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Whole-genome sequencing of toxigenic *Clostridioides difficile* reveals multidrug resistance and virulence genes in strains of environmental and animal origin

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Abstract

Background *Clostridioides difficile* has been recognized as an emerging pathogen in both humans and animals. In this context, antimicrobial resistance plays a major role in driving the spread of this disease, often leading to therapeutic failure. Moreover, recent increases in community-acquired *C. difficile* infections have led to greater numbers of investigations into the animal origin of the disease. The aim of this study was to evaluate the genetic similarities between 23 environmental and animal isolates by using whole-genome sequencing and to determine antimicrobial resistance and virulence factor genes in toxigenic *C. difficile* strains to provide important data for the development of diagnostic methods or treatment guidelines.

Results The most common sequence type was ST11 (87%), followed by ST2 (9%) and ST19 (4%). In addition, 86.95% of the strains exhibited multidrug resistance, with antimicrobial resistance to mainly aminoglycosides, fluoroquinolones, tetracycline and B-lactams; nevertheless, one strain also carried other resistance genes that conferred resistance to lincosamide, macrolides, streptogramin a, streptogramin b, pleuromutilin, oxazolidinone and amphenicol. In addition, a wide range of virulence factor genes, such as those encoding adherence factors, exoenzymes and toxins, were found. However, we observed variations between toxinotypes, ribotypes and sequence types.

Conclusions The results of this study demonstrated significant genetic similarity between ST11 strains isolated from environmental sampling and from animal origin; these strains may represent a reservoir for community-acquired *C. difficile* infection, which is becoming a growing public health threat due to the development of multridug resistant (MDR) bacteria and the number of virulence factors detected.

Keywords *Clostridioides difficile*, Antimicrobial resistance genes, Virulence genes, Toxin, Whole-genome sequencing, One Health

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Background

Clostridioides difficile (*CD*) is a gram-positive, anaerobic and spore-forming bacterium that can survive under adverse environmental conditions [1–3]. Its pathogenic action is performed through the production of toxin A (tcdA), toxin B (tcdB) and hypervirulent strains, which produce the binary toxin (CDT), expressed from the enzymatic component (cdtA) and binding component (cdtB) operons [4, 5]. There are two routes for developing *C. difficile* infection (CDI): first, gut microbiota disruption, which causes dysbiosis and favors CD proliferation; and second, contagion from exogenous sources [6, 7]. In humans, CDI can progress from diarrhea to fulminant pseudomembranous colitis, and if not treated appropriately, it can lead to toxic megacolon and even death [8].

In recent years, the epidemiology of CD has changed, and CD is now recognized as an emerging pathogen in both humans and animals [9]. In this context, antimicrobial resistance (AMR) plays a major role in driving the spread of some CD strains [10, 11] and represents an important threat to global health [12], leading to therapeutic failure and increasing cases of recurrence [13], above all because antibiotics represent both a treatment and a risk factor for CDI [14]. The European Centre for Disease Prevention and Control (ECDC) point-prevalence survey in 2016–2017 estimated that 189,526 patients annually developed health care-associated CDI in the EU/EEA [15], suggesting that there were 7864 fatal HA-CDI cases [16].

Recent increases in community-acquired CDI (CA-CDI) have led to increasing numbers of investigations into the animal origin of CDI [17–19]. These studies have identified animals as potential reservoirs of CD for human CDI, especially because strains typed as ribotype (RT) 078 and RT014 are commonly found in both animals and humans [20–27].

The aim of this study was to evaluate the genetic similarities between 23 environmental and animal isolates by using whole-genome sequencing and to determine antimicrobial resistance and virulence factor genes in toxigenic *C. difficile* strains to provide important data for the development of diagnostic methods or treatment guidelines.

Results

Taxonomic identification

Taxonomic identification confirmed that all the strains belonged to the *Peptostreptococcaceae* family, *Clostridioides* genus and *C. difficile* species (TAXID species: 1496), with percentage of reads similarity values of 90.98–97.07% from each sample covered by the clade rooted at this taxon. Pangenome analysis revealed 10,599 different genes overall, of which 1841 (17.4%) were core genes, common to all the samples, these genes are essential and conserved across all genomes; and among accessory genes: 144 (1.4%) were soft core genes, these genes were found in 95% of the genomes, 2804 (26.5%) were shell genes, present in 15% to 95% of the genomes, representing functions that are not necessary for all strains and 5810 (54.8%) were cloud genes, found in less than 15% of the genomes, are often indicative of niche adaptations or acquired traits, such as resistance genes or genes involved in specific environmental responses.

The isolates were classified into three different sequence types (STs) according to their multilocus sequence types (MLSTs). ST11 was the most common [20/23 (87%)], followed by ST2 [2/23 (9%)] and ST19 [1/23 (4%)].

The Single Nucleotide Polymorphism (SNP)-based approach used to establish the genetic relationship between isolates including external isolates (total $n=60$) identified two major clades (A and B) separated by 65,707–91,175 SNPs (Fig. 1). Clade A included the three isolates from this study belonging to ST2 (CD15 and CD16) and ST19 (CD19), and external isolates from different countries and sources, and were separated by < 9,841 SNPs (Fig. 1).

Clade B was divided into two different subclades (B1 and B2) separated by < 1,013 SNPs. Subclade B1 included mostly isolates from human CDI and the environment from external studies and two isolates from this study (CD23 and CD13) and were separated by 9–1149 SNPs (9–1014 SNPs if only strains from Spain are considered). All the isolates from this subclade for which the ST was available (7/60) were ST11. Subclade B2 (< 29 SNPs) was formed mostly by isolates from this study ($n=18$) and external isolates from Spain ($n=4$) and Ireland ($n=1$), mainly from animals and the environment. Strains from this subclade were separated by < 29 SNPs (< 25 SNPs considering only strains from Spain). All isolates in subclade B2 for which the ST was available (18/23) were also ST11.

Antimicrobial resistance genes

A high percentage of multidrug resistance (MDR) strains were detected: 21 (86.95%) strains carried more than three resistance genes that confer antimicrobial resistance, mainly to aminoglycosides, fluoroquinolones, tetracycline and B-lactams.

A total of 23 (100%) strains carried resistance-conferring genes against carbapenems (*CDD-1* and *CDD-2*) and fluoroquinolones (*cdeA*); 21 (91.3%) had resistance to aminoglycosides (*ant(6)-Ia_1, ant(6)-Ia_2, ant(6)-Ia_3, ant(6)-Ib_1, aac(6')-aph(2'')_1, (AGly)aadC and aph(3')-III_1*), of which 34.78% had two types of aminoglycoside resistance genes; 20 (86.95%) had resistance

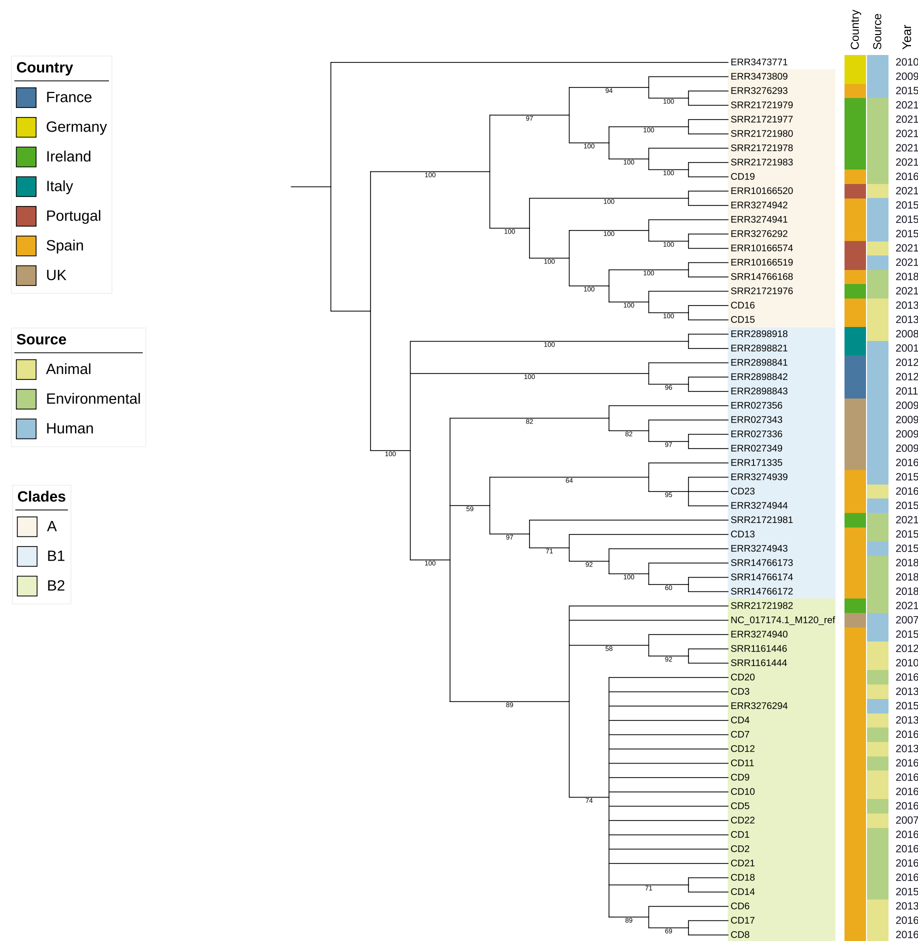

Fig. 1 Phylogenetic relationship of 61 C. difficile isolates. Maximum-likelihood phylogenetic tree (branches with bootstrap values > 50), based on whole-genome SNPs analysis, shows the following information to the left of the isolates: country, source and year of isolation. The coloured labels indicate the three subclades (A, B1, B2)

to tetracycline (*tet(M)_1, tet(M)_4, tet(M)_8, tet(M)_10, tet(44)_1 and tet(40)_1*), 39.13% of which had two types of tetracycline resistance genes; and 1 (4.34%) had resistance to nucleoside antibiotics (*Sat-4*). In one strain (4.34%), two resistance genes were detected: the *erm(B)* gene, which encodes resistance to lincosamide, macrolide and streptogramin b; and the *cfr(B)* gene, which encodes resistance to lincosamide, streptogramin a, pleuromutilin, oxazolidinone and amphenicol (Table 1).

Virulence factor genes

Among exoenzymes, 23 (100%) strains expressed the *zmp1* gene, and 22 (95.65%) expressed the *cwp84* gene; regarding adherence factors, 23 (100%) strains expressed the *CD0873, CD2831, fbpA/fbp68* and *groEL* genes; 2 (8.69%) strains had the *cbpA* gene; and 3 (13.04%) strains had the *CD3246* gene. Similarly, among toxins, regarding the most important virulence factors, 23 (100%) strains had the *tcdB* gene, 3 (13.04%) strains had the *cdtA* and *cdtB* genes, and 10 (43.47%) strains had the *tcdA* gene (Table 2).

Discussion

Despite having been collected across different years and different locations in Spain, the CD strains analyzed belonging to ST11 are closely related. An interconnected transmission pathway in the environment through which CD spreads has been suggested [28], which underlines the importance of the One Health approach in the management and control of CD [21]. Several studies have shown frequent zoonotic transmission of RT078 strains across geographic borders in numerous countries [21, 29], revealing high genetic similarity between human and animal isolates [30].

The predominant MDR patterns identified in this study included resistance to aminoglycosides, fluoroquinolones, tetracycline and B-lactams, but one strain also had other AMR genes that conferred resistance

Table 1 Screening for antimicrobial resistance genes in CD isolates

Product	Resistance gene	Accession Number	Antibiotic resistance	Nº of isolates
ant(6)-Ia	*ant(6)-Ia_1*	AF330699	Streptomycin	1
	ant(6)-Ia_2	KF421157	Streptomycin	18
	ant(6)-Ia_3	KF864551	Streptomycin	2
ant(6)-Ib	*ant(6)-Ib_1*	FN594949	Streptomycin	7
aac(6')-aph(2')'	*aac(6')-aph(2'')_1*	M13771	Streptomycin, isepamicin, gentamicin, tobramycin, kanamycin, fortimicin, dibekacin, netilmicin, amikacin	1
aph(3')-III	*aph(3')-III_1*	M26832	Amikacin, ribostamycin, paramomcycin, isepamicin, kanamycin, neomycin, lividomycin, butirosin	2
(AGly)aadC	*(AGly)aadC*	V01282	Aminoglycosides	2
CDD beta-lactamase	*CDD-1*	NG_065860.1	Carbapenem	23
	CDD-2	NG_065861.1	Carbapenem	1
cdeA	*cdeA*	AJ574887.1	Fluoroquinolone and disinfecting agents and antiseptic	23
tet(M)	*tet(M)_1*	X92947	Tetracycline, doxycycline, minocycline	1
	tet(M)_4	X75073		1
	tet(M)_8	X04388		1
	tet(M)_10	EU182585		17
tet(44)	*tet(44)_1*	NZ_ABDU01000081		7
tet(40)	*tet(40)_1*	FJ158002	Tetracycline, doxycycline	2
erm(B)	*erm(B)_18*	X66468	Erythromycin, quinupristin, pristinamycin IA, lincomycin, clindamycin, virginiamycin S	1
cfr(B)	*cfr(B)_1*	KM359439	Lincomycin, clindamycin dalfopristin, pristinamycin IIA, virginiamycin M, florfenicol, chloramphenicol, linezolid, tiamulin	1
sat-4	*sat-4*	U01945.1	Streptothricin	1

Table 2 Screening for virulence factors genes in CD isolates

Classification	Product	Virulence genes	Accession Number
Toxin	Toxin A	*tcdA*	WP_011860904
	Toxin B	*tcdB*	WP_009902069
	Binary toxin	*cdtA*	WP_004454925
	Binary toxin	*cdtB*	WP_021426623
Exoenzyme	Zinc metalloprotease	*zmp1*	WP_011861706
	Cell wall-binding cysteine protease	*cwp84*	WP_011861680
Adherence	Collagen-binding adhesin	*cbpA*	WP_011861847
	ABC transporter substrate-binding protein	*CD0873*	WP_011860976
	SrtB-anchored collagen-binding adhesin	*CD2831*	WP_011861707
	Cys-Gln thioester bond-forming surface protein	*CD3246*	WP_011861889
	Fibronectin-binding protein	*fbpA/fbp68*	WP_011861612
	Chaperonin	*groEL*	WP_003435012

to lincosamide, macrolides, streptogramin a, streptogramin b, pleuromutilin, oxazolidinone and amphenicol. The ST11 lineage has been characterized by phenotypic resistance to tetracycline, moxifloxacin, clindamycin, and erythromycin [27]. In RT078, antimicrobial genes conferring resistance against tetracycline and streptomycin are shared between human and animal strains [20], but it is suggested that the *ermB* gene is involved in an unknown type of selective pressure in human isolates [21]. Several studies have detected the *cdeA* gene in all strains, regardless of RT [21, 31, 32]. However, carbapenem resistance in CD strains has been reported to be high [3] and low [33–35].

Other studies have reported different MDR rates among countries, ranging from 77.55% ($n=49$) to 50.4% ($n=268$) in China [36, 37]; 60% in a meta-analysis

regarding Europe [38]; and 25.4% ($n=207$) in isolates from Australia, Asia, Europe, and North America [27], and 1.7% ($n=1091$) in Australia [35]. The MDR patterns include resistance to at least three of the following antimicrobial agents: clindamycin, erythromycin, moxifloxacin, cephalosporins, tetracycline, gentamicin and tedizolid.

In addition, we compared the WGS data with the in vitro antimicrobial susceptibility results previously obtained [3, 11, 39] for 40% ($n=9$) of the isolates screened in this study. The expression of the resistance genes was observed in vitro, except for one strain that was resistant to tetracycline in vitro, but it did not harbor the tetracycline resistance gene. Nonetheless, a comprehensive comparison is not feasible because we detected more resistance genes than the number of antimicrobial agents evaluated in vitro.

A wide range of virulence factor genes, such as those encoding adherence factors, exoenzymes and toxins, were found. However, CD virulence is mainly associated with the *tcdB* gene [31, 40], which was detected in all CD isolates analyzed in this study and encodes the same gene with an identity of 97–100%, although the strain belongs to a different ST and RT; the *tcdA* gene was not found in all isolates, despite PCR detection in previous analyses [3, 11, 39, 41]; and *cdtA* and *cdtB* were detected in 3 strains that belong to ST 2 and 19; however, they were not identified in isolates characterized as RT078 and RT126, belonging to the ST11 lineage, suggesting that the ST11 binary toxin is encoded by a variant other than that in ST19 and ST2.

We previously observed variations between toxinotypes and ribotypes [3, 11, 39, 41], and STs were determined via WGS in this study. For instance, WGS identified a strain that was previously described as RT010, which is nontoxigenic and contains the *tcdB* and *tcdA* genes. Additionally, two strains identified as RT078 were classified by MLST into ST2, which exhibited the binary toxin, and another strain identified as RT078 was classified into ST19. Moreover, two strains identified as RT014 were classified by MLST as ST11.

CD genotyping has largely been focused on ribotyping [42] but the results of this method do not match whole-genome sequence data Fig. 1) in agreement with previous studies [43–45] because MLST, ribotype, and toxin variants are not always consistent with one another [12, 46]. However, other authors have shown strong consistency between results of toxin variant identification and MLST [36]. These disparities between ribotyping and WGS results indicate issues with the ribotyping system, which drive us to develop more efficient taxonomic classification techniques.

Other CD virulence factor genes found were proteases, such as *zmp1*, which is capable of cleaving host proteins [47], and the *cwp84* gene, which is highly immunogenic [48] and is stimulated by ampicillin and clindamycin [2, 49]. Among adhesion factors, *CD2831* facilitates biofilm formation and enhances immune evasion [50]; *cbpA* promotes collagen interaction and extracellular matrix adherence [14, 51] *GroEL* improves CD adhesion to enterocytes when exposed to heat shock, acidic pH or low iron levels [52, 53]; *Fbp68* encodes capability of binding soluble fibronectin; and *CD0873* is a major adhesin of CD [47].

Conclusion

In conclusion, we found significant genetic similarity between ST11 strains isolated from environmental sampling and from animals of origin in Spain; these strains may represent a reservoir for CA-CDI, which is becoming a growing public health threat due to the development of high levels of MDR bacteria and the number of virulence factors detected.

Methods

Origin of isolates

A total of 23 isolates were recovered from the CD strain collection of the COVEMI research group of the Faculty of Veterinary Medicine, Complutense University of Madrid (UCM), Spain. The isolates came from animals ($n=12$, 5 fecal samples obtained from pigs [11]; 5 pig carcasses at different stages, such as prescalding and cecal content [41]; 2 dogs with digestive disorders [39]; and 11 environmental samples; 9 from an abattoir and processing plant located in southern Spain [41] and 2 from dog sandboxes located nearby in public playgrounds within the Madrid region [3]. See Additional file 1. C. difficile comprehensive strain database for more details.

These isolates were chosen at random from among the strains previously identified as RT078 (A+B+CDT+), considered hypervirulent; RT014 (A+B+CDT-), implicated in human diseases; and other ribotypes, such as RT126 (A+B+CDT+), RT 110 (A+B+CDT-) and RT 010 (A-B-CDT-) [3, 11, 39, 41].

Bacterial culture

CD strains were grown in duplicate on Columbia agar plates with sheep blood plates (Thermo Scientific) for 48 h at 37 °C in an anaerobic chamber with a GENbox Anaer (bioMérieux, Marcy l'Étoile, France). Anaerial indicator strips (BioMérieux, Marcy l'Étoile, France) were included inside the anaerobic chamber to confirm that the conditions were proper. Gram staining was performed to verify that the bacilli were not sporulated to ease DNA extraction.

DNA extraction

DNA was extracted and purified using a Gram-Positive Bacteria Genomic DNA Purification Protocol from the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) following the manufacturers' instructions. For the bacterial lysates, a gram-positive bacterial lysis buffer was prepared with 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100, and 20 mg/ml lysozyme (Sigma–Aldrich Chemie GmbH, Germany). The volume of the elution buffer added to the column was reduced to 50 µL to obtain more highly concentrated DNA. The DNA quality was checked using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). The extract was stored at − 80 °C until use.

Whole-genome sequencing and data analysis

The genomes were sequenced via Illumina technology in the genomics unit of the Research Support Center (CAI) of the Complutense University of Madrid. First, the DNA was quantified using a qubit fluorometer (Thermo Fisher Scientific, USA), and the DNA was subsequently analyzed via next-generation sequencing (NGS; NextSeq 1000/2000). The quality of the reads was evaluated by using fastqc_v0.12.1 [54], and the data were analyzed with TORMES, an automated pipeline for whole bacterial genome analysis [55].

This pipeline relies on many different software packages and databases; sequence quality filtering was evaluated by Prinseq [56], assembly was conducted using SPAdes [57], Quast was used for assessing the quality of assemblies [58], annotation was performed using Prokka [59], taxonomic identification was performed by Kraken2 [60], MLST software was used for typing by scanning contig files against traditional PubMLST typing schemes [61, 62], pangenome and core genome analysis was conducted using Roary [63], antibiotic resistance genes were identified by screening genomes against the Resfinder [64], CARD [65], and ARG-ANNOT databases [66] by using Abricate [67], and virulence genes were identified by screening the genome against the Virulence Factors Database (VFDB) [68] by using Abricate [67]. Any hit with coverage and/or identity less than 90% was removed for AMR genes and virulence genes.

Phylogenetic analysis

Thirty-two strains found in previous studies [20, 21, 27, 34, 69–71] and six strains available in Enterobase (July 2024) were selected in order to include isolates from different European regions and sources (Fig. 1). Reads from the strains isolated in this study ($n = 23$) and those from the strains mentioned before ($n = 38$) were mapped against the selected reference genome *C. difficile* strain M120 (ST11) (Genbank accession number NC_017174.1) using BWA [72], applying 'mem' option with defaults parameters. SAMtools [73] were used to sort and compress the resulting SAM files into BAM files. BCFtools [74] was used to perform the variant calling, applying mpileup' and 'call' options and excluding SNPs with a base quality < 30 and a mapping quality < 30. Consensus sequences were then generated from the corresponding VCF (variant call format) file by BCFtools.

A multi-fasta alignment created with the concatenated consensus sequences was used to filter out putative recombinant regions by Gubbins v3.1.4 [75]. The multi-fasta alignment file was used to generate a maximum-likelihood phylogenetic tree with RAxML v8.2.12 [76]. The tree was built using the general time-reversible substitution evolutionary model with gamma correction and 1,000 bootstrap replicates. The tree was then rooted with one of the external isolates (accession number ERR3473771) as outgroup and visualized with the iTOL editor [77].

Abbreviations

AMR	Antimicrobial resistance genes
CD	*Clostridioides difficile*
CDI	*Clostridioidesdifficile* Infection
CA-CDI	Community acquired- CDI
DNA	Deoxyribonucleic acid
HA-CDI	Healthcare-associated CDI
MDR	Multidrug resistance
MLST	Multilocus sequence typing
PCR	Polymerase chain reaction
RT	Ribotype
SNP	Single nucleotide polymorphisms
ST	Sequence type
UCM	Complutense University of Madrid
VFDB	Virulence Factors Data Base
WGS	Whole genome sequencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04332-0.

Additional file 1. *C. difficile* Comprehensive Strain Database.

Authors' contributions

DT-G, JLB, MH, MEG took part in study design. DT-G wrote the first draft. All authors took part in manuscript review and approved the definitive version. Funding: MEG.

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Data availability

Sequence data that support the findings of this study have been deposited in the European Nucleotide Archive with the primary accession code PRJEB71892.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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