deaths annually in the US [2]. *C. difficile* has been classified by the CDC as an urgent threat, the highest threat level, in both the 2013 and 2019 CDC reports,

Received 25 June 2021; Accepted 23 September 2021; Published 18 November 2021

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Keywords: antimicrobial resistance; *Clostridioides difficile*; genomics.

Abbreviations: AMR, antimicrobial resistance; CARD, comprehensive antibiotic resistance database; CDC, centers for disease control and prevention; CDI, clostridioides difficile infection; CDS, coding sequence; FQR, fluoroquinolone resistance; MDR, multidrug resistance; MLST, multilocus sequence typing; NI, nucleotide identity; RT, PCR ribotyping; ST, sequence type.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Supplementary material is available with the online version of this article.

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responsible for the highest number of annual deaths among the pathogens listed [2, 3]. In contrast to other pathogens, AMR in *C. difficile* has some unique features. AMR usually leads to difficulties in treating infections [5], and although the treatment of CDI is also a challenge [6], such challenge is not due to AMR per se as resistance to antimicrobials predominantly used for the treatment of CDI (vancomycin, metronidazole and fidaxomicin) remains rare [7]. Instead, AMR plays a significant role in the pathogenesis and spread of CDI [8], as it allows *C. difficile* to survive antimicrobial exposure in the host, while selective pressure allows the emergence and spread of AMR strains. Several AMR strains have been associated with outbreaks; PCR ribotype (RT) 017 with clindamycin [9], RTs 017 and 027 with fluoroquinolones [10, 11], RT 027 with rifampicin [12] and RT 078 with tetracyclines [13].

Using multi-locus sequence typing (MLST), the population of *C. difficile* can be divided into five major clades (C1 – C5) and three smaller cryptic clades (C-I, C-II and C-III). The three cryptic clades are extremely divergent (Figs 1 and 2a) and likely represent independent species or subspecies based on the genomic data [14]. Three of the five major clades contain epidemic sequence types (STs); C2 contains ST 1 (corresponding to RT 027), C4 contains ST 37 (RT 017) and C5 contains ST 11 (several RTs, including RT 078) [14]. To date, studies have been conducted on the role of AMR in the emergence and spread of two epidemic STs, 1 and 11 [10, 12, 13]. A few studies have focused also on *C. difficile* ST 37 [9, 11], a third epidemic lineage [15], which shows a high prevalence of resistance to many antimicrobial classes [8]. Although these studies provided insights on how AMR impacts the spread of *C. difficile*, they are limited to a few strain types in specific geographical regions, and there has not been any study of AMR prevalence in the species-wide population of *C. difficile*. Here, through detailed analysis of 10330 publicly-available genomes from *C. difficile* isolated worldwide, we provide the first species-wide snapshot of AMR genomic epidemiology in *C. difficile*.

METHODS

Genome collection and de-replication of clonal strains

The starting point for this analysis was an international collection of 12098 *C. difficile* Illumina paired-end sequence reads sourced from the NCBI Sequence Read Archive (SRA, <https://www.ncbi.nlm.nih.gov/sra/>) in January 2020. All sequence reads were screened for contamination using Kraken2 v2.0.8-beta and only reads with >85% of sequences classified as *C. difficile* were included. MLST was confirmed on these raw sequence reads by SRST2 v0.2.0 with the database available on PubMLST (<https://pubmlst.org/organisms/clostridioides-difficile>) as previously described [14, 16]. This dataset comprised a total of 270 STs spanning the eight currently described evolutionary clades with a relatively high number of reads from epidemic strains, particularly STs 1 (C2; $n=2,532$), 11 (C5; $n=1,185$) and 37 (C4; $n=786$), many of which were likely to be clonal. To adjust for

Impact Statement

Utilising a publicly-available database of 10330 sequence reads, this study provides the first species-wide evaluation of genotypic AMR in *C. difficile*. We report the most common AMR determinants and their genomic neighbourhood, associations between important AMR and MDR genotypes and specific clades or geographical regions, and rare AMR genotypes that may have been missed in earlier studies.

this strain selection bias, pairwise average nucleotide identity (ANI) of reads from these three STs, as well as ST 2 ($n=1153$), the most common strain in C1, were compared using the Sketch algorithm included in BBtools (<https://sourceforge.net/projects/bbmap/>). Reads with an ANI of 99.98% or higher were considered to be clonal and only one genome from each clonal complex was included in the final analysis. Based on a small dataset of 240 *C. difficile* reads (28680 possible pairs, 531 of which were clonal pairs), this cut-off point had a sensitivity of 70.1% and a specificity of 76.8% for the detection of clonal strains as defined by Didelot *et al.* (data not shown) [17]. The 10330 reads remaining in the dataset are summarised in Table 1.

Identification of multidrug-resistant *C. difficile*

Multidrug-resistant (MDR) *C. difficile* in this study refers to *C. difficile* strains with genotypic AMR determinants (both accessory genes and mutations in chromosomal genes) for at least three of the following antimicrobial classes: carbapenems, fluoroquinolones, glycopeptides (vancomycin), nitroimidazoles (metronidazole), oxazolidinones (linezolid), macrolide-lincosamide-streptogramin B (MLS_B), phenicols, rifamycins, tetracyclines and sulfa-containing agents. Resistance determinants for aminoglycosides and cephalosporins were excluded from this definition as *C. difficile* is intrinsically resistant to these agents [18, 19].

Detection of accessory AMR genes and associated transposons

To detect the presence of accessory AMR genes, raw sequence reads were interrogated against ResFinder/ARGannot databases, with an addition of two newly-characterised AMR genes found in *C. difficile*, *erm(52)* and *mefH*, using SRST2 with default settings [16, 20–22]. These databases contain over 500 different genes conferring resistance to 15 different antimicrobial classes, covering all AMR genes known to be carried by the *C. difficile* population analysed so far [20, 21]. The spectrum of β -lactamase enzymes detected was confirmed against the CARD 2020 database [23]. To further characterise the genomic context of the most common accessory AMR genes, *C. difficile* strains with *ermB*, *tetM* and *tet44* genes were interrogated using SRST2 against a database of *C. difficile* transposons carrying *ermB* (Tn5398 [GenBank accession AF109075.2], Tn6189 [MK895712.1], Tn6194 [HG475346.1], Tn6215 [KC166248.1] and Tn6218 [HG002387.1]), *tetM* (Tn916 [U09422.1], Tn5397

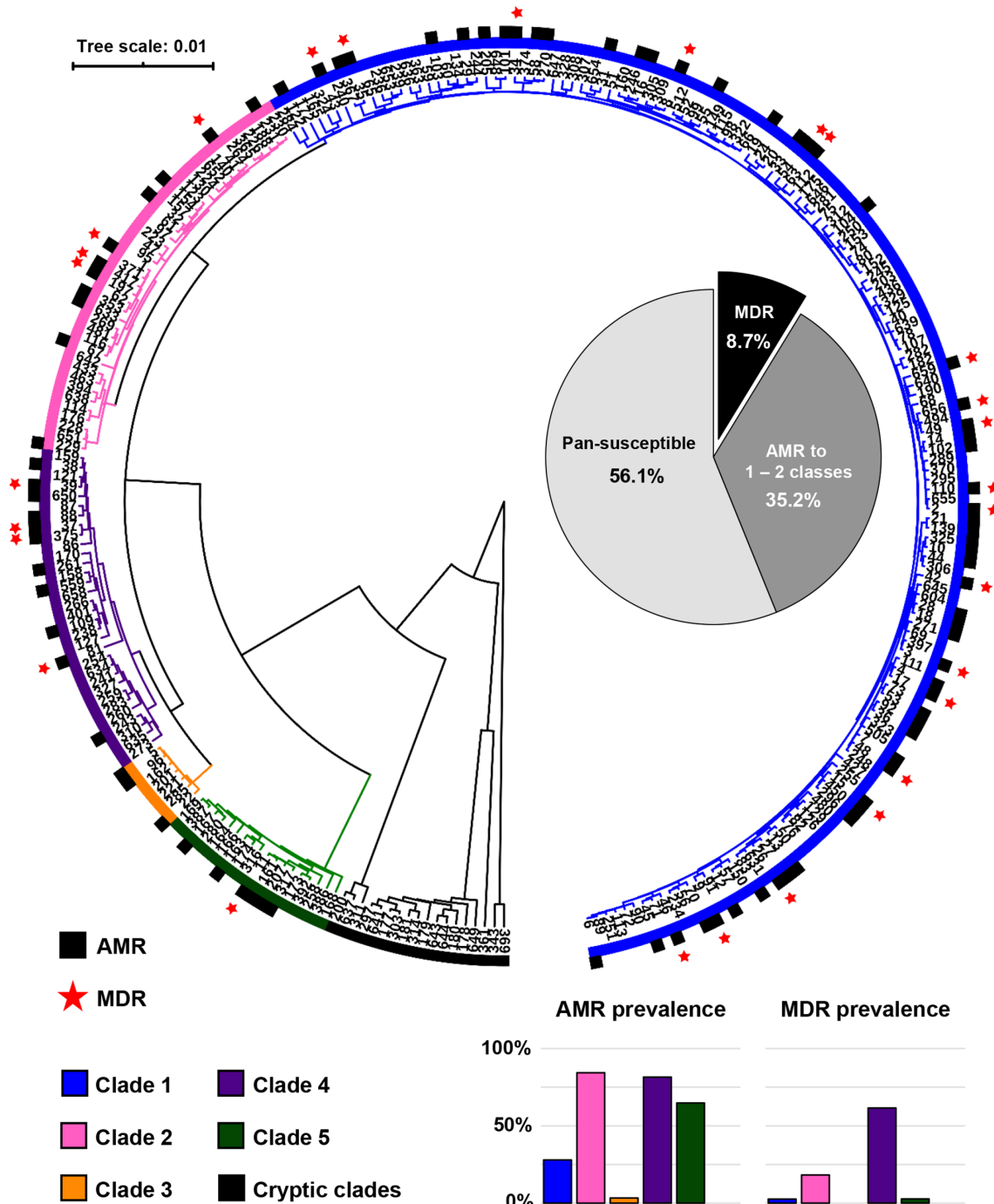


Fig. 1. Distribution of resistant and multidrug-resistant (MDR) *C. difficile*. The UPGMA phylogenetic tree represents a total of 270 STs included in this study. The black sections indicate that at least one strain in the ST had acquired resistance (AMR) to at least one antimicrobial class. The red stars indicate that at least one strain in the ST was MDR (i.e. had acquired resistance to at least three antimicrobial classes). The pie chart in the middle shows the overall prevalence of MDR *C. difficile* (black), *C. difficile* resistance to 1-2 antimicrobial classes (dark grey) and pan-susceptible *C. difficile* (light grey) among 10330 *C. difficile* strains. The bar charts below show the prevalence of resistant and MDR strains in each clade.

[AF333235.1] and Tn6190 [FN665653]) and *tet44* (Tn6164 [FN665653]) [24, 25] with 80% minimum coverage and 10% maximum divergence [16], corresponding with 72% minimum nucleotide identity (NI).

To detect the presence of a plasmid conferring metronidazole resistance (pCD-METRO) [26], a custom database was created consisting of all eight coding sequences (CDS) of pCD-METRO. SRST2 was used with default settings on all sequence reads

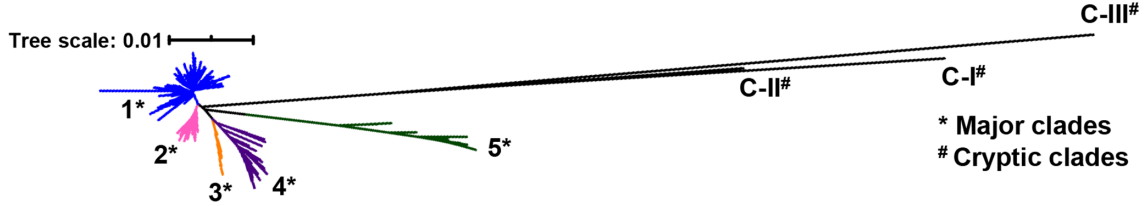
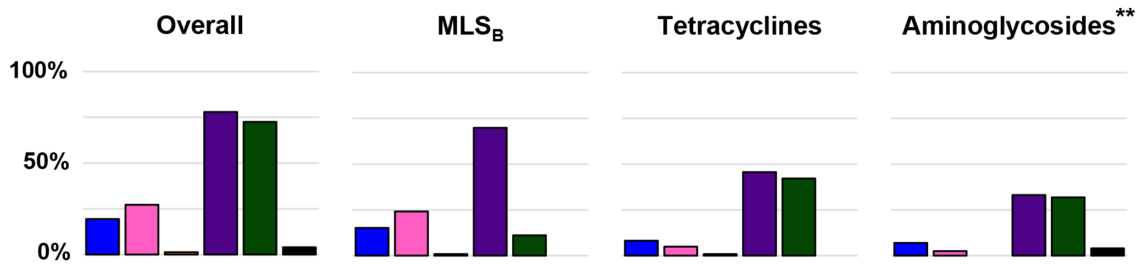
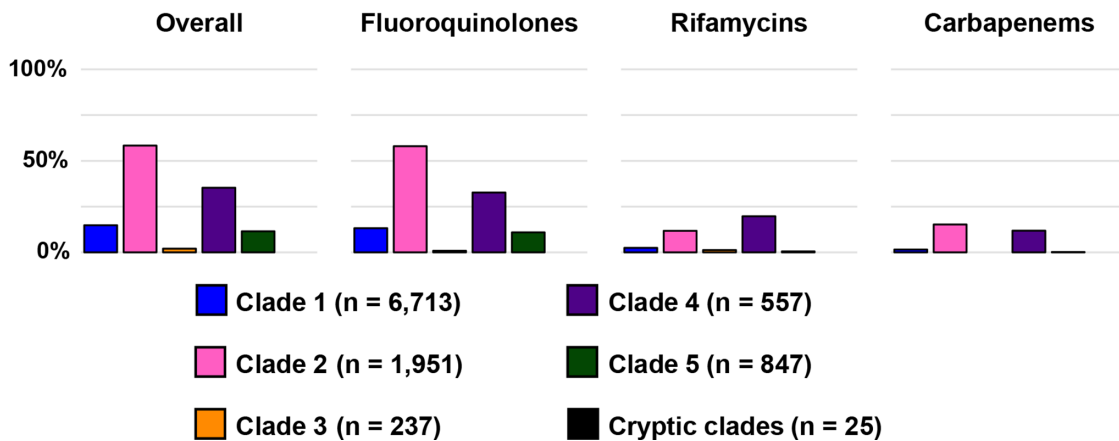
(a) Population structure of *C. difficile***(b) Accessory AMR genes****(c) Amino acid substitutions conferring AMR**

Fig. 2. Summary of antimicrobial resistance genotype of *C. difficile*. (a) For evolutionary context, a neighbour-joining phylogeny based on MLST shows the global population structure of *C. difficile*. (b) The prevalence of *C. difficile* strains harbouring accessory AMR genes across different clades (leftmost) and the prevalence of resistance to important antimicrobial classes conferred mainly by accessory AMR genes. The presence of an aminoglycoside resistance gene (**) does not contribute to the definition of MDR *C. difficile*. (c) The prevalence of *C. difficile* strains having significant amino acid substitutions associated with AMR across different clades (leftmost) and the prevalence of resistance to important antimicrobial classes conferred mainly by amino acid substitution.

against this customised database [16]. The 23 *C. difficile* genomes from the original study [26] were included in the analysis and used to evaluate the accuracy of the database.

Detection of amino acid substitutions conferring AMR

All genomes were screened for known point mutations in *gyrA*, *gyrB*, *rpoB*, *pbp1* and *pbp3* genes using customised databases in SRST2. The reference sequences for these genes were obtained from the PubMLST database (<https://pubmlst.org/organisms/clostridioides-difficile/>) as well as reference *C. difficile* genomes (CD630 [C1, GenBank accession AM180355], CD196 [C2,

FN538970], M68 [C4, FN668375] and M120 [C5, FN665653]). *C. difficile* strains were categorized as resistant to an antimicrobial if they carried a gene allele with at least one significant point mutation listed in Table 2 [24, 27, 28].

Assessment of AMR prevalence in different geographical areas

Data on geographical regions of isolation was available for 6227 (60.3%) *C. difficile* strains: Asia ($n=355$), Europe ($n=3548$), North America ($n=2212$) and Australia/New Zealand ($n=112$). The clade distribution was notably different in these regions (Table 3). Thus, multiple logistic

Table 1. *C. difficile* strains in the de-replicated NCBI database (January 2020)

<i>C. C. difficile</i> clade	No. of genomes (%)	Most prevalent STs
C1	6713 (65.0%)	ST 2 (9.2%)*
		ST 8 (6.0%)*
		ST 3 (5.4%)*
		ST 42 (4.1%)*
		ST 6 (3.2%)*
		ST 44 (2.5%)*
		ST 14 (2.4%)*
C2	1951 (18.9%)	ST 1 (16.6%)*
		ST 41 (0.8%)
C3	237 (2.3%)	ST 5 (2.0%)
		ST 22 (0.2%)
C4	557 (5.4%)	ST 37 (4.3%)*
		ST 39 (0.2%)
C5	847 (8.2%)	ST 11 (7.6%)*
		ST 167 (0.1%)
Cryptic clades	25 (0.2%)	ST 361 (<0.1%)
		ST 177 (<0.1%)
Total	10330	–

*Ten most prevalent sequence types (STs) in this dataset.
ST, sequence type.

regression analyses were performed using R to assess the clade-adjusted AMR prevalence for major antimicrobial classes (MLS_B, tetracyclines, fluoroquinolones and rifamycins), as well as MDR prevalence. From the initial analysis, the overall AMR prevalence was lowest in strains from Australia/New Zealand. Thus, they were used as the reference group in this analysis.

RESULTS

Summary of AMR and MDR prevalence

Of the 10330 *C. difficile* genomes evaluated, 4532 (43.9%) contained acquired resistance genes for at least one antimicrobial class, with 89 STs across five major clades having at least one resistant strain (Fig. 1). A total of 901 strains (8.7%) across 28 STs harboured resistance determinants for three or more antimicrobial classes and were therefore classified as MDR. Based on resistance prevalence, *C. difficile* could be divided into clades with an overall resistance prevalence of $\geq 50\%$, which included C2, C4 and C5, each of which contained an epidemic ST (ST 1 in C2, ST 37 in C4 and ST 11 in C5), and clades with an overall resistance prevalence of $< 50\%$, which included C1 and C3, as well as all three cryptic clades. The prevalence of MDR *C. difficile* was highest in C4 *C. difficile* (61.6% [343/557] compared

to an overall 5.7% [558/9,773] in other clades), over three times higher than in C2 which had the second-highest prevalence of MDR strains (356/1951; 18.3%). The overall resistance prevalence of important antimicrobial classes is shown in Fig. 2.

AMR prevalence in different geographical regions

Fig. 3 shows the results of logistic regression analyses of the clade-adjusted AMR and MDR prevalence compared to strains from Australia/New Zealand as the reference. Overall, strains from Asia, Europe and North America all had higher AMR prevalence ($P < 0.0001$). The difference in AMR prevalence was most pronounced for fluoroquinolones, where the prevalence of substitution associated with fluoroquinolone resistance (FQR) in the three continents (collectively 1491/6115; 24.4%) was estimated to be at least nine times higher than in Australia/New Zealand (3/112; 2.7%). In Asia, Europe and North America, AMR prevalence was not significantly different, with AMR prevalence in Asia (99/355; 27.9%) marginally higher than in Europe (814/3548; 22.9%) and North America (578/2212; 26.1%).

Fluoroquinolone resistance

Overall, 2959 *C. difficile* strains (28.6%) carried known DNA gyrase substitutions associated with FQR. The prevalence of FQR was highest in clade C2 (1606/1951; 82.3%), followed by C4 (296/557; 53.1%). Most resistance was conferred by point substitutions solely within the GyrA subunit of the enzyme (2771/2959; 93.7%), followed by point substitutions solely within the GyrB subunit (104/2959; 3.5%). Only 2.8% (84/2959) had substitutions on both gyrase subunits. The prevalence of GyrB subunit substitution (both alone and in addition to GyrA substitution) was highest in C4 (59/557; 10.6%). The most common GyrA substitution was Thr82Ile (2843/2855; 99.6% of strains with GyrA substitution) and the most common GyrB substitution was Asp426Asn (131/188; 69.7% of strains with GyrB substitution), followed by Asp426Val (44/188; 23.4%), the latter was almost exclusive to C4 (40/44; 90.9% of strains with Asp426Val substitution belonged to C4). Interestingly, a Ser416Ala substitution, a polymorphism that does not confer resistance, was found in a majority of C5 (825/847; 94.9%) and cryptic clades (20/25; 80.0%), but in only one clade C1 strain and none of the other major clades.

MLS_B resistance

Table 4 summarises the major genotypic determinants for MLS_B antimicrobials detected in our survey. The most common determinants were *ermB* (1775 strains, 17.2%) followed by *erm(52)* (145 strains, 1.4%) and *ermG* (25 strains, 0.2%). The *erm* class genes, which methylate 23S rRNA and prevent the binding of MLS_B antimicrobials, are associated with high-level resistance to all MLS_B antimicrobials, as shown by high-level resistance to both clindamycin and erythromycin [29]. The most common non-*erm* genes were *mefH* (156 strains, 1.5%), *mefA* (24

Table 2. Summary of known non-synonymous chromosomal point mutations conferring AMR

Protein	Substitution	Clade distribution*						Comment
		C1	C2	C3	C4	C5	Cryptic	
Fluoroquinolone resistance								
GyrA	Val43Asp	○	○	○	○	○	○	Absent in this dataset
	Asp71Val	●	○	○	●	○	○	Found in <10 strains in this dataset
	Asp81Asn	●	○	○	○	○	○	Found in <10 strains in this dataset
	Thr82Ile	●	●	●	●	●	○	Most common substitution
	Thr82Val	●	○	○	○	●	○	Found in <10 strains in this dataset
	Ala118Thr	●	○	○	○	●	○	Found in <10 strains in this dataset
	Ala384Asp	●	○	○	○	○	○	Found in <10 strains in this dataset
GyrB	Arg377Gly	○	○	○	○	○	○	Absent in this dataset
	Asp426Asn	●	●	○	●	○	○	Most common substitution
	Asp426Val	●	○	○	●	○	○	Mostly found in clade 4 <i>C. difficile</i>
	Arg447Lys	●	●	○	○	●	○	
	Glu466Val	○	○	○	○	●	○	Found in <10 strains in this dataset
Rifamycin resistance								
RpoB	Asp492Asn	○	○	○	○	○	○	Absent in this dataset
	Asp492Val	○	○	○	○	○	○	Absent in this dataset
	His502Asn	●	●	○	●	○	○	
	His502Arg	○	○	○	○	○	○	Absent in this dataset
	His502Leu	○	○	○	○	○	○	Absent in this dataset
	His502Tyr	●	○	○	○	○	○	Found in <10 strains in this dataset
	Arg505Lys	●	●	●	●	●	○	Most common substitution
	Ser550Phe	●	●	○	○	●	○	Found in <10 strains in this dataset
Ser550Tyr	●	●	○	●	○	○	Found in <10 strains in this dataset	
Fidaxomicin resistance								
RpoB	Gln1073Arg	○	○	○	○	○	○	Absent in this dataset
Carbapenem resistance								
Pbp1	Leu543His	●	○	○	○	○	○	
	Ala555Thr	●	●	○	●	●	○	Most common substitution
Pbp3	Tyr721Ser	●	○	○	●	○	○	

*Based on significant findings in this study. Solid circles refer to the presence of the substitution in the clade.

strains, 0.2%), *msrD* (21 strains, 0.2%) and *lnuC* (17 strains, 0.2%). In total, 1979 *C. difficile* strains (19.2%) across 65 STs (23.9%) in five major clades carried acquired MLS_B resistance determinants.

Among *ermB*-positive strains, known *ermB*-carrying transposons were identified in 1706 strains (96.5%) (range, 77.6–100.0% NI). Transposon diversity was highest in C1

(Table 4). The most common *ermB*-positive transposon was Tn6194 (788/1775; 44.4%; 81.9–100.0% NI), followed by Tn6189 (424/1775; 23.9%; 77.6–99.9% NI) and Tn6218 (216/1775; 12.2%; 85.3–100.0% NI). Tn5398, which contains two copies of the *ermB* gene, was found in 170 strains (9.6%; 81.2–100.0% NI), most of which belonged to clade C1 (168/170; 98.8%).

Table 3. Clade distribution in four major geographical regions

Region	Clade					
	C1	C2	C3	C4	C5	Cryptic
Asia	76.6%	3.4%	3.9%	15.2%	0.6%	0.3%
Europe	74.4%	11.0%	3.4%	2.7%	8.3%	0.2%
North America	68.9%	24.7%	0.1%	2.1%	4.0%	0.2%
Australia/New Zealand	39.3%	26.8%	1.8%	3.6%	28.6%	0.0%

Tetracycline resistance

Table 5 summarises the genotypic determinants found for tetracyclines. The most common tetracycline resistance determinant was *tetM* (1447 strains, 14.0%), followed by *tet40* (214 strains, 2.1%) and *tet44* (125 strains, 1.2%). These three genes encode ribosomal protection proteins which prevent the binding of tetracyclines to 16S rRNA. In total, 1645 *C. difficile* strains (15.9%) across 68 STs (25.0%) in five major clades carried at least one *tet* gene, with 333 strains (3.2%) carrying more than one gene, 81.4% of which (271/333) belonged to clade C5. Five ST11 *C. difficile* strains (C5) carried four different *tet* genes, the highest number of *tet* genes per genome in this dataset. Interestingly, *tet40* and *tet44* were almost exclusively found in clade C5 *C. difficile* (94.9 and 98.4% of *tet40*- and *tet44*-positive *C. difficile* belonged to C5, respectively).

Known *tetM*-positive transposons and their variants were detected in 1245 (86.0%) *tetM*-positive *C. difficile* (78.0–100.0% NI). Transposon diversity was highest in clade C1 (Table 5). The most common transposons were Tn916 (564/1447; 39.0%; 83.3–100.0% NI) and Tn6190 (456/1447; 31.5%; 81.5–100.0% NI). In contrast to the prevalence of *ermB*-positive transposons above, the distribution of *tetM*-positive transposons was different in clades C2, C4 and C5 (Fig. 4a). Known *tetM*-positive transposons could not be identified in 78.1% of *tetM*-positive clade C2 *C. difficile* (100/128). Analysis of the assembled genome of ST1 strain C00008355, a clinical isolate from the UK [SRA accession ERR347593], showed that the *tetM* gene was located on a 9013 bp element with an overall 37.1% GC which did not match any transposons in the NCBI database or published literature (Fig. 4b). The annotated sequence of this novel Tn, designated Tn6944 by the Liverpool transposon repository [30], was submitted to GenBank and is available in the DDBJ/ENA/GenBank databases under the accession number BK013348. Besides *tetM*, Tn6944 also carries *mefH* which encodes a macrolide efflux protein [22]. Tn6944 was identified in an additional 156 *C. difficile* strains (78.0–100.0% NI), 97 of which belonged to clade C2 (Table 5). All *tet44*-positive *C. difficile* harboured Tn6164 (80.3–100.0% NI), a 100 kbp genomic island containing *tet44* and *ant* [6]-Ib, a streptomycin resistance determinant [31].

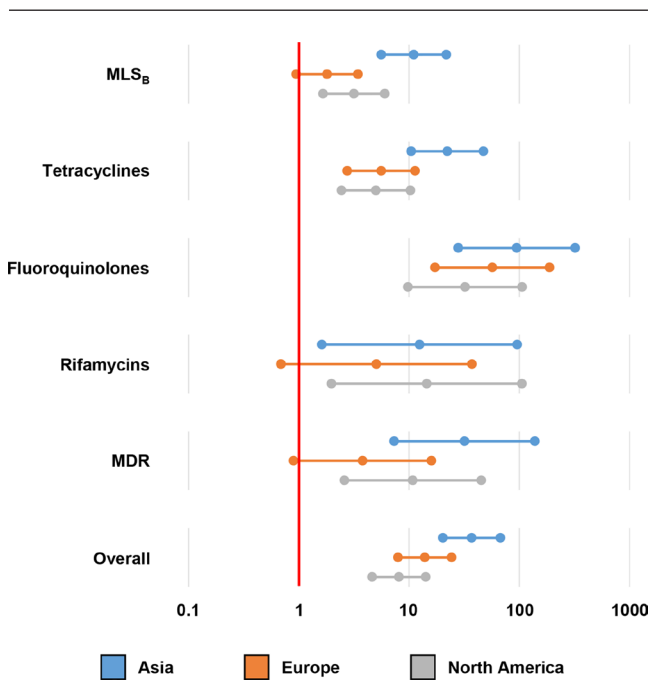


Fig. 3. Difference in antimicrobial resistance (AMR) prevalence in different geographical regions. Multiple logistic regression analyses were performed to compare the clade-adjusted AMR prevalence in four regions (Asia, Europe, North America and Australia/New Zealand). The Forest plot represents the estimated AMR prevalence in each continent compared to Australia/New Zealand.

Vancomycin resistance

A complete *vanB* operon (*vanR_B*, *vanS_B*, *vanY_B*, *vanW*, *vanH_B*, *vanB* and *vanX_B* genes) was identified in one *C. difficile* strain, belonging to ST 11 (clade C5). This *vanB* operon was previously described to be phenotypically silent due to a ~2.1 kbp disruption of the *vanR_B* gene which is a response regulator and part of a key two-component system [32, 33]. This strain was thus considered susceptible to vancomycin.

Metronidazole resistance

SRST2 with the customised pCD-METRO plasmid database correctly identified the plasmid in 14 *C. difficile* genomes from the Boekhoud *et al.* study [26] (nine belonged to ST 15 and five belonged to ST 2). Apart from these strains, the pCD-METRO plasmid was found in only one *C. difficile* strain belonging to ST15 (clade C1, RT 010, non-toxicogenic), the same RT reported in the Boekhoud *et al.* study [26].

Table 4. Summary of resistance determinants for MLS_B antimicrobials

Gene	Clade distribution [N (%)]						Overall
	C1	C2	C3	C4	C5	Cryptic	
<i>ermB</i>	953 (14.2%)	421 (21.6%)	0 (0.0%)	328 (58.9%)	73 (8.6%)	0 (0.0%)	1776 (17.2%)
Tn5398	168 (2.5%)	1 (0.1%)	0 (0.0%)	0 (0.0%)	1 (0.1%)	0 (0.0%)	170 (1.6%)
Tn6189	259 (3.9%)	104 (5.3%)	0 (0.0%)	44 (7.9%)	17 (2.0%)	0 (0.0%)	424 (4.1%)
Tn6194	204 (3.0%)	270 (13.8%)	0 (0.0%)	268 (48.1%)	46 (5.4%)	0 (0.0%)	788 (7.6%)
Tn6215	106 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.2%)	0 (0.0%)	108 (1.0%)
Tn6218	200 (3.0%)	4 (0.2%)	0 (0.0%)	10 (1.8%)	2 (0.2%)	0 (0.0%)	216 (2.1%)
Unknown	16 (0.2%)	42 (2.2%)	0 (0.0%)	6 (1.1%)	5 (0.6%)	0 (0.0%)	69 (0.7%)
Other <i>erm</i> genes	86 (1.3%)	17 (0.9%)	1 (0.4%)	66 (11.8%)	4 (0.5%)	0 (0.0%)	175 (1.7%)
Non- <i>erm</i> genes	76 (1.1%)	104 (5.3%)	1 (0.4%)	22 (3.9%)	18 (2.1%)	0 (0.0%)	222 (2.1%)

In total, only ten of 223 *C. difficile* ST 15 strains (4.5%) contained the pCD-METRO plasmid.

Rifamycin resistance

Points mutation in *rpoB* were found in 688 *C. difficile* strains (6.7%), with the highest prevalence in clade C4 (179/557; 32.1%), followed by C2 (327/1951; 16.8%). The most common substitution was Arg505Lys found in 68.0% of resistant strains (468/688), followed by His502Asn (340/688; 49.4%), with 44.5% of resistant strains (306/688) having both substitutions. Besides rifamycins, a Gln1073Arg substitution in RpoB was also reported to be associated with reduced susceptibility to fidaxomicin [28]. This substitution was not detected in this dataset.

Carbapenem resistance

A total of 643 *C. difficile* strains (6.2%) had substitutions in either Pbp1 or Pbp3 conferring imipenem resistance, with the prevalence slightly higher in clades C2 and C4 (21.6

and 19.4%, respectively, $P=0.2786$) than the other clades (collectively 1.4%, $P<0.0001$); 504 *C. difficile* strains had a substitution in Pbp1 (492 having A555T and 12 having L543H), 125 strains had a Y721S substitution in Pbp3 and 12 strains from ST 37 (C4) had substitutions on both Pbp1 (all A555T) and Pbp3.

In addition to the detection of point substitutions, carbapenemase-encoding genes were identified in two *C. difficile* strains; an unnamed strain [accession ERR2703875; ST 2, C1] carried SHV-1 and CD72 [accession SRR5367248; ST 81, C4] carried PER-1. By NCBI BLAST approach, the SHV-1 encoding gene was found on an element resembling a *Klebsiella pneumoniae* plasmid tig00001208_pilon [CP036443.1, 99.7% sequence identity, 35% coverage] and the PER-1 encoding gene was found on an element resembling *Acinetobacter haemolyticus* plasmid pAHTJR1 [CP038010.1, 99.8% sequence identity, 5% coverage].

Table 5. Summary of resistance determinants for tetracyclines

Gene	Clade distribution [N (%)]						Overall
	C1	C2	C3	C4	C5	Cryptic	
<i>tetM</i>	457 (6.8%)	128 (6.6%)	0 (0.0%)	402 (72.2%)	460 (54.3%)	0 (0.0%)	1447 (14.0%)
Tn916	146 (2.2%)	25 (1.3%)	0 (0.0%)	95 (17.1%)	298 (35.2%)	0 (0.0%)	564 (5.5%)
Tn5397	215 (3.2%)	1 (0.1%)	0 (0.0%)	1 (0.2%)	8 (0.9%)	0 (0.0%)	225 (2.2%)
Tn6190	7 (0.1%)	2 (0.1%)	0 (0.0%)	297 (53.3%)	150 (17.7%)	0 (0.0%)	456 (4.4%)
Tn6944	52 (0.8%)	97 (5.0%)	0 (0.0%)	6 (1.1%)	1 (0.1%)	0 (0.0%)	156 (1.5%)
Unknown	37 (0.6%)	3 (0.2%)	0 (0.0%)	3 (0.5%)	3 (0.4%)	0 (0.0%)	46 (0.4%)
<i>tet44</i>	2 (<0.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	123 (14.5%)	0 (0.0%)	125 (1.2%)
Tn6164	2 (<0.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	123 (14.5%)	0 (0.0%)	125 (1.2%)
Other <i>tet</i> genes	129 (1.9%)	12 (0.6%)	2 (0.8%)	14 (2.5%)	336 (39.7%)	0 (0.0%)	493 (4.8%)

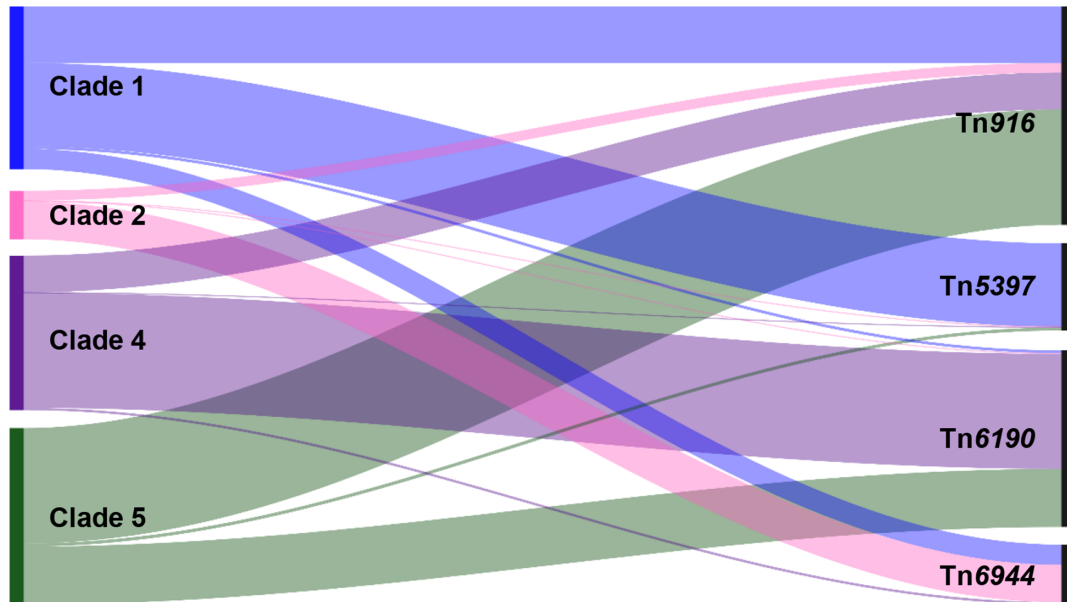
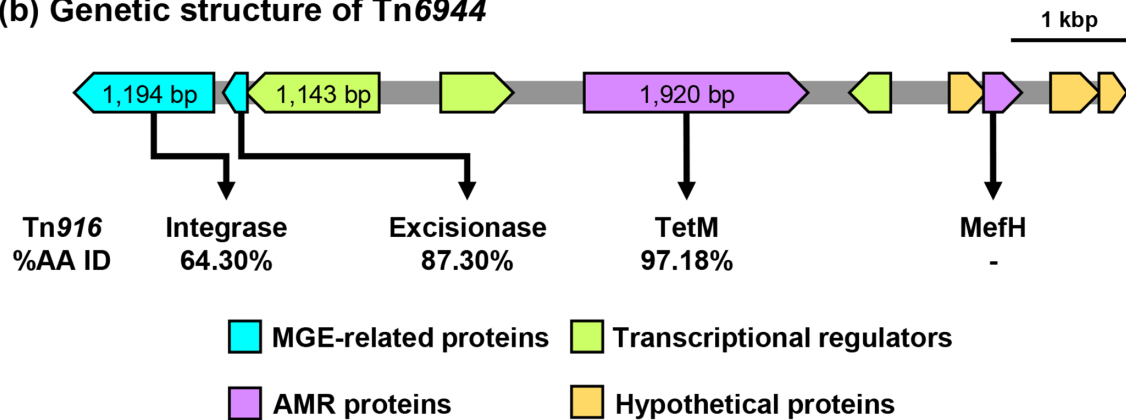
(a) Clade specificity of *tetM*-positive transposons**(b) Genetic structure of Tn6944**

Fig. 4. Clade specificity of *tetM*-positive transposons in *C. difficile*. (a) Sankey diagram shows the prevalence of four *tetM*-positive transposons commonly found in *C. difficile*. The left and right axes represent *C. difficile* clades and the transposons, respectively. The height of the left axis corresponds to the number of *tetM*-positive *C. difficile* strains in each clade, excluding strains with unknown transposons (clade 1, $n=419$; clade 2, $n=208$; clade 4, $n=711$; clade 4, $n=688$). (b) The genetic structure of the novel *tetM*-positive Tn, Tn6944 [BK013348]. The amino acid sequences of the key elements in this transposon were compared to the elements found in Tn916 [U09422.1].

Other resistance types

Genotypic resistance determinants for five other antimicrobials were also identified. First, 124 *C. difficile* strains (1.2%) were positive for the *cfrB* gene which confers linezolid resistance [34]. Resistance determinants for trimethoprim were identified in 147 (1.4%) *C. difficile* strains, six of which also harboured sulphamide resistance determinants. Ninety-eight *C. difficile* strains (1.0%) carried chloramphenicol resistance determinants. The most common determinant was *catP* (92/124; 93.9%).

In addition to the *C. difficile* class D β -lactamases which confer intrinsic cephalosporin resistance in *C. difficile* [18], a few *C. difficile* strains also had other classes of β -lactamases. Forty-three *C. difficile* strains carried genes encoding extended-spectrum β -lactamases (ESBL), the most common type belonging to the TEM family (36 strains), and five strains carried AmpC β -lactamase genes.

Finally, 1250 *C. difficile* strains (12.1%) carried various aminoglycoside-resistance determinants. The most common

determinants were *aac6-aph2* (666 strains, 6.5%), *aph-III* (279 strains, 2.7%) and *sat4* (271 strains, 2.6%) genes. Notably, 270 strains carried a locus containing *aph-III* and *sat4* genes adjacent to one another, 68.2% of which (184/270) belonged to clade C5 (183 ST 11 strains and one ST 163 strain). This locus had 99.91% nucleic acid identity to a gene cluster found in *Erysipelothrix rhusiopathiae*, as described in a previous study [35].

DISCUSSION

The success of several epidemic *C. difficile* strains is thought to be associated with an AMR phenotype which provides a survival advantage for these *C. difficile* strains in the presence of antimicrobials while imposing little fitness cost [36–38]. Resistance to several antimicrobial classes has been associated with specific *C. difficile* lineages: fluoroquinolone and rifamycin resistance and *C. difficile* ST 1 (C2) [10, 12], tetracycline resistance and *C. difficile* ST 11 (C5) [13], as well as resistance to various antimicrobial classes and MDR and *C. difficile* ST 37 (C4) [8]. This study provides genotypic evidence to support these associations, demonstrated by the higher resistance prevalence and, especially in the case of tetracycline resistance in *C. difficile* ST 11, a higher diversity of resistance determinants in the associated clades.

Although the metadata was not complete (only 60.3% of strains had information on geographical origin and there was inadequate information on host species), some interesting findings can be seen in this genome subset. Fig. 3 demonstrates the difference in AMR prevalence in different continents which may reflect the use of antimicrobials in these regions. The most prominent example is fluoroquinolones which are strictly regulated in Australia and New Zealand but widely used elsewhere [39]. Consequently, there was a stark difference in the prevalence of FQR between Australia and the other three regions. Besides fluoroquinolones, the high prevalence of MLS_B and tetracycline resistance, especially in Asia, is suggestive of the overuse of these antimicrobials in the region [40]. We compared the prevalence of AMR genotypes in Australia/New Zealand with a surveillance study from the same region and found that the prevalence in this study correlates with the phenotypic data ($P > 0.05$ for clindamycin [high-level resistance], moxifloxacin and rifaximin resistance) [41]. A similar correlation was seen when comparing the AMR prevalence in Asia and North America with studies from Thailand [22] and the United States [42], respectively. On the contrary, this study underestimated the AMR prevalence in Europe [43, 44]. It should be noted that there was a difference in the number of sequenced strains from various regions. For instance, there were 3548 strains from Europe, many of which were from non-clinical sources, and only 112 strains from Australia/New Zealand in this dataset. As next-generation sequencing (NGS) becomes more accessible [45] and the collection of metadata becomes more systematic, a future study should represent a more complete picture of AMR in the global *C. difficile* population.

Based on a large sample size, which should give an accurate representation of the *C. difficile* population, this study provides a global atlas of genotypic AMR determinants in *C. difficile*. In general, one resistance determinant appeared to dominate in most antimicrobial classes. For example, *ermB* and *tetM* genes were found in almost 90% of *C. difficile* strains with genotypic resistance to MLS_B and tetracycline, respectively. Fluoroquinolone and rifamycin resistance was also mainly determined by a single substitution in GyrA (Thr82Ile) and RpoB (Arg505Lys), respectively. This is similar to other Gram-positive bacteria, such as *Staphylococcus aureus* [46], where one genotypic determinant is responsible for a resistance phenotype in a majority of the bacterial population and is in contrast to many Gram-negative bacteria, such as several members in the *Enterobacteriaceae* [47], where resistance to an antimicrobial class can be conferred by several genotypic determinants. The dominance of a single genotypic determinant accommodates the development of genotype-based rapid detection kits for drug-resistant *C. difficile*, similar to real-time PCR assays for methicillin-resistant *S. aureus* [48]. Such tools can be beneficial for surveillance for *C. difficile* outbreaks in the future.

Another benefit of large sample size and NGS is the power to detect rare genotypic determinants. The most notable finding was the detection of carbapenemase-encoding genes in two *C. difficile* strains, STs 2 and 81, comprising approximately 0.02% of the population. Previously, carbapenem resistance in *C. difficile* has been mainly associated with point substitutions on Pbp1 and Pbp3 which cannot be transferred horizontally and only confer imipenem resistance [27]. On the contrary, many carbapenemases provide resistance to a wide range of carbapenem antimicrobials and are capable of horizontal transfer [49]. The detection of carbapenemase-encoding genes is concerning, as *C. difficile* mainly resides in the colon, the same habitat as many pathogenic *Enterobacteriaceae*, and transfer of these genes could give rise to carbapenem-resistant *Enterobacteriaceae* (CRE), another urgent threat in AMR [3]. Conversely, *C. difficile* can also serve as a reservoir of these resistance genes. Indeed, the gene encoding SHV-1, one of the carbapenemases found in this study, was found on an element similar to a *K. pneumoniae* plasmid (tig00001208, GenBank accession CP036443.1; 99.7% NI), suggesting a possible inter-phylum transfer event between these two organisms, although this plasmid was classified as an IncF plasmid according to PlasmidFinder [50]. Generally, the host range for IncF plasmids is limited to only within the Family *Enterobacteriaceae* [51]. Further study is thus needed to confirm that this horizontal transfer is possible.

Recently, two novel resistance determinants for MLS_B antimicrobials were found in Asian *C. difficile* isolates; *erm(52)* and *mefH* [22]. In a larger population of *C. difficile*, these two genes were found in 1.4–1.5% of *C. difficile* strains, approximately six times more prevalent than *ermG*, a gene previously believed to be the second most prevalent resistance determinant in *C. difficile* [8]. Failing to detect these two determinants could partially explain the discrepancy between resistance genotype and phenotype in earlier studies [24]. Indeed, the inclusion

of *erm(52)* improved the concordance between clindamycin resistance genotype and high-level clindamycin resistance phenotype to 100% and *mefH* provided concordant genotype to *C. difficile* strains with isolated erythromycin resistance [22]. Further characterisation of *mefH* revealed that the gene was located adjacent to *tetM* on a newly defined transposon Tn6944 (Fig. 4b). This transposon has also escaped detection and characterisation despite being present mainly in ST 1 (clade C2), a strain that has been extensively studied [10, 52]. Interestingly, even though tetracycline resistance was a key factor in the evolution of the epidemic *C. difficile* ST 11 due to its use in agricultural practices [13], this antimicrobial was not included in the antimicrobial susceptibility panel in a pan-European study [43, 44]. Tetracycline resistance was also never mentioned in studies involving *C. difficile* ST 1, perhaps because the prevalence in this lineage was much lower than that of FQR mutations (7.1 vs 82.3%, respectively).

A recent study explored the genomic architectures of several accessory AMR genes in 2190 publicly-available *C. difficile* assemblies and suggested that horizontal gene transfer played a crucial role in the spread of AMR both within *C. difficile* and among intestinal bacteria in general [53]. This study provides more supporting evidence, as there was a high diversity of *ermB*-positive transposons throughout the four major clades, suggesting a constant exchange of genes among the population. Evidence of gene transfer could also be seen among *tetM*-positive transposons. For instance, Tn6190 was shared between C4 and C5, despite their divergence over a million years ago [14].

We also identified key antimicrobials, resistance to which can potentially lead to outbreaks of CDI; fluoroquinolones, MLS_B, rifamycins and tetracyclines, as well as the specific *C. difficile* clades associated with such resistance. This provides an opportunity to develop a focused antimicrobial stewardship policy, targeting specific antimicrobial classes based on the prevalent *C. difficile* strains in the region. A real-world example can be seen in the US, where the reduction of fluoroquinolone use led to a significant reduction in the number of CDI cases and the associated cost [3].

As an obligate anaerobe, *C. difficile* is intrinsically resistant to aminoglycosides. Additional resistance determinants to these antimicrobials are not beneficial to the bacterium and are unlikely to be conserved in the genome. Thus, the presence of aminoglycoside resistance determinants should reflect recent, and likely continuous, inter-species gene transfer with taxa in diverse environments such as the animal gut and soils. The most common aminoglycoside resistance determinant was *aac6-aph2*, a bifunctional gene found in *Staphylococcus* spp. and *Enterococcus* spp. [54], commensal species commonly found in the human and animal gut. Interestingly, many ST 11 (C5) strains also carried an *aph-III* and *sat4* cluster, a gene cluster found in *E. rhusiopathiae* which inhabits the porcine gut [55], supporting the animal origin and One Health importance of this lineage [35]. Indeed, aminoglycosides have been heavily used in both agricultural and veterinary practices [56]. The presence of aminoglycoside resistance determinants in *C.*

difficile highlights another aspect of AMR in *C. difficile*; the role of *C. difficile* as a reservoir of AMR genes. Aminoglycosides remain a key treatment option for serious staphylococcal and enterococcal infections, such as infective endocarditis, in conjunction with β -lactams antimicrobials [57]. Resistance to aminoglycosides in these pathogens complicates treatment of these infections which may result in adverse clinical outcomes. Thus, colonisation with *C. difficile* carrying these resistance determinants may pose an additional risk of treatment failure in these patients.

This study utilised the direct analysis of raw sequence reads without the need for genome assembly which enabled the characterisation of a large dataset within a relatively short time (approximately 5 min of CPU time [16 cores] per strain as opposed to more than 30 min of CPU time per strain for a *de novo* assembly pipeline). SRST2 provides rapid MLST and AMR genotyping [16]. SRST2-based AMR genotyping can be performed using three types of databases: well-characterised databases of accessory AMR genes [20, 21, 23], species-specific gene allele databases (e.g. the PubMLST database), as well as customised databases. The latter was used in a previous study on a smaller dataset, the results of which were similar to a standard approach using BLAST on annotated draft genomes [58].

Besides the lack of complete metadata, another limitation of this study was the lack of comparative phenotypic data, as the study was performed on a publicly-available genome dataset. However, many key AMR genotypes were reported to have a high correlation with phenotypic characteristics [24, 58]. Thus, the prevalence values reported in this study should reflect the resistance prevalence in *C. difficile* population. Also, this study only reports the presence or absence of genotypic AMR determinants and does not take into account the different alleles of the genes, as the alleles were not included in the databases used in the analyses [20, 21]. Further analyses on the allelic distribution across *C. difficile* population may provide additional information on the spread of AMR genes.

In conclusion, almost half of *C. difficile* strains studied carried at least one genotypic resistant determinant. The resistance prevalence was higher among clades C2, C4 and C5 which have been associated with epidemic *C. difficile* STs 1, 37 and 11, respectively. Though resistance to antimicrobials for treatment of CDI is rare, this study provides evidence to support the role of AMR in the spread of *C. difficile*, as well as the role of *C. difficile* as a reservoir of accessory AMR genes, most notably aminoglycoside resistance determinants and carbapenemase-encoding genes.

Funding information

This work was supported, in part, by funding from The Raine Medical Research Foundation (RPG002-19) and a Fellowship from the National Health and Medical Research Council (APP1138257) awarded to D.R.K. K.I. is a recipient of the Mahidol Scholarship from Mahidol University, Thailand. This research used the facilities and services of the Pawsey Supercomputing Centre [Perth, Western Australia].

Conflicts of interest

The authors declare that there are no conflicts of interest.

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