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Antimicrobial Resistance Progression in the United Kingdom: A Temporal Comparison of *Clostridioides difficile* Antimicrobial Susceptibilities

Vernon Jon J [1*], Wilcox Mark H [1, 2], Freeman Jane [1, 2]

Running Title: Historical *C. difficile* Resistance

1. Healthcare-Associated Infections Research Group, Molecular Gastroenterology, Leeds Institute for Biomedical and Clinical Sciences, University of Leeds, Old Medical School, Leeds General Infirmary, LS1 3EX, West Yorkshire, UK.

2. Microbiology, Leeds Teaching Hospitals Trust, Leeds, UK.

*corresponding author

Email: j.j.vernon@leeds.ac.uk

Abstract

Objectives

Clostridioides difficile (CD) is widely reported as one of the most prevalent multi-drug resistant (MDR) organisms. Assessment of temporally disparate isolate collections can give valuable epidemiological data to further the understanding of antimicrobial resistance progression.

Methods

A collection of 75 CD isolates (1980-86) was characterised by PCR ribotyping, cell cytotoxicity assay and susceptibility testing with a panel of 16 antimicrobials and compared to a modern surveillance collection consisting of 416 UK isolates (2012-2016). Agar-incorporation was performed to ascertain susceptibility data for vancomycin, metronidazole, rifampicin, fidaxomicin, moxifloxacin, clindamycin, imipenem, chloramphenicol, tigecycline, linezolid, ciprofloxacin, piperacillin/tazobactam, ceftriaxone, amoxicillin, tetracycline and erythromycin. Genomes were obtained using Illumina HiSeq3000 sequencing and assembled using CLC Genomics Workbench. Resistance genes were identified using the Comprehensive Antibiotic Research Database's Resistance Gene Identifier and ResFinder3.0.

Results

Twenty-six known and one previously unobserved ribotype (RT) were detected. RT015 and RT020 dominated; 21.3% and 17.3%, respectively. Three moxifloxacin resistant (16-32 mg/L) RT027 isolates were recovered, pre-dating the earliest reports of this phenotype/genotype. Phenotypic resistance was observed to moxifloxacin (9.3% of isolates), ciprofloxacin (100%), erythromycin (17.3%), tetracycline (9.3%), linezolid

and chloramphenicol (4.0%). Phenotypic comparisons with modern strains revealed increasing minimum inhibitory concentrations (MIC), with MIC_{50} elevations of one doubling-dilution for the majority of compounds, excluding clindamycin and imipenem. Moxifloxacin MIC_{90} comparisons revealed a two doubling-dilution increase between temporal isolate collections. Historical genomes revealed twenty different resistance determinants, including *ermB* (8.0% of isolates), *tetM* (9.3%), *cfr* (5.3%) and *gyrA* substitution Thr-82→Ile (9.3%). Seventeen isolates (22.7%) were resistant to ≥3 compounds (MDR), demonstrating ten different combinations. Intra-RT diversity was observed.

Conclusions

Antibiotic resistance in CD has increased since the early 1980s, across the majority of classes. Moxifloxacin resistance determinants may pre-date its introduction.

Keywords: *C. difficile*, Antimicrobial resistance, Multi-drug resistance, Ribotype 027, antimicrobial susceptibility testing

Introduction

The development of antimicrobial resistance, often multi-drug resistance (MDR), in *C. difficile* is considered a key factor in the emergence and spread of this pathogen. [1-4] Susceptibility studies on *C. difficile* isolates from the 1980s, when *C. difficile* first came to prominence, are rarely linked to modern ribotyping data. In addition, antibiotic susceptibility panels are not always comparable between temporally separated studies. These factors make comparisons between historical and modern phenotypic and genotypic data difficult and are barriers to our understanding of *C. difficile* epidemiology and resistance development.

High prevalence of MDR in *C. difficile* isolates has been reported amongst recent surveillance, with one study reporting a rate as high as 55%. [3] Common ribotypes associated with multiple resistance were 001, 012, 017, 018, 027, 078, 106 and 356, where reduced susceptibilities to erythromycin, clindamycin, moxifloxacin and rifampicin constituted the majority of instances. [3, 5] In contrast, studies of earlier isolate collections have found lower MDR rates, including low moxifloxacin resistance. [6] In this study, Taori et al described only 7.8% MDR among 179 UK isolates, with high tetracycline resistance rates noted. Whilst determination is highly dependent on the panel of agents tested, knowledge of any MDR acquisition is central to understanding CDI therapeutics and antimicrobial stewardship.

Ultimately, knowledge of *C. difficile* epidemiology and resistance progression over time provides an opportunity to respond quickly to emerging resistance and outbreak situations. Here, we interrogated a collection of *C. difficile* isolates from 1980-86 for ribotype (RT) prevalence, antimicrobial susceptibility patterns and genomic resistance determinants, in order to provide valuable comparators to modern epidemiological data.

We contrasted findings with the modern surveillance data of the *Clos*ER study [7], using the same methods for enhanced confidence in the analyses. This investigation sought to reveal the progression of antimicrobial resistance (AMR) in UK *C. difficile* isolates over a period of circa 30 years. By understanding resistance progression in this important nosocomial pathogen, we may improve therapeutic and prescribing approaches, reducing the burden of disease.

Materials and Methods

Strains

Strains were obtained from an historical collection of ~2000 clostridia spp. isolates, established as the first national diagnostic and surveillance collection between 1980 and 1986 (Prof. Peter Borriello, Public Health England). Strains were selected on the following criteria: original *C. difficile* identification, of human origin and reported as toxin positive by cell cytotoxicity assay. Of the retained isolates, 1,253 were matched to specific laboratory records, constituting 476 patients. Original specimens were enriched in Schaedler's anaerobic broth (Oxoid, UK) ± sodium taurocholate (0.1/1%) and glycine (0.4/4%) and isolated on cycloserine-cefoxitin Brazier's agar (Oxoid, UK). Seventy-five *C. difficile* strains were recovered from unique patient samples and designated the prefix JV, e.g. JV01.

The comparator *Clos*ER isolate collection, consisted of 416 *C. difficile* isolates collected between 2012 and 2016 from five UK regional referral laboratories, selected through the European Study Group on *C. difficile* network. Isolates originated from faecal

samples associated with *C. difficile* infection (CDI) and toxin positive confirmations, from de-duplicated CDI cases.

PCR Ribotyping

Isolates were ribotyped by the *Clostridium difficile* Ribotyping Network (CDRN); Leeds, UK; [8] based on the methods of Stubbs *et al*, [9] and identical to those used for typing the comparator *Clos*ER collection.

Agar Incorporated Minimum Inhibitory Concentration Testing

Wilkins Chalgren-based agar incorporation susceptibility testing was performed in accordance with previous surveillance. [1] A panel of 16 comparator antimicrobials; vancomycin (VAN), metronidazole (MTZ), rifampicin (RIF), fidaxomicin (FDX), moxifloxacin (MXF), clindamycin (CLI), imipenem (IPM), chloramphenicol (CHL), tigecycline (TIG), linezolid (LZD), ciprofloxacin (CIP), piperacillin/tazobactam (TZP), ceftriaxone (CRO), amoxicillin (AMX), tetracycline (TET) and erythromycin (ERY) was investigated. These compounds were selected to span a range of antibiotic classes, correspond to data available from the *Clos*ER study [7] and to include the standard treatment options for *C. difficile* infection. Minimum inhibitory concentrations (MICs) were defined as the lowest concentration at which marked inhibition of growth was observed after 48-hour culture. A panel of five control organisms (*C. difficile* ATCC 700057, *C. difficile* E4, *Bacteroides fragilis* ATCC 25285, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212) and several *Clos*ER isolates of known MIC were tested concurrently to ensure consistency of results. To establish a direct comparison with the *Clos*ER MIC data, an identical methodology was used here, performed by the same operator, at the same facility.

Determination of *C. difficile* toxin status

Toxigenicity of *C. difficile* isolates was determined by inoculation (1:10) of 48-hour brain heart infusion (BHI) broth culture supernatants (10 minutes at 12,000 g) into Vero cell (Sigma, USA) cytotoxicity neutralisation assays. *Clostridium sordellii* anti-toxin acted as a neutralising control. Results were defined after 48 hours, with positive results assigned with the identification of >50% cell rounding.

Next Generation Sequencing

DNA was extracted from 0.5 McFarland suspensions of 24-hour *C. difficile* culture on Columbia Blood Agar plates. The QIAamp Fast DNA Kit chemistry (Qiagen) was used on a QIAxtractor, with extended lysis stages (2 x 10 minutes). Double-stranded DNA (dDNA) was quantified via a PicoGreen fluorescence assay. Briefly, DNA extracts were diluted 1:50 in a Nunclon 96 Flat Bottom Black Tray (Thermo Fisher Scientific, USA) and mixed with 0.5% PicoGreen (Life Technologies, USA) in TE Buffer (Sigma-Aldrich, USA), prior to ten-minute incubation at ambient temperature. Fluorescence was excited at 585nm and measured at 535nm, using a Tecan infinite F200 pro. Absorbance readings were converted to dDNA quantifications via a calibration curve of lambda DNA (Sigma-Aldrich). A cut-off of 1 µg/µL dDNA was implemented before proceeding with library preparation. Sequencing was performed by the University of Leeds, Next Generation Sequencing Facility using NEBNext® Ultra™ chemistry (New England Biolabs) and Illumina HiSeq3000 technology. Contigs were assembled with CLC Genomics Workbench (Qiagen) and annotated using the Rapid Annotation using Subsystem Technology (RAST) web server (rast.nmpdr.org). Antibiotic resistance genes were identified using the Comprehensive Antibiotic Research Database's Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi) and ResFinder 3.0, whilst SNPs in

common resistance-conferring genes; *rpoB, gyrA* and *gyrB*, were assessed through multiple sequence alignment with Clustal Omega 1.2.4. (www.ebi.ac.uk/Tools/msa/clustalo). Historical genome sequences are accessible under BioProject number PRJNA704635. No sequence analysis was performed on the contemporary *Clos*ER collection.

Results

PCR Ribotyping

Twenty-six known and one previously unobserved RT (RT862) were detected in the historical collection. RTs 015 and 020 dominated; 21.3% and 17.3%, respectively (Table 1). RT027 was recovered on three occasions from specimens collected in 1981, 1983 and 1986. Two instances of the binary toxin-producing RT078 were observed, in addition to RTs 012 (*n*=4) and 017 (*n*=3).

Breakpoint Analysis

Based on phenotypic analyses, all isolates were sensitive to FDX and MTZ, with the majority susceptible to VAN (94.7%). Four isolates (5.3%) demonstrated intermediate VAN resistance (4 mg/L) attributed to a collection of VAN resistance determinants, only present in these strains (*vanRG*, *vanSG*, *vanUG*, *vanYG*, *vanG*, *vanXYG*, *vanWG* and *vanTG*). These strains represented three ribotypes: RT001 (*n*=2), RT041 (*n*=1) and RT200 (*n*=1). Seven isolates (9.3%), including all RT027 isolates exhibited MXF resistance (>16 mg/L) in agar incorporation susceptibility assays, correlating with the common Thr-82→Ile polymorphism in *gyrA*, whilst CIP resistance was universal. Resistance was observed

against CLI (66.0%), IPM (1.3%), CHL (4.0%), LZD (4.0%), CRO (12.0%), TET (9.3%) and ERY (17.3%); (Table 2). Breakpoints are listed in Table 2.

Multi-Drug Resistance

Twenty different antibiotic resistance encoding elements were detected amongst fifty (66.7%) isolates; (**Error! Reference source not found.**). Seventeen (22.7%) demonstrated resistance to ≥3 antimicrobials; (MDR). Eight (10.7%), four (5.3%) and five (6.7%) isolates were resistant to three, four and five antibiotics, respectively. RT012 (*n*=4), RT027 (*n*=3) and RT078 (*n*=2) represented the most prevalent MDR ribotypes. Ten different combinations of AMR were observed, with three permutations predominating; (TET, CLI, CIP, LZD & CHL; CLI, CIP, ERY, MXF & CRO; and CLI, CIP & ERY; Table 3). Combined ERY and CLI resistance was apparent in eight (10.7%) isolates, whilst LZD, CLI and CHL resistance was demonstrated in four (5.3%), conferred by the *cfr* gene located on a Tn*6218* transposon.

ermB was detected in six (8.0%) genomes, but only three demonstrated combined CLI and ERY resistance. Four variant configurations of the upstream regulatory regions of *ermB* genes were detected, clustered by ribotype (Figure 1). TET resistance was detected in seven (9.3%) strains, all of which harboured the *tetM* gene on either a Tn*5397* or Tn*916*-like transposon. These strains represented three RTs; 012 (*n*=4), 078 (*n*=2) and 015 (*n*=1).

Discussion

Overall RT prevalence amongst isolates originating from 1980-1986 was similar to those reported in England in 2015 and other contemporaneous surveillance, notwithstanding the limited size of our historical collection. [1, 8] While previous studies

showed significant fluctuations in epidemic strain prevalence across several years, [1, 10] our data suggest that there may be a baseline of RT distributions that is reverted to as epidemic strains disappear or revert to background prevalence, possibly as selection pressure for particular epidemic strains subside. It is possible that historical strain re-establishment could be because these RTs are the "fittest" for long term dormancy and survival.

The isolation of RT027 strains from this historical collection potentially provides two of the earliest instances of this epidemic RT. Originating from 1981 and 1983; isolates JV02 and JV73 pre-dated the CD196 strain recovered from a French patient in 1985. [11] Intriguingly, all three historical RT027 isolates in our collection demonstrated MXF resistance. This represents evidence of resistance prior to existing reports in the literature, [4, 12] demonstrating the potential for cross-resistance across different (older and newer) fluoroquinolones. [13] We previously described an increased bacterial fitness in RT027 with these polymorphisms, [14] which may contribute to the retention of fluoroquinolone resistance even in the absence of selective antimicrobial pressure. The detection of fluoroquinolone resistance in this important RT, decades prior to the major clonal expansion of the early 2000s, potentially offers new insights into the understanding of resistant lineage emergence. Possibly, therefore, the increasing clinical use of fluoroquinolones following their introduction in the 1980s, acted as a selection pressure for pre-existing resistance determinants in *C. difficile*.

Interestingly, two less common binary toxin gene carrying strains, RT023 and RT033 were both isolated singly from the historical collection. RT033 is commonly reported in zoonotic infections [15], but is increasingly being identified in human cases. [16-18] The isolate recovered in this study only demonstrated CIP resistance. Antimicrobial

resistance data on clinically isolated RT033 antimicrobial susceptibilities are scarce, but Knight et al found a relatively low level of antimicrobial resistance in 28 clinical and environmental RT033 isolates. [19] Of note, Androga et al also described difficulties in accurately diagnosing CDI due to this strain, since the characteristic truncated pathogenicity locus may result in false negatives where diagnostic assays rely on a complete sequence. [16]

Comparisons with modern UK strains from a large-scale surveillance study (2012-2016) [1, 7] revealed increases in the MICs of all comparator compounds with the exception of CLI and IPM; (Table 2). Since both sets of MIC data were acquired through identical methodologies in the same institution and by the same operator, confidence was established in these results as direct comparators. These findings were upheld even after comparisons were normalised by RT (data not shown). Interestingly, the MXF MIC_{90} was two doubling-dilutions lower in the historical isolates (2 mg/L), suggesting a substantial expansion of resistance amongst modern strains (8 mg/L). Unfortunately, genomic data is not available for the contemporary collection, which may reveal the quinolone-resistance determining region SNPs responsible for this increase. This increase may result in an elevated risk of CDI onset in colonised patients with fluoroquinolone exposures; potentially due to improved bacterial survival and proliferation in these cases.

The 9.3% prevalence rate of *tetM* was similar to the 13.0% resistance rates amongst UK isolates from 1979-86. [6] Although data from Taori *et al*. indicated that TET resistance rates in the UK reduced between 1979-86 (13.0%) and 1996-2004 (2.0%), [6] European frequencies were reported as high as 17.1% in recent MDR isolates. [3] Interestingly, a recent report from Dingle *et al.* revealed the presence of the *tetM* gene in 76.9% of

RT078 isolates, [20] identifying the determinant in pre-1990 strains, but describing major clonal expansion from 2000 onwards. The research postulated that the most plausible explanation for this, in the wake of reduced clinical use, was agricultural prescribing and zoonotic transmission.

While there are several known mechanisms of fluoroquinolone resistance, unfortunately, the data presented cannot identify a mechanism for the widespread CIP resistance observed both here and extensively in the literature. [2] Evidence for the involvement of putative efflux or gyrase protective homologues, such as *CD2068* [21] or *qnr*-like genes [22] is undermined by lack of absolute genotypic/phenotypic correlation in this collection, whilst no relationship was associated with any extra-quinolone resistance-determining region mutations.

This study demonstrated a MDR rate of 22.7%, largely constituting RT012, RT027 and RT078; (23.4%, 17.6% and 11.8%, respectively). Higher frequencies of MDR are documented in modern strains, with 26.0% reported in European surveillance, [3] and up to 59.7% demonstrated amongst North American isolates. [23] Nonetheless, increased prevalence may be related to localised expansions of particular epidemic strains, so considerations of epidemiological context are essential.

Nearly half (47.1%) of the historical MDR strains exhibited a combination of ERY, CLI and fluoroquinolone resistance, in concordance with a recent large-scale review reporting this as a prevalent combination (in 61.5% of international studies). [2] Whilst significant increases of a combined ERY and fluoroquinolone resistant phenotype were reported in a retrospective analysis of German isolates (13.0% in 1990 and 63.0% in 2008), [24] the collection was dominated by RT001, further highlighting the influence of local epidemiology on antibiotic susceptibility rates. The abundance of genetic

267 determinants in modern strains is not surprising, given the frequency and
268 heterogeneity of genetic arrangements reported in *ermB* determinants, which suggests
269 frequent transposition and genetic exchange amongst *C. difficile* and other species. [25]

270 Since *ermB* presence usually confers ERY resistance [3, 26, 27], the unexpected finding of
271 two strains (JV33 and JV40) harbouring the gene, but not indicating the resistant
272 phenotype was intriguing. This may be due to expression complexities associated with
273 promotor or regulator sequences or even a mutation within the *ermB* gene, leading to
274 loss of functionality. The latter notion has been previously reported in an *ermB* positive,
275 ERY sensitive *C. difficile* strain [28]. Nonetheless, although four different *ermB* gene
276 configurations were observed (Figure 1), these did not correlate with the resistant
277 phenotypes and cannot explain the differences detected. Further investigations would
278 be required to determine the mechanism behind these discrepancies.

279 Bacterial compulsion for evolutionary survival dictates that resistance often rapidly
280 follows the introduction of new antibiotics. However, there is a paucity of data
281 comparing *C. difficile* AMR across decades to underline this notion. By examining this
282 unique, historical collection, we highlight the trends in antibiotic susceptibility of UK *C.*
283 *difficile* isolates. The finding of MXF-resistant RT027 isolates from the early 1980s may
284 provide valuable information to further inform the emergence of this RT.

285

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Transparency declarations

The data presented in this article was generated as part of J. J. V.'s PhD studies at the University of Leeds.

All other authors: none to declare.

Author contributions

All authors contributed to the conception of the project and data interpretation. J. J. V. led on manuscript construction, whilst J. F. and M. H. W. contributed to critical revision. All data were generated and analysed by J. J. V.

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	Ribotype	Isolates (*n*)	% total	CCTA	Original Specimen Collection Dates	*Clos*ER UK Isolates		
						Ribotype	*n*=	% total
	015	16	21.3	+	1981 - 1984	002	42	10.1
	020	13	17.3	+	1980 - 1983	015	42	10.1
	001	6	8.0	+	1981 - 1983	014	39	9.4
	012	4	5.3	+	1981 - 1982	078	37	8.9
	014	4	5.3	+	1981 - 1983	005	32	7.7
	027	3	4.0	+	1981; 1983; 1986	106	23	5.5
	002	3	4.0	+	1981; 1981; 1983	020	22	5.3
	017	3	4.0	+	1981; 1982; 1982	023	21	5.0
	078	2	2.7	+	1981; 1983	001	14	3.4
	070	2	2.7	+	1980; 1983	026	13	3.1
	200	2	2.7	+	1981; 1982	011	9	2.2
	061	2	2.7	+	1983; 1983	027	9	2.2
	220	1	1.3	+	1982	018	8	1.9
	056	1	1.3	+	1983	012	7	1.7
	103	1	1.3	+	1983	081	5	1.2
	137	1	1.3	+	1983	087	5	1.2
	041	1	1.3	+	1981	045	4	1.0
	626	1	1.3	+	1983	054	4	1.0
	341	1	1.3	+	1983	056	4	1.0
	032	1	1.3	+	1981	070	4	1.0
	862	1	1.3	-	1983	554	4	1.0
	619	1	1.3	+	1983	039	3	0.7
	033	1	1.3	-	1983	050	3	0.7
	242	1	1.3	+	1981	053	3	0.7
	003	1	1.3	+	1986	076	3	0.7
	023	1	1.3	+	1983	126	3	0.7
	010	1	1.3	-	1982	Other	53	12.7
Total	**27**	**75**	**100.0**			**68**	**416**	**100.0**

Table 1: PCR ribotype prevalence amongst the UK historical C. difficile isolate collection (1980-86) and UK ClosER isolates (2012-2016). *Toxigenic strain status is reported for historical isolates, CCTA - cell cytotoxicity assay, performed with Vero-cells incubated with 48 hour brain heart infusion broth culture supernatants. >50% cell rounding after 48 hours was demonstrative of cytopathic effect.*

Antimicrobial Compound	S	I	R	MIC Interpretive Criteria (mg/L)			Historic UK Isolates (1980-1986) *n=75unless stated*			*Clos*ER Study UK Isolates (2012-2016) *n=416*		
				S	I	R	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Geometric $\overline{x}$ (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Geometric $\overline{x}$ (mg/L)
VAN	71 (94.7%)	4 (5.3%)	0	≤2	4	≥8	0.5	1	0.704	1	2	0.839
MTZ	75 (100%)	0	0	≤2	4	≥8	0.125	0.25	0.171	0.25	0.5	0.283
RIF	74 (98.7%)	1 (1.3%)	0	≤0.004	0.008-16	≥16	0.001	0.002	0.001	0.002	0.004	0.002
FDX	75 (100%)	0	-	≤0.5	1	*	0.03	0.06	0.028	0.06	0.125	0.050
MXF	68 (90.7%)	0	7 (9.3%)	≤2	4	≥8	1	2	1.617	2	8	2.021
CLI	35 (46.7%)	13 (17.3%)	27 (36.0%)	≤2	4	≥8	4	16	3.154	8	16	4.880
IPM	51 (68.0%)	23 (30.7%)	1 (1.3%)	≤4	8	≥16	4	8	4.553	4	8	3.940
CHL	71 (94.7%)	1 (1.3%)	3 (4.0%)	≤8	16	≥32	4	8	4.768	4	8	5.462
TGC	75 (100%)	0	-	≤0.125	0.25	*	0.03	0.06	0.035	0.06	0.06	0.050
LZD	72 (96.0%)	0	3 (4.0%)	≤2	4	≥8	2	2	1.678	*ND*	*ND*	*ND*
CIP	0	0	75 (100%)	≤2	4	≥8	16	32	20.022	*ND*	*ND*	*ND*
TZP (*n=42*)	42 (100%)	0	0	≤32	64	≥128	8	8	6.672	*ND*	*ND*	*ND*
CRO	23 (30.6%)	43 (57.4%)	9 (12.0%)	≤16	32	≥64	32	64	27.601	*ND*	*ND*	*ND*
AMX	75 (100%)	0	0	≤2	4	≥8	1	2	0.920	*ND*	*ND*	*ND*
TET	68 (90.7%)	0	7 (9.3%)	≤0.5	1	≥4	0.06	0.125	0.085	*ND*	*ND*	*ND*
ERY	62 (82.7%)	0	13 (17.3%)	≤0.25	0.5	≥2	16	16	16	*ND*	*ND*	*ND*

Table 2: Antimicrobial susceptibility data from UK C. difficile isolates (1980-1986) against a panel of 16 antibiotics compared to modern UK isolate (2012-2016) surveillance. *VAN-vancomycin, MTZ-metronidazole, RIF-rifampicin, FDX-fidaxomicin, MXF-moxifloxacin, CLI-clindamycin, IPM-imipenem, CHL-chloramphenicol, TGC-tigecycline, LZD-linezolid, CIP-ciprofloxacin, TZP-piperacillin/tazobactam, CRO-ceftriaxone, AMX-amoxicillin, TET-tetracycline, ERY-erythromycin. S- sensitive, I – intermediate, R – resistant. Breakpoints based on the U.S. Clinical & Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) as utilised by Freeman et al. (2018); [1] or Freeman et al. (2016). [29] * - No resistant breakpoint defined.*

PCR ribotype (*n*=)	Number of *C. difficile* isolates resistant to different antimicrobial combinations									
	CLI, CIP, ERY	CLI, CIP, TET	CIP, TET, ERY	CIP, CRO, ERY	MXF, CLI, CIP, ERY	MXF, CIP, CRO, ERY	CLI, CIP, CRO, TET, ERY	MXF, CLI, CIP, CRO, ERY	CLI, CHL, LZD, CIP, TET	MXF, IPM, CIP, CRO, ERY
012 (4)		1	1				1		1	
027 (3)						1		1		1
078 (2)									2	
137 (1)	1									
041 (1)					1					
001 (1)								1		
200 (1)								1		
014 (1)	1									
017 (1)				1						
010 (1)	1									
015 (1)									1	
Total (17)	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**3**	**4**	**1**

Table 3: *Multi-drug resistance characteristics of C. difficile isolated from the UK (1980-1986).3*

CLI - clindamycin, CIP – ciprofloxacin, MXF – moxifloxacin, CHL – chloramphenicol, LZD – linezolid, IPM – imipenem, CRO – ceftriaxone, TET – tetracycline, ERY – erythromycin. PCR – polymerase chain reaction. Resistant breakpoints based on the U.S. Clinical & Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) as utilised by Freeman et al. (2018); [1] *or Freeman et al. (2016).* [29]

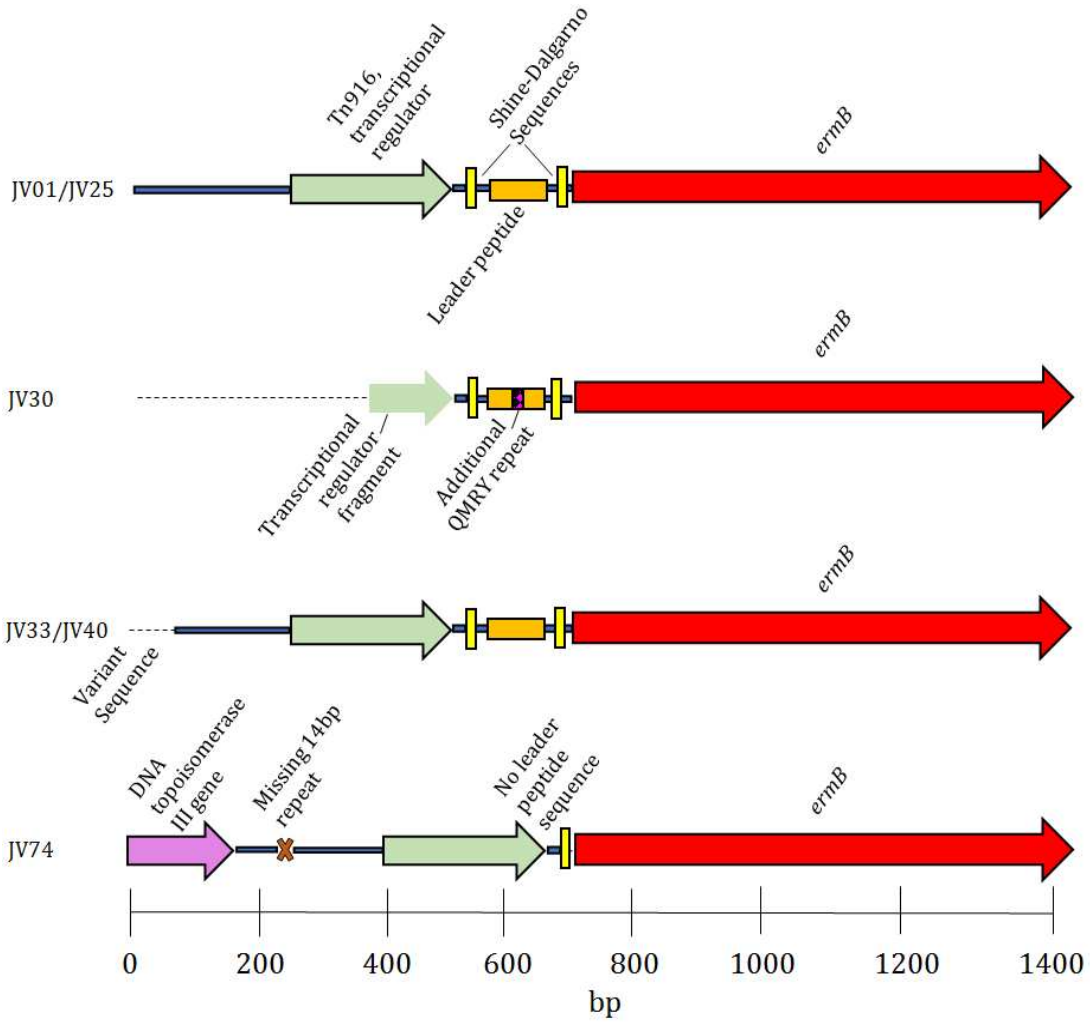

Figure 1: Schematic representation of the configuration of regulatory sequences upstream from the ermB gene of historical C. difficile genomes. *Thick blue lines represent identical nucleotides to the consensus sequence, whilst dashed lines represent sequence variation.*