Multidrug Resistant *Clostridioides difficile*: The Presence of Antimicrobial Resistance Determinants in Historical and Contemporaneous Isolates, and the Impact Of Fluoroquinolone Resistance Development on PCR Ribotype 027 Fitness

by

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Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

The University of Leeds
Leeds Institute of Medical Research,

Faculty of Medicine & Health

January, 2019

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter Two was prepared using data elements jointly acquired by Daniel Simon Pickering (DSP) and the author during the preparation of the following publication:

Pickering, D.S., Vernon, J.J., Freeman, J., Wilcox, M.H. and Chilton, C.H. Investigating the effect of supplementation on Clostridium difficile spore recovery in two solid agars. Anaerobe. 2018, ***50****, pp.38-43.*

Specifically, DSP performed the solid agar comparison assays, with phase-contrast microscopy and spore production jointly performed by DSP and the candidate, due to the time sensitivity of the method.

Regarding the publication, I (the candidate) was responsible for the MIC testing investigation and was also involved in elements of manuscript authoring (specifically susceptibility testing method and MIC results sections), whilst contributing to scientific discussion and critical revision. All other data presented in this chapter was generated, analysed and discussed by the candidate.

Bioinformatic assembly of *C. difficile* genomes presented in Chapter Four was advised on and partly performed by Dr Chris Randall, University of Leeds. Maximum likelihood phylogenies presented in Chapter Four were performed by Dr David Eyre (Nuffield Department of Medicine, University of Oxford, John Radcliffe Hospital, Oxford), all trees were created and interrogated by the candidate.

Elements of Chapter Six have been published in the Journal of Antimicrobial Chemotherapy:

Vernon JJ, Wilcox MH, Freeman J.

Effect of fluoroquinolone resistance mutation Thr-82 →Ile on Clostridioides difficile fitness. Journal of Antimicrobial Chemotherapy. 2018, pp.535-535.

The candidate was responsible for all experimental work and data analysis. The manuscript was prepared by the candidate, with critical revision contributions from the other authors.

These elements have also previously been presented by the candidate as conference papers (#P0386) at the 28th European Congress of Clinical Microbiology and Infectious Diseases, Madrid, 2018 and the 6th International *C. difficile* Symposium, Bled, Slovenia, 2018.

Acknowledgements

I wish to offer my sincere gratitude to my primary supervisor, Dr Jane Freeman, for all of her guidance, mentoring and support, particularly with the periodic morale boosting pep talks. I have enjoyed researching with you and have learnt a great deal during this time.

I also wish to thank Professor Mark Wilcox for his support and critical direction during periods of uncertainty. The necessary support was always available when required. I have developed a greater understanding of this field under to your tutelage, especially regarding the politics of academic research.

I would like to acknowledge my friend and PhD colleague Daniel Pickering for the joint work contributing to Chapter Two, both in the laboratory and in scientific discussions helping to shape the chapter. Our (often inane) conversations kept me sane amongst the ups and downs of this research project.

The contributions of both Dr Chris Randall and Dr David Eyre have been integral to the bioinformatic and phylogenetic analyses performed in this thesis. I thank them for their contributions and advice in this field, which have enabled me to learn the necessary bioinformatic skills to independently generate and interrogate my own data.

Similarly, I would like to recognise Dr Warren Fawley and Dr Emma Best for their guidance on MLVA and next generation sequencing, as well as the CDRN team for all of the ribotyping support, making these elements of the study painless. It was much appreciated.

I also wish to thank the entire Healthcare-associated Infection Research Group for the many scientific discussions helping me to shape the investigations presented in this thesis.

I would also like to give my appreciation to Dr Sally Fairweather and the Leeds Next Generation Sequencing Facility, who gave up their time to guide me through the library preparation and genome sequencing protocols.

I must thank my family, particularly my parents for the years of endless support and belief in me.

Finally, I must thank my wife for all of her loving support during my studies leading to the creation of this thesis. You always believe in me, even at times of self-doubt. Without this support, particularly in the amazing job you've done during this time in raising for our two wonderful children, I would not have been able to complete this thesis.

Abstract

Clostridioides difficile is the major cause of infectious antibiotic-associated diarrhoea, imparting a substantial clinical and financial burden on healthcare facilities. Resistance development, particularly to fluoroquinolones, has been implicated in major, international epidemics, predominantly associated with the hyper-virulent ribotype 027. The development of multiple antimicrobial resistance may contribute significantly to the considerable clinical challenges associated with this organism.

In this study, optimised germination environments, antimicrobial susceptibility testing and next generation sequencing were utilised in the recovery and characterisation of an historical *C. difficile* collection (1980-86). Epidemiological comparisons of ribotype distribution and susceptibility patterns with modern surveillance data (2012-2016) sought to reveal antimicrobial resistance variance between two distinct periods. By correlating phenotypic resistance and genetic determinants, the dissemination of resistance genes was evaluated. Contributions of bacterial mutability to resistance propagation were investigated in response to fluoroquinolone exposure amongst seven prevalent, clinical ribotypes; (*n*=44). Through *in vitro* batch and continuous co-culture modelling, the impact of resistance-conferring *gyrA* and *gyrB* mutations on the fitness of ribotype 027 (*n*=7) was assessed.

The majority of test antimicrobials (*n*=8/9) were less active against modern vs historical isolates. This is potentially due to increased antimicrobial exposure and subsequent selection/expansion of resistant strains. Moxifloxacin testing demonstrated the largest increase in resistant populations, reinforcing the notion of reduced susceptibility to modern fluoroquinolones as a potential contributory factor in disease. Phylogenetic analyses highlighted the complexity of molecular clock predictions, with 69% of historical genomes correlating with a 95% prediction interval.

Elevated mutability was observed amongst ribotype 027, suggesting greater propensity for resistance evolution in this type. Common fluoroquinolone resistance-conferring substitutions revealed advantages to bacterial fitness. Continuous, competitive co-culture modelling of a Thr82>Ile mutant, 027 strain emphasised the fitness benefits of this polymorphism, retained in the absence of fluoroquinolone pressure. These findings indicate a potential contribution to the success of this ribotype.

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List of Abbreviations

AMX	Amoxicillin
ABC	ATP-Binding Cassette
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BWA	Burrows Wheeler Aligner
CA	Community-Acquired
CA-HCFA	Community Acquired Healthcare Facility Associated
CARD	Comprehensive Antibiotic Resistance Database
CBA	Columbia Blood Agar
CCCP	Carbonyl Cyanide 3-Chlorophenylhydrazone
CCEY	Cycloserine Cefoxitin Egg Yolk
CCEYL	Cycloserine Cefoxitin Egg Yolk Lysozyme
CCFA	Cycloserine Cefoxitin Fructose Agar
CCNA	Cell Cytotoxicity Neutralisation Assay
CDC	Center for Disease Control and Prevention
CDI	*Clostridioides difficile* Infection
CDRN	*Clostridium difficile* Ribotyping Network
CDS	Coding DNA Sequences
CDT	*Clostridioides difficile* Binary Toxin
cfr	Chloramphenicol Florfenicol Resistance
CFU	Colony Forming Unit
CHL	Chloramphenicol
CI	Competition Index
CIP	Ciprofloxacin
CLI	Clindamycin
*Clos*ER	*Clostridium difficile* European Resistance
CLSI	Clinical and Laboratory Standards Institute
CO	Community-Onset
CRO	Ceftriaxone
dDNA	Double Stranded DNA
DNA	Deoxyribonucleic Acid
DPA	Dipicolonic acid
ECDC	European Centre for Disease Prevention and Control
EIA	Enzyme Immunoassay
erm	Erythromycin Resistance Methylase
ERY	Erythromycin
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EUCLID	European, Multicentre, Prospective, Biannual, Point-Prevalence Study of *Clostridium difficile* Infection in

	Hospitalised Patients with Diarrhoea
FDX	Fidaxomicin
FQR	Fluoroquinolone Resistant
FMT	Faecal Microbiota Transplant
GATK	Genome Analysis Tool Kit
GDH	Glutamate Dehydrogenase
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
GLY	Glycine
GUI	Graphical User Interface
gyrA	DNA Gyrase Subunit A
gyrB	DNA Gyrase Subunit B
HO	Hospital Onset
IDSA	Infectious Diseases Society of America
IPM	Imipenem
LZD	Linezolid
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation - Time of Flight
mar	Multiple Antibiotic Resistance
MATE	Multidrug and Toxic Compound Extrusion
MDR	Multidrug Resistance
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MLS$_B$	Macrolide-Lincosamide-Streptogramin B
MLST	Multi-Locus Sequence Typing
MLVA	Multi Locus Variable Number Tandem Repeat Analysis
MMR	Mismatch Repair
MTZ	Metronidazole
MXF	Moxifloxacin
NAAT	Nucleic Acid Amplification Testing
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NICE	National Institute for Health and Care Excellence
ORFs	Open Reading Frames
PaLoc	Pathogenicity Locus
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDR	Pan Drug Resistant
PFGE	Pulsed-Field Gel Electrophoresis
PFOR	Pyruvate-Ferredoxin Oxidoreductase
PhLOPS$_A$	Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins and Streptogramin A
PMC	Pseudomembranous Colitis
PPI	Proton Pump Inhibitors
PPV	Positive Predictive Value

QRDR	Quinolone Resistance Determining Region
RAST	Rapid Annotation using Subsystem Technology
REA	Restriction Endonuclease Analysis
RGI	Resistance Gene Identifier
RIF	Rifampicin
RND	Resistance Nodulation Cell Division Superfamily
rpoB	RNA Polymerase Beta Subunit
rRNA	Ribosomal Ribonucleic Acid
SAM	Sequence Alignment and Map
SHEA	Society for Healthcare Epidemiology of America
SLP	Surface Layer Protein
SMR	Small Multidrug Resistance Family
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
STR	Streptomycin
TC	Taurocholate
TcdA	*C. difficile* Toxin A
TcdB	*C. difficile* Toxin B
TE	Tris Ethylenediamine Tetraacetic acid
TET	Tetracycline
TGC	Tigecycline
TMS	Transmembrane Segments
tRNA	Transfer RNA
TVC	Total Viable Count
TZP	Piperacillin/Tazobactam
USER	Uracil Specific Excision Reagent
UV	Ultraviolet
VAN	Vancomycin
VRE	Vancomycin-Resistant Enterococci
XDR	Extensively Drug Resistant

Chapter 1 Introduction

1.1 *Clostridioides difficile*

Clostridioides difficile is a Gram-positive, spore bearing, obligate anaerobe. This highly motile, rod-like bacterium is ubiquitous in nature, often found in soil and aquatic environments (1, 2). The existence of *C. difficile* spores in both hospital (3, 4) and community cohorts (5) demonstrates the abundance of this organism, whilst asymptomatic colonisation (6-8) and zoonotic carriage (9, 10) further facilitate transmission. Pathogenicity is toxin-mediated and directly related to antibiotic-associated microbiota depletion and the creation of colonic niches (11).

1.2 History

Though the importance was not appreciated at the time, the first recorded isolation of this significant pathogen was published by Hall and Toole in 1935 (12). They reported the isolation of this bacterium from the stool of a healthy infant, originally giving it the name *Bacillus difficilis*, due to difficulties associated with its culture. A dearth of research followed this discovery, with no significant literature published until the 1960s, by 1970 the organism was renamed *Clostridium difficile* (13). Evidence of human infections soon followed (14).

Pseudomembranous colitis (PMC) was first discovered by an American surgeon, John Finney in 1893 (15). He identified complications arising with a post-operative patient, whom developed haemorrhagic diarrhoea after gastro-enteric surgery. Nonetheless, associations between *C. difficile* and this severe outcome were not made until much later (16, 17).

By 1973 the first links between PMC and antibiotic exposure were made, through a study connecting seven out of eight PMC patients with prior lincomycin use (18). Clindamycin use was first associated with PMC in 1973 (19, 20), with a plethora of cases reported in the following years (21-24). By 1974 the initial discovery of a cytopathic toxin associated with clinical manifestations of PMC was made, as described in guinea pig stools (25).

Hamster models proved significant in initiating further breakthroughs in the understanding of the disease. *C. difficile* was established as the aetiological agent of PMC in 1978, when it was isolated from the caecal contents of diseased animals, and the presence of neutralisable toxins were demonstrated (26-28). Work by Bartlett and colleagues was crucial in demonstrating the link between *C. difficile* and PMC, suggesting a cross-reactivity with clostridial anti-toxins (16, 17). Subsequently antibiotics were identified as specific risk factors, with clindamycin and cephalosporins deemed the agents of highest risk and the main antimicrobial classes associated with infection (20, 29). The awareness of *C. difficile* infection (CDI) risk associated with quinolone compounds rapidly followed their introduction and wide spread usage (30).

Recently, the classification of *C. difficile* has been under review, with phylogenetic analyses first suggesting a proximity to the Peptostreptococcaceae family (31). However, further research assessing phenotypic behaviour in conjunction with phylogenomics has led to a reclassification into a novel genus, and should be officially referred to as *Clostridiodes difficile* (32). Nonetheless, this bacterium is still commonly referred to a *C. difficile* and will herein be referred to by that name.

1.3 *Clostridioides difficile* infection

C. difficile is the leading cause of antimicrobial-associated diarrhoea in hospital, and increasingly, community settings (33). CDI primarily affects the elderly, with incidences and mortality dramatically increasing at >50 years and risk further escalating at >65 years (34). *C. difficile* produces resilient spores that can remain dormant in the environment for prolonged periods, before potentially being ingested by a host. Subsequent exposure to broad-spectrum antibiotics has a deleterious effect on the host gut flora, depleting colonisation resistance; a mechanism where the resident gut bacteria inhibit invading pathogens from colonising (35). This subsequently allows *C. difficile* to proliferate, release toxin and initiate infection (11). Most recent UK figures from 2012 indicated that 1,646 deaths were attributable to *C. difficile* (36), with an estimated potential healthcare cost of up to £8,542 per CDI patient (37). Financial impact on healthcare providers is significant, with annual healthcare costs have been estimated as more than five billion US dollars, in North American alone (38).

1.4 Clinical manifestations

CDI is characterised by a wide range of clinical symptoms, from mild, self-limiting antibiotic-associated diarrhoea to colitis, PMC and toxic megacolon (39). Diarrhoea is often watery, voluminous and commonly emanates a distinctive odour (40), whilst additional clinical markers include fever, low serum albumin levels (<3 g/dL), raised leukocyte count (>15,000 cells/μL) and high serum creatinine levels (41, 42). Severe cases resulting in PMC and toxic megacolon are often associated with abdominal cramping, distention, vomiting, lethargy, nausea and white blood cell counts as high as 50,000 cells/μL (40, 43, 44). PMC is characterised by necrosis of the epithelial lining, producing lesions that can erupt exudate, effecting further necrosis (45). Other complications associated with CDI are tachycardia, hypotension and extensive dehydration leading to renal failure (40). Fulminant disease is reported in <5% of CDI patients, where toxic megacolon can result in bowel perforation and death (40), with fulminant CDI associated with an all-cause, 30 day mortality rate as high as 36% (46).

1.4.1 Recurrence of infection

Post symptomatic CDI resolution, 13-35% of patients suffer recurrent episodes of infection (47-50). Of these cases 45-65% experience further episodes of relapse or reinfection (51, 52), with extrapolated rates reported in the United States of 83,000 recurrent infections in 2011 (53). Retention of *C. difficile* spores or the reintroduction of new strains into a sustained diminished colonic environment can result in germination, proliferation and disease. Several studies have reported an increased risk of mortality associated with recurrent CDI, with 9.3% and 16.4% of cases resulting in death after 30 and 365 days, respectively (54-56). Many of the common risk factors associated with primary instances of CDI are further correlated with recurrent infections, including advancing age, use of antacids and prolonged antibiotic treatments (57-60). Immune response is important in the prevention of recurring infections, with poor IgG antibody responses to *C. difficile* toxins associated with elevated primary and recurrent CDI risk (61, 62).

Recurrent episodes of CDI can be differentiated into relapses of disease with the same strain, and reinfections with a different strain from the environment. Figueroa *et al.* reported higher proportions of relapse over reinfection in early (0-14 days) compared to late (15-31 days) recurrences, 86.7% and 76.7% respectively (63). Ribotype 027 cases were significantly more likely to result in relapses (93%), than non-027 strains (75%), indicating further challenges associated with this hyper-virulent ribotype. In support of these findings, data from molecular surveillance of 102 patients revealed that 88% of secondary episodes occurring within 8 weeks were a result of a relapse, whilst only 65% of episodes recurring after 8 weeks were attributed to the same strains (64). This suggests that retained spores may be the most likely aetiology of recurrent infection in the majority of instances, even two months after initial disease resolution.

Secondary to the clinical implications, the cost of recurrent infections is a major burden to healthcare providers, and has been estimated as $2.8 billion per annum in the USA alone (65). Spiralling costs are predominantly attributable to increased lengths of hospital stay and additional intensive care admissions (58), with one study reporting 68% of cost associated with increased hospitalisation, 20% with surgeries and 8% constituted of additional drug expenditures (65). The true cost of recurrent CDI is difficult to calculate, due to many confounders, such as associations with co-morbidities. However, a large scale, retrospective observational study by Zhang *et al.* proposed an estimated average cost of $49,456/£37,289 per case, $10,580/£7,977 more than a primary CDI instance (58).

Antimicrobial resistance is not thought to be a factor in CDI recurrence (66), and recommended therapies often involved repeat courses of either metronidazole or vancomycin, although efficacy of this approach was observed in only 50% of cases (67). Therefore, recent guidelines focus on treatment with vancomycin, administered via a tapered or pulsed regimen (68). These alternative dosing regimens were reported as significantly improved over standard therapies, with recurrence reported in 44.8%, 31.0% and 14.3% of cases, for repeat, tapered and pulsed vancomycin treatments respectively (69). Due to the evident success of fidaxomicin in clinical trials (50, 70), current recommendations also suggest the use of this narrow spectrum antimicrobial, however this remains a far more costly drug (71). In cases of multiple recurrences, the use of faecal microbiota transplants has demonstrated superior efficacy (72-74).

1.4.2 CDI risk factors

Identification and understanding of the risk factors associated with CDI acquisition and severity are crucial to the effective diagnosis and management of potential patients. Several important patient characteristics have been linked with increased incidence and severity of CDI.

1.4.2.1 Increased age

The most commonly reported risk factor for CDI is increased age (34, 75-80). As outlined in the Society for Healthcare Epidemiology of America (SHEA) guidelines, incidence of CDI is highest in patients aged over 65 (68). One prospective study encapsulating over 4,000 patients across six Canadian hospitals, further outlined this risk, demonstrating a 2% increase in risk for every additional year beyond 18 years of age (34). The same research group further delineated their findings, indicating an elevated incidence amongst a population of over 50 years of age, whilst mortality rates correlated significantly with those over the age of 60 (78). Whilst age demographics strongly correlate with CDI rates, the association with disease severity is less clear. A recent retrospective chart review revealed significant correlation between advanced age (>70), severe CDI and all-cause mortality (81). Nonetheless, other studies have found no links to outcome severity (82-85). The plethora of confounding factors that are associated with age further complicate this picture. Increased contact with healthcare settings, greater exposure to antimicrobial treatments, additional co-morbidities and potential physiological alterations impairing immune response efficacy, may all contribute to an overall risk (80, 86, 87). Changes in microbial diversity of the gut have been observed in aging populations (88, 89), with Rea *et al.* identifying a distinct reduction in Bacteroidetes and Clostridium species amongst this population (88). Ultimately, these alterations to the gut microbiota may affect the level of colonisation resistance and susceptibility to CDI onset.

1.4.2.2 Predisposing antimicrobials

Understanding the complex relationship between antimicrobial usage and CDI is critical to reducing incidence of this disease. The majority of antimicrobial classes have previously been linked with predisposition to CDI (90), however, early research implicated particular broad-spectrum antimicrobials as CDI inducing agents, including clindamycin, ampicillin and cephalosporins (29). Further studies supported these findings, indicating those compounds with greater anti-anaerobic activity were associated with greater CDI risk (91, 92). Conversely, several antibiotic classes, including ureidopenicillins and tetracyclines have been associated with a lower risk (93). Exposures to both prolonged therapies and multiple, concurrent antimicrobials have also been correlated with an increased risk of severe CDI (80, 94, 95), undoubtedly due to the severe deleterious effect on intestinal microbiota.

1.4.2.2.1 Clindamycin

This broad spectrum lincosamide has a range of indications for use, including respiratory, soft skin, bone and joint infections (96). Clindamycin has long been established as a risk for the onset of antibiotic-associated diarrhoea (20), with many subsequent studies confirming the high level of risk related to use of this antimicrobial (28, 80, 95, 97-99). Three large scale meta-analyses comprising of almost 50,000 subjects (100-102) all determined clindamycin as one of the primary risk factors for CDI (with odds ratios as high as 20.4), whether in hospital or community environments. As awareness of this association increased, restrictions on this antibiotic have led to decreased incidence of CDI, attributable to more considered prescribing, as opposed to declining resistance development (97, 103, 104). Consequently, current UK guidelines recommend the avoidance of clindamycin in elderly patients (105). High-level resistance development to this agent has been reported as characteristic of epidemic strains and may be considered a potential risk factor (92, 97).

1.4.2.2.2 Cephalosporins

Cephalosporins, particularly second and third generation compounds, have demonstrated some of the strongest associations with CDI risk (100-102). Increasingly reported in the aftermath of clindamycin restriction (29), these agents were regularly described as significant risk factors for *C. difficile* disease in both hospital and community settings (78, 80, 95, 98, 106, 107). A systematic review revealed cephalosporins as, by far, the class of antibiotics with the greatest risk of CDI, more than four-fold higher than clindamycin (80). One prospective study of Canadian cohorts indicated cephalosporins as a risk of disease (OR 3.8; 95% CI 2.2-6.6) even after adjustment for confounders, such as age and co-morbidities (34). The high degree of CDI risk associated with cephalosporin use may partly be due to their low-level of activity against *C. difficile* (108-110). This intrinsic resistance to many agents in this class, may exacerbate the associated risk, by enabling the bacteria to survive and proliferate where other gut flora do not.

1.4.2.2.3 Fluoroquinolones

Fluoroquinolones were previously considered as low risk antibiotics with regards to CDI development (111). However, more recent work has revealed an increasing risk association (34, 92, 99). Crucially, the emergence of the hyper-virulent, PCR ribotype 027 as a major cause of epidemic disease at the beginning of the century was strongly attributed to the development of fluoroquinolone resistance (34, 112). In the context of this outbreak situation, Loo *et al*. observed a strong, independent correlation between prior fluoroquinolone exposure and *C. difficile*-associated diarrhoea (OR 3.9, 95% CI 2.3-6.6) (34). Pepin *et al.* demonstrated that one quarter of CDI patients received fluoroquinolones prior to onset, with over a third of these featuring a quinolone as contributory to the onset of infection (99). Gaynes *et al*. reported associations amongst a long-term care facility cohort, where prescribing policies changed from levofloxacin to a predominance of gatifloxacin (91). This shift in third generation fluoroquinolone was correlated significantly with CDI acquisition ($p<0.0001$), as 30% of patients receiving gatifloxacin developed CDI. Equally, prolonged treatment was associated with significant increases in CDI risk, with those developing disease averaging a length of fluoroquinolone exposure of 13.5 days vs 6.9 in the non-

CDI cohort. The distinct difference in risk may be explained by the markedly improved activity of gatifloxacin against anaerobic bacteria (113), potentially conveying considerable deleterious effects against the gut microbiota. In support of these data, another study demonstrated similar findings (114), with Gaynes *et al*. further demonstrating significance with a reversion in the formulary back to levofloxacin. This resulted in a considerable decrease in CDI cases, although other factors, such as differing illness severity amongst the cohorts and potential ascertainment bias from clinicians are possibly involved (91). Conversely, one North American study revealing fluoroquinolone use as the only significant risk, demonstrated vastly more levofloxacin use (60%) than gatifloxacin (15%) (115), further indicating the complexity of drug-microbiota interactions.

Interestingly, work by Cain and O'Connor indicated that the infection risk increased, where the resident *C. difficile* strain displayed existing resistance to the administered drug (111). With rates of asymptomatic carriage described as high as 51% within certain populations (116), it may be proposed that administration of fluoroquinolones could have greater implications if resistance increased in colonising strains. Intriguingly, Saxton's research determined that a combination of ribotype 027 and moxifloxacin in an *in vitro* model of the human gut, indicated toxin production prior to notable germination and the generation of fluoroquinolone resistant colonies (117). This may be due to a pre-existing sub-population of resistant colonies connected with the hyper-virulent strain. The risk is not always straight forward; in the aforementioned *in vitro* models, levofloxacin displayed a reduced impact on gut flora, although spore germination still occurred. These data highlight the potential involvement of additional factors in disease onset, such as direct stimulatory effects of antibiotic molecules or the relevance of elimination of specific metabolites (117)

Although fluoroquinolones were attributed to CDI risk to a lesser extent than clindamycin and cephalosporin in a large meta-analysis, the association was described in more studies than any other antibiotic class (100). With the increased prevalence of fluoroquinolone resistant strains (118), the risk associated with this broad spectrum class of antimicrobials is considerable.

1.4.2.2.4 Other CDI eliciting compounds

In the 1980s broad spectrum penicillins, such as amoxicillin, were the second highest antibiotic CDI risk factor behind clindamycin (29, 119, 120), largely due to the extent of usage. Current opinions reflected in several meta-analyses, report them as moderate risk (OR 1.45), above macrolides and sulphonamides/trimethoprim (100-102), whilst carbapenem use has also been reported as a significant risk factor (79). A further review suggested the length of exposure as more important in penicillin risk, with treatments prolonged over one week carrying a significant risk (Relative Risk 3.62; 95% CI 1.28-8.42) (121). In contrast, a recent analysis of randomised-controlled trials demonstrated penicillins and fluoroquinolones as involved in equal numbers of CDI cases (122).

1.4.2.2.5 Low risk antimicrobials

There does not appear to a simple relationship between broad-spectrum compounds and onset of disease. Antimicrobial combinations, such as piperacillin/tazobactam have demonstrated relatively low CDI risk compared to other antibiotic classes (101, 123, 124). A large meta-analysis of clinical CDI risk factors recently identified an odds ratio of 1.45 (1.05-2.02) associated with penicillins (100). Interestingly, in an *in vitro* model of the human gut, piperacillin/tazobactam combination therapy effected a major deleterious influence on gut microbiota populations, yet *C. difficile* spores remained dormant (125). Nonetheless, the literature does correlate strongly for some antimicrobial classes. All three large scale meta-analyses of healthcare and community settings concurred in the observation of no significant CDI risk increase relating to tetracyclines treatment, whilst assessment of aminoglycosides also demonstrated no elevation of risk (100, 101, 126).

1.4.2.3 Length of hospitalisation

Length of hospital stay has regularly been associated with an increased risk of CDI acquisition (76, 80, 82, 107, 127). This may be as anticipated, as prior exposure to healthcare settings often serves as a proxy for an increased period of potential contact with spores in the clinical environment, raising the chance of colonisation. Equally, patients suffering extended hospitalisation are often burdened with co-morbidities and are exposed to further risk factors, such as multiple antibiotic treatments. Length of hospital stay is not only associated with increased incidence of CDI, but an elevated severity. Dudukgian *et al*. reported significance associated with mortality, observing a prior hospitalisation of 12 days vs. 6 days in deceased and living CDI patients; respectively (82). Ultimately, this risk is difficult to separate from other risk factors, but demonstrates a compounding effect of several high risk characteristics.

1.4.2.4 Proton pump inhibitor use

One area of considerable debate is the impact of proton pump inhibitors (PPI) on CDI acquisition. Several studies have reported gastric acid suppressants as significantly associated with CDI (128-130), while others have found no correlation (131, 132). These widely consumed gastric acid supressing drugs, used in the treatment of ulcerations and gastroesophageal reflux disease (133) were first independently associated with *C. difficile* colonisation risk by Dial *et al*. in 2004 (128). This study of a large scale Canadian cohort also revealed an increasing risk associated with prolonged PPI use. The mechanism behind this elevation in risk is likely to revolve around an increased potential for ingested spores to reach the lower gastrointestinal tract intact. Research has demonstrated that the reduction of gastric acid production associated with these agents has been demonstrated to lead to overgrowth of bacteria in the gastrointestinal tract (134), with considerable microbiome alterations reported with prolonged PPI use (135, 136). Nonetheless, not all studies have observed an increased risk associated with PPIs (99, 131).

1.4.2.5 Nasogastric intubation

The association between nasogastric tube feeding and CDI has been well documented (80, 137, 138). This relationship is generally attributed to the extra involvement of clinical staff during insertion (137, 139), in addition to the risk of feed contamination (140). A recent large scale meta-analysis demonstrated a 1.8-fold increase in risk associated with enteral feeding tubes, although no significance was linked to recurrence (138). Bliss *et al*. observed a significant risk of nasogastric tubes, with involvement reported in 20% of colonisation cases vs. 8% of the control (137). Disease was established in 9% of intubated patients in comparison with only 1% of control cases. Again, the literature is not always consistent, with an increased severity of disease reportedly associated with enteral feeding in some studies (85, 139), whilst others observed no significant link

(76, 81, 94). Nonetheless, Bignari's major systematic review did report an overall significance associated with this clinical practice (80).

1.4.2.6 Additional risk factors

The severity of underlying disease and additional comorbidities, including inflammatory bowel disease, renal failure, cardiac disease, diabetes and hematologic cancer have all been correlated with CDI risk (49, 141, 142). Host factors such as immune response have also been linked to *C. difficile* colonisation and disease. Loo *et al*. reported a link between the level of toxin B antibodies and healthcare-associated colonisation (78). This association has been supported by recent findings of trials of monoclonal antibodies to *C. difficile* toxins, where antibody response to toxin B was deemed as protective to recurrent CDI (143).

1.4.1 Diagnosis of CDI

Diagnostic algorithms for CDI vary across country, study and even hospital (144). Although recommendations of "gold standard" methodologies have been outlined (145, 146), not every institution can access the finance or expertise required for best practice. This variation impacts on infection rate surveillance (147) and could lead to falsely reported CDI cases, due to the use of sub-optimal testing algorithms.

1.4.1.1 Detection of clinical manifestations

The foundation of any diagnostic assay is the accurate determination of clinically relevant symptoms. Since, the primary indicator of CDI is diarrhoeal stools, reliable and consistent definitions of what constitutes diarrhoea are necessary. Current SHEA and Infectious Diseases Society of America (IDSA) guidelines recommend that more than three episodes of loose stools in 24 hours, which take the shape of the container, should be further tested for the presence of *C. difficile*, unless explained by an underlying reason (68). Confirmation of disease should then be sought through histopathological evidence of PMC or subsequent laboratory testing. The absence of correct diarrhoeal diagnosis may result in wasted financial and time resources.

Two methodologies have been considered as the "gold standard" for the identification of toxigenicity; cell cytotoxicity neutralisation assays (CCNA) and cytotoxigenic culture (68, 146, 148). CCNAs rely on the identification of a cytopathic effect on a cell culture line, associated with the action of *C. difficile* toxin. Confirmation of cell death can be attributed to *C. difficile* toxins, through the simultaneous demonstration of the neutralising effect of *Clostridium sordellii* anti-toxin. While this method is highly specific and sensitive (148-150), it is not ideal for rapid clinical diagnosis, as it is time consuming and requires constant maintenance of cell lines. Cytotoxigenic culture involves an initial culture step on selective agar, and subsequent CCNA of broth culture to identify the presence of a toxin producing strain (151). Where the latter has been demonstrated as more sensitive (152), questions are raised as to the validity of this method, as it only tests the potential for the colonising strain to produce toxin and not the actual presence of toxin in faeces (148). Therefore, since these tests identify different targets, they demonstrate differing sensitivities and specificities (148, 153, 154).

1.4.1.2 Glutamate dehydrogenase and toxin screening

Many commercially available kits exist for the detection of glutamate dehydrogenase (GDH) and *C. difficile* toxin (151, 155). Based on enzyme immunoassay (EIA), lateral flow (immunochromagraphic) and PCR techniques, these kits enable the simple and rapid detection of surface enzymes, free toxin or the presence of the *tcdB* gene in stool. The detection of *C. difficile* in faeces is routinely carried out with an EIA, which detects the GDH enzyme present on the outer surface of all *C. difficile* cells (156). Although this method is unable to distinguish between toxigenic and non-toxigenic strains, it acts as a highly sensitive screening method for *C. difficile* detection (155-159). Interestingly, GDH sensitivity has been demonstrated as strain dependent, with significantly lower sensitivities observed for PCR ribotypes 002, 027 and 106 (160, 161). Due to the low specificity of GDH assays and the lack of direct association with disease (162), positive GDH specimens must be subjected to subsequent toxin testing to diagnose CDI (68, 146).

With the increasing prevalence of CDI associated with toxin A negative, toxin B positive strains (163-165), EIA is now widely used for the detection of both toxins A and B. Where commercially available toxin detection kits provide a rapid result and reduce the level of expertise required, they have been demonstrated to exhibit extensive variability and, in some cases, suboptimal sensitivities/specificities (151, 155). In one large scale comparison of nine toxin detection kits, sensitivity ranged from 66.7 to 91.7%, whilst specificity ranged between 90.9 to 98.8%, with the Premier toxin A+B and Techlab toxin A/B kits amongst the most sensitive EIAs (155). Both of the membrane assays demonstrated greater specificity than the EIAs tested, with highest positive predictive values (PPVs) observed for these kits. Nonetheless, PPVs for commercial kits are generally reported as low, with a systematic review by Planche *et al.* suggesting that due to poor results, single assay kits were insufficient as independent diagnostic tests (151).

1.4.1.3 Nucleic acid amplification testing

The advent of PCR based, nucleic acid amplification testing (NAATs) methods, has led to a reconsideration of recommended testing algorithms (68, 146). There are multiple different commercial testing platforms available for these molecular tests, which are used to identify the presence of *tcdA* and/or *tcdB* genes in a specimen, but none are able to differentiate between expression of toxin and toxigenic potential (166). PCR techniques have been demonstrated as far more sensitive than CCNA approaches (167), with Berry *et al.* reporting sensitivity of 99.1% vs 81.0%; respectively (168). However, since asymptomatic carriage of toxigenic strains has been reported as approximately 6% (169), the use of NAATs could potentially over diagnose CDI (166, 170) and a multiple step algorithm may be necessary for accurate diagnosis (166, 171). Therefore, both the European and North American guidelines recommend a two-step algorithm of GDH and toxin detection or toxin detection and PCR identification, never NAATs alone (68, 146).

1.4.2 CDI treatment

There are several therapeutic options for the treatment of CDI, with antibiotic administration often the primary response (68, 146). Nonetheless, this paradox of treating an antibiotic-associated disease with further antimicrobial therapies has led to the consideration of other approaches, including faecal microbiota transplant and monoclonal antibody therapies.

1.4.2.1 Metronidazole

Metronidazole has been used to treat anaerobic infections for over fifty years (172), with a range of diseases, including respiratory, bone and joint, intra-abdominal, oral and skin and skin structure infections treatable with this antimicrobial (173). This nitroimidazole agent has demonstrated good activity against a variety of anaerobes, with a spectrum covering Gram positive and negative bacteria (174, 175). Metronidazole is well-absorbed and can be given intravenously and topically, but since the CDI target is in the gastrointestinal tract, oral administration is most common (173). Alongside vancomycin, metronidazole is a primary treatment for CDI, previously considered as the antibiotic of choice (176, 177), due to the low cost (178) and perceived non-inferiority to vancomycin (179-181). A ten year surveillance program revealed oral metronidazole clinical cure rates of CDI as superior to vancomycin (91% vs 88%), with only 7% recurrence associated with the former (181). However, considerably more patients were treated with this antibiotic, potentially skewing the data. Another study by Wenisch *et al.* revealed comparable rates of clinical cure and recurrence with vancomycin (180).

Recently the efficacy of metronidazole has come into question, with several studies indicating treatment failures and inferior outcomes compared to those with vancomycin (84, 122, 182, 183). A study by Musher *et al.* revealed only 50% of 207 patients treated with metronidazole were cured, with 22% demonstrating no symptomatic relief at all (182). They also reported significantly higher mortality rates associated with those demonstrating minimal treatment response, compared to cure (33% vs 21%; $p<0.05$). In a randomised-controlled study by Zar *et al.* clinical cure was observed as 90% and 98%, for metronidazole and vancomycin respectively (183), while others found failure rates as high as 22% (84). Whilst seemingly efficacious in the treatment of mild to moderate CDI, metronidazole has demonstrated poor success in cases of severe disease (183-185). A retrospective cohort of almost 50,000 patients showed that treatment of CDI with metronidazole was significantly more likely to result in death than if vancomycin was used (184). Recurrence rates associated with metronidazole treatment have also been reported as elevated above other therapeutic options (84, 182, 185, 186); in some instances recurrent infection was described in as many as 25% of patients (84, 186). Although resistance to metronidazole is scarce, isolates with reduced susceptibility have been reported (187, 188). Whilst resistance is rare, since faecal concentrations are relatively low (189), any small reduction in susceptibility may contribute towards treatment failures. The true reasons behind metronidazole treatment failures are likely to be multifaceted, with some suggesting correlations between increased age, severity of underlying disease (141, 190), broad spectrum antimicrobials (191), resistance development (not primarily to metronidazole) (91, 115, 191) and the emergence of hyper-virulent epidemic clones (112, 192). Taken together these results have led to a consideration of a shift in CDI treatments away from metronidazole.

1.4.2.2 Vancomycin

Vancomycin is a widely used antibacterial agent, particularly in cases of severe Gram positive infections, often where resistance to other frontline antibiotics is encountered (193). Due to the poor absorption of vancomycin, high faecal concentrations are achieved (>1,000 mg/L) (194), which are ideal for the treatment of gastrointestinal diseases. As one of the recommended primary treatments for mild to moderate cases of CDI (68, 146), vancomycin has demonstrated significant superiority of clinical cure rates, over metronidazole (183, 195, 196). Equally, for severe CDI, significant differences (p=0.02) in cure rates were observed for vancomycin (97%) compared to metronidazole (76%) (183). However, these differences are not always observed. Several early studies have exhibited comparable efficacies of vancomycin and metronidazole (179, 180), and since the latter agent is considerably less expensive (178), it is still used as a frontline treatment. Recurrence of CDI is comparable between vancomycin and metronidazole (183, 184), with approximately 16% of cases resulting in further episodes. However, although the study was not powered for the investigation of recurrence, Johnson *et al.* identified vancomycin superiority amongst the total study population, including 20% with recurrent infections (195).

Although vancomycin resistance is uncommon in *C. difficile* (118, 197, 198), instances of resistant isolates are gradually being reported (199-201). Whilst these are unlikely to contribute to treatment failures, due to high colonic concentrations, it demonstrates the potential for resistance acquisition. Furthermore, the selective potential on concomitant bacteria must be considered, as the emergence of vancomycin-resistant enterococci (VRE) has been associated with use of the compound (202). Evidence of co-existing VREs and *C. difficile* populations has been reported (203, 204) and both correlate with many of the same risk factors (190).

1.4.2.3 Fidaxomicin

Fidaxomicin is a macrocyclic antibiotic demonstrating strong activity against *C. difficile* (118, 205, 206), with high concentrations of the compound accumulating in the faeces (>1,000 μg/g) (207). Exhibiting a narrow spectrum of activity, focussed on Gram positive anaerobic bacteria (208), fidaxomicin has a lower deleterious effect on the concomitant gut microbiota than vancomycin. After phase III clinical trials revealed its safety and a non-inferiority to vancomycin (50, 70), fidaxomicin was introduced to the European market in 2012 for the treatment of severe CDI (50). Clinical cure rates have been demonstrated as 88%-92%, compared to 86-91% for vancomycin (50, 195). Fidaxomicin demonstrates superiority over other antimicrobials, with reduced incidence of recurrent episodes of CDI reported. Clinical trial evidence revealed that fidaxomicin reduced the risk of recurrence, with only 15% of cases relapsing, compared to 25% with vancomycin (50, 70). Nonetheless, the significant impact on recurrence rate was only observed in non-ribotype 027 strains (50). One proposed mechanism contributing to the reduction in recurrent infections is the adherence of the compound, via electrostatic interactions, to spore surfaces (209). This could mean that vegetative forms emerging after spore germination are rapidly met with direct antibiotic action.

The main drawback of fidaxomicin use is expense (71), however, the clinical benefits of reduced recurrent infections and hospital stay can result in cost savings (210, 211). Whilst this compound may not currently be routinely used as a first line treatment, it may be beneficial in the treatment of the first episode of recurrence. Until recently, fidaxomicin resistant isolates have not been reported in clinical specimens (188, 206, 212), but laboratory mutants have demonstrated the potential for resistance development (213). However, a contemporary study of Floridian isolates has identified one of the first clinical strains, resistant to up to 16 mg/L fidaxomicin (214), whilst a single French isolate demonstrated a fidaxomicin MIC of >8 mg/L, amongst >3,000 susceptible surveillance strains (215).

1.4.2.4 Faecal microbiota transplant

Failure of antimicrobial therapies and recurrent infections often require alternative approaches to treatment. Since dysbiosis of the gut microbiota is essential to the onset of CDI, replenishment of a healthy and diverse colonic microbial population is considered as a novel preventative method for chronic relapses of infection. The instillation of healthy faeces directly into the gut through a naso-gastric tube, colonoscopy or even via the ingestion of frozen faecal capsules (216) act to redress the balance of micro-organisms in the colon. Thus, restoring the benefits associated with a healthy gut microbiome, including "colonisation resistance" and a proficient metabolic environment (217).

The first randomised, controlled clinical trial to investigate the efficacy of faecal microbiota transplants (FMT) in the resolution of CDI symptoms was performed in the Netherlands, between 2008 and 2012 (72). Van Nood *et al.* randomly assigned each of 43 CDI patients to one of three treatment regimen; vancomycin alone, vancomycin followed by bowel lavage, or vancomycin, bowel lavage and subsequent FMT instillation through naso-duodenal tube. With a primary endpoint of diarrhoeal resolution and no recurrent infection for up to ten weeks, 13 out of 16 FMT patients (81%) demonstrated clinical cure and a further two patients achieved symptomatic resolution with a second instillation. Results were significantly superior to vancomycin treatment, which achieved only 31% cure rate (p<0.0001). Importantly, no significant differences in adverse events were observed. Furthermore, one major systematic review of FMT studies identified a treatment resolution of over 90% (74).

Although the success of faecal bacteriotherapy is not wholly understood, associations have been observed with increased microbial diversity in the gut, particularly with elevated populations of Bacteroidetes and Clostridium clusters IV and XIVa (72, 73, 218). The search for the combination of fundamental species necessary to prevent *C. difficile* proliferating in the human gut continues through the use of modern metagenomic techniques, such as 16S sequencing (219). Although a successful treatment option, it is not without limitations and controversy. There may be a stigma associated with the nature of this treatment, with 41% and 24% of patients reportedly concerned about safety and cleanliness, respectively (220). Crucially, there are no studies demonstrating the longitudinal outcomes of FMT, and whilst there are suggestions of potential links between intestinal microbiota composition and diseases such as obesity and diabetes (221, 222), the National Institute for Health and Care Excellence (NICE) guidelines advise caution (223).

1.4.2.5 Novel therapeutics

The search for new anti-CDI antibiotics is ongoing, with several compounds reaching clinical trial stages. Equally, attempts to circumvent the treatment paradox of using antimicrobial therapies to treat an antibiotic elicited disease have generated several novel approaches to CDI.

1.4.2.5.1 Novel antimicrobials

Ridinilazole is a novel, bactericidal antimicrobial demonstrating potent activity against *C. difficile* (224, 225). Although its mechanism of action is unknown, it has been demonstrated to effect cell division and septum formation (226). Due to its narrow spectrum of activity (227-229), demonstrating superior retention of gut microbial diversity than fidaxomicin (228), the gut microbiota is spared any major deleterious effects, potentially preserving colonisation resistance (228). During phase II clinical trials ridinilazole demonstrated a non-inferiority to vancomycin treatments in rates of sustained clinical cure over 30 days (66% vs. 42.4%; p=0.0004) (230). The strong potential of the agent was further outlined by the indication of statistical superiority over vancomycin in the primary analysis population (230). Furthermore, Snydman *et al.* revealed a lessened risk of VRE acquisition, compared to vancomycin (23.7% vs. 29.7%), although this was not significant (231).

Another promising anti-*C. difficile* compound was the oxazolidinone, cadazolid. This protein synthesis inhibitor demonstrated potent activity against *C. difficile*, preventing toxin and spore formation (232, 233). Phase II clinical trials revealed increased rates of diarrhoeal resolution compared to vancomycin (60.0% vs. 33.3%), with marked reductions in recurrent infections (50% vs. 25%) (234). Unfortunately, following mixed phase III trial results, development was discontinued (235).

An alternative approach to reducing the impact of antimicrobial therapies is employed by ribaxamase. This oral β-lactamase is administered concurrently with intravenous β-lactams antibiotics (236). Ribaxamase administration is aimed at reducing the impact on gut microbes by degrading excess β-lactam compound reaching the gastrointestinal tract, thus reducing the risk of CDI development (236). Ribaxamase has performed well in phase II clinical trials, demonstrating both significant reductions in relative CDI risk (p=0.045) and VRE acquisition (p=0.0002) (236, 237).

1.4.2.5.2 Microbiota therapeutics

Based on the perceived success of faecal transplantations in the treatment of recurrent CDI (72), probiotic capsules derived from faecal microbiota are under clinical testing as a potentially more accessible treatment option. SER-109, created by Seres Therapeutics, Inc. is a formulation of purified spores (predominantly Firmicutes) from healthy faecal donors, promoted as an anti-CDI recurrence treatment. Initial clinical trials demonstrated safety and clinical success, with 86.7% of patients sustaining resolution of diarrhoea for 8 weeks (238). Significant increases in microbial diversity were observed and deemed important to the success of this formulation, however, no placebo control cohort was used in this study. Disappointingly, the phase II, randomised, placebo controlled trial results failed to meet primary efficacy end points (239), but further analyses suggested that higher dosages may improve the results (240). Phase III trialling of SER-109 is currently underway (241).

A similar microbial formulation, designed for the treatment of recurrent CDI is RBX-2660. Phase II clinical trials revealed 87-89% success in prevention of recurrent infection (242, 243), with comparable efficacy to FMT treatments (72). Nonetheless, the most recent trial did not meet the primary end point, as the efficacy of two doses was not significant, with only single doses demonstrating significance over placebo (242). This serves to highlight the complexity of microbiota suspension treatments, with further trialling necessary. As with FMT, the long term consequences of microbiota alterations are yet to be determined and therefore caution must be taken with these approaches.

Knowledge of the role of both primary and secondary bile acids in *C. difficile* spore germination (244) has also led to proposed novel therapies, such as those altering the bile acid milieu, either through instillation with probiotic, bile acid metabolising microbiota (245) or synthetic bile acid analogues (246). Other approaches include the controversial targeted colonisation with non-toxigenic strains (247), and largely unsuccessful toxin-sequestering adjunctive compounds such as tolevamer (195).

1.4.2.5.3 Immunotherapeutics

A considerably elevated risk of developing severe CDI has been associated with low IgG levels in the serum (86). Therefore, to counteract this lack of antibody response, artificially increasing immunoglobulin concentrations is one approach to reduce the risk of CDI recurrence. The intravenous administration of immunoglobulins, containing anti-toxin antibodies against *C. difficile* has demonstrated efficacy in preventing infection relapse (248-251). Nonetheless, mixed results have been observed with these passive immunotherapies. In a retrospective review of immunoglobulin treatments, McPherson *et al.* reported sustained resolution of disease in 64% of cases (252), and Abougergi *et al.* indicated that beneficial effects were achieved in four non-controlled trials (253). However, the only controlled study reported, revealed no significant differences in all-cause mortality rates associated with post-antibiotic immunotherapy (254).

One promising monoclonal antibody therapy is Bezlotoxumab. Targeting the clostridial toxins A and B, Bezlotoxumab inhibits the binding of toxin to host cell, neutralising the threat and conferring host immunity. Phase III clinical trials have revealed a significant reduction in recurrent CDI episodes associated with this treatment (7% compared to 25%) (255). Interestingly, further analysis by Gerding *et al.* revealed that the largest reduction in CDI recurrence associated with Bezlotoxumab use was observed in patients with more than three risk factors (256).

1.4.3 Asymptomatic colonisation

Highly discriminatory molecular techniques, such as next genome sequencing have been utilised to map nosocomial transmissions of *C. difficile* (257-259). Due to large proportions of unexplained transmissions in these studies, the role of asymptomatic carriers has become a focus of recent infection control considerations (6, 8). Where *C. difficile* spores are ingested by subjects with healthy gut microbiomes, the organism can either colonise asymptomatically or transiently pass through the gut (8). Although these carriers have no direct clinical concerns, they can potentially act as transmission sources for CDI (257, 258). Reported colonisation rates differ extensively and seem dependant on the study setting. Asymptomatic colonisation rates range from 3-21% amongst hospitalised patients (4, 78, 259-261) and 4-15% in healthy adults (262-264), with highest rates observed in long term healthcare facility cohorts, (4-51%) (88, 265-267). Nonetheless, instances of colonisation are difficult to separate from CDI cases, with testing algorithms important to differentiation (8). These issues could be reflected in the wide range of colonisation rates described. The lowest rates of colonisation are reported by some of the largest studies (78, 259), however, Kong *et al.* suggest that this may be due to the high prevalence of epidemic strains within these study populations (268). Nonetheless, there is evidence suggesting that asymptomatic carriage may act as a potential transmission source for *C. difficile* and the value of additional screening at hospital admission should be considered.

1.4.4 *C. difficile* in the community

CDI is traditionally regarded as a hospital-acquired infection, associated with well-defined risk factors, such as antibiotic and healthcare exposure. However, increasing numbers of case reports of community acquisition and onset of disease associated with individuals not previously deemed at risk, have been reported (269). This has highlighted the importance of these cases and is currently considered essential to the holistic understanding of epidemiology and infection prevention. Community-acquired (CA)-CDI is generally defined as symptomatic onset within 48 hours of admission or more than 12 weeks post discharge (270) (Figure 1), although as highlighted by Wilcox *et al.* (271), many authors use varying definitions further complicating the determination of true acquisition rates. Rates of CA-CDI vary extensively, with ranges reportedly between 2-46% of CDI cases (53, 77, 271-273). Whilst these cases are generally considered to elicit less severe clinical outcomes with fewer reported instances of recurrent infections (41, 272), CA-CDI has been associated with a greater severity of early symptoms (274), likely due to late diagnoses. Although still primarily a nosocomial disease, the incidence of community onset infections remains high, whilst strict adherence to classical risk factors may result in a significant proportion of CA-CDI cases being overlooked (271). Interestingly, in the UK ribotype prevalence varies between hospital and community settings, with the latter dominated by the toxigenic 002 type (275).

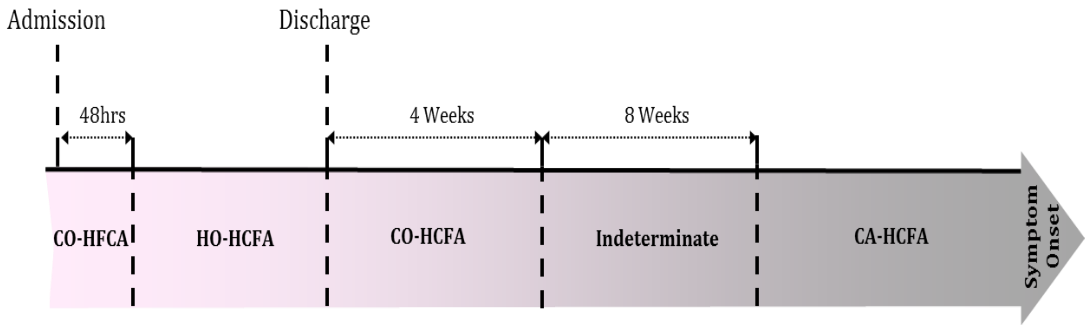


Figure 1: Definitions of hospital vs community onset of CDI. *CO-community onset, HO – hospital onset, CA-HCFA – Community acquired healthcare facility associated. Adapted from McDonald (2007) (270)*.

1.5 Virulence factors

Symptoms of CDI are elicited by the action of up to three distinct, proinflammatory toxin molecules, toxin A (enterotoxin), toxin B (cytotoxin) and binary toxin (CDT) (276).

1.5.1 Large clostridial cytotoxins A and B

The main virulence components of *C. difficile*, toxin A (TcdA) and toxin B (TcdB) are large clostridial cytotoxins, 308 kDa and 270 kDa in size, respectively (276). TcdA weakens the junction between epithelial cells, whilst TcdB further disrupts the actin cytoskeleton (277). These toxins, encoded by genes within the pathogenicity locus (PaLoc), act by glycosylating Rho GTPases, inhibiting essential transcription proteins and disrupting cell integrity. Subsequently, membrane permeability is increased and characteristic diarrhoea is instigated (278). TcdA was initially considered as the key virulence factor, demonstrating direct causation of mucosal damage in rats in the absence of TcdB, whereas TcdB alone had no effect (279). This stance has recently become more ambiguous, with evidence of disease-causing, TcdA negative TcdB positive strains (280) and work from Riegler and colleagues indicating TcdB as ten times more potent, with increased electrophysiological and permeability alterations in Ussing chamber experiments (281). Furthermore, Lyras *et al.* were able to demonstrate the necessity of TcdB through the production of isogenic *C. difficile* strains with *tcdA* and *tcdB* gene knockouts (282). Interestingly, Warny *et al.* identified a greater proportion of TcdB production in hyper-virulent ribotype 027, demonstrating toxin production as 23 times higher when compared to twelve other types (283). Nonetheless, the results of this experiment should be treated with caution, as the findings were only representative of batch culture experiments, lacking the complexities of immune response *in vivo*. Strains lacking the *tcdA* and *tcdB* genes are deemed as non-toxigenic (280).

1.5.2 Pathogenicity Locus

The pathogenicity locus is a well characterised, 19.6 kb section of the *C. difficile* genome, which encodes the TcdA/B elements of the organism. The absence of the PaLoc is also well documented in non-toxigenic isolates, where it is replaced with a 115-bp fragment (284). The PaLoc consists of *tcdA* and *tcdB* genes, corresponding to the toxins of the same name and additional accessory genes, *tcdC*, *tcdD*, and *tcdE* (285, 286). Hundsberger and colleagues analysed the transcriptional pattern of the PaLoc and determined that *tcdD* and *tcdC* are positive and negative transcriptional regulators for *tcdA and tcdB*, respectively (285). By determining high levels of *tcdC* expression during the exponential growth phase and its depletion at stationary phase, in correlation with toxin production, they were able to indicate the impact of this negative regulatory gene. Whilst the PaLoc is highly stable and conserved (287), variation in the small open reading frames (ORFs), containing the accessory genes, have been reported (288). Interestingly, Spigaglia and Mastrantonio demonstrated 25% PaLoc divergence, with all strains variant in *tcdC*, offering further support for this transcriptional regulator as influential in virulence potential (288).

1.5.3 Binary toxin

The first binary toxin was identified by Popoff *et al.* in 1988, who discovered the production of a novel ADP-ribosyltransferase in the hyper-virulent *C. difficile* strain, CD196 (289). Subsequent studies have identified an association with hyper-virulence in selected ribotypes (34, 290). As an ADP-ribosyltransferase, it blocks actin polymerisation (276) and has been linked to increased pathogenicity due to greater cell adherence capabilities, enabling a more efficient delivery of its inhibitory elements (278). CDT is unrelated to the large clostridial toxin group and consists of two independent protein chains, CDTa and CDTb (289). Encoded by *cdtA* and *cdtB*, which demonstrate >80% sequence identity with the iota toxin produced by other clostridia (291), these components are required in combination for functionality. The larger, binding component enables the translocation of the enzymatic component, preventing polymerisation and effecting modifications to the cytoskeleton (292). The expression of CDT genes is reportedly controlled by a regulator, *cdtR*, demonstrating influence on CDT production with the insertion and deletion of this putative gene (293). CDT prevalence amongst *C. difficile* isolates has been reported as around 6%, but variable rates have been demonstrated (1.6-34.7%), depending on sample size and outbreak context (283, 294-297).

The precise relevance of the binary toxin to *C. difficile* virulence remains unclear. Case-control studies have exhibited a significant increase in diarrhoeal severity (76.9% liquid stools compared to 59.5% in non-CDT producing strains) and mortality associated with binary toxin strains (28% deaths within 30 days of infection vs 17% in binary toxin negative strains) (298, 299). Nonetheless, differentiating between the impact of TcdA/B and binary toxin has proven difficult, since they are often expressed concomitantly (296). The demonstration of a TcdA/B negative, CDT positive toxinotype XI failing to cause disease in a hamster model, suggests that the role of binary toxin may be more interconnected, exhibiting an additive effect (300, 301).

1.5.4 *C. difficile* Spores

The ability of this anaerobic bacterium to sporulate, allows survival in aerobic atmospheres, extreme temperatures and chemical pressures (302). This environmental persistence through resilient *C. difficile* spores ensures an effective pathway for onward transmission of this successful pathogen. Importantly, spores in isolation are unable to cause disease and therefore must germinate in order to reach a vegetative state, where they can produce the toxins essential to elicit disease (11).

1.5.4.1 Structure

The ultrastructure of *C. difficile* endospores has been elucidated through transmission electron microscopy. Similar in structure to other well characterised species, such as *Bacillus subtilis* and *Clostridium perfringens*, they consist of an inner peptidoglycan core containing dipicolonic acid, germ cell wall (which forms the outer wall of the nascent vegetative cell), thick spore cortex and a spore coat (303). Further to these classical spore components, *C. difficile* spores exhibit an additional surface layer, the exosporium (303, 304). Although less than 25% of *C. difficile* spore coat proteins demonstrate homology to *B. subtilis*, research has suggested their similar roles in protection from external stressors and potential links to virulence (305, 306).

1.5.4.2 Sporulation

The fundamental dogma of *C. difficile* transmission is its ability to sporulate and repeat the cycle of infection. Although not fully defined, its sporulation pathway shows many similarities to other Bacilli (304, 307). Spo0A is reported as the master regulator for sporulation, and is activated by a sequence of phosphorylation in *B. subtilis* (308, 309). While there is a distinct lack of homologous phosphorelay transferases, research suggests direct kinase activation of Spo0A as the initiating factor for *C. difficile* sporulation (307, 310). A collection of key sigma factors activate specific transcriptional pathways leading to endospore formation (307, 311). As outlined in other species, sporulation commences with asymmetrical division of the mother cell with septum formation, creating a large mother cell and smaller forespore compartment. This division is influenced by sigF^{-} and sigE^{-} (307). Under the influence of sigG^{-} and sigK^{-}, the mother cell then engulfs the forespore by a process akin to phagocytosis, forming membrane and outer coat layers. Finally, mother cell autolysis releases the endospore into the environment (307). Whilst these pathways are relatively conserved in *C. difficile*, Pereira and colleagues reported variant sigma factor pathways, indicating that the order of sigma factor expression may not be crucial (311).

1.5.4.3 Germination

Proliferation and outgrowth of quiescent *C. difficile* spores requires reactivation of this form to a metabolically active state. This complex process of germination is necessary to initiate the outgrowth of the toxin producing, vegetative cell form.

1.5.4.3.1 Mechanisms

Germination pathways for *C. difficile* are not as well defined as in other spore-forming organisms, where mechanisms are highly conserved. In the model organism, *B. subtilis*, germination triggers (which are species dependant) signal *ger* receptors to initiate the germination process. Briefly, nutrient germinants, ʟ-alanine, ʟ-valine and ʟ-asparagine bind to *ger* receptor, protein complexes in the inner-membrane, leading to a change in permeability and the release of Ca^{2+}DPA (dipicolonic acid) through SpoVA channels. This triggers cortex lysis enzymes to hydrolyse the spore cortex, allowing rehydration, activation of cell metabolism and eventual outgrowth (312). However, through analysis of the complete *C. difficile* 630 genomic sequence, Sebaihia and colleagues discovered a marked lack of germinant receptor homologues, as described in Bacillus and other clostridial species (313, 314). This absence of the tricistronic *ger* operon is indicative of a novel response mechanism to external germination stimuli utilised by *C. difficile*.

Since ingested *C. difficile* spores inhabit the gastrointestinal tract, the key to germination initiation can also be discovered there. A combination of the primary bile acid, taurocholate and glycine (co-germinant) has been identified as an important germinant trigger in *C. difficile* (244, 315, 316). Bile secreted by the gall bladder, to assist with digestion and absorption of fat and cholesterol, consists of cholate and deoxycholate molecules conjugated with taurine or glycine. Metabolism of these complexes by the indigenous gut flora releases potential *C. difficile* germinants into the ileum. Also, bile salt hydrolases produced by the gut flora alter glycine conjugates as they passes through the lower gastrointestinal tract, releasing free glycine for germination interactions (317). Adding to the complexity of the germination mechanics, other primary bile acids have been implicated in the direct inhibition of *C. difficile* germination, with chenodeoxycholate demonstrated as inhibitory by Sorg and Sonenshein (318). In the aforementioned study they observed direct competition for receptor sites between primary bile acids, with chenodeoxycholate exhibiting a greater affinity than taurocholate. This correlated convincingly with the knowledge of a reduction in chenodeoxycholate concentration in the colon (319), enabling directed germination in the anaerobic environment of the lower intestine. The availability of both taurocholate and amino acid co-germinants allow interactions with inhabiting *C. difficile* spores and the initiation of the complex germination process. Interestingly, Buffie *et al* recently discovered the significance of the bile acid 7α-hydroxylating action of *Clostridium scindens* to an enhanced protective effect to CDI (320). This was deemed to be due to the generation of inhibitory secondary bile acids and could be considered as a novel pre-treatment. Nonetheless, as with any therapy affecting the balance of gut microflora, caution would be advised when considering this mechanism as a potential method of prophylaxis, as high levels of bile acids have been linked with several cancers (321).

Detailed mechanisms for these interactions are only gradually being elucidated. The current working model (Figure 2) implicates CspC, a pseudoprotease, as the initial receptor for cholic acid derivatives. This interaction stimulates a protease, CspB (322), to activate SleC via cleavage of a small prodomain, leading to lysis of the spore cortex. Ca^{2+}DPA is released and the germination process proceeds as in other organisms (323-325). Further work on this model suggested that CspC, although essential in signalling CspB, actually inhibits the core lysis enzyme (323). How the nutrient signal of glycine presence interacts with this model remains unclear. Since several other amino acids have also been implicated as effective germinants for *C. difficile* spores, including L-alanine, L-phenylalanine, L-arginine and serine (326-328), further mechanistic elucidation is required. Interestingly, Shrestha and Sorg highlighted the importance of lower-level temperature differences in germinant potential, indicating a series of amino acids previously considered as ineffective at 25°C, as germination stimulating at 37°C (327). This broad spectrum of potential germinants is suggestive of either the presence of multiple specific receptor sites or a more complex, pliable interaction at a single site.

Other factors must also be considered in this complex process, as additional elements have been implicated in *C. difficile* germination response. Intriguingly, Kochan and colleagues reported the influence of Ca^{2+} ions on the *C. difficile* germination process (329). By demonstrating a 90% germination reduction in murine models with depleted ileal calcium levels, they related this deficiency to the use of proton pump inhibitors, a known CDI risk factor (130). They postulated that the presence of glycine triggers the release of endogenous calcium, stimulating the germination cascade.

Research is ongoing to complete the understanding of *C. difficile* germination pathways, with modern molecular techniques such as forward and reverse genetics demonstrating vast potential in the elucidation of all the relevant proteins involved (330). Whilst determination of the intricacies of the *C. difficile* germination pathway is valuable, identification of optimal germination conditions is of importance in the practical and diagnostic implications of this knowledge. Since the factors affecting germination are multifaceted and often appear contradictory in the literature, further investigation is necessary to improve *in vitro* recovery and research.

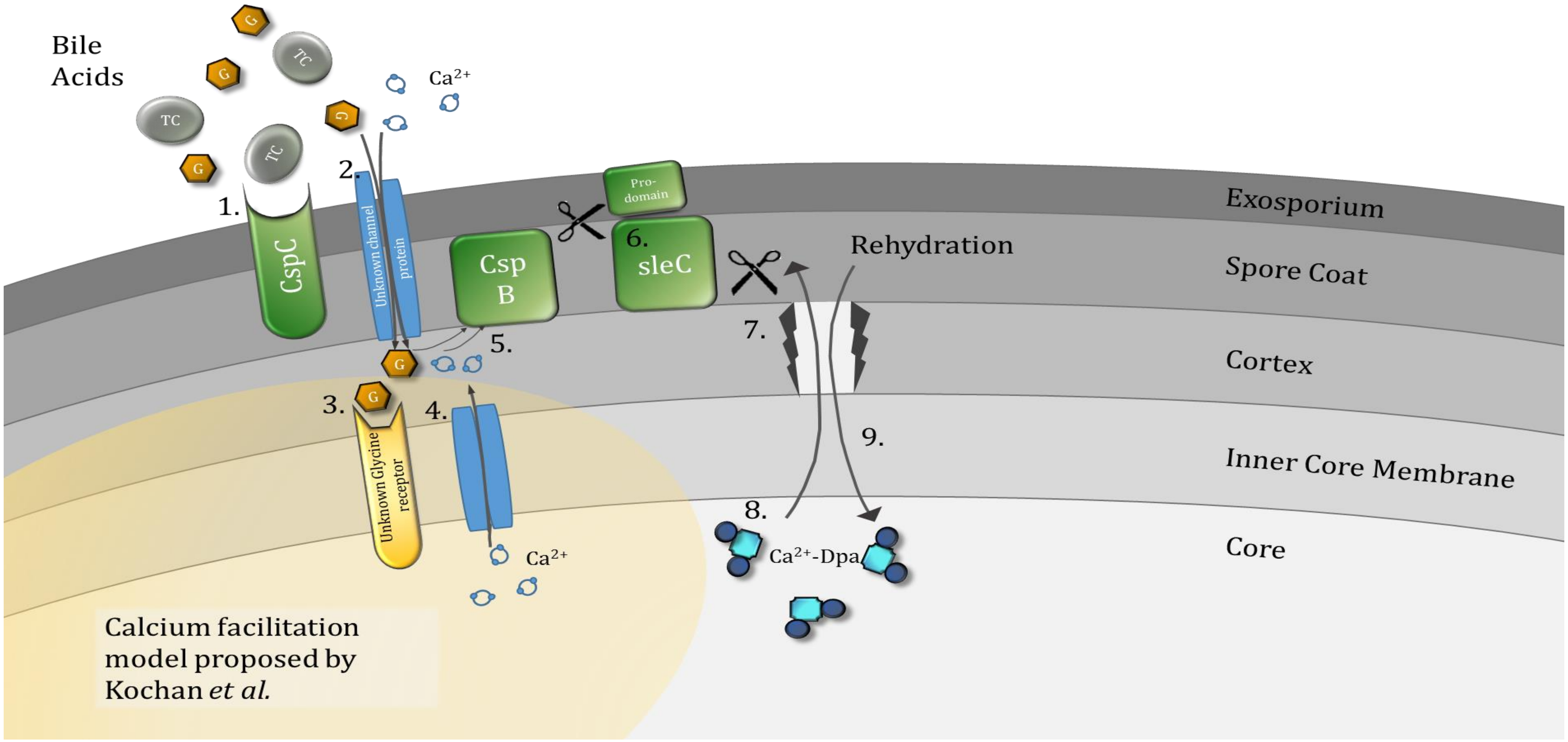


Figure 2: Proposed C. difficile germination model by Francis et al.* (324, 325)*, additional calcium involvement reported by Kochan et al.* (329) *(Orange circle). *Diagram adapted from Kochan et al.* (329)*. 1. Taurocholate (TC) and glycine (G) from bile acid interact with CspC, 2. Enabling glycine and* Ca^{2+} *transport through the spore coat. 3. Glycine binds to an unknown receptor, 4. Releasing* Ca^{2+} *from the core, which interacts with CspB, 6. Cleaving the pro-domain of SleC. 7. Active SleC lyses the cortex, 8. Releasing* Ca^{2}*-Dpa, 9. Exchanging with water to rehydrate the core metabolism.*

1.5.4.3.2 *In vitro* germination

To increase the efficacy of *C. difficile* diagnostics and research, careful selection of culture environment is essential to the optimisation of germination and outgrowth of spores. Comparisons of agar and broth media, along with potential germination enhancing supplements have previously been investigated, with varied results (244, 331-335).

Supplementation of taurocholate into solid agar has been shown to improve recovery rates of *C. difficile* spores, with addition to cycloserine-cefoxitin-fructose agar (CCFA) consistently demonstrating improved recovery (332). Nonetheless, the concentration of cholate utilised in culture media is important to the efficacy of *C. difficile* germination. Studies have indicated that although a 0.1 mmol/L taurocholate concentration is the minimum requirement for germination, a greater concentration of 1-10 mmol/L produces a more complete recovery of spore populations (244, 331). The latter concentration is comparable to the physiological concentrations found in the jejunum (1.2 mmol/L) (336) and duodenum (6.9 mmol/L) (337), suggesting an evolutionary, clinical relevance. Conversely, there are reports in the literature suggesting that lesser concentrations produce comparable levels of recovery, with 0.05% taurocholate recommended as an equally effective, cheaper alternative (338). Nevertheless, variable strain responses are often observed with bile acid supplementation (339), so it may not be achievable to identify a *C. difficile* culture media, optimised for recovery of all strains.

The germination process appears to be more convoluted, with Sorg and Sonenshein suggesting exposure period impacts on bacterial propagation (244). They determined that prolonged exposure to low levels of taurocholate, coupled with the co-factor glycine, acted as a more efficient germinant combination than high concentrations over a short exposure period. Exposure to 10% sodium taurocholate for a ten minute period led to germination of only 60% of the spores recovered through continuous exposure at 0.1% concentration. This would suggest that a prolonged exposure to the bile salt provides a more suitable environment for efficient spore germination and recovery than a transient exposure (244). However, this is contradictory to the understanding that bacterial spores have been demonstrated to commit to germination (340), suggesting that prolonged exposure to germinant signals is unnecessary.

The purity of taurocholate is also considered as a potential factor affecting *C. difficile* germination. Earlier studies reported inefficiencies with some crude reagents lacking purity (315, 341). However, manufacturing processes have vastly improved in recent times and synthetic analogues are likely to negate this variance.

Lysozyme has also been associated with increased *C. difficile* spore germination and recovery from environmental samples (342). Deemed an artificial germinant, lysozyme acts to digest the spore cortex releasing Ca^{2+}DPA and initiating germination, as opposed to interacting with species specific germination receptors (312, 343).

As with many core functions, strain variability inevitably affects germination, with research indicating significant divergence in the germination efficiency between *C. difficile* ribotypes (304). Interestingly, Kamiya's work identified distinct differences between toxigenic and non-toxigenic *C. difficile* strains, with the latter demonstrating lesser germination efficiency (341).

1.5.5 Biofilms

Biofilms are ubiquitous in nature and the clinical environment is no different. Formation on medical devices and prosthetic implants can be a serious challenge to clinicians, and their presence in dental and gastrointestinal niches are universal (344-346). *C. difficile* has been demonstrated to form biofilms in the gut environment (347, 348), offering protection from external stressors and survival assistance in a hostile environment. Biofilms often consist of multiple, co-existing species prompting symbiotic relationships. *C. difficile* has been reported in these integrated bacterial communities (349, 350). Formation and arrangement of planktonic cells into sessile structures is a multifactorial process, which is not fully understood in *C. difficile*. The influence of quorum sensing on biofilm formation is often reported (351, 352), with evidence of a *luxS* homologue implicated in cell assembly (352, 353). Surface layer proteins have demonstrated involvement in early stage biofilm formations (353), whilst type IV pili have been shown to mediate cell surface adhesion (354). Flagella have been implicated in maintenance of mature biofilms, with *fliC* deficient mutants demonstrating decrease efficacy (353). The production of extra-cellular matrix contributes large portions of biofilm structure (353, 355), whilst acting as a physical buffer for the sessile cells within (356). Implicated in recurrent infections, biofilms are of extreme importance when considering antimicrobial susceptibility. Phenotypes of cells within biofilms have been reported as divergent from planktonic populations(350), with reports of up to 1000-fold increase in resistance levels (356). Dapa and colleagues demonstrated resistance to higher concentrations of vancomycin in *C. difficile* sessile populations (353), whilst *in silico* modelling predicted treatment failures after a growth density threshold was reached (357).

1.6 *C. difficile* epidemiology

1.6.1 Typing methods

Molecular typing of *C. difficile* is essential to epidemiological investigation and the identification and control of potential hyper-virulent, epidemic strains. Numerous typing methods have been utilised globally, with geographic preferences highly apparent (358-361). Different methodologies present distinctive nomenclature, making direct comparisons challenging (E.g. PCR ribotype 027 is also referred to as North American pulsed-field gel electrophoresis type 1 (NAP1) and restriction endonuclease analysis group BI).

Restriction endonuclease analysis (REA) utilises the *HindIII* enzyme to digest the entire bacterial genome, with fragments resolved via gel electrophoresis (362). Pulsed-field gel electrophoresis (PFGE) is similar to REA, but exploits the *SmaI* enzyme to generate larger fragments and specialist equipment to apply pulsed-field separation for greater fragment resolution. Whilst PFGE was historically considered as the gold standard for outbreak investigation, these gel-based methods are time consuming, require technical expertise and are limited by the inter-laboratory comparability of profiles (358).

PCR ribotyping is the most widely applied method throughout Europe (360, 361), implemented as the technique of choice by the UK's national surveillance service, the *Clostridium difficile* Ribotyping Network (CDRN) (363). Since *C. difficile* demonstrates significantly greater variability in the 16S - 23S intergenic spacer regions than other organisms (364), ribotyping takes advantage from this variability to discriminate between strains. Demonstrating strong concordance with PFGE types, this methodology provides highly accurate and reproducible groupings (365). Originally developed in conjunction with gel electrophoresis (366-368), the profile comparability was improved by the addition of fluorescently labelled primers and the use of capillary electrophoresis resolution (369). This development enabled direct transferability of ribotype profiles across laboratories with searchable databases allowing rapid and reproducible comparisons.

More discriminatory methods such as multi-locus variable number tandem repeat analysis (MLVA), fragment DNA based on the highly variable number of non-coding tandem repeats. Due to its high discriminatory power, MLVA has applications in sub-typing (370) and outbreak investigations (371). Nonetheless, it cannot be used to infer ribotype and is not used routinely in the UK, due to its requirements in cost and expertise (359, 361).

Several other techniques provide typing of *C. difficile* strains into distinct groups aligned by a specific phenotype. Toxinotyping amplifies and digests specific regions of the PaLoc, identifying variability in toxin genes. Currently 34 toxinotypes have been identified outlining strains' toxigenic potentials, although they are less discriminatory than other methods and binary toxin identification cannot be determined (13, 372). Serotyping methods rely on bacterial surface layer proteins and the variation amongst the antigens present. Initially relying on immunoassay techniques, they were proven as reliable and reproducible in grouping *C. difficile* isolates (373). Modern surface layer protein (SLP) gene analysis methods have largely superseded these (374). However, this typing method is uncommon and SLP types differ within ribotypes, presenting comparability challenges (358).

Multi-locus sequence typing (MLST) utilises sequence differences in seven or more housekeeping genes, assigned to distinct alleles to designate sequence types (ST). This allows some phylogenetic relationships to be identified, as well as accurate translatability between testing laboratories (375). Single nucleotide polymorphism (SNP) analysis such as MLST, although highly discriminatory, is also expensive (360). Nonetheless, the viability of typing by sequencing common genes through next generation sequencing (NGS) has already been demonstrated effectively (257, 376). The advent of high-throughput sequencing technologies and sequencing by synthesis methods is generating greater access to bacterial genomes. Where previous typing methods utilise a select number of genes to calculate sequence type, NGS allows for a considerably greater number of target genes or even the whole genetic sequence to be assessed in typing algorithms (377). Providing far superior resolution, NGS typing is able to distinguish between previously indistinct sub-groups (377), as well as predict antimicrobial resistance (378, 379).

1.6.2 *C. difficile* epidemiology in the United Kingdom

Prior to the emergence of hyper-virulent ribotype 027, *C. difficile* epidemiology in the UK was dominated by ribotype 001 (367, 380, 381). As the first reported epidemic strain, ribotype 001 was implicated in 55% of all UK CDI cases (380), and 93% of all UK outbreaks (382). Other prevalent UK ribotypes prior to the twenty-first century were 010, 014, 106, 012 and 020 (367). The first UK outbreak of CDI caused by the PCR ribotype 027 strain, epidemic in North America (34, 112), occurred between 2003 and 2004 at Stoke Mandeville hospital, where it was involved in 174 cases and 19 deaths (383). As this hyper-virulent strain spread through the country, the number of deaths associated with CDI rose sharply, from 2238 in 2004 to 8324 in 2007 (105). By 2008 ribotype 027 was implicated in 55% of all UK incidences of CDI (384). In 2007, the introduction of a national surveillance service, the CDRN, alongside mandatory cases reporting (105), enabled a valuable insight into the epidemiology of *C. difficile* in the UK (363). With ribotyping data for more than two-thirds of reported cases, this national service acts as a vital tool in outbreak assessment and control, allowing a greater understanding of UK epidemiology and the ability to track ribotype prevalence longitudinally.

Two recent, large scale studies reported CDI incidences in the UK of 3.7 and 10.6 cases per 10,000 patient days (154, 385). Bauer *et al.* reported clustering of ribotype 106 in the UK (13 isolates), whilst it was not detected in any of the other 33 countries involved in the study (385). A recent comparison of discharge data from the USA and UK revealed a considerably lower rate in the latter (115.1 vs 19.3 cases per 100,000 population; $p<0.0001$) (386). These vast differences were reflective of, and potentially attributed to, the introduction of CDI management policies in the UK (105), resulting in a reduction of pre-disposing antibiotic use. This finding was supported by Dingle *et al.*, who used national *C. difficile* data in conjunction with NGS to report a decrease in CDI cases caused by fluoroquinolone resistant strains in Oxfordshire. Correlating with a reduction of fluoroquinolone use, CDI rates from resistant strains declined from 67% in 2006, to 3% in 2013 (387).

A major pan-European study into *C. difficile* epidemiology and antimicrobial resistance revealed that in 2014 the most prevalent UK ribotypes were 014, 106, 015, 020, 078 and 002 (188). Ribotype 027 only constituted 3% of all UK CDI reported in the study. The latest CDRN report (2013-15) confirmed similar results as the large European studies (154, 188), identifying emergent ribotypes as 078, 002, 005, 014 and 015 (363). The longitudinal data also indicated considerable decreases in UK epidemic strains, 027, 001 and 106, correlating with decreased usage of high CDI risk antibiotics, cephalosporins and fluoroquinolones (384). Compensatory increases in sporadic strain types led to a more heterogeneous spread of ribotypes (363), potentially reflecting improved infection control and antimicrobial stewardship.

1.6.3 *C. difficile* epidemiology in Europe

The emergence of PCR ribotype 027 in North America at the beginning of the twenty-first century resulted in rapid dissemination into Europe. As observed in the UK (383), by 2005-06 outbreaks were being reported across several European countries (388-392). In the Netherlands, considerable increases in CDI between 2004 and 2005 were attributed to the emergence of ribotype 027, with incidence rising from 4 to 83 cases per 10,000 patient admissions (390). To combat this rise, restrictions of cephalosporins and a ban on fluoroquinolone use were instated. In conjunction with increased infection control procedures and ward separation, Debast *et al.* reported the cessation of one large, Dutch outbreak (393).

In 2003, Barbut and colleagues reported a mean incidence of CDI in one pan-European study, as 11 cases per 10,000 patient days (144). This rate was considerably lower when reported from 97 hospitals across 34 European countries in 2008, where a mean incidence of 4.1 per 10,000 patient days was observed (385). However, this rate varied widely between individual countries and hospitals, with Turkey and Poland demonstrating polarised incidence of 0.0 and 36.3 per 10,000 patient days, respectively. Two large scale studies reported comparable numbers of ribotypes amongst European populations, with 66 reported in 2007 and 65 in 2008 (385, 394). In 2007 Barbut *et al.* reported a distinct lack of diversity, with a predominance of 12 ribotypes (mostly 001, 014, 027, 020, and 017) constituting over 65% of the total (394). All of the ribotype 027 isolates demonstrated resistance to modern fluoroquinolones, but were clustered in only three hospitals. Bauer reported similar ribotype prevalence, with 014 (16%), 001 (9%), 078 (8%) and 027 (5%) the most abundant (385). Instances of ribotypes 106 and 078 were increased over early findings (144), reflecting the emergence of these ribotypes in other countries (290, 395), with the latter potentially implicating the role of zoonotic transmission (10). In support of Barbut's findings, ribotype 027 demonstrated clustering in certain countries, only being detected in six out of 34 nations. This may be a result of improved management and infection control practices in response to major outbreaks of previous years. Potential outbreaks of other ribotypes were reported by Barbut *et al.*, with ribotype 017 observed in up to 80% of cases from Poland and Ireland (394). Equally, ribotype 001 was detected in 73% of CDIs across three hospitals in Madrid.

More recent, large scale pan-European surveillance reported that PCR ribotypes 027, 001, 078 and 014 were prominent, comprising of over one third of all infections (118, 188). Ribotype 027 prevalence remained stable between 2011-14, constituting between 11.8% and 12.6% of all CDI cases. Nonetheless, these instances were highly country dependant, and were particularly abundant in Denmark, Hungary, Italy and Poland. This reinforced the findings of the earlier, European, multicentre, prospective, biannual, point-prevalence study of CDI in hospitalised patients with diarrhoea (EUCLID) study, which reported similar prevalence rates, with the noticeable lesser frequency of ribotype 078 (154). In the EUCLID study, 19% of infections were attributed to ribotype 027, although, again these were isolated primarily in four countries, with isolates from Germany, Hungary, Poland and Romania comprising 88% of total cases. Considerably more ribotypes were reported in these recent studies than those of Barbut and Bauer *et al.*, with 144 and 138 reported in the *Clos*ER and EUCLID studies respectively (118, 154). The *Clos*ER study also revealed the presence of ribotypes 198 and 356 emerging in Italy and Hungary, respectively; potentially due to further diversification of CDI strains (118). Nonetheless, as highlighted by Davies *et al.,* only 32% of hospitals involved in the EUCLID study utilised optimal diagnostic methods (154). It was conservatively projected that this could lead to more than 40,000 missed instances of CDI per year, representing a vast potential impact on CDI rates. Through the sequencing of the EUCLID isolates, Eyre *et al.* suggested that there were two distinct patterns of *C. difficile* dissemination across Europe, one health care transmitted (027 and 001) and one diverse lineage, widely spread group (002, 014, 020) (396).

1.6.4 *C. difficile* epidemiology in North America and the rest of the world

Historical CDI outbreaks in the USA, were attributed to a PCR ribotype 001, clindamycin resistant “J-strain”, which was implicated in multiple cases across four hospitals, between 1989 and 1992 (97). CDI rates rapidly increased at the turn of the century, with the emergence of the hyper-virulent ribotype 027, implicated in several outbreaks across Canada and North America (34, 112, 397). The first 027 reports from the multi-institution, case-controlled study by Loo *et al.* revealed 22.5 CDI cases per 1,000 admissions (34), whilst rates in the USA more than doubled between 1997 and 2001, due to of clonal expansion of this epidemic strain (92, 398). A review of Canadian cases by Pépin *et al.* indicated a four-fold increase in CDI incidence between 1991 and 2003, with a ten-fold increase observed in the population of over 65 year olds (397). In support of these findings, a multi-centre study comprising eight American institutions revealed the presence of the BI/NAP1/027 type in 51% of CDI cases (112). The success of these isolates was attributed, in part to a resistance to modern fluoroquinolones (78, 99) and postulated the superior toxin producing ability of this ribotype (283).

Adjusted estimates of CDI cases in ten geographical distinct regions of the USA indicated 453,000 instances and approximately 29,000 deaths in 2011 (53). Increasing community cases (53), in addition to reports of disease onset in previously low risk populations, such as paediatrics (399) and peri-partum women (400) all contributed to this large burden of disease. A recent, nationwide database review including ten years of CDI case data from the USA (2005-2014), revealed an annual increase in infections of 3.3%, with a larger proportional increase in community acquired disease (401). Although rates continued increasing, mortality rates decreased from 9.7% in 2005 to 6.8% in 2014; (p<0.0001). This may be attributable to improved awareness of antimicrobial restriction and infection control requirements, as observed by Evans *et al.* (402). The aforementioned study of CDI in veteran affairs long-term care facilities in the USA demonstrated a 36% decrease in CDI following the introduction of a CDI prevention initiative in 2014 (402).

The clonal expansion of the epidemic ribotype 027 has reached beyond Europe and North America. Although this ribotype is not as prevalent in the rest of the world, cases have been reported across Asia (403-405) and into Australia (406). Nonetheless, in a collection of 474 Australian isolates typed by Knight *et al.,* 37 different ribotypes were detected, but none were ribotype 027 (407). Historically, high levels of CDI in Western Australia were attributed to widespread use of third generation cephalosporins in the 1980s (408). Rates decreased as the usage of this high risk class of antimicrobials lessened, with 2-3 cases per 1,000 discharges reported in 1993-98, compared to 0.87 in 1999. A five year study of Japanese cases revealed an epidemiological shift from a predominance of ribotype *a* in 2000 (45%), to domination by ribotype *f/smz* in 2004 (409). Meta-analysis of 51 studies of Asian *C. difficile* infections revealed similar instances to Europe and the USA, 5.3 cases per 10,000 patient days (410). CDI was highest in East Asia (19.5%), with the Middle Eastern and South Asian regions demonstrating rates of 11.1% and 10.9%, respectively. There is a paucity of epidemiological data from Latin America, with data from Argentina suggesting an increase in CDI from 37 cases per 10,000 admissions in 2000, to 84 in 2005 (411), and one review suggesting a 4% mortality rate, lower than more developed countries (412). However, caution must be taken as these results may be attributed to sub-optimal diagnostics implemented in some of these regions.

Where global epidemiology often reflects divergent patterns of strain prevalence, comparisons are made more challenging due to the lack of consensus on typing methods (413). Geographic isolation, diverse infection control measures and varied prescribing regulations can lead to differing strain dominance (186, 198, 201, 407, 414-416).

1.6.5 PCR ribotype 027

As previously mentioned, hyper-virulent ribotype 027 emerged as an epidemic strain in North America in 2004 and has been implicated in multiple international outbreaks since first reported (34, 112). The discovery of the 027 strain correlated with a 400% increase in CDI cases in Canada alone, with 82% of Canadian cases attributed to this hyper-virulent strain. Ribotype 027 emerged as a cause of CDI outbreaks in the UK shortly thereafter (383), followed by a dramatic increase in *C. difficile* associated deaths (417). This important ribotype has also been linked to increased rates of recurrent disease (418, 419), although other risk factors such as increasing age and antibiotic use may be stronger predictors of recurrence. (420).

The success of this particular strain has been linked to superior toxin production (283), although not all evidence supports this finding (421). Initially assumed to be caused by an 18bp deletion in a putative toxin regulator gene, *tcdC*, (112, 283), Matamouros *et al.* demonstrated no detriment to TcdC harbouring the 18bp deletion (422). A single base pair deletion at the 117 position of the *tcdC* gene, resulting in truncation of the negative regulator protein, has also been implicated in the elevated production of TcdA and TcdB (422, 423). However, there may be other factors involved in hyper-virulence, as Curry *et al.* observed other distantly related, non-hyper-virulent *C. difficile* strains exhibiting the same 1bp *tcdC* deletion (423).

Although generally described as hyper-virulent, there are reports demonstrating no correlation between 027 isolates and superior toxin levels or disease severity (424-426), whilst not all ribotype 027 strains have demonstrated equivalent behaviour (424, 427). A study of Swedish ribotype 027 isolates discovered several genotypically distinct strains containing the wild-type *tcdC* gene, revealing a 13-fold reduction in *in vitro* toxin production (424). Sporulation assays have demonstrated the increased rate of spore production in hyper-virulent vs non-hyper-virulent strains, with considerable intra-ribotype variation observed amongst 027 isolates (421, 427). Akerlund *et al.* demonstrated sporulation rates ranging between 25% and 45% of *C. difficile* cells after 48 hours of *in vitro* culture (427). Conversely, Burns *et al.* observed no significant association between increased sporulation and ribotype 027, reporting wide variation within the population (428). These phenotypic differences could be factors in differing disease severity and recurrence potential.

Other potential explanations for the success of ribotype 027 have been proposed. Stabler *et al.* reported instances of novel *tcdB* variants, suggesting putative N-terminal domain alterations were impacting the binding potential of the toxin molecule (429). Merrigan and colleagues suggested that excess toxin production may be attributed to RNA polymerase sigma factor, *tcdR*, and polymorphisms in the ribosomal binding site, leading to potential transcriptional read-through of *tcdA* and *tcdB* (421).

Robinson *et al.*, used *in vivo* murine modelling to observe the ability of ribotype 027 strains to outcompete non-027 isolates in the gut (430), suggesting this competitive advantage as a potential contributor to its success. Recent work using stochastic simulations of infection models tested several healthy, colonised and diseased states for the probability of an invading *C. difficile* strain to proliferate amongst established intestinal microbiota (431). Here they observed a propensity for more infectious/virulent strains to supersede other resident microbes.

Nonetheless, increased resistance to antibiotics, particularly fluoroquinolones remains strongly linked to the success of this ribotype, which has become synonymous with a *gyrA*, Thr82>Ile mutation (34, 99, 112). Resistance to these widely used, broad spectrum antimicrobials can result in *C. difficile* survival in severely diminished gut microbial environments and a high risk of disease onset. Nevertheless, recent increased awareness and infection control governance, along with the implementation of national surveillance systems has led to a reduction in ribotype 027 prevalence in countries such as the UK (188, 363).

1.7 Antimicrobial resistance in *C. difficile*

In order to fully comprehend the effects of both direct and ancillary antibiotic exposures on the evolution of *C. difficile* strains, the mechanisms of action and resistance must be fully understood. Whilst resistance to CDI treatment drugs is minimal, the development of reduced susceptibilities to other antimicrobial classes is of high importance to the onset of disease.

1.7.1 Quinolones

1.7.1.1 Mechanism of action

Quinolones are a class of synthetic, broad spectrum antibiotics that have been widely used for over 50 years. Their extensive spectrum of activity allows for an abundance of clinical applications in the treatment of skin and soft tissue, respiratory, sexually transmitted and urinary tract infections (432). Fluoroquinolones are modern generation quinolone derivatives containing an additional fluorine atom at position C-6. This modification provides increased efficacy of DNA gyrase sub-unit A binding, interfering with the DNA cutting and resealing process (433).

The mechanism of action employed by quinolones involves the inhibition of two essential bacterial DNA enzymes, DNA gyrase and topoisomerase IV. By targeting specific regions of both these enzymes, antibiotic-enzyme complexes form and binding elicits conformational changes, enzymatic inactivation and eventual DNA replication inhibition (434). The impact on these key elements of the DNA replication process enables quinolones to effect bactericidal properties (435).

DNA gyrase and topoisomerase IV are the two type II topoisomerase enzymes involved in the essential processes of bacterial DNA replication. The DNA gyrase molecule consists of two sub-units, A and B (436). Hydrolysed adenosine triphosphate (ATP) binds to the molecule, effecting a conformational change, which allows the gyrase enzyme to begin a process of negative supercoiling. The enzyme binds to positively supercoiled bacterial DNA enabling a transient break of the double-strand (a process carried out by sub-unit A) and a negative supercoiling to allow replication (associated with sub-unit B) (437).

Topoisomerase IV is the essential affiliated enzyme to DNA gyrase for DNA replication in most bacteria. Similar in structure to gyrase, it contains 2 ParC and 2 ParE sub-units. Its primary function is to allow the separation of interlinked daughter chromosomes in the final stages of DNA replication. A secondary function is to enable the positive supercoil to relax in preparation for gyrase enzymes to carry out negative superhelical twisting of the DNA. An absence of topoisomerase IV genes has been outlined in the *C. difficile* genome and therefore eliminates it as a quinolone target in this organism (438).

1.7.1.2 Mechanism of resistance

Resistance to the quinolone class of antimicrobials is commonly mediated by mutations in the targeted, DNA gyrase genes (*gyrA* & *gyrB*). Located within the quinolone resistance determining region (QRDR) (439), mutations of the *gyrA* coding region reduce the binding affinity of the transcribed gyrase molecule to quinolone molecules, whilst chromosomal changes in the *gyrB* region often exacerbate binding affinity reductions (440). If the quinolone molecule cannot bind to its target effectively, its efficacy will reduce and the organism will benefit from reductions in susceptibility.

Mutational hotspots (regions of high mutational frequency (441)) have been implicated in resistance to fluoroquinolones. Cambau and Gutmann highlighted the clustering of substitutions around the Ser-83 codon of the *gyrA* gene in *E.coli* as a predisposition for quinolone resistance (442). Due to phylogenetic closeness, mutations in this region can also be associated with quinolone resistance in *C. difficile* isolates.

Ackermann and colleagues identified moxifloxacin resistance in 19 out of 72 (26%) clinical *C. difficile* isolates, with 14 harbouring single point mutations in codon 82 (equivalent to *E. coli* numbering 83) (443). Single point mutations were demonstrated in 13 of these isolates, ACT (Thr) to ATT (Ile). The remaining resistant isolate displayed two base changes, GTT, leading to the expression of valine. All susceptible strains exhibited the same, wild type sequence, reinforcing the significance of these mutational substitutions. Data from Spigaglia *et al*. supported this finding; by determining 73 of 82 multidrug resistant isolates exhibited the same single point mutation in the *gyrA* gene, in conjunction with the moxifloxacin resistant phenotype (444). Nonetheless, Spigaglia's study indicated quinolone resistance in five isolates without modification in this region of the genome. This sustains the comprehension that quinolone resistance can be multifaceted with additional mechanisms, such as mutations in *gyrB* genes or an increase in drug efflux being influential.

Mutations in the *gyrB* gene have also been implicated in quinolone resistant *C. difficile* strains, with the critical importance of the Asp-426 codon reported in *E. coli* and *S. aureus* (432). Drudy *et al.* demonstrated a comparable mutation repeatedly occurring in *gyrB* across all fluoroquinolone resistant *C. difficile* isolates in one study (445). This group hypothesised that the relationship of mutations in codon 426, from aspartic acid to valine, and fluoroquinolone resistance is potentially due to a physical hindrance of the binding process. As valine is a branched chain amino acid, this additional bulk in the binding pocket region could partially inhibit the antimicrobial molecule forming the necessary complex with the organism. Dridi and colleagues identified mutations in the same Asp-426 codon in five isolates with moxifloxacin minimum inhibitory concentrations (MICs) of 8-16 mg/L (438). However, unlike in previous studies, these isolates reflected a mutation into an asparagine amino acid, as opposed to valine. This may indicate the influence of charge on the binding complex, as Asp426>Asn reflects a replacement of a negatively charged amino acid with an uncharged residue. The same alteration in charge would apply to the valine mutation in Drudy's work.

Barrett *et al.* suggested that quinolones bind to a pocket created by a complex of the QRDR of gyrase sub-unit A and a similarly influential region of sub-unit B (446). One proposed model of this binding pocket implicated Asp-426 and Lys-447 of *gyrB* as key regions, demonstrating interactions with both the phosphate backbone of DNA and the C-7 group of ciprofloxacin (447). This proposal suggested that the antibiotic molecule acts as an intercalating agent, inhibiting the enzyme. Therefore, any mutations at the *gyrB* encoding region leading to structural changes, may affect the solidity of the complex and the activity of the quinolone molecule. Although this provides a detailed insight into the ciprofloxacin-gyrase interface, there is a requirement for this proposed mechanism of resistance to be further validated. By identifying interactions between an array of fluoroquinolone compounds, greater confidence in these defined complexes can be established.

1.7.2 Nitroimidazoles

1.7.2.1 Mechanism of action

Metronidazole is a low molecular weight compound which diffuses across the cell membrane and imparts bactericidal effects via intracellular reduction within anaerobic organisms (448). Reduction occurs through interactions with the nitro-group of metronidazole and Pyruvate-Ferredoxin oxidoreductase (PFOR) creating toxic derivatives that covalently bond to DNA. This disrupts the helix, inhibiting DNA synthesis and instigating cell death (449).

1.7.2.2 Mechanism of resistance

Since sensitivity to metronidazole is dependent on PFOR activity, primary resistance mechanisms have been identified as involving the alteration of enzyme efficiency and reductase pathways. In Bacteroides spp. the involvement of *nim* genes has been identified in the reduction of the nitro-group of nitroimidazoles and the creation of an inefficiently active amine group (450), leading to the conversion of nitroimidazoles into non-toxic derivatives (448). Gal & Brazier discovered 24% carriage of *nim* genes amongst Bacteroides spp., with 11.6% displaying resistance above therapeutic levels (>16 mg/L) (451). However, they did also indicate that seven isolates not carrying the *nim* genes were resistant to 5 mg/L, suggesting alternative mechanisms, such as decreased uptake or increased efflux.

Nitroimidazole resistance is multifaceted, and since analysis of metronidazole resistance in *C. difficile* presented no evidence of *nim* genes (452, 453), other mechanisms must be considered. In *C. difficile,* Lynch *et al.* identified mutations in the *nifJ, fur* and *rsbW* genes, encoding for part of the PFOR pathway, regulation of ferric uptake and an anti-sigma factor, respectively (454). These are involved in bacterial stress reactions, potentially reducing oxidative stress and nitroimidazole activation (455). Disruption of electron transport pathways (456), over expression of efflux pumps (457) and the DNA repair protein, RecA (458), have all been implicated in reduced susceptibility to metronidazole in other organisms, although these are yet to be reported in *C. difficile*.

A recent series of work from Wu & Hurdle *et al.* suggested a strong correlation between stable metronidazole resistance and presence of heme in *C. difficile* cultures (459). They demonstrated resistance instability by reducing metronidazole MICs from ≥8 mg/L to between 1-2 mg/L, via simple passage (460). The addition of heme maintained the resistant phenotype *in vitro*. Not only this, but the group were able to demonstrate the association, both through a reduction in MIC with the exclusion of hemin from growth media and the dramatic increase in MIC in its presence. Ribotype 027 strains were specifically influenced by hemin inclusion in culture media, with 4-10-fold increases in metronidazole MIC observed (459). The group went on to test hemin metabolite action on metronidazole MIC, indicating no correlation between resistance and the presence of hemin breakdown products (Biliverdin, Protoporphyrin IX, Bilirubin and FE3+), only evidencing a correlation with the complete hemin molecule. One hypothesis is that hemin may be acting as a co-factor for an unknown enzyme. Further work is required in this area to identify the potential involvement of iron regulatory genes, *nifJ*, *feoB* and *Iscr* (460). Emerging research from Smits *et al.* has implemented *in silico* analyses to find a correlation between the presence of a pCD630 plasmid and a metronidazole resistant phenotype (461).

1.7.3 Glycopeptides

1.7.3.1 Mechanism of action

As a primary treatment option for CDI, vancomycin is the key glycopeptide of interest when considering resistance in *C. difficile*. Its mechanism of action involves interfering with the process of cell wall biosynthesis via the binding of this bulky molecule to D-Ala-D-Ala peptidoglycan precursors, physically blocking the transpeptidation process (462). Without this cross-linked formation the nascent cell’s rigidity is absent and it cannot survive intra cellular pressures.

1.7.3.2 Mechanism of resistance

The high specificity of the glycopeptide class of antimicrobials allows for a targeted toxicity, whilst the physical inhibition of cell wall synthesis ensures that resistance acquisition is difficult. Vancomycin does not directly target the cell wall biosynthesis enzymes, its efficacy comes from effecting the substrate specificity of peptidoglycan precursors synthesising enzymes (463). Therefore, resistance to this class is determined by gene clusters working to simultaneously synthesise modified precursors and eliminate pre-existing high-affinity peptides (464). A VanH dehydrogenase enzyme, usually located on a Tn*1546* transposon, reduces pyruvate to D-Lac, while VanA ligates this to D-Ala to generate the modified precursor (464). Different configurations of *van* genes have been identified among other genera (465-467), where similar mechanisms generate variant precursors containing D-Ser or D-Ala components. Simultaneous activity of a D-D-dipeptidase encoded by *vanXY* genes results in the removal of usual precursors, subsequent uptake of modified peptides and an intermediate resistant phenotype.

In *C. difficile* a *vanG*-like cluster was identified in the 630 reference genome, containing five open reading frames, *vanR, vanS, vanG, vanXY* and *vanT* (314). However, no resistant phenotype was observed. Later Amman *et al.* demonstrated the functionality of this operon, identifying the presence of modified precursors (468). Whilst it is unclear why these do not lead to phenotypic expression, the lack of regulatory genes may be contributory.

1.7.4 Rifamycins

1.7.4.1 Mechanism of action

The rifamycins class originates from the fermentation product of *Amycolatopsis mediterranei*, rifamycin B (469). Rifampicin and rifaximin are semi-synthetic derivatives of this natural product with increased antimicrobial activity. The mechanism of action they employ involves inhibition of RNA synthesis through allosteric hindrance of polymerase activity. Physical binding to the β sub-unit of RNA polymerase blocks phosphodiester bonding early in the elongation process of the RNA back-bone, effectively inhibiting the synthesis of essential bacterial proteins (470-473).

1.7.4.2 Mechanism of resistance

As with fluoroquinolones, rifamycin resistance generally arises from mutational events reducing the binding affinity of the antimicrobial agent and target molecule, RNA polymerase (471). Several studies have reported over 20 SNP variations in the 350bp *rpoB* gene of *C. difficile*, in direct correlation with rifamycin resistance (444, 474-477). Studies by Huhulescu *et al.*, Curry *et al.* and Pecavar *et al.* all identified Arg505>Lys as the predominant sequence variant, in 74%, 48% and 46% of resistant strains, respectively (475-477). Combinations of multiple SNPs in the *rpoB* gene were also reported by these groups, with the majority located within a "hot spot" region of *rpoB* (477). The mechanism was further delineated through the use of X-ray crystallography, which identified the proximity of key RpoB amino acids and the rifamycin binding pocket (473).

Since the presence or absence of an *rpoB* mutation directly affect susceptibility phenotype, the distribution of susceptibilities to this class of antibiotics is generally reported as bimodal (478, 479). Whilst geographic bias impacts resistance epidemiology, due to over-use of rifamycins in certain countries (480), often epidemic ribotypes reveal rifampicin resistance (474, 475).

C. difficile research identifies a strong correlation between resistance to rifampicin and rifaximin, once considered for its potential as an alternative CDI treatment option (474, 476). One *in vivo* study reported the rapid development of rifaximin resistant *C. difficile* in a patient exposed to rifampicin (481). Furthermore, a rifampicin resistant, ribotype 046 clone has also been implicated in an outbreak amongst tuberculosis patients in Poland (482). Interestingly, seven out of eight patients exposed to a rifamycin in one North American study harboured resistant strains (475).

1.7.5 Tetracyclines

1.7.5.1 Mechanism of action

Antimicrobials of the tetracycline class function via protein synthesis inhibition. By binding to a single, high affinity site of the 30S ribosomal subunit, they interfere with tRNA complex formation (483).

1.7.5.2 Mechanism of resistance

The mechanism of resistance to tetracyclines revolves around ribosomal protection. Resistant determinants were first discovered in Streptococci spp. (now *E. faecalis*), indicating that *tetM* genes express a soluble protein, which protects the ribosome, preventing translation interference and protein synthesis inhibition (484). The majority of tetracycline resistance determinants have been demonstrated to reside on mobile elements (485).

Tetracycline resistance in *C. difficile* is usually mediated by *tetM* genes found on conjugative transposons (486-488). The most prevalent of these mobilisable elements is Tn*916* (487, 488). Nonetheless, reports of *tetM* positive isolates that are susceptible to tetracycline suggest a more complex relationship between genes and phenotype (201, 489). Interestingly, Spigaglia *et al.* identified confluent resistance in strains containing the Tn*5397*-like determinant, whilst those harbouring Tn*916* exhibited a spectrum of susceptibilities. Wang *et al* provided the first reported instance of a clinical *C. difficile* strain containing the Tn*916* insertion (490). The discovery of this transposon in *S. aureus*, Enterococcu*s* spp. and Streptoccocus spp. suggests that transfer is highly likely to occur between a range of organisms, widening the potential sources for clostridial acquisition (491).

The use of cryo-electron microscopy to study the detailed interactions of tetracyclines and TetM, ribosomal protection proteins indicated potential interactions between the C-terminal of the TetM protein and 70S ribosomal subunit (492). Further interactions with the 16S rRNA binding site, leading to conformational changes and eventual drug release from the active site were observed. Contemporary research furthered this work by imaging the complex during translation (493), reporting no alteration of nucleotide C1054, as previously proposed (492).

1.7.6 Resistance to other antimicrobial classes

Resistance to chloramphenicols in *C. difficile* is often mediated by a *catD* gene, encoding for a chloramphenicol acetyltransferase (494). Carried on a Tn*4435a* or Tn*4451* transposon, this enzyme adds an acetyl group to chloramphenicol molecules, effectively inhibiting its complex formation with the ribosome (495). This gene has been found in combination with other mobile genetic elements, such as *ermB* and *tetM*, and is typically lineage associated (496).

β-Lactam compounds such as agents in the cephalosporin class, act upon the penicillin binding proteins during cell wall synthesis. Resistance to this action is associated with the production of β-Lactamases, which hydrolyse the β-Lactam ring and degrade the antibiotic (497). Several putative β-Lactamase genes have been identified in *C. difficile*, but at present no functional activity has been determined (498).

1.7.7 Antimicrobial susceptibility testing methodologies

In vitro susceptibility testing provides a valuable prediction of bacterial response to antimicrobial compounds in clinical settings. MIC data can be used as an indicator of novel agent efficacy or as essential surveillance to track the intrinsic levels of susceptibility of an organism. Although the Clinical and Laboratory Standards Institute (CLSI) recommend the agar dilution technique for anaerobes (499), there are multiple methodologies in use across clinical and research settings. Whilst considered as the "gold standard" method, agar dilution is laborious and time consuming, making it unsuitable for routine analysis of clinical specimens (500). Nonetheless, comparisons of more rapid methods, such as Etest and disk diffusion, have identified discrepancies between MIC findings. In support of previous findings (501), a recent comparative study demonstrated a negative bias for MIC data generated using a broth microdilution method, as opposed to agar dilution (500), also observing poor reproducibility of the former technique. Conversely, Igawa *et al.* demonstrated concordance between the methods, when testing a panel of Japanese isolates (502). This serves to highlight the ambiguity of method accuracies. Whilst discrepant disk diffusion and Etest results have incited debate as to where the resistant breakpoints should lie, due to the subjective nature of zone boundaries (503), research has demonstrated their inferiority to agar dilution methods (187, 504). Despite the fact that comparisons largely favour the guideline method, there remains a debate as to which is the optimal agar for *C. difficile* testing. Baines *et al.* suggested that metronidazole MICs may be affected by the testing method

(187), whilst others have supported the notion of differing media constitution affecting growth density and the generation of variable results (503, 505). Use of Wilkins Chalgren agar has been demonstrated to display greater reproducibility over Brucella agar (187), and has been used successfully in a major European surveillance study (188).

1.7.8 Multidrug resistance

1.7.8.1 Epidemiology

A consortium, comprising of experts from both the European Centre for Disease Prevention and Control (ECDC) and the Center for Disease Control and Prevention (CDC) outlined standardised definitions of multidrug resistance (506). In these guidelines the development and acquisition of resistance to three or more antimicrobial classes was defined as multidrug resistance (MDR). Resistant organisms were further delineated as extensively drug-resistant (XDR), where resistance is demonstrated to all but two antimicrobial classes and pan drug-resistant (PDR), where non-susceptibility to all of the agents tested is observed. (506).

Large scale surveillance studies have begun to highlight the extent of the problem in *C. difficile* (188, 444). The *Clostridium difficile* European Resistance (*Clos*ER) study indicated the predominant MDR strains as PCR ribotype 001, 017, 012 and 027, in agreement with Spigaglia's research demonstrating the former three types as 39%, 18% & 12% of all MDR isolates, respectively. However, no ribotype 027 isolates were reported in the latter work, possibly due to a small collection period in this prospective study. The *Clos*ER study also highlighted regional prevalence, with high levels of MDR linked to the related ribotype 018 and emergent ribotype 356 strains in Italy. Further work by Freeman and colleagues expanded the antibiotic test panel of the *Clos*ER study, indicating that ribotypes previously linked to MDR (including ribotype 027) also displayed resistance to linezolid and/or ceftriaxone (225). Adding additional concern, these already MDR ribotypes demonstrated the highest MICs for fidaxomicin and a novel treatment compound (*SMT19969*). This strengthens the suggestion of the presence in specific ribotypes of determinants that are able to influence resistance to multiple antimicrobial classes.

Where Spigaglia's surveillance reported almost 50% of 316 European clinical *C. difficile* isolates as resistant to at least one antibiotic, 54 isolates displayed resistance to two antimicrobials, whilst 82 were resistant to three or more (444). Of these, 39 (12% of total) showed resistance to four different classes, lincosamides, macrolides, fluoroquinolones and rifamycins. A recent review of 12 studies encapsulating 370 MDR isolates, indicated class prevalence in these strains, with combined clindamycin, erythromycin and fluoroquinolone resistance comprising almost 30% of all MDR (507).

Recent large scale, retrospective surveillance across >7,000 inpatients indicated *C. difficile* as the most frequently reported MDR pathogen (1.66%) (508). As the principal aetiological agent of antibiotic-associated diarrhoea, development of MDR in *C. difficile* has important implications with regards to the instigation of infection. However, resistance to multiple classes of antimicrobials implicates complex mutational and transposable mechanisms, many of which remain unidentified.

1.7.8.2 Mechanisms

The factors involved in expressing resistance are multifaceted (Figure 3), with numerous elements often working in synergy. Conformational changes in a drug's target site, along with antibiotic structure altering enzymes, often work in combination with mutable influx and efflux determinants (509). Although not fully understood, several of these multiple resistance mechanisms have been described in *C. difficile*.

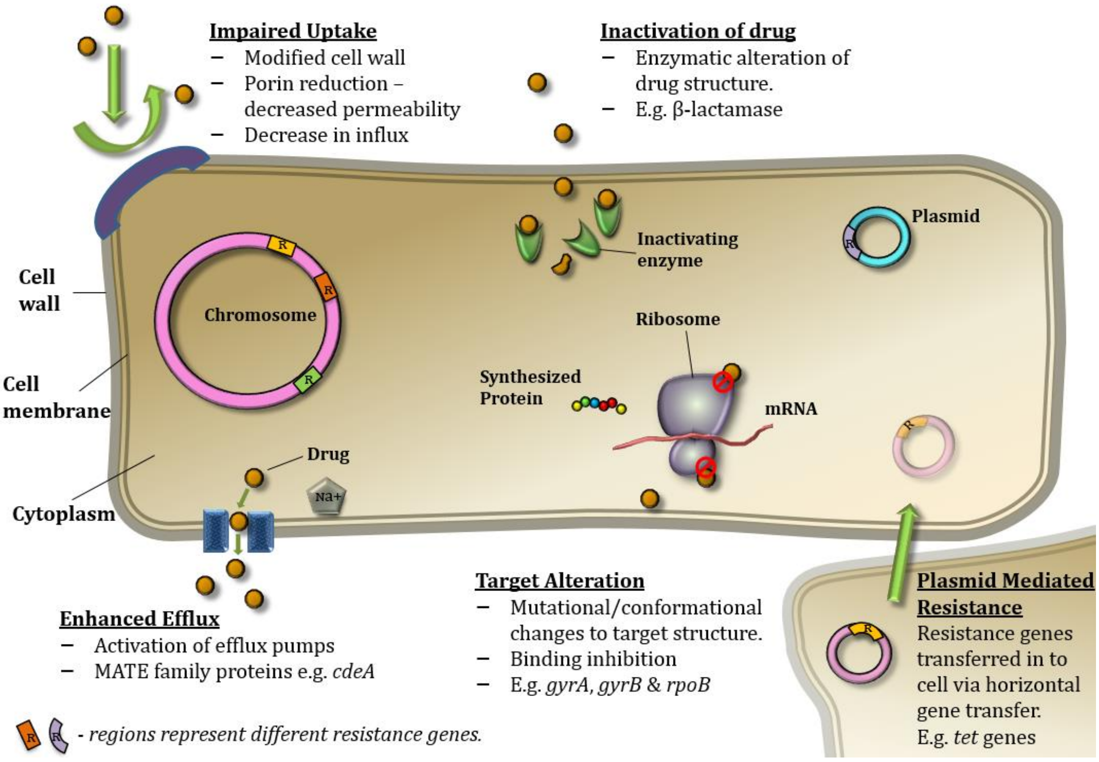


Figure 3: Antibiotic resistance mechanisms overview. *Created by J. Vernon*

1.7.8.2.1 Erythromycin resistance methylase (*erm*) genes

The primary determinants implicated in the cross-resistance of the macrolide-lincosamide-streptogramin B (MLS_B) classes of antibiotics are the erythromycin resistance methylase genes; *erm* (444, 489, 510). Whilst most Erm determinants are genus specific, *ermB* is widespread throughout bacterial genera and are reported extensively in *C. difficile* populations (511, 512). Ribosomal RNA methyltransferases encoded by *erm* genes, methylate the adenine residue at position 2058 of the 23S rRNA molecule, part of the 50S ribosomal unit (513-515). This effectively blocks antibiotic binding complexes forming and allows DNA synthesis to proceed as normal. Since the active sites for MLS_B antimicrobials overlap, resistance can develop simultaneously for multiple agents (514).

Surveillance studies have reported *ermB* frequencies of 28% and 19% in European and American *C. difficile* isolates, respectively (444, 510). With all isolates demonstrating the cross-resistant phenotype to erythromycin and clindamycin (444). The widespread distribution of these resistance determining elements suggests that genetic transfer is rife amongst *C. difficile* populations. Early work identified that *C. difficile* carried *ermB* genes on a conjugative-like transposon, Tn*5398*, demonstrating the capability for bi-directional genetic transfer, but lacking the excision and integration genes necessary for independent conjugation (516, 517). Nonetheless, Wasels and colleagues later successfully implemented conjugation assays with these mobilisable elements, demonstrating the potential of genetic transfer in the absence of plasmid DNA (518). Furthermore, one recently identified arrangement of *ermB* determinant, Tn*6215*, has been demonstrated as bacteriophage mediated (519). Interestingly, transconjugants have been demonstrated to suffer from a fitness cost associated with the insertion of *ermB* carrying transposons (518). Those strains harbouring the transduced DNA exhibited significant growth deficiencies, although it cannot be discounted that this was due to insertional site disruption.

Seventeen genetic variants of the *ermB* arrangement have been reported to date, termed E1 – E17 (444, 489, 517). The most prevalent *ermB* variant is reportedly transferred on the Tn*6194* conjugative transposon (444, 520). Interestingly, the MLS$_B$ resistant, CD630 genome contains two copies of the *ermB* gene on a Tn*5398* transposon, a novel arrangement proposed to be the product of homologous recombination (517). Whilst no direct correlation between genetic arrangement and PCR ribotype have been identified (444), the E2 arrangement demonstrated resistance to a lesser extent (489).

Although ErmB elements appear to drive the majority of MLS$_B$ resistance in *C. difficile*, repeated findings of erythromycin and clindamycin resistant strains in the absence of *erm* elements are apparent (444, 521-523). These underline the potential contribution of other mechanisms in the expression of this resistance phenotype, such as efflux and mutation of the 23S rDNA target (521, 524).

1.7.8.2.2 Chloramphenicol-florfenicol resistance gene

Resistance to the phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (PhLOPS$_A$) group of antimicrobials is associated with the presence of a chloramphenicol-florfenicol resistance gene, *cfr* (525, 526). First observed in *Staphylococcus scuiri* (525), this MDR determinant expresses a methyltransferase, leading to the methylation of the A2503 residue of the 23S rRNA sub unit. Since the target of these antimicrobial classes overlap, a simple methylation interferes with drug binding and allows synthesis to proceed (527). Homologues of this plasmid-mediated determinant have since been reported in

C. difficile, with direct associations with a concurrent linezolid and chloramphenicol resistant phenotype (528-530).

Whilst oxazolidinone insusceptibility is rare in *C. difficile*, resistant strains have been reported (225, 444, 531). Marin's study of Spanish strains revealed seven of the nine linezolid, clindamycin and chloramphenicol resistant strains harboured a *cfr*-like gene (528), with findings reinforced by the absence of a *cfr* gene in ten susceptible strains. This gene indicated 89% sequence identity with *cfr* elements from *Bacillus amyloliquefaciens*, suggesting a similar epigenomic modification in clostridia. Equally, the absence of point mutations/deletions in the 23S rRNA sub unit, indicated that, although linezolid resistance is often caused by this, additional mechanisms, such as *cfr* genes must be involved.

Later designated as Tn*6218*, due to its mobilisable characteristics, *cfr* demonstrated a concrete relationship with the PhLOPS_A resistant phenotype through its expression in *E. coli* and insertion/deletion in a *C. difficile* genome (529, 532). Confirmation of the *cfr* homologue's expressed function was indicated through significantly elevated MICs and the indication of both methylation at A2503 and reduction at the Cm2498 stop codon.

Contemporary research has indicated the presence of a novel *cfr*-like gene, *cfr(C)*, and a direct correlation with PhLOPS_A resistance (530). Whilst ten percent of *C. difficile* isolates and seven percent of genomes analysed *in silico* harboured this gene, it is primarily found in other commensal gut bacteria (533). Ultimately, these plasmid mediated *cfr* genes demonstrate high potential for dissemination and the expansion of problematic MDR strains (534).

1.7.8.2.3 Efflux pumps

Efflux pumps are active transporters, requiring chemical energy to transport toxic compounds including antibiotics across the cell membrane and out of the cell. These transmembrane pumps are grouped into two main categories, primary ATP-binding cassettes (ABC) and secondary multidrug transporters. The former rely on the hydrolysis of ATP to provide energy for transport, whilst the latter generate a difference in electrochemical potential by pumping ions in and out of the cell (535). The expelled molecule can then travel along the electrochemical gradient, either out (uniporters/symporters) or in and out simultaneously (antiporters) (536, 537).

Efflux pumps are a major factor associated with MDR in prokaryotic cells (537). Where some transporters mediate the extrusion of a specific class of drug, others are able to efflux multiple, structurally unrelated compounds. Therefore, these multidrug transporters are of major interest in the identification and treatment of MDR infection.

It is not only the presence of efflux encoding genes that must be considered. Mutations in these sequences have been demonstrated to reduce antimicrobial susceptibility in other organisms (538, 539), whilst adaptation to environmental stimuli, such as pH and iron availability have been reported to affect expression regulation (540, 541).

1.7.8.2.3.1 Major facilitator superfamily (MFS)

The major facilitator superfamily group consists of several subgroups, including the multi antimicrobial extrusion protein superfamily (MATE), small multidrug resistance family (SMR) and resistance nodulation cell division superfamily (RND) (536). To date, there is no evidence of the latter two subgroups in *C. difficile* reported in the literature.

All MFS efflux pumps consist of membrane proteins involved in uniport, symport and antiport. These efflux processes are divided into two main categories, those with 12 transmembrane segments (TMS) and those with 14. Within the 12-TMS cluster, the *S. aureus* protein NorA has been implicated in resistance to hydrophilic compounds, such as antimicrobials from the fluoroquinolone and methicillin classes (542, 543). Further work by Neyfakh *et al.* suggested that NorA mediates resistance to a range of structurally diverse drugs in *S. aureus* with structural homologues identified in other species, including *B. subtilis* (544, 545).

Lebel *et al.* observed five ORFs in *C. difficile* with homology to NorA determinants, transforming the *cme* gene into *E. faecalis* to demonstrate a link between its expression and erythromycin resistance (546). Equally, the use of reserpine, an efflux pump inhibitor, facilitated enhanced resistance to ethidium bromide and safranin O in *C. difficile* (547). In support of this work, the presence of the *cme* gene in one draft *C. difficile* genome was putatively identified as the cause of erythromycin resistance (548).

1.7.8.2.3.2 Multi antimicrobial extrusion protein superfamily

The first multidrug transporter determinant reported in *C. difficile* was *cdeA* *(509)*. Dridi *et al.* determined that this gene encoded a protein with homology to known proteins from the MATE family of efflux pumps present in other organisms. Through PCR detection methods, this work highlighted that *cdeA* is present in the majority of *C. difficile* strains *(509)*.

Research has indicated that MATE family proteins are Na^+ coupled efflux pumps, dependent on the presence of sodium for transportation (549, 550). Dridi's group confirmed this with CdeA, by observing weak efflux activity in the absence of Na^+ and an eight-fold increase in its presence (509). Although *cdeA* harbouring strains demonstrated high levels of efflux for ethidium bromide, the extrusion of ciprofloxacin and norfloxacin remained low. However, by overexpressing the protein with the *Plac* promotor driving the transcription, resistance to ciprofloxacin and norfloxacin increased. Therefore, mutations in the *cdeA* regulatory gene have the potential to markedly increase resistance to fluoroquinolones.

Further mechanisms have been determined in other organisms. The *norM* gene of *Vibrio parahaemolyticus* has been identified, along with its homologue in *E.coli* (*ydhE*), as encoding an energy dependent efflux system (551). These mediate resistance to hydrophilic fluoroquinolones and aminoglycosides. Nonetheless, although NorM has 12 transmembrane segments, which would generally classify it as part of the major facilitator superfamily, no other similarities in sequence to this family were determined.

1.7.8.2.3.3 Multiple antibiotic resistance (*mar*) gene

Although not clearly defined in *C. difficile*, the *mar* regulon, consisting of transcriptional regulators, influences resistance to a plethora of compositionally diverse compounds, including antimicrobials, organic solvents and disinfectants (552). Associations have been determined between the *mar* locus in *E.coli* and decreased susceptibility across a range of antibiotic classes including;

chloramphenicol, cephalosporins, rifamycins, tetracyclines, fluoroquinolones and penicillins (553).

The sequence of this multiple antibiotic resistance locus has revealed the involvement of a series of fundamental regulatory genes in both *E. coli* and *Salmonella typhimunium* (554). Martin & Rosner first described the involvement of MarR, a repressor that binds to the operator, MarO (555). This down regulates expression of the repressor itself and MarA, a regulator in efflux mechanisms and outer membrane proteins. Therefore, mutations in the repressor gene, *marR*, result in inactivation and subsequent expression of *marA*. The multiple resistance phenotype occurs due to *marA* up-regulation of a series of genes involved in the activation of the AcrAB efflux pump, coupled with the down regulation of outer membrane protein F (556, 557).

Cohen *et al.* indicated a propensity for *E. coli* strains containing mutations in the *mar* loci to have a lower susceptibility to fluoroquinolones (558). By identifying a 4-8-fold decrease in fluoroquinolone susceptibility in *mar* mutants, when compared to an OmpF only mutant, they highlighted the involvement of other resistance mechanisms. Underlining the impact of non-gyrase or topoisomerase gene mutations on fluoroquinolone resistance, Kerr and colleagues described up to a ten-fold decrease in fluoroquinolone susceptibility in strains containing both *gyrA* and *mar* mutations in *E. coli* (559). This highlights the potential for the *mar* gene to impact heavily on multiple antimicrobial resistance, compounding existing prevalent mechanisms.

There is a paucity of data available relating to *mar* genes in *C. difficile,* but the presence of MarR encoding genes have been identified in the 630 genome (560). Although the *C. difficile marR* gene has reported low sequence similarities with *E. coli* and *S. aureus marR* genes, investigation of its crystal structure identified highly similar dimer structures (560). One recent investigation into fidaxomicin resistance in the organism, through *in vitro* induction by serial passage, identified a mutation in a *marR* homologue (213). Discovered in a laboratory mutant displaying a 64-fold increase in fidaxomicin MIC, the homologue exhibited a deletion in CD22120 resulting in a frame shift after amino acid 117 of the *mar* loci. This significant finding implies the presence of non-RNA polymerase based mutations that appear to directly affect *C. difficile* resistance phenotype. Nevertheless, no direct evidence of resistance to other antimicrobial classes was reported to correlate with the mutation at CD22120.

1.7.8.2.3.4 ATP-binding cassette superfamily (ABC)

There are currently no reports of ABC transporters present in *C. difficile*, but work by Harnvoravongchai and colleagues suggested that expression of *E. coli* primary transporter genes in *C. difficile* reduced susceptibility to several compounds (535).

1.8 *C. difficile* evolution

1.8.1 Evolutionary analysis methodologies

Epidemiological distributions and *C. difficile* strain transmission are routinely investigated using several well defined genotyping methods; PFGE, REA and PCR ribotyping (360). Where further strain differentiation is required, more advanced genomic techniques, such as MLVA prove valuable (371, 561). However, since the rapid progression of NGS technologies, high throughput apparatus has brought affordability and widespread accessibility to near complete genomic sequences. Subsequently, analysis of species phylogeny has enabled a greater understanding of bacterial evolutionary lineages (562-564).

1.8.2 Phylogeny

Comparative analysis of the *C. difficile* genome has demonstrated the existence of at least eight phylogenetic clades (2, 375, 564, 565). Clade 1 represents a highly heterogeneous collection of clinically relevant strains; clade 2 includes the hyper-virulent PCR ribotype 027 as well as closely related ribotypes 176, 198 and 244 (566), clade 3 includes RT023, whilst clade 4 comprises the atypical toxin B only type, RT017 (564). Reported by Griffiths and colleagues (375), the fifth clade includes the genetically distinct, binary toxin producing, RT078, which has been linked to zoonotic transmission (567). The final clades, C-I, C-II and C-III consist of rare, environmental, often non-toxigenic strains of even greater genetic distinction than clade 5, potentially considered as novel subspecies of *C. difficile* (565, 568).

Evolution occurs through mutational or transposable alterations to a genome that do not significantly disrupt the core survival processes of the mutant. Since mutation rates for the *C. difficile* genome have been determined to lie between 0.74 and 1.4 SNPs per genome, per year (257, 569), it is apparent that major genomic evolution is likely to be driven by transfer of large coding sequences.

A study of 75 representative strains covering clades 1-4 demonstrated that only 19.7% of genes were homologous throughout all *C. difficile* isolates, comprising those responsible for essential cell processes, such as metabolism and replication (564). Since around 11% of the comprehensively annotated 630 genome consists of mobile genetic elements, indicative of longitudinal interactions with gut microbiota (314), it can be concluded that the *C. difficile* genome is highly adaptable to change. With a genome over 40% larger than closely related clostridia (up to 4.3kb) and variability amongst core coding sequences (570), it is highly suited to adaptation and species survival (571, 572). In support of this notion, the *C. difficile* pan-genome has been conservatively estimated to contain 9,640 coding DNA sequences (573), suggesting ultra-low levels of conservation.

Such diversity in the *C. difficile* gene pool suggests an abundance of horizontal gene transfer, affecting evolutionary change. Whilst investigating the evolutionary dynamics of *C. difficile*, He and colleagues identified large coding regions distinctly unrelated to *C. difficile* origins, as well as determining that the majority of SNPs were limited to distinct areas of the genome. These findings potentially signified large fragments of homologous recombination, up to 300kb (563). In assessing the relative ratio of recombination and mutation, they determined a moderately high ratio, supporting the concept of homologous recombination asserting a substantial effect on genome expansion. Analysis of the PaLoc of 1,693 *C. difficile* isolates strongly supported this hypothesis, highlighting 26 independent evolutionary events of acquisition or loss of full loci and the involvement of homologous recombination in this species' evolution (565). The actions of bacteriophages are likely to contribute to extensive horizontal transfer and drive evolutionary change in *C. difficile* (568).

1.8.3 PaLoc evolution

Investigation of the PaLoc indicated high conservation of this operon, essential to disease potential (565). Similar phylogeny of this region across PCR ribotypes indicated evolution post-clade divergence, strongly suggesting the involvement of homologous recombination between clades. Similarly, evidence of related *tcdA* and *tcdB* genes in other clostridia suggests the strong probability of the contribution of inter-species transfer on the *C. difficile* pangenome (574).

1.8.4 Molecular clock

The determination of a theoretical rate of evolution is essential to the phylogenetic analyses of any organism. Approximations of the number of mutational events per genome, per year allow lineages to be approximately dated and transmission events to be linked through microevolutionary analyses. Based on Bayesian phylogenetics, for *C. difficile* the number of SNPs has been estimated at between 0.74 and 1.4 per genome, per year (257, 562, 569, 575). This figure correlates strongly with other bacterial species (576).

A key factor potentially affecting the accuracy of *C. difficile* molecular clock calculations is the time spent in the quiescent spore form (569). This state of evolutionary suspension is difficult to assess, potentially impacting the mutation rate, leading to a substantial underestimation. Nonetheless, the rate applied in any given investigation must be carefully considered, as several factors could impact on the analysis. Short term estimates of the molecular clock, calculated through the sequencing of serial samples, have demonstrated rates elevated above historical approximations (563, 576, 577). Whilst short-term rates are useful for microevolutionary analysis of transmission, extrapolations of these estimates may lead to inaccurate long-term rates. Also, the emergence of recombination events must be considered, so not to vastly overestimate the SNP rates (569). Software algorithms are available to include variable elements, such as these (578).

1.8.5 PCR ribotype 027 evolution

The evolution of PCR ribotype 027 is of acute interest to enable a greater understanding of the important genetic alterations that have led to the emergence of hyper-virulence. Phylogenetic analysis has suggested that this ribotype experienced a population expansion period around the turn of the century, with evidence of horizontal gene transfer across multiple points of phylogeny (563). This was demonstrated through evidence identifying complementary SNPs between isolates with large evolutionary distances. Comparisons of whole genome sequences between modern, epidemic and "historic", non-epidemic 027 strains have indicated five large genomic regions of difference, suggesting recent acquisitions in evolutionary terms (579). However, no genetic differences were identified in the PaLoc between 027 isolates from pre or post outbreak eruption of 2003 (562). This lends greater weight to the argument that excess fluoroquinolone use and subsequent resistance in this ribotype was the major influential factor driving its emergence (99, 417). He's study utilised maximum-likelihood models and Bayesian statistics to strongly indicate the presence of two main lineages for PCR ribotype 027, both acquiring Thr82>Ile mutations through separate evolutionary events, leading to fluoroquinolone resistance. Offering the nomenclature of FQR1 and FQR2, He and colleagues discovered that FQR1 originated in North-East USA, whereas FQR2 was more widespread across Canada and North America, and was the source of international dissemination of the original outbreak. Similar patterns of lineage divergence have recently been determined in the toxin B only ribotype, 017 (580).

Additionally, transcriptomic analysis of different strains in a murine model of infection has highlighted the differential expression of *C. difficile* genes (581). The well characterised 027 strain, R20291 demonstrated upregulation of different genes to CD630 (PCR ribotype 012), including many proteins of unknown function, which may have involvement in the success of this strain. This finding correlates with the previous discovery of several point mutations upstream of coding sequences in ribotype 027 isolates (564). All of these findings further highlight the evolutionary divergence of ribotype 027 and its direct impact on gene expression.

1.8.6 *C. difficile* mutation

1.8.6.1 Spontaneous mutation

Mutations are spontaneously occurring, permanent modifications in the nucleotide sequence of DNA. Any mutational event has the potential to alter the translational output of amino acid chains and result in divergent phenotypes. This capacity for the generation of genetic variation is crucial for bacterial populations to survive changeable environments and exogenous stresses. As conventional DNA replication occurs, errors will arise in the base pairings created by DNA polymerase enzymes. Internal DNA repair systems ordinarily correct these mistakes, but not with 100% efficiency (582). Spontaneous mutagenesis is understood to be a product of leakage through the error repair pathways resulting in these errors being permanently incorporated into the bacterial genome. (583)

The fundamentals of evolution dictate that under stable conditions, bacterial genomes will spontaneously mutate at a given rate, which is generally accepted to lie between 10^{-9} and 10^{-10} mutations per genome, per generation (584). The natural variation in mutagenesis rate is dependent on a multiplicity of factors, including organism and environment (585). In the *in vivo* experiments by Giraud *et al.*, population dynamics within a murine model were shown to affect the mutation frequency (585). When the environment remained stable and bacterial populations thrived, mutation frequencies were low. In contrast, the presence of exogenous stressors or environmental pressures elevated mutant generation rates. Since spontaneous genomic mutations are considered to be stochastic, with no bias toward advantageous changes (586), increased mutagenesis in stable populations has the potential to disadvantage the population through increased risk of introducing deleterious effects. Conversely, where populations are under threat, an increased mutation rate presents the opportunity to generate beneficial adaptations and survive the “selective bottleneck” (585, 587, 588).

One key external stressor implicated in increased formation of bacterial mutations that has been well reported in the literature, is exposure to antimicrobial agents (589-591).

1.8.6.2 Mechanisms (DNA SOS, *mut* genes, mismatch repair)

Bacterial SOS response was first outlined over 40 years ago, with further elucidations of precise mechanisms following (592). LexA and RecA have long since been recognised as the key proteins involved in bacterial SOS response, protecting the stability of the genome (593). LexA is a transcriptional regulator, which binds to DNA sequences near gene promotor regions, obstructing RNA polymerase and the transcription of SOS response genes. RecA acts as the inducer protein, cleaving LexA and enabling cell survival responses, such as increased mutagenesis. These genes are ubiquitous in the bacterial kingdom, with homologues identified across most species (594). Whilst studies have reported mechanisms in other clostridia (595, 596), minimal evidence has been reported in *C. difficile*. Walter and colleagues have undertaken comprehensive analyses of this mechanism, identifying its role in a host of crucial pathways, including sporulation, biofilm formation and sensitivity to antibiotics (597, 598). They discovered amino acid substitutions amongst a collection of strains, but none were associated with the active site. Interestingly, they determined that LexA disassociation from *recA* genes was twenty times slower in *C. difficile* than that of *E. coli* equivalents. This indication of late expression of key SOS genes was deemed suggestive of *C. difficile* potentially prioritising upregulation of other stress response genes, located on the same regulon, such as those involved with transporters and sporulation (598).

The bacterial methyl-directed mismatch repair (MMR) system is also understudied in *C. difficile*, but *mutS*/*mutL* operon knockouts have demonstrated an increased mutability in other clostridial strains (599). One *C. difficile* focussed study discovered high conservation of the MMR genes across genomes (573), whilst Eyre and colleagues discovered comparable data (600). Nonetheless, any evidence of genomic differences in these loci may lead to reduced stability of the genome and high rates of mutation, as observed in other genera (582, 589, 601).

The *hfq* gene is pleiotropic in nature, demonstrating effects on multiple core processes, including sporulation, growth rate and stress response (602). Its involvement in the unification of small RNAs and mRNA targets has demonstrated impact on expression of a multitude of areas. Hfq deficiency in other organisms has provided evidence of differing stress responses and therefore may have involvement in mutation propensity (603).

1.8.6.3 Antimicrobial mutagenesis

The principal mechanisms of antibiotic resistance are the presence of endogenous mutations in active target sites, drug uptake/efflux systems and exogenous horizontal gene transfer of resistant determinants (604, 605). Since hypermutable strains demonstrate inefficiencies in DNA repair systems and increased capacities for inter-species gene transfer (588, 606), the potential for these phenotypes to promote MDR is vast. Consequently, the study of these hypermutable strains can be extremely valuable in researching worst case scenarios for resistance acquisition.

Ground-breaking work by Mao *et al.* demonstrated the potential of mutagenesis associated with a single selection stage, with mutator proportions elevated from 0.001% to 0.5% (607). Miller *et al.* demonstrated large increases in mutant *E. coli* generation with independent exposure to both rifampicin and ciprofloxacin (608). Since development of resistance to both of these compounds require only a single point-mutation in either *rpoB* or *gyrA* respectively; mutant phenotypes are likely to occur. Nonetheless, they also indicated that resistance to antimicrobials requiring mutations in multiple target regions (cefotaxime & D-cycloserine) were more prevalent in mutator strains than normomutators. Although resistance to fluoroquinolones can be demonstrated with single mutations, multiple DNA gyrase mutations have been linked to further reductions in susceptibilities to this class of antibiotic.

Studies have shown that increased mutation frequencies of fluoroquinolone resistance in other genera are associated with pressure from compounds such as salicylate (609). However, research has indicated that, as well as being strain dependent, mutational frequencies vary within the class of quinolone agents (610, 611). Also, the exposure concentration appears to impact the generation of mutants, with polarised antibiotic concentrations both demonstrating elevated levels of mutation in different organisms (591, 612).

Stress-induced mutagenesis occurs when damaged DNA is identified by a signalling molecule, which initiates a cascade of reactions leading to the de-repression of DNA SOS system (613). In the case of fluoroquinolones, single stranded DNA acts as the stimulatory element, and is identified by the RecBCD enzyme, which activates RecA resulting in LexA cleavage (614).

The capability of any antimicrobial compound to select resistant, mutant progeny is a consideration in its clinical value and dosing practices. Studies have demonstrated that selection pressure from exposure to increased fluoroquinolone concentrations can lead to increased resistant populations. This correlation suggests a link between higher mutation frequencies and exposure (615).

Determination of a compound-dependant, mutation prevention concentration potentially minimises resistance development and onward transmission, as well as aiding prescribing guidelines. However, since an agent such as moxifloxacin is not generally used as a treatment option for CDI, a flaw in the mutation prevention contingency may be exposed. Effective treatment of an unrelated bacterial infection could potentially lead to the propagation of resistant *C. difficile* populations.

1.8.6.4 Mutation rate vs mutation frequency

Mutation frequency and mutation rate have distinctly different definitions, since the measureable phenomena they refer to differ in both concept and determination. Where mutation rate denotes an estimated probability of the number of nucleotide changes over a designated time period, frequency refers to acquisition or loss of a specific, quantifiable phenotype, such as resistance to a class of antibiotics (589). Since the majority of mutations are likely to occur in non-coding DNA regions or result in no expressible differences, the mutation rate is not always directly relevant to an evolutionary advantage. Determination of an actual frequency of phenotypic expression transformation may be more useful when considering clinical impact. Consequently, mutation frequencies determined for a designated antibiotic class must only be considered as relevant to that specific phenotype. Ultimately, since heterogeneous quinolone susceptibilities have been associated with combinations of mutational events in *gyrA* and *gyrB* regions (616), before even considering other QRDR-independent mechanisms, such as enhanced efflux systems, mutation rates are often of lesser relevance than mutation frequency.

As all progeny of a mutant cell will carry the same phenotype (disregarding reverse mutations at the same nucleotide) standard mutation frequency calculations will be wholly dependent on the generation at which the mutation occurred. “Mutational jackpots” can arise if a phenotype altering mutation transpires in an early generation replication, resulting in an almost entirely homogeneous bacterial population reflecting the mutant type (617). In order to suppress this potential effect, methodologies have routinely adopted the use of multiple biological replicates to generate an average frequency (618, 619).

1.8.6.5 Mutator strains

Bacterial strains demonstrating the capacity to mutate at an elevated rate are referred to as “mutator” strains and have classically been utilised to aid the understanding of DNA error-repair systems (583). Defects in these pathways are often reported in mutator strains, resulting in increased mutagenesis (582, 583, 606). The MMR system involves the identification of an erroneous nucleotide addition, cleavage and correction by DNA polymerase. If any of the *mutS*, *mutL* or *mutH* genes involved in the repair process exhibit reduced functionality, then elevated mutability (100-1000-fold increase) can been observed (583, 606, 608). Although the majority of research into DNA repair systems reflects the *E. coli* genome, studies have identified defective homologues in other genera producing increases in mutation rate (620-623). Natural mutator populations amongst *E. coli* and *Salmonella* isolates have been reported as up to 1% (622, 624), whilst *Pseudomonas aeruginosa* and *S. aureus* proportions are reported as high as 20% in the persistent environment of the lungs of cystic fibrosis patients (623, 625).

Baquero *et al.* proposed further delineation of terminology when categorising strains of *E.coli* populations based on their mutation frequencies (582). Frequencies in proximity to the modal distribution point (8×10^{-9}–4×10^{-8}) were defined as “normomutable”, with strains with lower and higher mutation frequencies termed “hypomutators” and “hypermutators”, respectively.

Mutators have been demonstrated to confer an early advantage under new stress environments (585). However, they are generally accepted to become a hindrance when external pressures abate (587, 588). Mutators can lead to decreased bacterial fitness through impairment of growth rates, additional temperature sensitivities and reduced motility (587). As indicated previously, a stable genome is beneficial to a stable population. Constant genetic alteration is proposed to maintain the rarity of mutator populations, due to the inevitable high proportion of deleterious mutations impacting on strain fitness. This is known as Muller's Ratchet (626). Therefore, it has been suggested that it is efficacious for elevated mutation frequencies to be transient in nature and that natural populations may lose the mutator phenotype, due to mutational reversion or recombination of functioning DNA repair genes (588). SOS repair has been implicated as the mechanism involved in transient switching of mutator status (627). Transience may negate the deleterious potential of constant mutation, enabling survival and stability.

Research in *E. coli* suggests an inversely proportional correlation between rifampicin resistance and population density, demonstrating reductions in density causing a three-fold increase in mutation rate (628). This work has highlighted links between the quorum-sensing gene, *luxS*, and the transitory nature of mutation frequencies.

There is a paucity of research into hypermutability of *C. difficile* strains. One large study interrogated the genomes of 184 PCR ribotype 027 isolates of both clinical and *in vitro* origin documented no evidence of deviations in MMR homologues, previously described in other organisms (600). Interestingly, in the absence of any classical mutator gene homologues, *S. pneumoniae* has demonstrated 3.4-fold increases in *rpoB* mutations after ciprofloxacin exposure, suggesting the involvement of other unknown mechanisms (618).

1.8.7 Antibiotic resistance and *Clostridioides difficile* fitness

The impact of antimicrobial resistance conferring mutations has been extensively studied in other organisms, with a particular focus on fluoroquinolone substitutions (629-636). However, there is a paucity of data investigating their influence in *C. difficile*.

Recent work by Kuehne *et al.* demonstrated the fitness cost of mutations imparting fidaxomicin resistance in *C. difficile* (637). By introducing substitutions in the Val-1143 codon of the *rpoB* gene by allelic exchange, this group observed deficiencies in virulence and competitive growth rates compared to isogenic parent strains. This burden upon bacterial fitness could contribute to the reasons behind fidaxomicin resistance scarcity in the clinic. Interestingly, rifamycin resistance conveying substitutions in other regions of the same gene did not impose any burden on fitness in the majority of cases (638).

Wasels *et al.* have reported the detrimental effects of the uptake of transferable elements, with all isolates receiving a transposon containing the *ermB* gene demonstrating deterioration of growth rates (518). However, crucially, further work by the same group reported a lack of fitness cost associated with gyrase alterations conveying fluoroquinolone resistance (639). The most common chromosomal substitution, Thr82>Ile, demonstrated no impairment to fitness, although amino acid replacement of the same codon with valine did indicate a significant disadvantage. Although this study focussed on several resistance imparting variants, all originated from only one ribotype 012 strain. Therefore, more work is necessary to further elucidate the intricacies of the relationship between fitness and resistance in *C. difficile* in other key ribotypes, such as 027.

Chapter 2 Optimising *Clostridioides difficile* Germination and Recovery Methodologies – The Inhibitory Effect of Glycine

2.1 Introduction

Spores are fundamental to *C. difficile* transmission, but cannot produce the toxins necessary for disease. Therefore, germination and subsequent vegetative outgrowth are essential to disease aetiology. Consequently, knowledge of germination pathways and signalling molecules is important to the optimal recovery of this bacteria. *C. difficile* spore germination mechanisms are gradually being elucidated (323, 324, 640), but remain less well defined than that of the model organism, *B. subtilis.* In *B. subtilis ger* genes act as nutrient germinant receptors initiating the germination cascade (343, 641). Since the discovery of an absent tricistronic *ger* receptor operon from the CD630 genome (314), an alternative germination mechanism was sought for *C. difficile*.

Bile acid components have been strongly implicated in the *C. difficile* germination process, of which taurocholate is the most effective germinant (244, 315, 316). Notable work by Sorg and Sonenshein revealed a significant increase in spore germination in the presence of 0.1% sodium taurocholate, with a 10^5-fold increase observed in broth culture (244). Optimal taurocholate concentrations were reported between 0.1-1%, whilst prolonged exposure to low levels demonstrated greater germination efficacy than short exposures at higher concentrations. They also identified the involvement of glycine, as a germination co-factor. By isolating individual nutrient components from the culture media, they were able to demonstrate the germination of *C. difficile* spores in a glycine buffer with taurocholate, but not in its absence (244). Further work into the influence of physical and chemical factors on *C. difficile* germination, supported this glycine association (331). Other amino acids have also been implicated as germination co-factors, with L-alanine, L-histidine and L-serine reported amongst compounds increasing the efficiency of *C. difficile* germination (244, 327, 642). One study reported the importance of histidine as a co-germinant, observing consistent spore germination and recovery of between 97.9 and 99.9% (642).

Nonetheless, germination interactions with bile acid components are more complex. The primary bile acid chenodeoxycholate has been demonstrated to inhibit *C. difficile* germination, potentially through direct competition with taurocholate for receptor sites (244, 318). Interestingly, primary bile salt analogues have demonstrated efficacy as CDI prevention treatments in murine models (643). Furthermore, Buffie *et al.* revealed the concept of a bile acid-mediated CDI resistance, highlighting the presence of *C. scindens* as potentially protective due to its bile acid hydrolysing ability (320).

The most recently proposed model involves bile acid analogues stimulating a spore coat-bound pseudoprotease, CspC. This interaction initiates an enzymatic cascade comprising a protease, CspB (322), in the activation of a spore lysis enzyme, SleC. This reportedly occurs when a small prodomain is cleaved, allowing subsequent disruption of the spore cortex. Dipicolinic acid in the spore core can then be released in an exchange with water. This process of hydration enables the reactivation of spore metabolism and results in vegetative outgrowth (323-325). Further additions to the model implicate Ca^{2+} in the activation of CspB (329). Kochan *et al.* reported an absence of germination during *in vitro* assays deficient in Ca^{2+}, highlighting its involvement in the germination mechanism. Co-germinant involvement remains unclear, however, interactions between Ca^{2+}, glycine and CspB suggest a co-factor relationship (329).

Recently, additional characterisation has implicated further genes in *C. difficile* germination, with Fimlaid *et al.* reporting evidence of the involvement of a GerS lipoprotein regulator (307). By observing defective germination and cortex lysis in *gerS* knockout strains they indicated the requirement for GerS in the activation of SleC. Furthermore, the *gerG* gene has been implicated in the incorporation of CspA, CspB and CspC into the spore, and thus, the efficacy of germination (644). Using strains containing *gerG* deletions Donnelly *et al.* highlighted the influence of this gene, observing a detrimental effect to both germination efficiency and response to germination triggers.

Recovery of aged spores presents further challenges to *C. difficile* germination investigations. The concept of super dormancy describes bacterial spores with attenuated capacities for germination under normal conditions, and has been reported in several species (645-648). Nonetheless, it can only be considered as a relative notion, where germination environment plays an important role. Observations of small proportions of spore populations demonstrating superdormancy may represent a risk reducing, non-committal strategy, in case the exogenous environment is not tolerable. Spore aging has been related to superdormancy in *C. difficile*, potentially associated with a range of other factors linked to longitudinal storage (647). This concept must be considered as a potential complicating factor in the attempted recovery of *C. difficile* spores from historical catalogues.

Optimisation of culture media is essential to the reliable germination and recovery of *C. difficile*, whether for diagnostic or research purposes. Many comparisons of culture media, agar and broths, have resulted in a general consensus for taurocholate supplementation to increase germination efficiency (332, 649-653). Cycloserine-cefoxitin fructose agar (CCFA), cycloserine-cefoxitin egg yolk agar with lysozyme (CCEYL) and *C. difficile* ChromID agars (bioMérieux, France) are amongst those widely favoured (332, 342, 654-657). Although chromogenic ChromID agar has demonstrated superior sensitivity and recovery rates (657-659), deficient detection of ribotype 023 colonies due to an inability to hydrolyse esculin indicates a major pitfall with this novel agar (660, 661). A further reason for the reluctance to adopt ChromID agar may be because of the high cost of these colorimetric plates. The CDRN utilises CCEYL for national surveillance, since the addition of lysozyme has been demonstrated to increase the recovery rates of environmental samples (342, 363). Enrichment broths, particularly those supplemented with germinants have demonstrated beneficial increases in *C. difficile* germination, although a range of broth bases are often used (649, 650, 653).

While previous studies have indicated the germinant actions of taurocholate and co-germinant, glycine (244, 315, 316), inconsistent reports of optimal germination concentrations have been proposed (244, 315, 338). A continued search for optimised culture approaches is necessary to further define highly sensitive and robust culture methods to promote optimal diagnostic testing.

2.2 Rationale

By assessing the *C. difficile* germination potential of two solid agar and two anaerobic broth bases, supplemented with a range of known germinant compounds at varying concentrations, a model algorithm for spore germination and recovery was sought. This was acknowledged as critical to the maximal recovery of *C. difficile* spores from a historical collection of isolates (Chapter Three), essential for the further investigation of MDR development in this thesis. Without recovery of isolates through optimised germination protocols, downstream epidemiological and genomic analyses would not be achievable.

Whilst the synergistic effects of glycine, as a co-germinant to sodium taurocholate, have been demonstrated at low concentrations (331, 642), here elevated levels revealed inhibitory effects on *C. difficile* growth. Further examination of this finding was necessary to improve the understanding of this concept. This initial chapter attempts to define an optimal combination of solid and broth media, in order to maximise the germination and recovery of aged *C. difficile* spores, whilst concurrently investigating inhibition of germination or outgrowth by high concentrations of glycine.

2.3 Methodologies

Two solid agar bases and two broth culture media, supplemented with contrasting combinations and concentrations of germinant compounds, were assessed for *C. difficile* germination capabilities. Spore and vegetative population dynamics were evaluated through culture assays and phase-contrast microscopy, with an optimal algorithm sought to facilitate recovery of historical *C. difficile* spores.

All *C. difficile* incubations were carried out in an A95 anaerobic workstation (Don Whitley Scientific, UK) at 37°C for 48 hours, unless otherwise stated.

2.3.1 Pilot investigations

2.3.1.1 Germinant exposure pilot

Pre-reduced 5 mL Schaedlers anaerobic broths (Oxoid, UK) supplemented with 0.1% sodium taurocholate (MP Biochemicals, USA) and 0.4% glycine (Sigma-Aldrich, USA) were inoculated with PCR ribotype 027 *C. difficile* spores (2.5 $x10^7$); (spore preparation and harvesting is described in 2.3.2.2) to achieve a broth concentration of 5.2 $x10^5$ CFU/mL. Broths were incubated and *C. difficile* vegetative cells were enumerated after 30, 60, 90 & 120 minutes. Enumeration was performed through serial dilution (10^{-7}) in pre-reduced, sterile PBS (Oxoid, UK) prior to plating of the subsequent dilution series onto Brazier's agar (LabM, UK) supplemented with cycloserine (250 mg/L); (LabM, UK), cefoxitin (8 mg/L); (LabM, UK), 2% defibrinated horse blood (E&O Laboratories, UK) lysed with Saponin (50 mL/L); (Sigma-Aldrich, USA) and 5 mg/L lysozyme (Sigma-Aldrich, USA); (CCEYL). Aliquots of 200 μL were collected at each time point and "shocked" in ethanol/water (50% v/v) for spore population enumeration, as described previously. Germination differences were assessed based on the proportions of spores and vegetative cells. Testing was performed in biological triplicate.

2.3.1.2 Phase-contrast microscopy pilot

Pre-reduced 5 mL Brain Heart Infusion (BHI) broths (Oxoid, UK) supplemented with 0.1% sodium taurocholate and 0.4% glycine were inoculated with 50 μL of PCR ribotype 001 *C. difficile* spores (2.3 $x10^7$) to achieve an initial broth concentration of 2.3 x10^5. After 90 minutes incubation, broth aliquots of 125 µL, 250 µL, 500 µL & 1,000 µL were transferred into 1.5 mL Eppendorf tubes (in duplicate) and centrifuged at 12,000 g for one minute. The supernatant was decanted and the cell pellets re-suspended in 50 µL of M9 minimal salt media, before transferring onto a glass slide (26mm x 26mm). Slides were fixed on a heat plate at 50°C for 30 minutes, prior to the addition of 70 µL of molten Wilkins Chalgren anaerobe agar (Oxoid, UK) and a glass coverslip. Fixed slides were dried at 50°C for a further period of 30 minutes.

Outcomes were assessed based on an ability to clearly identify entities on a single plane of view, whilst ensuring sufficient, countable numbers (10-100) to allow accurate quantification. *C. difficile* spores appeared oval in shape with a thick, dark outer coat. Ca^{2+}DPA in dormant spores, presented an inner white glow or phase-bright spore core, whilst germinating, phase-dark spores appeared far darker. Vegetative cells appeared as dark, elongated rods; (Figure 4).

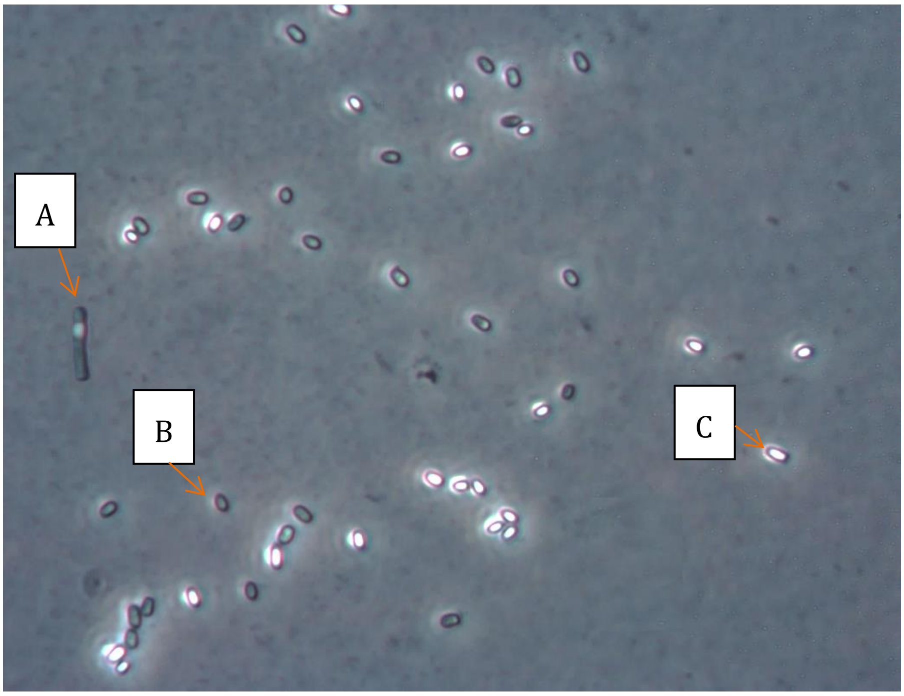


Figure 4: Phase-contrast microscopy image of alternative C. difficile forms. *A – vegetative cell (containing fore-spore), B – phase-dark spore, C - phase-bright spore. Image recorded using a Leica DM2000 phase-contrast microscope (100x Hi Plan objective) and an Optika™ Vision Pro Digital USB Camera.*

2.3.2 Optimising growth media for spore germination

The efficacy of ten solid agar and nutrient broth formulations were investigated for their potential germination capabilities on *C. difficile* spores. Total viable counts and spore counts, in addition to phase-contrast microscopy techniques were performed to facilitate the determination of germination response amongst different PCR ribotypes; (Figure 5).

2.3.2.1 Test isolates

Single isolates of *C. difficile* from five PCR ribotypes; 001, 015, 020, 027 and 078, were assessed for germination response to all test media. These were library strains assigned by the CDRN, Leeds, UK. Further data is available via the National Centre for Biotechnology Information BioProject database (NCBI), *http://www.ncbi.nlm.nih.gov/bioproject/248340*.

2.3.2.2 Spore preparation and harvesting

All strains were inoculated onto single CCEYL agar plates and cultured for 48 hours, before sub-culture onto a further eight CCEYL agars. Growth from each CCEYL plate was inoculated onto a further ten Columbia Blood Agar (CBA) plates (E&O Laboratories, UK) by spread plating technique and cultured, anaerobically for 10-14 days. After incubation, cultured plates were removed from the anaerobic cabinet, checked for purity and exposed to aerobic conditions for a minimum two hour period. Subsequent sporulated growth was removed from the plates with a dry swab and emulsified into ethanol/water (50% v/v), at a rate of 20 plates of growth per millilitre of ethanol (209, 662).

Spore preparations were enumerated before use, through a serial dilution plating method, standardised via ethanol dilution to a cell density of ~6 x 10^5 CFU/mL and stored at ambient temperature in sealed universal tubes.

2.3.2.3 Solid media comparisons

Laboratory produced agar plates of ten solid growth media combinations were compared for their spore recovery capabilities. BHI and cycloserine-cefoxitin egg yolk agar (CCEY) were used as base media, with each also supplemented with a series of germinant compounds; (Table 1). Egg yolk was omitted from CCEY agar to provide a direct comparison to the media used by the CDRN.

Standardised *C. difficile* spore preparations were serially diluted with sterile PBS in 96-well microtitre trays (10^{-7}). Twenty microlitres of each dilution was inoculated onto quartered agar plates and spread. Inoculated plates were incubated and individual colonies were counted.

Identifier	**Base Media**	**Supplements**
BHI	BHI + Agar Technical No.3 (15 g/L)	None
BHI(L)		Lysozyme (5 mg/L)
BHI+0.1%		0.1% taurocholate + 0.4% glycine
BHI+1%		1% taurocholate + 4% glycine
BHI+1% (0.8%)		1% taurocholate + 0.8% glycine
CCEY	CCEY (without egg yolk)	None
CCEYL		Lysozyme (5 mg/L)
CCEY+0.1%		0.1% taurocholate + 0.4% glycine
CCEY+1%		1% taurocholate + 4% glycine
CCEY+1% (0.8%)		1% taurocholate + 0.8% glycine

Table 1: Constituents of solid agar media used in C. difficile spore germination experiments. *Taurocholate and glycine added prior to autoclaving, lysozyme was added subsequently. BHI – brain heart infusion, CCEY – cycloserine-cefoxitin egg yolk.*

2.3.2.4 Broth media comparisons

Eight broth culture media combinations were compared for germination efficacy; (Figure 5). Schaedlers anaerobic broth and BHI broths were tested as base media, without the addition of Agar Technical No.3 (Oxoid, UK) to the either broth. A series of broths supplemented with differing germinant combinations were tested; (Table 2). Wassermann tubes containing 5 mL of pre-reduced broth were inoculated with 50 µL of fresh (<2 days old) *C. difficile* spore preparation and allowed to germinate for 90 minutes; as determined by a previous exposure experiment (2.3.1.2). Broths were serially diluted as previously (2.3.2.3) with pre-reduced PBS and 20 µL of each dilution spread on quartered CCEYL. Individual colony forming units were counted, post incubation.

After 90 minute germinant exposure, two 500 µL aliquots of each broth were transferred into 1.5 mL Eppendorf tubes; one for phase-contrast microscopy (see 2.3.2.5) and one shocked with 500 µL ethanol/water (50% v/v) for spore population determination. Ethanol shocks were mixed and left at ambient temperature for a minimum of one hour, prior to serial dilution to 10^{-4} and plating as previously (2.3.1.1).

All broths were tested in duplicate, with triplicate serial dilutions. Repeat testing of the PCR ribotype 001 and 078 spore preparations was performed after a six week period to identify any temporal differences.

Identifier	**Base Media**	**Supplements**
SCH	Schaedlers anaerobic broth	None
SCH(L)		Lysozyme (5 mg/L)
SCH+0.1%		0.1% taurocholate + 0.4% glycine
SCH+1%		1% taurocholate + 4% glycine
BHI	BHI	None
BHI(L)		Lysozyme (5 mg/L)
BHI+0.1%		0.1% taurocholate + 0.4% glycine
BHI+1%		1% taurocholate + 4% glycine

Table 2: Constituents of broth media used in C. difficile spore germination experiments. *Taurocholate and glycine added prior to autoclaving, lysozyme was added subsequently**.** BHI – brain heart infusion, SCH – Schaedlers anaerobic broth.*

2.3.2.5 Phase-contrast microscopy

As part of the broth media comparison investigation, *C. difficile* spores were processed for phase-contrast microscopy after 0 and 90 minutes incubation. Cultured broth aliquots of 500 µL were centrifuged at 12,000 g for one minute, and slides were prepared as previously described (2.3.1.2). Ten fields of view were imaged for each slide, using a Leica DM2000 phase-contrast microscope (100x Hi Plan objective) and an Optika™ Vision Pro Digital USB Camera, Italy. Entities included in the counts were vegetative cells, phase-bright spores and phase-dark spores, with proportionate data evaluated; (Figure 5). All test were performed in triplicate.

2.3.2.6 Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics v.21.0.0.1. The spore recovery on solid agar data was compared using a one-way ANOVA with Tukey comparison, after logarithmic transformation. Individual PCR ribotype and broth germination data were compared using a non-parametric Kruskal-Wallis test, with Dunn's *post hoc* testing. *P* values <0.05 were classed as significant, whilst *p*<0.001 were defined as highly significant.

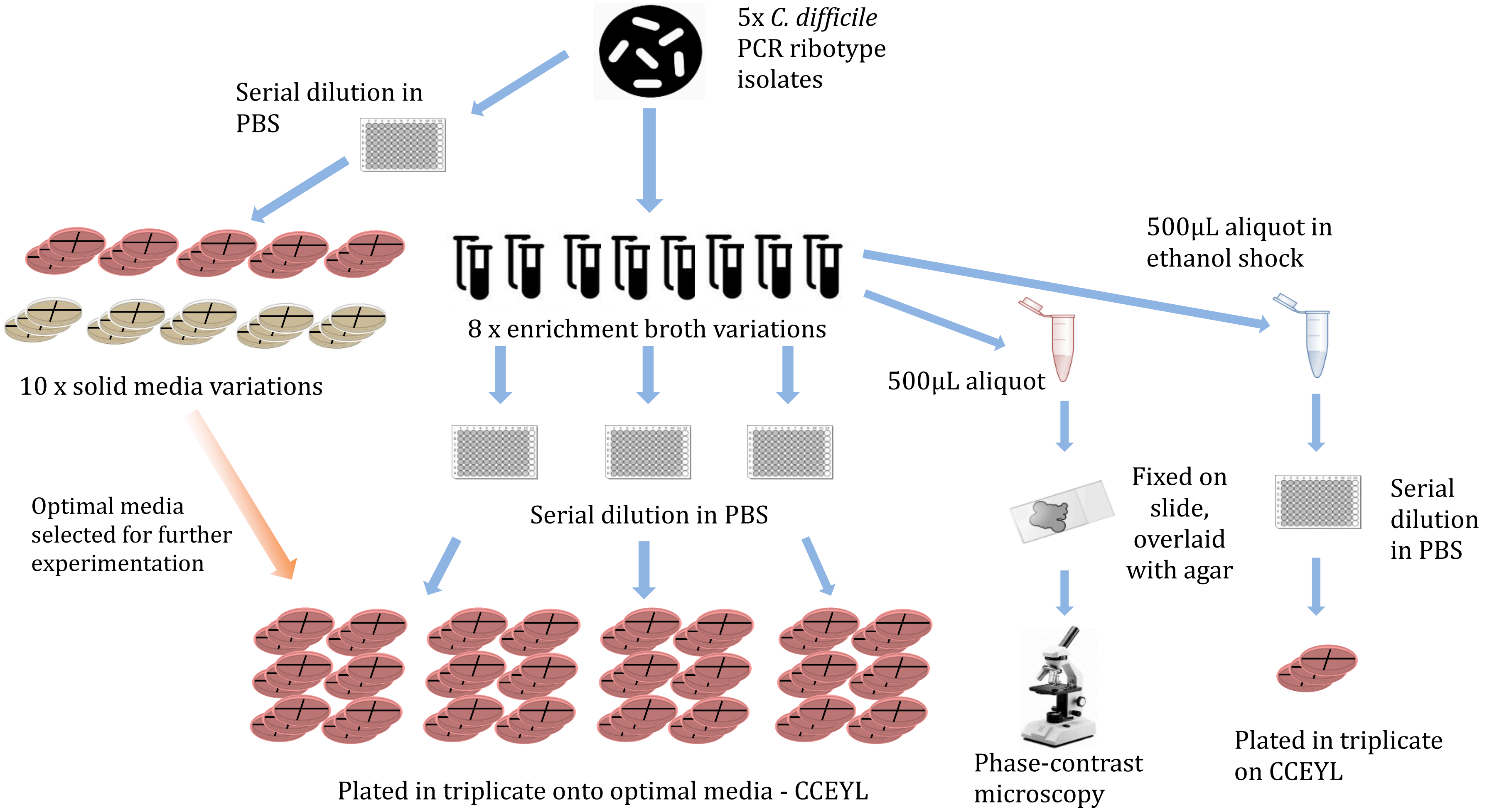


Figure 5: Flow diagram of germination investigation methodologies. *CCEYL – cycloserine-cefoxitin Brazier's agar supplemented with 5 mg/L lysozyme, PBS – phosphate-buffered saline.*

2.3.3 Susceptibility testing

2.3.3.1 Agar incorporation testing

A panel of the same five *C. difficile* isolates (ribotypes 001, 015, 020, 027 and 078) tested in the germination assays (2.3.2.1) was utilised to determine any antimicrobial effects of the supplementing germinant concentrations. All strains were tested for detrimental effects on both vegetative and spore forms of *C. difficile*. Glycine and sodium taurocholate were tested both independently and in combination using an agar incorporation minimum inhibitory concentration method, as previously described (187, 480). Briefly, concentration ranges were selected to correspond with broth supplementation (Table 3), with compounds dissolved and diluted in sterile water to achieve a doubling dilution series. Supplementary solutions of 5 g/L increments were tested to further delineate the MIC, where necessary. Two millilitres of each concentration of test compound solution was added to 18 mL of molten agar, mixed, set and dried at 37°C for 20 minutes. Vegetative cell response was investigated using isolates cultured on CBA for 48 hours, prior to inoculation into 4 mL Schaedlers anaerobic broth and further culture for 24 hours. Broth cultures were diluted 1 in 10 with pre-reduced saline (Oxoid, UK), before a 1 µL inoculation (~1×10^4 CFU) of the compound-incorporated agars using a multi-point inoculator (Denley Hydraulics, UK). Spore response was investigated using a standardised inoculum of each spore preparation (~1×10^7 CFU/mL) directly onto the agar series. Susceptibilities were tested on both Wilkins Chalgren and Brazier's CCEY agar, supplemented with 2% lysed, defibrinated horse blood. Due to the insolubility of glycine in high concentrations, testing of >30 g/L was carried out with supplementation directly into individual agar aliquots, prior to autoclaving. Inhibition of growth was assessed after anaerobic incubation, with the MICs defined as the lowest concentration at which growth was markedly inhibited. All test compound concentrations were assessed in duplicate.

Compound	Concentration Range (g/L)
glycine	1.25 – 40
sodium taurocholate	0.3 – 10
glycine & (sodium taurocholate)	1.25 (0.3) – 40 (10)

Table 3: Test compound concentration ranges for minimum inhibitory concentration determination. *Compounds were tested in duplicate at doubling concentrations.*

2.3.4 Germination inhibition assay (PCR ribotypes 015 and 020)

Two *C. difficile* strains, PCR ribotype 015 and 020 (2.3.2.1), were utilised to test the effects of increasing glycine and sodium taurocholate concentrations in broth culture, on both vegetative and spore populations. As with agar incorporation testing (2.4.5), both compounds were tested independently and in combination for the same concentration ranges; (Table 3). BHI broths supplemented with doubling concentrations of test compound were aliquoted (180 µL) into duplicate wells of a Sterilin Microplate U, 96-well tray. Spore preparations of approximately 5 x 10^6 CFU were added (20 µL) to the broth-containing wells, in order to achieve a final spore concentration of ~5 x 10^5 CFU, as per CLSI guidelines (663). Vegetative inocula were created using 0.5 McFarland preparations of overnight BHI broth cultures. These were further diluted by 1 in 100 in fresh broth, prior to the addition of 20 µL of culture each test well, achieving a final cell concentration of ~5 x 10^5. Test wells were created in duplicate with a further set of biological duplicates used to validate the results. Blank, uninoculated wells containing each compound concentration were used as negative controls, enabling a normalisation process of the absorbance data; (Absorbance readings for uninoculated wells containing the equivalent compound concentrations were averaged and subtracted from those of test wells). Absorbance measurements at 595nm were taken at multiple time points (0, 1.5, 3, 6, 24 & 48 hours) using a Tecan Infinite F200 pro (Tecan, Switzerland) and plotted on growth curves.

2.4 Results

2.4.1 Germinant exposure pilot

Total viable counts (TVC) were comparable across all time points, while after 90 minutes of germinant exposure, an approximate 1 log reduction in spore counts was observed; (Figure 6). Exposure for longer periods (120 minutes) demonstrated no further substantial decrease in spore recovery. Therefore, further experimentation progressed with a 90 minute exposure period.

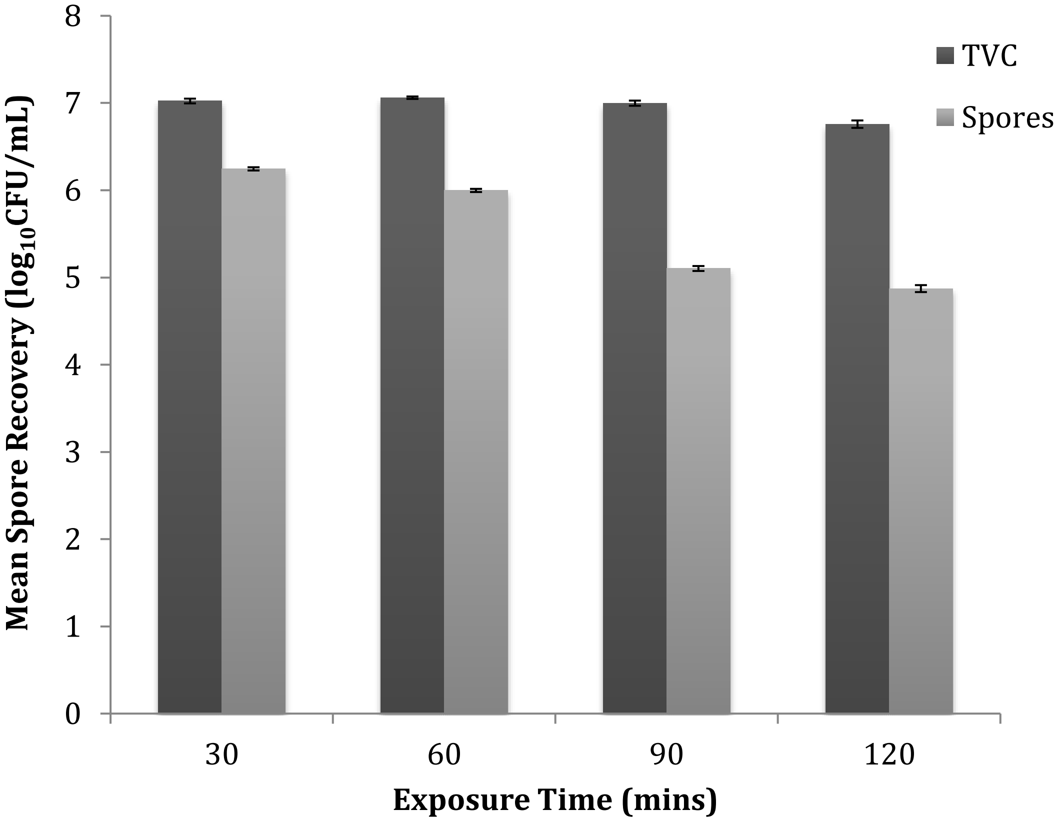


Figure 6: C. difficile spore germination vs exposure time in broth culture. *Spores were cultured in Schaedlers anaerobic broth, supplemented with 0.1% sodium taurocholate and 0.4% glycine. Differences in TVC (total viable counts) and spore count were indicative of germination.*

2.4.2 Phase-contrast pilot

Centrifugation of a 500 µL aliquot was deemed to be the optimal volume for clear visualisation of broth culture populations by phase-contrast microscopy. Entities appeared in sufficient quantities (10-100) for reliable proportional data to be determined, whilst background cell debris was minimised; (Figure 4). Greater volumes (1,000 µL) produced slides that proved over populated, with reliable entity counts obscured by cell debris. Smaller samples (<500 µL) conveyed insufficient total spore populations to achieve accurate estimates.

2.4.3 Solid media comparisons

Four variations of solid media demonstrated equivalent peak levels of *C. difficile* spore recovery;

- **BHI 0.1%:** (7.93-8.45 $\log_{10}$CFU/mL, $\bar{x} = 8.16 \pm 0.10$),
- **BHI 1% (0.8% GLY):** (7.80-8.59 $\log_{10}$CFU/mL, $\bar{x} = 8.25 \pm 0.08$),
- **CCEY:** (8.15-8.32 $\log_{10}$CFU/mL, $\bar{x} = 8.20 \pm 0.05$),
- **CCEYL:** (8.15-8.38 $\log_{10}$CFU/mL, $\bar{x} = 8.26 \pm 0.08$).

No significant differences were observed between the four optimal variations; ($p > 0.05$); (Figure 7). Slightly elevated spore recovery rates were observed after supplementation of 0.1% taurocholate into BHI ($\bar{x} = 0.88$ $\log_{10}$CFU/mL), but this was not significant ($p > 0.05$), whilst the equivalent addition into CCEY demonstrated a significant decrease in recovery ($\bar{x} = 2.73$ $\log_{10}$CFU/mL, $p < 0.001$). Increased concentrations of 1% taurocholate (TC) and 4% glycine (GLY) effected complete inhibition of spore outgrowth for both media types. Reduction in glycine concentration (to 0.8%) with 1% taurocholate exhibited significantly different effects on recovery with BHI and CCEY ($p < 0.001$). In CCEY, the addition of 1% TC/ 0.8% GLY led to a significantly reduced recovery ($\bar{x} = 5.34$ $\log_{10}$CFU/mL, $p < 0.001$), whilst supplementation into BHI demonstrated no significant effect ($p > 0.05$). The addition of lysozyme to either media base produced no significant effect on spore recovery ($p > 0.05$). No significant variation in recovery performance was observed across the five PCR ribotypes tested ($p > 0.05$).

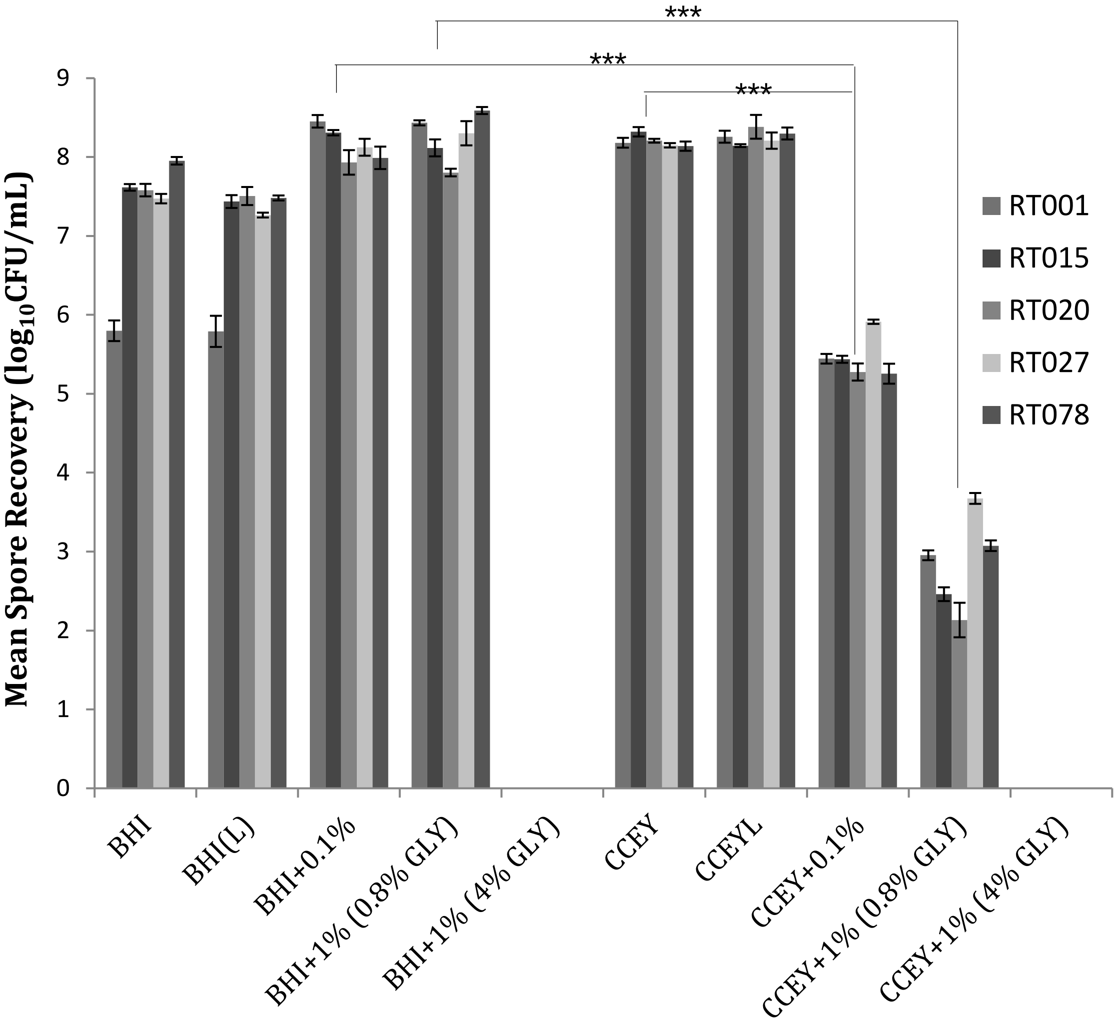


Figure 7: Comparison of germinant supplemented agar for C. difficile spore recovery. *Percentage concentrations refer to sodium taurocholate, unless stated. BHI – Brain Heart Infusion, SCH – Schaedlers anaerobic broth, L – lysozyme (5mg/L), GLY – glycine. Data was based on mean average (±SE) of triplicate counts. *** $p<0.001$.*

2.4.4 Broth media comparisons

2.4.4.1 Agar plate growth counts

Total viable counts across all broth variations remained consistent within each isolate/ribotype tested, only differing between 0.08 and 0.48 log$_{10}$CFU/mL; (Figure 8). TVC and spore count differences were comparable between all BHI and Schaedlers anaerobic broth variations, ranging from 0.08 – 0.64 ($\bar{x}$ = 0.22 log$_{10}$CFU/mL, $p>0.05$). Supplementation with 5 mg/L lysozyme displayed minimal variance in recovery (0.002 – 0.07, $\bar{x}$ = 0.03 log$_{10}$CFU/mL, $p>0.05$). Broths containing bile acid germinants showed notably wider distinctions between TVC and spore counts, compared to those without (1.60-3.30 log$_{10}$CFU/mL, $\bar{x}$ = 2.52 vs 0.03–1.38 log$_{10}$CFU/mL, $\bar{x}$ = 0.42 respectively); ($p<0.001$). Increased taurocholate concentration in both BHI and Schaedlers broths demonstrated a very slight decrease in spore recovery, $\bar{x}$ = 0.11 and 0.21 log$_{10}$CFU/mL, respectively ($p>0.05$). In BHI only, reduction of glycine concentration (from 4% to 0.8%) indicated further slight reductions ($\bar{x}$ = 0.35 log$_{10}$CFU/mL) in spore recovery ($p>0.05$), (data not shown).

PCR ribotype variance was apparent, with ribotype 001 demonstrating the largest difference in vegetative population (TVC minus spore count) between cultures, with and without germinant supplementation ($\bar{x}$ = 2.87 and 2.88 log$_{10}$CFU/mL for BHI and Schaedlers broths, respectively, $p>0.001$). This contrasted to ribotype 078, which demonstrated far lower differences of $\bar{x}$ = 1.44 and 1.12 log$_{10}$CFU/mL in BHI and Schaedlers broths. Ribotype 015 displayed the lowest decrease in spore population after taurocholate addition, whilst all other ribotypes indicated differences within 0.3 log$_{10}$CFU/mL of the mean difference ($\bar{x}$ = 2.08 and 2.11 log$_{10}$CFU/mL, BHI and Schaedlers broths, respectively).

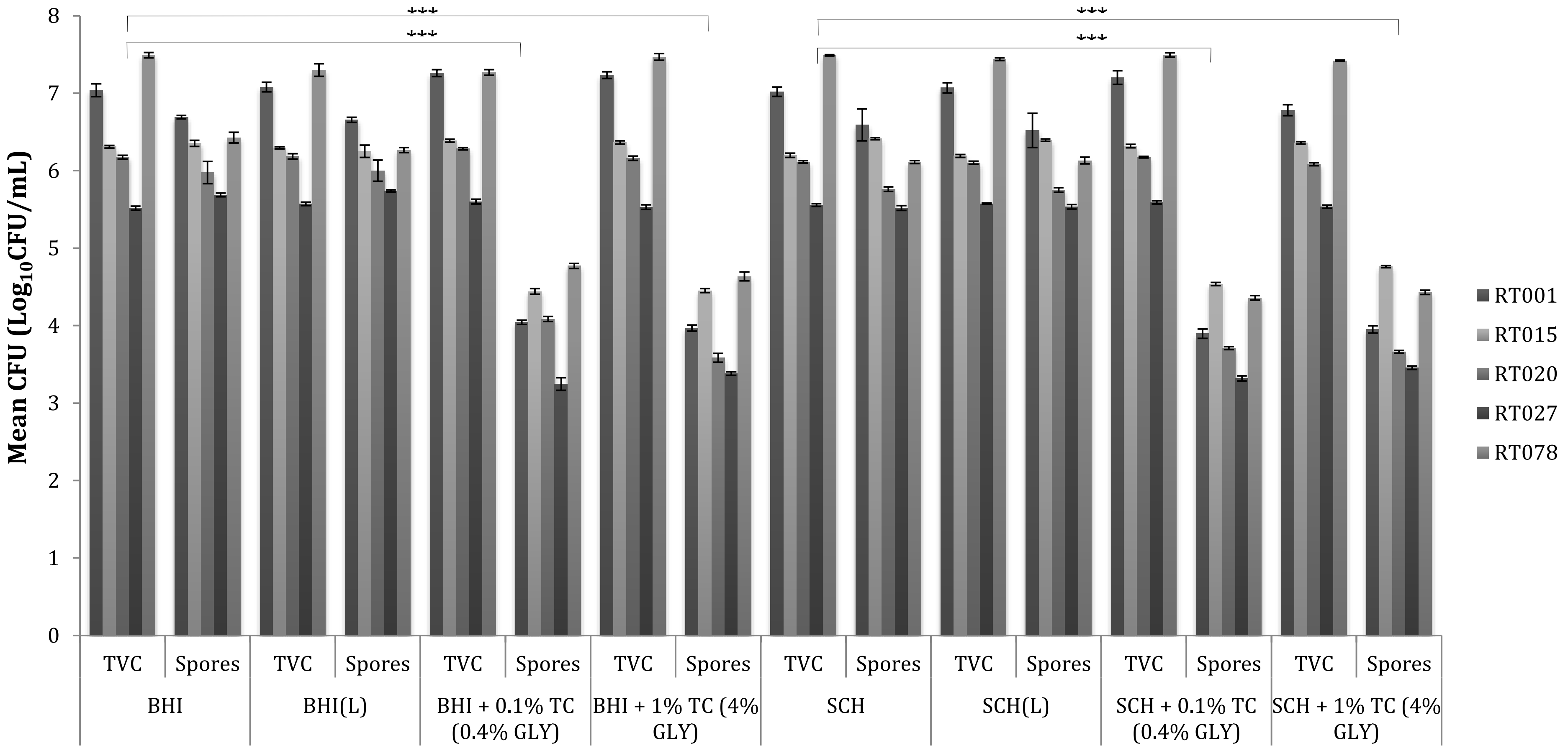


Figure 8: Germination of five different PCR ribotype (RT) C. difficile strains in broths supplemented with various germinant concentrations. *Germination efficiency is represented by differences in mean (±SE) total viable counts (TVC) and spore counts. Broths were exposed to germinants for 90 minutes. BHI – Brain Heart Infusion broth, SCH – Schaedlers anaerobic broth, L – lysozyme (5mg/L), GLY – glycine, TC – sodium taurocholate. Counts are based on triplicate broths. *** P<0.001.*

2.4.4.2 Phase-contrast microscopy results

Proportions of entities observed with phase-contrast microscopy were comparable across all ribotypes tested, with the exception of ribotype 078; (Figure 9). Excluding the data for this ribotype, proportional entity counts at time of spore inoculation (zero) revealed phase-bright spore populations ranging between 70-91%, ($\bar{x}$ = 82%), with phase-dark spores and vegetative cells comprising of between 2-25%, ($\bar{x}$ = 11%) and 5-10%, ($\bar{x}$ = 8%) respectively. Visualisation of ribotype 078 at the zero time point revealed proportions of phase-bright, phase-dark and vegetative cells as 8%, 85% and 7%, respectively. Excluding ribotype 078, comparable entity proportion data was observed for both base media, varying by 0-5%, ($\bar{x}$ = 2.4%). Therefore, average proportional changes associated with broth supplementation are reported from here in. Phase-contrast data correlated with broth germination colony counts, with the same trends observed in both assays. Minimal differences were observed between base media and supplementation with lysozyme, with an average of 3% of phase-bright spores shifting to phase-dark state. As with agar plate enumerations, bile acid and co-germinant addition considerably altered the population dynamics, with 0.1% taurocholate conferring significant changes from phase-bright to phase-dark spores (p<0.0001). Phase-bright spore populations reduced to between 0-10%, ($\bar{x}$ = 3%), a decrease in the range of 62-68%, ($\bar{x}$ = 65%). Phase-dark spore proportions elevated to 48-49%, ($\bar{x}$ = 48%), an increase ranging between 44-49%, ($\bar{x}$ = 48%). Vegetative cell percentages raised by 13-21%, ($\bar{x}$ = 18%) to a range between 17-41%, ($\bar{x}$ = 30%). Exposure to an increased concentration of taurocholate (1%) resulted in an even greater percentage of phase-dark spores (61-88%, [$\bar{x}$ = 72%]), with phase-bright proportions almost eliminated (0-6%, [$\bar{x}$ = 1%]) and an increasing vegetative cell population, (11-38%, [$\bar{x}$ = 26%]). In both BHI and Schaedlers broth assays with ribotypes 020 and 027, phase-bright spores were undetectable (0%).

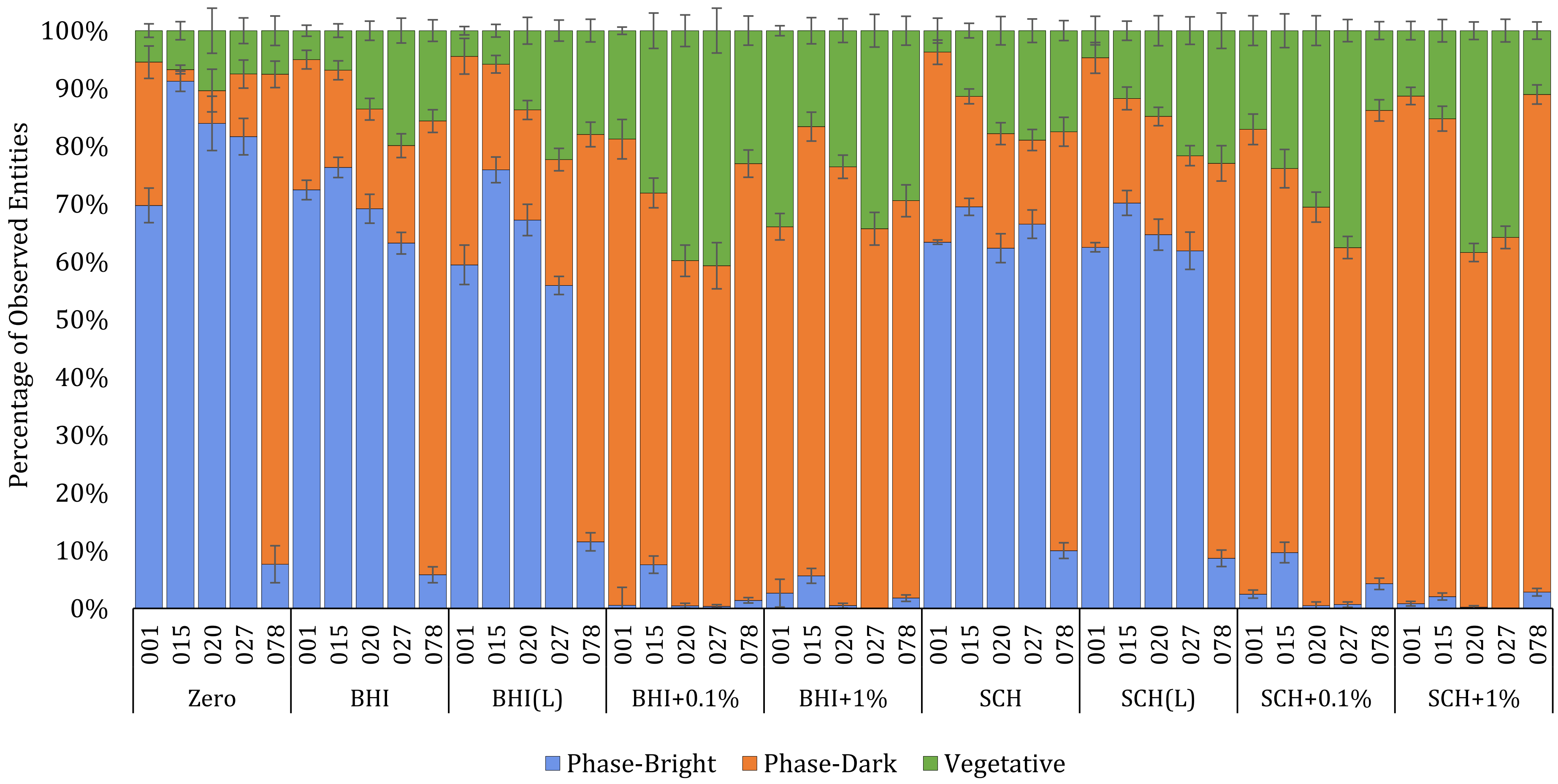


Figure 9: Broth germination comparisons of five different PCR ribotype C. difficile strains by entity proportion determination with phase-contrast microscopy. *Mean proportions (±SE) of entities after 90 minute incubation are displayed. Entities were enumerated in 10x fields of view, per biological replicate. X-axis numbers (e.g. 001) refer to PCR ribotype designations. Percentages refer to taurocholate.*

2.4.4.3 Six week aged spore preparations

The trend of TVC and spore population differentiation remained comparable between fresh and six week aged spores; (Appendix). Findings for BHI and Schaedlers broth cultures produced equivalent counts, ranging from -1.24 – 0.97, ($\bar{x} = 0.31$) $\log_{10}$CFU/mL, $p>0.05$. The differences between broth cultures with and without germinant supplementation remained significant, ranging from 1.60-3.30 $\log_{10}$CFU/mL, ($\bar{x} = 2.52$) and 0.03–1.38 $\log_{10}$CFU/mL, ($\bar{x} = 0.42$), respectively); ($p<0.001$). All but two broth variants demonstrated differences between TVC and spore populations slightly elevated over fresh spore observations ($\bar{x} = 0.38$ $\log_{10}$CFU/mL); (Table 4). Whilst the reduction in TVC and spore difference was minimal in ribotype 001 Schaedlers broths with 0.1% taurocholate (0.28 $\log_{10}$CFU/mL), the same spore preparation germinated in BHI with 0.1% taurocholate exhibited an outlying finding of a 1.24 $\log_{10}$CFU/mL reduction compared to fresh spores.

	Mean CFU ($\log_{10}$CFU/mL) difference between TVC and spore populations							
	BHI	**BHI(L)**	**BHI + 0.1% TC (0.4% GLY)**	**BHI + 1% TC (4% GLY)**	**SCH**	**SCH(L)**	**SCH + 0.1% TC (0.4% GLY)**	**SCH + 1% TC (4% GLY)**
RT001 (Fresh)	0.35	0.42	3.22	3.26	0.43	0.55	3.31	2.83
RT001 (6 weeks)	0.85	0.80	1.98	3.62	1.39	1.58	3.03	3.14
Difference	0.50	0.38	-1.24	0.36	0.96	1.03	-0.28	0.31
RT078 (Fresh)	1.07	1.03	2.50	2.83	1.38	1.31	3.14	2.99
RT078 (6 weeks)	1.87	2.00	2.79	3.17	2.00	2.08	3.16	3.19
Difference	0.80	0.97	0.29	0.33	0.62	0.77	0.02	0.20

Table 4: Mean CFU differences ($\log_{10}$CFU/mL) between TVC and spore counts for fresh and six week old spore preparations of PCR ribotypes 001 and 078. *Data represents the mean average of triplicate values. RT – ribotype, BHI – brain heart infusion, SCH – schaedlers anaerobic broth, TC – taurocholate, GLY – glycine, CFU – colony forming units. Figures reported to 2 decimal places.*

2.4.5 Susceptibility testing

2.4.5.1 Agar incorporation testing

Triplicate testing of five *C. difficile* PCR ribotypes demonstrated marked inhibition of vegetative growth by glycine at 20 g/L concentration (with no colony formation at 25 g/L); (Figure 10). Three ribotypes (001, 020 and 078) exhibited noticeably inhibited growth at 20 g/L, whilst the other two (015 and 027) demonstrated complete inhibition. Results were comparable across both agar types, as well as with vegetative and spore forms. Minimal differences were observed between vegetative and spore assays, with 86.7% and 80.0% MIC concordance demonstrated with Wilkins Chalgren and Brazier's agar, respectively (Table 5). All MIC disparities between vegetative and spore populations were within one doubling dilution. No detrimental effect to *C. difficile* vegetative growth was observed in the presence of sodium taurocholate, up to 10 g/L concentration. The combination of both compounds in a 4:1 ratio indicated comparable results to glycine alone, demonstrating inhibition of growth at 20 g/L glycine: 5 g/L sodium taurocholate, although three strains exhibited marginally elevated MICs (<1 doubling dilution).

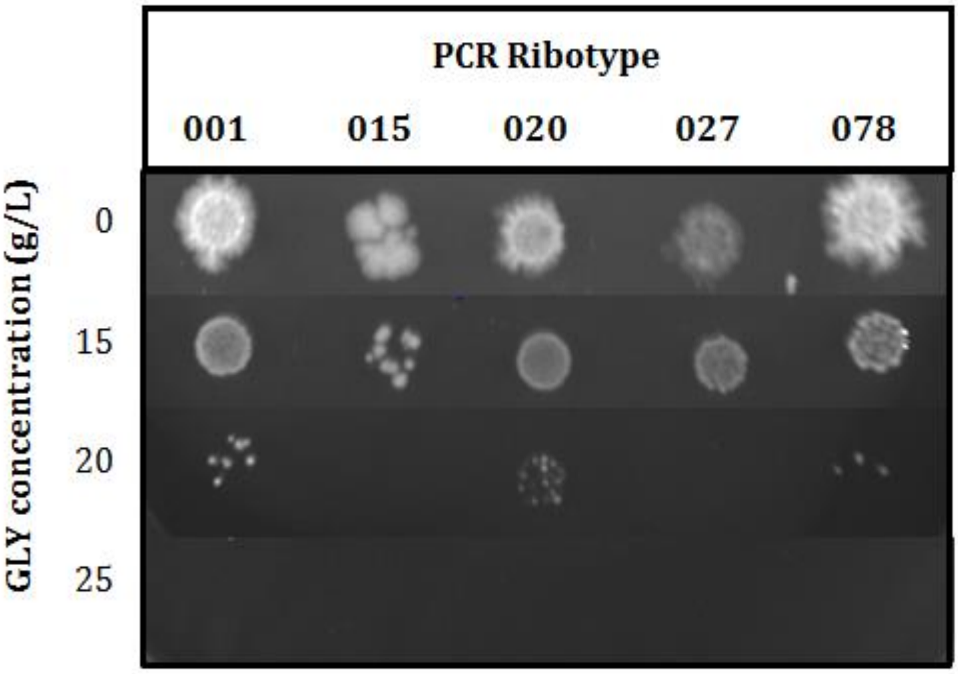


Figure 10: Photographic representation of the growth and inhibition response of five different C. difficile PCR ribotype strains cultured on Wilkins Chalgren anaerobe agar incorporated with increasing concentrations of glycine (GLY).

	Minimum Inhibitory Concentration (g/L)					
	GLY veg (spores)		TC veg (spores)		GLY + TC* veg (spores)	
PCR Ribotype	Wilkins Chalgren	Brazier's	Wilkins Chalgren	Brazier's	Wilkins Chalgren	Brazier's
001	20 (25)	25 (25)	>10 (>10)	>10 (>10)	20 (20)	20 (25)
015	20 (20)	25 (25)	>10 (>10)	>10 (>10)	20 (20)	20 (20)
020	20 (25)	25 (25)	>10 (>10)	>10 (>10)	20 (20)	20(20)
027	20 (20)	25 (25)	>10 (>10)	>10 (>10)	20 (20)	25 (20)
078	20 (20)	25 (25)	>10 (>10)	>10 (>10)	20 (20)	20 (25)

Table 5: Summary table of minimum inhibitory concentrations for glycine (GLY) and taurocholate (TC) of five different C. difficile PCR ribotypes, tested in both vegetative (veg) and spore forms by agar incorporation method, on two agars. ** GLY + TC combined in a 4:1 ratio, values displayed refer to GLY concentration.*

2.4.6 Germination inhibition assay (PCR ribotypes 015 and 020)

Absorbance readings at 48 hours were consistent (within ~0.1) for all glycine concentrations up to 10 g/L, including a glycine-free control broth; (Figure 11A). Substantial reductions in absorbance were demonstrated at 20 & 40 g/L concentrations (from ~0.8 to 0.02). The combination of glycine and taurocholate produced a similar finding; (Figure 11C). Absorbance levels reduced from ~0.8 to 0.02 between 5 g/L glycine (1.25 g/L taurocholate) and 10 g/L glycine (2.5 g/L taurocholate). Parallel analyses of taurocholate concentrations indicated no inhibition of growth, but a notable detrimental effect on absorbance was observed with the addition of the compound (0.95-0.77, 29% reduction), further decreasing to 0.60 (37% reduction) as the concentration increased to 2.5 g/L. Absorbance at higher concentrations remain stable at ~0.60. All findings were consistent across both PCR ribotypes (Figure 11B), as well as with vegetative and spore forms.

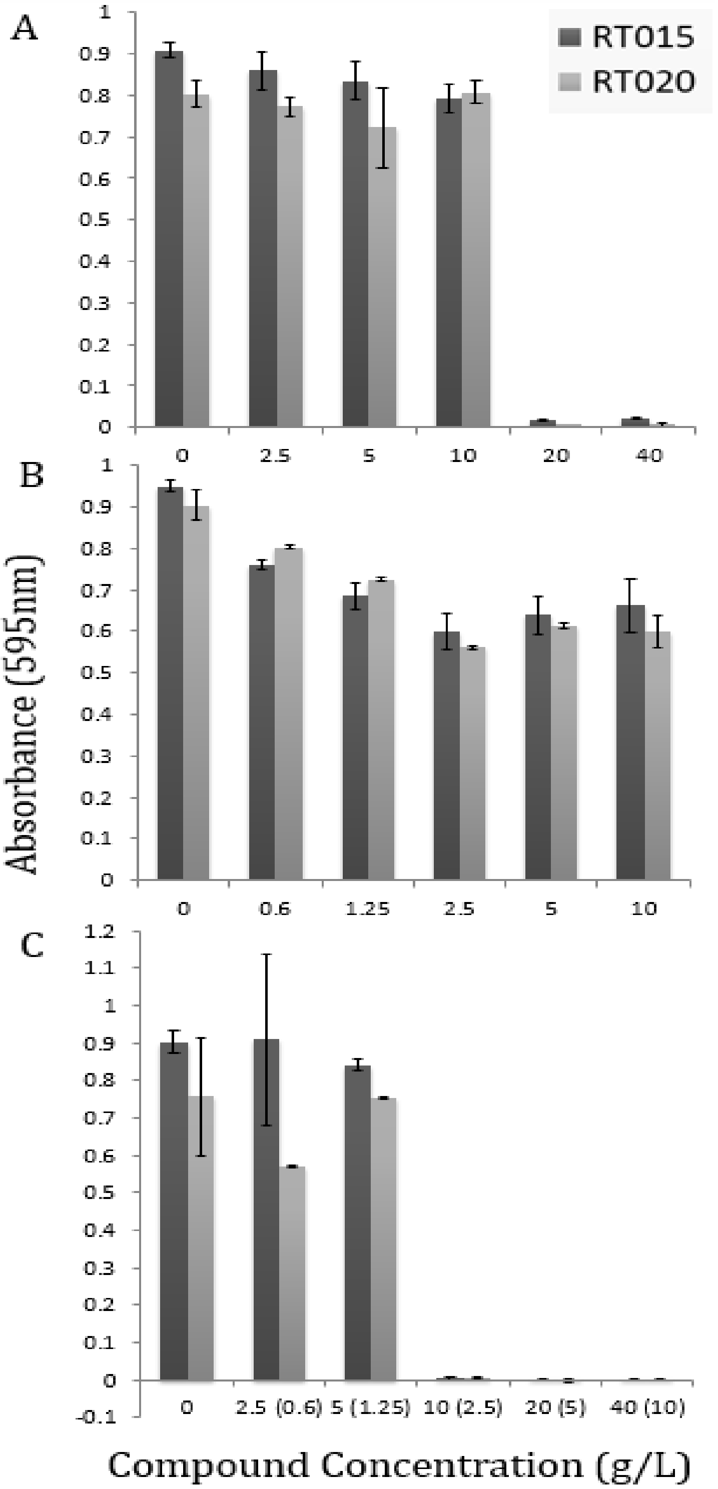


Figure 11: Germinant compound minimum inhibitory concentrations of PCR ribotype 015 and 020 C. difficile spores, as measured by absorbance at 595nm after 48 hour incubation. *A –glycine, B – sodium taurocholate, C – glycine and sodium taurocholate in 4:1 ratio (sodium taurocholate concentration show in parenthesis). Data represents mean averages (±SE) of two biological replicates per test.*

Absorbance measurements from multiple time points highlighted a delayed outgrowth in broth cultures containing 10 g/L glycine (Figure 13 A-B) compared to those with 5 g/L concentration (Figure 12). Whilst absorbance increased in 5 g/L glycine culture after 24 hours (range 0.29-0.48), 10 g/L broth readings remained low (0.015-0.049). However, by 48 hours the absorbance readings of both sets of cultures were similar (0.47-0.87 and 0.56-0.76 for 5 and 10 g/L, respectively). Whilst 24 hour reads for 10 g/L glycine remained low (0.02-0.07) for both PCR ribotypes tested, ribotype 020 exhibited elevated absorbance levels at 48 hours (0.76), surpassing the growth of 0-5 g/L cultures (0.46-0.65); (Figure 13B). However, ribotype 015 demonstrated a considerably decreased absorbance level of 0.35 at the same concentration; (Figure 13A).

Growth curves were similar for all taurocholate concentrations and both ribotypes, expanding exponentially from six hours onwards; (Figure 13 C-D). Interestingly, 5 g/L (0.74-0.79) and 10 g/L (0.74-0.81) concentrations closely matched the final absorbance measurements for cultures without taurocholate supplementation (0.85-0.76), whereas lower concentrations peaked between 0.52-0.60.

Whilst low concentrations of glycine alone (2.5-5 g/L) reached comparable levels at 48 hours as cultures containing no glycine, in combination with taurocholate the absorbance was reduced (0.26-0.34) in comparison to 0 g/L (0.56); (Figure 13 E-F).

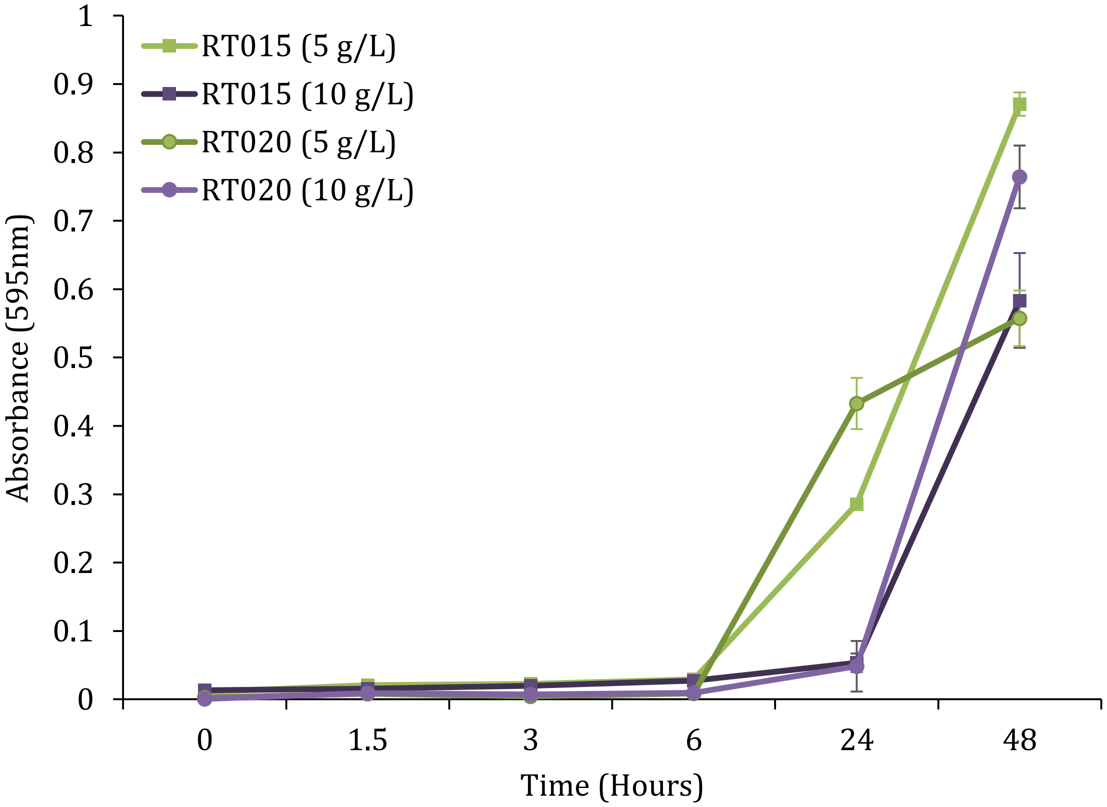


Figure 12: Comparison of absorbance measurements (595nm) from BHI broths supplemented with 5 and 10 g/L GLY, inoculated with C. difficile spores of two PCR ribotypes (015 and 020). *Data was calculated as mean averages (±SE) of quadruple replicates, calibrated against blank (uninoculated) media controls.*

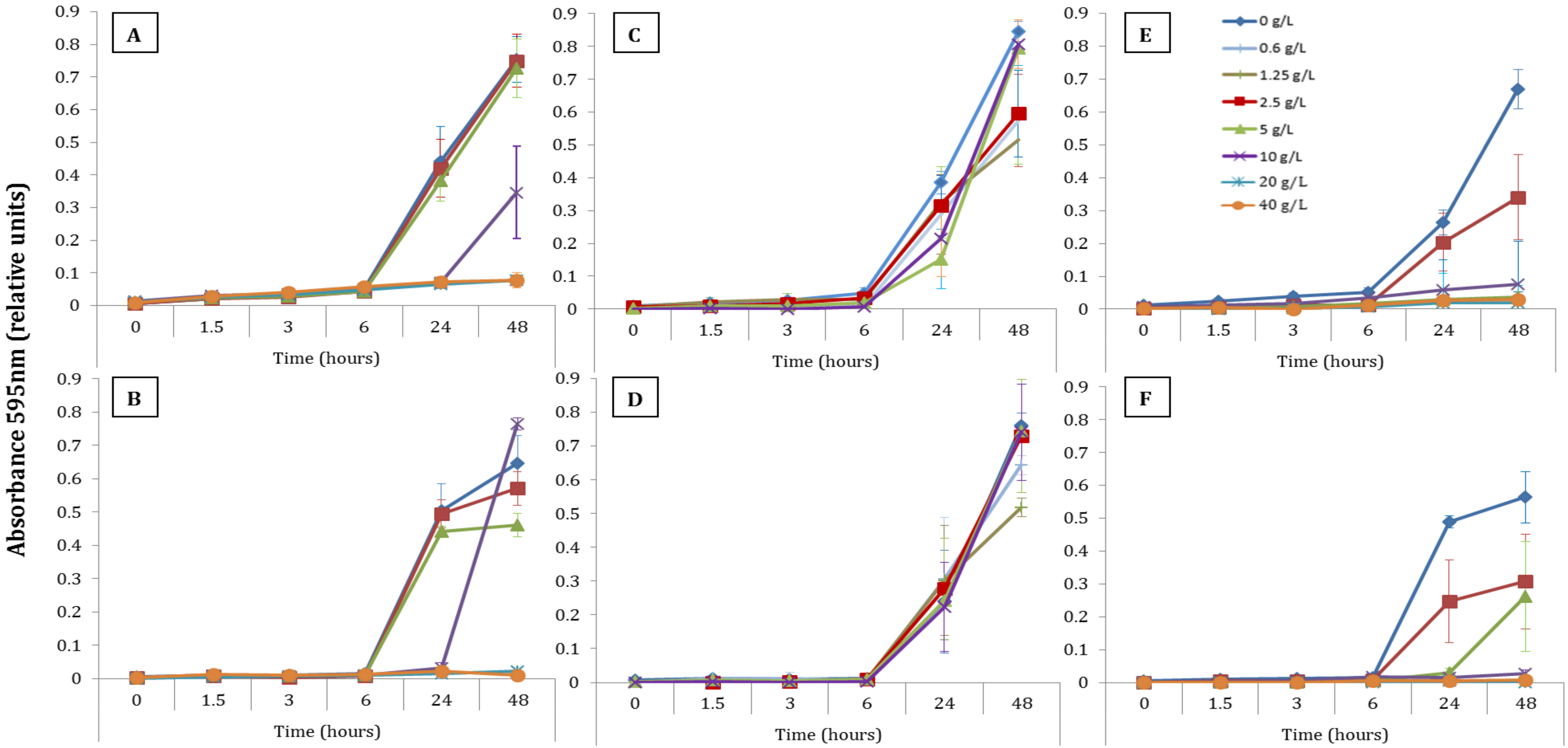


Figure 13: The effect on absorbance (595nm) of glycine (A and B), sodium taurocholate (C and D) and glycine/taurocholate combined (4:1) (E and F) on PCR ribotype 015 (A, C and E) and 020 (B, D and F) C. difficile spore outgrowth over time. *Spores were cultured in brain heart infusion broths supplemented with doubling concentrations of test compound. Data represents mean averages (±SE) of quadruple replicates, calibrated against blank (uninoculated) media controls.*

2.5 Discussion

Many commercially produced agars are available for the culture of *C. difficile*, whilst various non-selective broths exist for enrichment of recovery. However, there is no conclusive agreement as to which media provides the greatest germination recovery and sensitivity, with different studies favouring CCFA, CCEYL (utilised by the CDRN (363)), and ChromID *C. difficile* agars (333, 342, 658, 659, 664, 665). Often the recovery targets differ, with fresh, aged and environmental spores amongst the variable targets tested. Here we investigated selective (CCEY) and non-selective (BHI) solid media and two commonly used broths (BHI (244, 341, 666) and Schaedlers anaerobic broth (187, 280, 480)), supplemented with varying additives and concentrations in an attempt to identify optimal spore germination and recovery. Determination of the most efficient method for spore recovery and germination was sought with the intention for use in the optimal recovery of aged *C. difficile* spores from an historical collection of isolates.

2.5.1 Solid media comparisons

Ten combinations of common solid growth media were tested for their efficacy in triggering germination in *C. difficile* spores. Whilst CCEY exhibited superior recovery over BHI alone, when supplemented with 0.1% taurocholate, BHI was equivalent to CCEY in demonstrating the greatest levels of recovery. This complements evidence from Lister *et al*., who also established the latter media as the most sensitive, when compared to four other selective agars (667). In the aforementioned study, CCEY was able to detect levels as low as 10^2 CFU/mL, whereas other media could only detect levels above 10^4. Although ChromID *C. difficile* agar has been reported as the most effective in recovery by several studies (657-659), the high sensitivity reported for CCEY is of greater importance with regards to aged isolates, since they are likely to contain diminished spore levels. This may also translate to diagnostic applications, where clinical specimens may have been exposed to antibiotic treatment prior to sample collection and are liable to contain reduced bacterial loads.

Whilst the primary bile acid, taurocholate, in combination with a co-germinant, glycine, has been implicated in increased germination and recovery, Sorg and

Sonenshein proposed that germination with taurocholate may be enhanced by a semi-solid support, implying an efficacy in solid media (244). We might, therefore have expected to see a universal increase in recovery using taurocholate, however, this was not the case in CCEY. Supplementation with low concentrations (0.1%) of taurocholate demonstrated polarised effects on the recovery of spores with both agar bases. Whilst an increased efficacy was observed in BHI, bringing its recovery potential in line with unmodified CCEY, a dramatic reduction was demonstrated with its addition to CCEY; (Figure 7). It is hypothesised that these diverging findings are due to the pre-existing cholate content in CCEY, absent from BHI, which provides an existing germination efficiency in this previously optimised media.

Strikingly, enhancement of either media with high concentrations of taurocholate (1%) and the equivalent 1:4 ratio of glycine (4%) resulted in complete inhibition of vegetative growth. Since reduction of glycine concentration to 0.8% enabled spore recovery, comparable to lower concentrations (0.4%) in BHI and to a reduced extent in CCEY, this potentially identifies it as the implicating factor in inhibition. In order to further elucidate the source of inhibition, additional experiments were undertaken to investigate the antimicrobial potential of elevated concentrations of both compounds (2.3.3).

Notably, the addition of lysozyme to either agar type demonstrated no beneficial effect to recovery, supporting work by Nerandzic (338), with others even finding detrimental effects when assessing 24 hour lysozyme exposed cultures (659). However, these data are in contrast with previous work by Wilcox *et al.*, where supplementation of CCEY agar with lysozyme was shown to improve recovery of *C. difficile* spores from environmental swabs (342). Nonetheless, the latter experiment focussed on the recovery of environmental spores, which are likely damaged through desiccation and exposure to external physical and chemical stressors, such as heat and detergents. This may potentially precede a state of dormancy and therefore a reduced germination rate, presenting a greater propensity for enhancement by lysozyme action. Although no beneficial impact on germination was demonstrated with the relatively new spores tested in this investigation, since lysozyme addition showed no detrimental effect, its use in recovery of highly aged spores maybe more pertinent.

Since ChromID agar also contains taurocholate, it might be expected to be effective in spore recovery, however it is expensive, at almost three times the cost of CCEYL (658). However, issues have been reported regarding lack of colouration in certain genotypes (660, 661) and since the chromogenic nature of the media is not necessary for pure recovery applications, CCEY provides a reasonable alternative.

The findings of this investigation suggest the comparable efficacy of three solid media variants, CCEY, CCEYL and BHI supplemented with 0.1% taurocholate and 0.4% glycine. Whilst any of these formulations would provide optimised spore germination, selection of a particular agar must be assessed based on the specific purpose. For pure culture recovery, all would suffice, although assessment of the costings per plate suggest that the BHI option (19 pence) would be less expensive than CCEY (32 pence). Although these calculations are highly estimated, as pricing will vary on quantity and supplier etc., an obvious preference can be seen for this application. However, if faecal specimens or potentially impure samples are to be tested, the additional selection factor of cycloserine and cefoxitin would be necessary. Whilst BHI was not tested with the addition of these antibiotics, it seems unlikely that this would provide equivalent levels of selectivity, as the constituent elements of Brazier's agar have been optimised for the selection of *C. difficile*.

2.5.2 Broth media comparisons

Determining a sufficient length of exposure time for the effects of germination to be distinguishable was essential. The pilot data described in Figure 6 showed that spore counts decreased by almost 1 log between 60 and 90 minutes exposure to 0.1% taurocholate and 0.4% glycine. This represented the largest single step decrease in spore population, whilst TVCs remained stable, indicative of the exposure time with the greatest increase in germination. Equally, this remained a short enough period to eliminate the potential for vegetative overgrowth to mask the data. Therefore, a 90 minute incubation was utilised in all broth comparison assays.

There were no apparent differences in germination efficiency between the commonly used BHI and Schaedlers broths. Taurocholate incorporation into broths supported previous findings (332, 655, 668), demonstrating significantly elevated germination ($p<0.001$); although increasing the concentration yielded no advantage. This directly contradicted work by Heeg *et al.* who found the majority of isolates tested demonstrated a greater germination rate when broths were supplemented with 1% taurocholate, as opposed to 0.1% (339). However, one strain did exhibit no difference in germination response, suggesting inter-strain variation, as demonstrated significantly in the different ribotype 027 isolate responses to both primary and secondary bile acids. Inter-strain variation was demonstrated here, with as much as a 2 log difference observed between ribotypes; (Figure 8). Nonetheless, these disparities were consistent across broth variations, potentially suggesting some inconsistency in the initial inocula. Recently, Weingarden *et al.* investigated the germination efficacy of clinically relevant bile acid concentrations from CDI patients on *C. difficile* spores, noting PCR ribotype variance (669). Interestingly, two ribotype 078 isolates demonstrating more efficient germination responses were shown to contain several amino acid substitutions in the *cspC* receptor gene. The elevated recovery of 078 spores in this study supports previous findings, although no genomic analysis has been undertaken to correlate CspC mutations. It is possible that these receptor polymorphisms may contribute to the increased success of this ribotype in clinical settings. Nonetheless, the slight variance observed amongst individual ribotypes may be related to slight differences in growth rate, as opposed to germination efficiency. Inter-ribotype growth rate differences have previously been reported in *C. difficile* (670), and may contribute to the variances detected in this study.

Interestingly, in this study, the inhibitory effects of high glycine concentration observed in solid media was not replicated in broth investigations. This finding may have been a consequence of a larger initial inoculum (50 µL) in the broth enrichment experiments, or simply that the spores were unculturable in direct contact with high glycine concentrations, only stimulating the initiation of the germination process. Consequently, when aliquots of broth were enumerated on less inhibitory media, germinating spores were able to outgrow as expected. The differences between supplemented and non-supplemented broths may be a result of pre-exposure to the necessary germinants.

Phase-contrast microscopy is a vital tool in the evaluation of germination data (671), since it allows a visual assessment of spore response at a cellular level, providing support to colony counting data (672). Initial tests showed the density of spores within the broths to be insufficient to allow a comprehensive analysis of entity population by phase-contrast microscopy. Therefore a small pilot study was implemented in order to optimise the protocol to allow the inclusion of this method.

Phase-contrast data from the optimised methodology (Figure 9) proffered strong support for the colony count findings, demonstrating high proportions of phase-bright (dormant) spores in the broths exhibiting comparable TVC and spore counts, whilst a predominance of phase-dark (germinating) spores were observed in broths containing taurocholate. Dominance of phase-dark spores in conjunction with a reduction in phase-bright spores is indicative of germination, since the release of $Ca^{2+}DPA$ associated with spore cortex lysis (673) causes a marked reduction in a spore's refractive index (674). These alterations in spores can be differentiated effectively by phase-contrast microscopy.

Comparator investigations of broth germination in six week aged spore preparations were implemented to identify any variance between germination response of nascent and more established spores; (Table 4). Whilst no major differences were identified, a far lengthier aging of spores may be necessary to elicit different responses, as outlined by several studies (645, 647, 675). Segev *et al.* reported variable RNA production in fresh *B. subtilis* spores exposed to diverse environments, including polarised temperatures and prolonged dormancy (675), whilst Ghosh and colleagues described a decrease in germinant receptors in super dormant spores (645). In *C. difficile* Rodriguez-Palacios identified diverging responses of fresh and aged spores to germination via heat shock (647). Unfortunately, due to extensive experimental testing with these preparations during a series of investigations into *C. difficile* spore heat response, where no benefit was observed with heat shocking (676), insufficient volumes of the necessary spore suspensions remained for this further testing. Interestingly, differences were amplified in the aged spore investigation for broths without supplemented by the classical germinants. This could potentially be due to the slightly larger inoculum in the aged spore experiment, exacerbating the difference, whereas the germination efficiency of taurocholate broth optimisation may negate any discrepancies.

2.5.3 Glycine mediated inhibition

The inhibitory effects of glycine, along with other amino acids, has been documented since the 1940s (677), with hindrance to bacterial growth reported in multiple genera, irrespective of Gram status (678-681). Hishinuma *et al.* identified substantial variation in the inhibitory action of high concentrations, demonstrating a spectrum of responses from sensitive through to resistant. Although described in other clostridia (678, 682), to our knowledge this is the first report in the literature linking glycine with inhibition of *C. difficile* outgrowth. The findings of this study, that glycine in high concentrations (>2%) entirely inhibits bacterial growth, coincide strikingly with other studies demonstrating complete inhibition at 2% concentrations, in *C. acetylbutylicum*, *L. lactis* and *Helicobacter pylori* (678, 679, 681). Interestingly, Hishinuma *et al.* reported minimal disruption at 0.5% glycine, similar to the concentration achieving the best recovery in this study (0.4%) (678).

One study reported that *C. difficile* germination significantly reduced in slightly acidic conditions (pH 5.56-6.32) (331). Since glycine buffers to pH 6.0, high concentrations of this compound could bring the pH environment within this range, potentially inhibiting germination. Nonetheless, since the inhibitory effect of glycine was observed in both vegetative and spore assays, it seems more likely that the inhibition is related to vegetative cell reproduction, as opposed to a germination. As glycine is considered a co-germinant for *C. difficile*, this may not be unexpected. Glycine, the simplest amino acid, is a protein precursor metabolised from serine (683). One of its primary functions in bacteria, is as constituent part of the peptidoglycan cell wall, where it forms pentaglycine cross linkages to bridge tetrapeptides, as part of the transpeptidase reaction of cell wall biosynthesis (684). One putative explanation for glycine mediated inhibition of *C. difficile* is the substitution of alanine isomers with glycine, in residue positions 1 and 4 of the peptidoglycan subunits, as demonstrated in other organisms (678, 685). Incorporation of these deficient tetrapeptide precursors into the cell wall structure has been confirmed to reduce cross-linking and result in an inability to withstand the pressure of cell turgidity and eventual cell lysis; (Figure 14). The observed reversibility of glycine inhibition with the addition a L-alanine by Hishinuma *et al.* strongly supports this hypothesised mechanism in other bacteria (678). Nonetheless, a recent study has highlighted the novel structure of *C. difficile* peptidoglycan, reporting a prevalence of 3-3 cross bridging, compared to the typical 4-3 linking (686). Therefore, the relationship between glycine and *C. difficile* peptidoglycan formation may potentially differ. An additional explanation suggested by Minami *et al.* involves the interaction of glycine and carboxypeptidase enzymes, essential to cell wall biosynthesis (679). Since glycine has been demonstrated to inhibit this enzymatic process, it is plausible that cell disruption is effected by this interaction. A more simplistic explanation could involve the greater glycine concentrations leading to a hypertonic environment and the potential loss of water into the highly concentrated media. Nonetheless, this explanation does not account for the lack of inhibition at relatively high glycine concentrations below 2% or the decrease in recovery in CCEYL plates with 0.4% glycine supplementation; (Figure 7). Equally, the aforementioned reversibility demonstrated by Hishinuma contradicts this hypothesis, suggesting the involvement of other mechanisms.

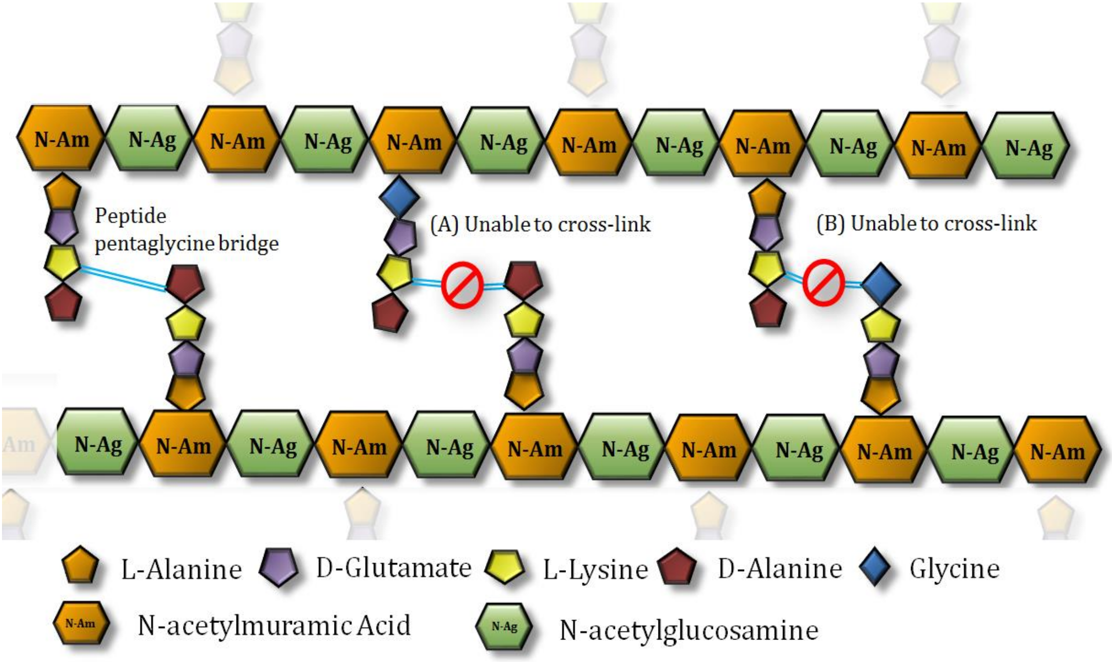


Figure 14: Representation of deficient peptidoglycan formation caused by alanine substitutions with glycine in peptide subunits. *(A)- Glycine replacement of L-alanine in position 1. (B) – Glycine replacement of D-alanine in position 4.*

The delayed outgrowth observed in cultures containing 1% glycine compared to 0.5% (Figure 12) potentially indicates an inhibitory concentration boundary. Since the final absorbance measurements mask this development, the growth curve assays were crucial in elucidating these findings. Here, the actual MIC is likely to fall between these concentrations and the exponential outgrowth observed cannot occur until the glycine concentration is reduced to below the MIC. It can be speculated that glycine within the nutrient broth is being metabolised between the six and 24 hour period, after which the concentration becomes tolerable to log phase growth. The absence of differentiation between final absorbance readings at 48 hours may be a result of a prolonged culture, offering the slow starting cultures an opportunity to catch up. Interestingly, inhibition at high glycine concentrations was demonstrated by micro broth assay, but not at larger volumes during enrichment broth germination comparisons; (Figure 8). As previously discussed, this may potentially be due to difference in exposure length and the additional recovery step on non-selective agar, necessary to enumerate the broth cultures.

2.5.4 Response to high levels of taurocholate

Since previous studies found minimal detrimental effects of taurocholate (244, 687), it may be as expected that these data demonstrated no absolute inhibition of *C. difficile* vegetative growth at high concentrations (<=10 g/L); (Table 5). However, as observed spectrophotometrically, a reduced absorbance, reflective of diminished cell density, was identified with supplementation of concentrations above 0.6 g/L; (Figure 11). Although cultures remained viable, the decreased absorbance connotes demonstrable impairment. Taurocholate mediated inhibition in *C. difficile* vegetative growth has been reported, with crude formulations reducing viable counts by a factor of 3 x 10^3 compared to pure compound (315). This work suggested potential impurities in the preparation, including the known inhibitor, deoxycholate (244). However, unlike in the present study, Wilson *et al.* found no detrimental impact of pure taurocholate preparations (315). Due to the vast expense in the formulation of taurocholate, it is not inconceivable that there may be an element of impurity in the compound used in this study. Other organisms have exhibited impairment in the presence of taurocholate, with Tannock *et al.* observing a reduction in lactobacilli viability with exposure (688). They discovered high levels of cholic acid accumulating in cultures, due to the deconjugation of the primary bile acid by bile salt hydrolases. Whilst it seems self-defeating for *C. difficile* to produce these enzymes, homologues have been identified in its genome, as well as in other clostridia (689, 690). Although Darkoh *et al.* observed no complete inhibition with taurocholate, they demonstrated some inter-strain variability in response. It may be plausible that expression of these bile salt hydrolase encoding genes is repressed in *C. difficile*, until unnecessarily high concentrations of primary bile acids are encountered. Nevertheless, this did not appear to impact on the germination and recovery of spores in more voluminous broths and could represent an artefact of the micro broth assay.

2.5.5 Study limitations

An obvious limitation of this study was the focus of germination testing on only two bases each, for both solid (BHI and CCEY) and broth (BHI and Schaedlers anaerobic broth) media. Whilst other bases are widely used (334, 335, 505, 657, 658, 667), those tested here represented the commonly used options within our research group, inclusive of the CCEYL media type utilised by the national reference centre. The focus of these investigations remained on the impact of the addition of varying bile acid and co-germinant concentrations.

One drawback of this investigation surrounds the application of these findings to the actual test specimens, which the experimental design was determined for. Whilst it was never feasible to replicate spores between 32 and 38 years old, the spores tested in the germination assays were only aged by six weeks. Since, variation has been identified amongst spores of diverse ages (645, 647), further aging may have enabled closer comparisons to the ultimate application. However, minimal differences were observed after six week aging.

Although five prevalent disease causing ribotypes were tested for germination responses, multiple isolates from within each type would have further substantiated findings, as well as potentially revealing some inter-ribotype variability, as previously detected (339, 669). Nonetheless, the experimental protocols, particularly the broth methods, were considerably time and labour intensive and the addition of multiple strain variables would have proved too costly.

Another possibility for future testing would be to use highly pure taurocholate, since the preparation tested was classified as >97% pure, and variation in purity has previously been associated with disparity of germination efficacies (341), testing of highly pure compound may have provided additional confidence in the results.

Heat activation and ethanol pre-treatments were not considered as part of this investigation, as they were beyond the scope of this work and would have detracted from the main focus of this research. Nevertheless, some of these hypotheses were investigated within our research group, with no beneficial effect to germination of heat treatment observed (Pickering *et al.* 2018, publication under review).

With regards to the discovery of the inhibitory impact of glycine, only five isolates were assessed. Whilst determination of susceptibilities of a larger panel of isolates would provide a better indication of MIC distributions, highlighting any strain variation, the results served to identify the source of the inhibition in *C. difficile* and create an awareness of the burden of high concentrations in solid media.

2.6 Conclusion

Based on the common laboratory media tested, these data suggested an optimal, two-step algorithm for *C. difficile* germination and recovery. This would involve an initial enrichment protocol with either BHI or Schaedlers broth supplemented with 0.1% taurocholate and 0.4% glycine, followed by recovery on a standard Brazier's CCEY agar plate. Nonetheless, the spore recovery environment should be considered and the addition of lysozyme to the solid media may yield benefits for spores exposed to high levels of environmental stress (342). This also correlates with the media currently in use by the CDRN (363).

Supplementation with greater concentrations demonstrated no advantage to spore germination and is therefore deemed an unnecessary expense, particularly in high throughput diagnostic lines. Whilst, lysozyme exhibited no beneficial effects on fresh spore recovery.

These findings are the first reports of glycine mediated inhibition in *C. difficile*, with concentrations greater than 2% proving inhibitory. Whilst the detrimental effect appears related to vegetative outgrowth, the exact mechanism requires further elucidation.

Chapter 3 Phenotypic Characterisation and Typing of Historical *Clostridioides difficile* Strains

3.1 Introduction

Phenotypic characterisation and bacterial typing are highly important to the surveillance of epidemiological trends in CDI. Identification of the circulating strain types in a given setting facilitates the monitoring of endemicity and transmission of epidemic strains (257, 371, 376, 691). While a range of typing techniques are available, standardisation is essential to the production of a coherent epidemiological picture across multiple time points and geographic locations (358-361, 367). The introduction of a national typing service in the UK, the CDRN (363), enabled *C. difficile* strain distribution and prevalence to be longitudinally monitored and epidemiological shifts tracked, through the use of PCR ribotyping (367). Unfortunately, there are few studies providing typing data using this high resolution technique for *C. difficile* isolates from the early 1980s (381, 496, 692-694). This poses challenges when comparing historical and modern prevalence data. Whilst the CDRN data is able to highlight decreasing rates of ribotype 027 infection and the emergence of ribotypes 005 and 023, it can only provide data back to 2008. Prior to the launch of the CDRN, an isolate collection existed at the Anaerobe Reference Unit, University Hospital of Wales, Cardiff, UK dating back to 1993 (367). This collection was used as a typing service for England and Wales for the purpose of outbreak investigation. Whilst the collection at Cardiff was invaluable in the formation of the CDRN, the strains described in this chapter pre-date these isolates, often by more than a decade. Reports of these earlier isolates reveal a dominance by ribotype 001 and 106 isolates, with John and Brazier discovering 55% of all isolates tested representing the former strain type (692). This correlated with data from outbreaks, caused by clindamycin-resistant ribotype 001 strains in the USA (97). *C. difficile* populations have fluctuated frequently, whether due to the introduction of epidemic strains, change in antibiotic usage and infection control behaviours or clonal expansion of resistant strains (363, 417). Analysis of CDRN data revealed considerable decreases in endemic ribotypes 001 and 106 between 2007 and 2010. This correlated with the reduction of high risk antibiotic use, as well as with infection control behavioural changes brought about by the introduction of mandatory reporting (384). As outlined by one pan-European surveillance study of isolates from 2014, ribotype prevalence was often region/country dependent (188). Whilst hyper-virulent ribotype 027 remained the predominant type across Europe, UK distribution demonstrated greater heterogeneity, reporting a predominance of ribotypes 014, 106, 015, 020, 078 and 002 (188). In support of these data, other large European studies discovered associations between ribotype diversity, incidence of epidemic strains and antimicrobial resistance (118, 154, 695).

Further to strain typing, the elucidation of antimicrobial resistance patterns gives valuable information, revealing correlations between antibiotic use and reductions in susceptibilities (91, 97, 103, 115). These are of importance to the potential prediction of clinical response and risk of disease onset. Antibiotic susceptibility data from the late 1980s and 1990s is more readily available than ribotyping data. The available data from *C. difficile* studies temporally tracking susceptibilities, generally reveals increases in resistance over time (381, 521, 693), presumably due to prolonged antibiotic exposure. A study of 179 Scottish isolates revealed MXF resistance increases, with reported rates of 0, 3.3 and 10.2% resistance in 1979-86, 1987-95 and 1996-04, respectively (381).

However, it is not always the case that resistance levels are reported to increase over time. Whilst, reductions in susceptibility to modern agents were observed in an analysis by Taori *et al.*, decreases in resistance associated with older compounds, clindamycin and tetracycline, correlated with declines in their use. Metronidazole and vancomycin susceptibilities are commonly reported to remain stable, with minimal resistance development (521, 696). Conversely, Barbut *et al.* demonstrated reductions in the number of isolates resistant to clindamycin, tetracycline, erythromycin, rifampicin and chloramphenicol between 1991 and 1997 (696). This serves to highlight the relevance of type prevalence to resistance levels, since decreases in the aforementioned study were strongly related to a distinct shift in the predominance of serogroup C isolates.

Recent large scale, retrospective surveillance across >7,000 inpatients indicated *C. difficile* as the most frequently reported MDR pathogen (508). Since *C. difficile* is the primary cause of antibiotic-associated diarrhoea, resistance to multiple agents is of high importance, as it may result in elevated risk of disease acquisition through increased pathogen survival to antimicrobial exposure for alternative infections (387, 697, 698). Determination of the development of MDR, by assessing prevalence pre and post introduction of antimicrobial compounds is important to the understanding and management of these organisms. High prevalence of MDR in *C. difficile* has been reported amongst recent surveillance, with one study reporting a rate as high as 55% (444). 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 (188, 444). Contrasted with historical data discovering only 7.8% MDR amongst 179 UK isolates, tetracycline resistance was elevated and moxifloxacin resistance minimal (381). 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. Equally, lessons can be learnt from putative *mistakes* from the past, and prescribing behaviours optimised. In this chapter an historical collection of isolates was interrogated and assessed regarding ribotype distribution and phenotypic response to a panel of antimicrobial agents. Understanding toxin status may also help to uncover cases of asymptomatic carriage or instances of alternative enteric disease, wrongly attributed to *C. difficile*. Comparisons to modern UK isolates enabled an assessment of resistance development, particularly regarding MDR development.

3.2 Rationale

Considering the relationship between the introduction of new antibiotic agents and the progression of antimicrobial resistance in *C. difficile* is essential to understanding the development of MDR strains. Investigations in this chapter sought to recover and characterise *C. difficile* isolates from the early 1980s (pre-dating isolates stored in existing national collections) to establish strain distribution and susceptibility profiles, prior to the addition of modern generation antibiotics to the formulary. This baseline comparative data could be used to further the understanding of the expansion of certain PCR ribotypes and their propensities for acquisition and development of multiple antimicrobial resistances.

3.3 Methodology

3.3.1 Isolation of *C. difficile* from an historical strain collection

3.3.1.1 Historical *C. difficile* collection properties

An historical collection of ~2000 clostridia spp. isolates was established by Professor Peter Borriello and retained at Public Health England laboratories, Colindale, UK. Strains were collected (September 1980 – September 1986) as part of a first national diagnostic service and were originally seeded into cooked meat broth at point of isolation. Log books were retained with the specimens, containing basic demographic data. Prof. Borriello gave his specific consent and encouragement for his isolate collection to be studied in Leeds.

3.3.1.2 Sample selection

Upon transfer of this collection to the Healthcare-Associated Infection Research group in Leeds, UK, specimens were paired with clinical data where possible. Due to the large volume of retained material, an initial cohort selection process was implemented. Specimens with the following demographics were removed from selection: not specifically identified as *C. difficile*, of non-human origin, cell cytotoxicity negative and/or an absence of original *C. difficile* positive confirmation. Of the retained isolates, 1,253 were matched to specific laboratory records, constituting 476 patients. These provided the final sample selection to be subjected to recovery and isolation methods.

3.3.1.3 *C. difficile* recovery and isolation

Historical samples were subjected to multiple recovery methodologies in order to isolate the maximum number of strains for further analysis; (Figure 15). The progression of methodological approaches was based on assessments of recovery rates and the contemporaneous findings of the spore germination optimisation investigations from the previous chapter. Where patients were associated with multiple specimens, testing was ceased after initial *C. difficile* isolation (unless tested for repeat isolation confirmations). All incubations were carried out in an A95 anaerobic workstation at 37°C for 48 hours, unless stated.

3.3.1.3.1 Direct plating method (*n*=270)

Historical specimens (cooked meat broths) were vortexed for ten seconds, prior to inoculation of 100 µL of isolate suspension onto CCEYL. Where specimens were too dry or viscous to pipette, an arbitrary inoculum of a one millilitre loop full of material was transferred to the agar. Inocula were streaked in order to isolate individual colonies, prior to incubation. Where positive growth was observed, individual colonies were sub-cultured to CBA and re-incubated. Where multiple colony morphologies were present, each type was sub-cultured to CBA for further differentiation by PCR ribotyping (3.3.2.1). *C. difficile* colonies were identified through their grey, feathery appearance and characteristic horse-manure odour, or by Matrix-Assisted Laser-desorption Identification – Time of Flight Mass Spectrometry (MALDI-TOF); (Bruker Daltonik, Germany); see 3.3.1.4. Recovery batches were controlled using a positive growth control plate, consisting of 10 µL of PCR ribotype 001 spore suspension, created as previously described (699), streaked onto CCEYL agar. A CCEYL plate streaked with a sterile loop acted as a negative control. A CCEYL settle plate was left on the bench during culture preparations, representing an environmental control. All control agars were incubated alongside test cultures. Recovered *C. difficile* isolates were assigned a unique laboratory identification number (e.g. JV01) and retained in duplicate (-20°C), in 1 mL aliquots of 17% glycerol nutrient broth.

3.3.1.3.2 Broth enrichment (*n*=20)

Schaedlers anaerobic broth was prepared according to the manufacturer's instructions and supplemented with lysozyme (5 mg/L) post autoclaving. Twenty millilitre aliquots were pre-reduced overnight in an anaerobic atmosphere to remove any oxygen. Original cooked meat broth specimens were vortexed for ten seconds with 1 mL transferred into a sterile universal, containing 1 mL of ethanol/water (50% v/v). Samples were shaken for five seconds and left for one hour at ambient temperature (alcohol shock). A 1 mL aliquot of the alcohol-shocked sample was transferred into the pre-reduced Schaedlers broth and shaken for ten seconds, prior to incubation. CCEYL plates were inoculated with 100 µL of the broth cultures, streaked for individual colonies and incubated. A pre-reduced Schaedlers broth inoculated with 10 µL *C. difficile* spore suspension acted as a positive experimental control. An uninoculated broth was incubated alongside test samples, as a negative growth control. A further uninoculated broth with the lid removed, located alongside test plates for the duration of testing, acted as an environmental control. All control plates were incubated as previously described; (3.3.1.3.1).

3.3.1.3.3 Broth enrichment with multiple germinants (*n*=739)

The method was as described in 3.3.1.3.2 (excluding the ethanol shock stage), with the addition of either 4 g/L (0.4%) glycine and 1 g/L (0.1%) sodium taurocholate; or 40 (4%) and 10 g/L (1%) to the Schaedlers broth, prior to autoclaving. Positive and negative controls were as previously described (3.3.1.3.2).

3.3.1.3.4 Broth enrichment – whole sample method (*n*=389)

Schaedlers broth was prepared at double the manufacturer's strength recommendation and were supplemented with the same concentration combinations of glycine and sodium taurocholate as previously; (3.3.1.3.3). (Broth enrichments using spore germinants were evaluated in Chapter Two. Based on the conclusions of this work regarding the potential inhibitory effects of high concentrations of glycine, a modified version of this method was also performed with glycine supplemented at 4 g/L in conjunction with 10 g/L taurocholate). The entire original cooked meat broth specimen (~20 mL) was added to a 100 mL flask containing 20 mL broth and agitated prior to incubation. Enriched cultures were plated and incubated as previously described; (3.3.1.3.2). Positive and negative controls were created as previously (3.3.1.3.2), using double-strength Schaedlers broth.

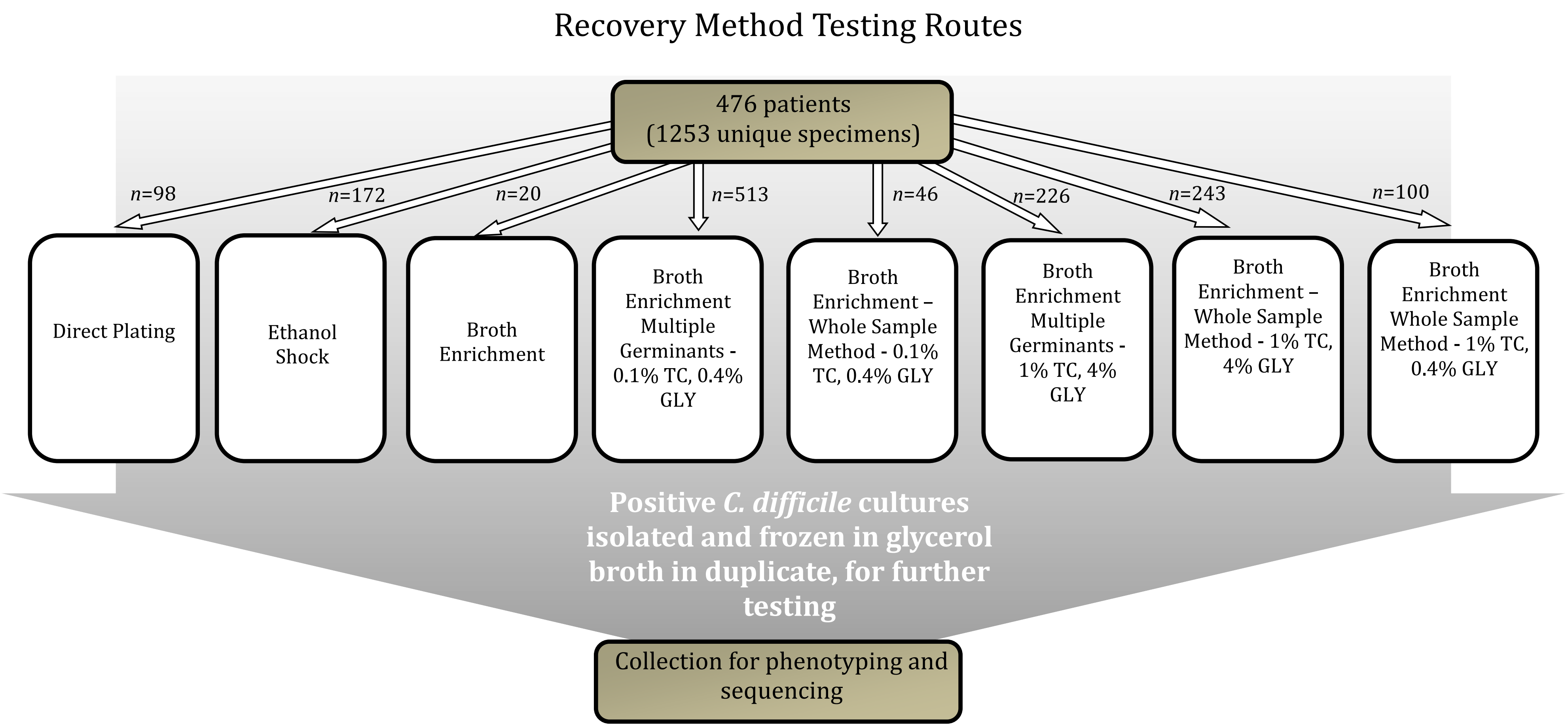


Figure 15:* *Germination and recovery methods for C. difficile recovery from the historical collection (1980-86). *Individual samples were subjected to multiple recovery methods. Specimens were not tested if C. difficile was isolated from another sample associated with the same patient. n sizes for each methodology are totals from multiple test batches. GLY – glycine, TC – taurocholate.*

3.3.1.4 Confirmation of *C. difficile* identification by MALDI-TOF

MALDI-TOF was performed for the rapid identification and confirmation of *C. difficile*. Principally, a UV-absorbing matrix assists the ionisation of biomolecules by laser excitation and subsequent separation based on charge. Electropherogram peaks determine standardised molecular weight fragments that can be compared against a database on known profiles (700). Identification was carried out using single colonies of fresh growth (24-48 hours) from non-selective agar (CBA). Colonies were emulsified onto a clean MALDI-TOF plate, overlaid with 1 µL of matrix, air dried and analysed on a MALDI Biotyper (Bruker Daltonik, Germany). Tests with a “score-value” of >2.0 were accepted as species designation, whilst a value >1.7, confirms the genus only. Specimens with scores less than this cut-off were re-tested to acquire an organism classification.

3.3.2 *C. difficile* strain characterisation

Recovered *C. difficile* isolates were subjected to an array of phenotypic and genotypic typing methods in order to elucidate individual strain characteristics; (Figure 17).

3.3.2.1 PCR ribotyping

C. difficile isolates were genotyped by the CDRN; based on the methods of Stubbs *et al*. (367), with the addition of capillary electrophoresis. The CDRN is the UK’s national typing service for *C. difficile*, consisting of eight regional laboratories, including a reference centre in Leeds, UK. Providing surveillance for the mandatory reporting of this pathogen, the CDRN currently tests over 8,000 specimens per year (363). Briefly, single colonies from non-selective agar (CBA) were emulsified in sterile water (McFarland 0.5) and loaded into an extraction block for automated DNA extraction by a QIAxtractor (Qiagen, Germany). Extracts were digested and specific 16S rRNA regions amplified by PCR. Subsequent amplicons were separated by fragment length via capillary electrophoresis (ABI Genetic Analyser 3130xl; Applied Biosystems, USA) and peak data assigned by GeneMapper software v4.1 (Applied Biosystems, USA). Electropherograms were compared against the CDRN database using BioNumerics software (701) (Applied-Maths, USA) and ribotypes were designated.

3.3.2.2 Inferred multi-locus sequence type

Next generation sequence read data (FASTQ format) was obtained as described in Chapter Four and was used to generate an inferred MLST sequence type for one strain (JV59), due to its novel ribotype determination. EnteroBase Clostridiodes v1.1.2 (702) was used to assemble raw Illumina reads and determine MLST sequence type, based on the seven housekeeping loci described by Griffiths *et al* (375).

3.3.2.3 Determination of *C. difficile* toxin status by cell-cytotoxicity assay

Forty-eight hour growth of *C. difficile* colonies on CCEYL agar was transferred into 4 mL pre-reduced BHI broth and incubated. After pipette mixing, a 1 mL aliquot of each broth culture was transferred into sterile Eppendorf tubes and sedimented by centrifugation for 10 minutes at 12,000 g. Vero cell cultures (African Green Monkey Kidney cells; Sigma-Aldrich, USA) were diluted (2:18 mL) in Dulbecco's modified eagle's medium supplemented with 10% new born calf serum (Gibco, Life Technologies, USA), 1% antibiotic/antimycotic solution (Sigma-Aldrich, USA) and 1% ʟ-glutamine (Sigma-Aldrich, USA). 180 µL was aliquoted into 96-well microtitre trays (Sigma-Aldrich, USA) and incubated at 37°C for 48 hours in a 5% CO_2 cabinet (Panasonic, Japan). All preparation and passage of Vero cell lines was carried out in a laminar flow hood (Holten LaminAir; Holten, USA). Confluency of Vero cell monolayers were confirmed via inverted microscopy (Leica DM IL, Germany). For each test sample, two wells were inoculated with 20 µL of test supernatant, before dilution (1:10) in two further wells. The addition of 20 µL of *Clostridium sordellii* anti-toxin to one set of wells, acted as a neutralising control. Inoculated trays were incubated in 5% CO_2 at 37°C. Cells were examined after 24 hours and results were confirmed 48 hours post inoculation. Positive results were assigned with the identification of >50% rounding of Vero cells and neutralisation (no cytopathic effect) in the corresponding antitoxin well, indicating the presence and action of *C. difficile* toxin; (Figure 16).

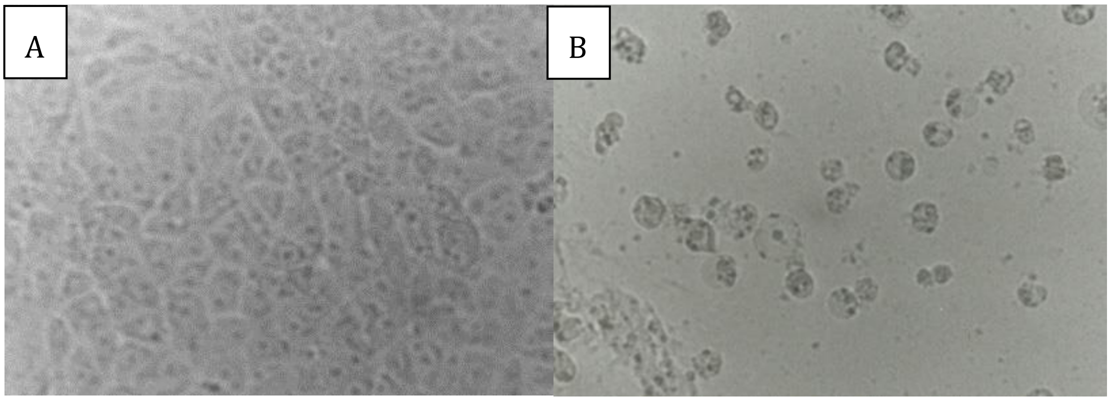


Figure 16: Vero cell response to cytopathic effect of C. difficile toxin as viewed under inverted microscopy. *A - Confluent growth of living Vero cell monolayer. B – Cell rounding with deterioration of the cell monolayer.*

3.3.3 Agar incorporated minimum inhibitory concentration testing

Wilkins Chalgren based agar incorporated susceptibility testing of the historical *C. difficile* isolates was performed in accordance with previous studies (187, 188). A panel of 16 comparator antimicrobials; vancomycin (VAN), metronidazole (MTZ), rifampicin (RIF), fidaxomicin (FDX), moxifloxacin (MXF), clindamycin (CLI), imipenem (IPM), chloramphenicol (CHL), tigecycline (TGC), linezolid (LZD), ciprofloxacin (CIP), piperacillin/tazobactam (TZP), ceftriaxone (CRO), amoxicillin (AMX), tetracycline (TET) and erythromycin (ERY) was investigated. Briefly, antibiotic dilution series were created (Table 7), with 2 mL of each added to 18 mL molten Wilkins Chalgren anaerobe agar and mixed. Wilkins Chalgren agar was used at a concentration higher than the manufacturer's recommendation, with 10% more agar powder, to account for the additional diluent from the antibiotic solutions. Overnight Schaedlers broth cultures were diluted (1:10) in sterile saline and added to an inoculation block. A multi-point inoculator was used to transfer 1 μL of diluted cultures (~1 x 10^4 CFU) onto antibiotic-incorporated agar of doubling concentrations, which were subsequently incubated anaerobically for 48 hours. Minimum inhibitory concentrations were defined as the lowest concentration at which growth was markedly inhibited. All antibiotic concentrations were tested in duplicate. The panel of antimicrobial compounds were selected to span a range of antibiotic classes, including the standard treatment options for *C. difficile*. Control strains were tested across all batches to evaluate quality and consistency of results; (Table 6).

Organism	**Strain Identifier**	**Initial culture conditions (°C, hours)**	**Culture media**
C. difficile	ATCC 700057	Anaerobic, 37, 48	CCEYL
C. difficile	E4 (PCR Ribotype 010)	Anaerobic, 37, 48	CCEYL
Bacteroides fragilis	ATCC 25285	Anaerobic, 37, 48	CBA
Staphylococcus aureus	ATCC 29213	Aerobic, 37, 24	CBA
Enterococcus faecalis	ATCC 29212	Aerobic, 37, 24	CBA

Table 6: List of control strains for susceptibility testing. *CCEYL – cycloserine-cefoxitin egg yolk agar with lysozyme, CBA – Columbia blood agar, ATCC – American Type Culture Collection.*

Antimicrobial Agent	Supplier	Antimicrobial Class	MIC range (mg/L)	Solvent	Diluent	Stock Conc^{n}	Powder Quantity (mg)	Volume of solvent	H_2O dilution to achieve working stock solution
Vancomycin	Acros Organics	Glycopeptide	0.125-32	Water	Water	6400mg/L	32	5 mL	1 in 10 (640 mg/L)
Metronidazole	Fluka Biochemika	Nitroimidazole	0.125-32	DMSO	Water	6400mg/L	32	5 mL	1 in 10 (640 mg/L)
Rifampicin	Sigma-Aldrich	Rifamycin	0.001-16	DMSO	Water	3200mg/L	16	5 mL	1 in 10 (320 mg/L)
Fidaxomicin	Astellas Pharma	Macrolide	0.002-4	DMSO	10% DMSO	800mg/L	4	5 mL	1 in 10 (80 mg/L)
Moxifloxacin	Cayman Chemical Company	Fluoroquinolone	0.125-64	Water	Water	12800mg/L	32	2.5 mL	1 in 10 (1280 mg/L)
Clindamycin	Santa Cruz Biotechnology	Lincosamides	0.125-64	Water	Water	12800mg/L	32	2.5 mL	1 in 10 (1280 mg/L)
Imipenem	Merck Sharp & Dohme	Carbapenem	0.125-64	1M MOPS	Water	12800mg/L	32	2.5 mL	1 in 10 (1280 mg/L)
Chloramphenicol	Sigma-Aldrich	Chloramphenicol	1-256	Ethanol	Water	51200mg/L	256	5 mL	1 in 10 (5120 mg/L)
Tigecycline	Pfizer	Tetracycline	0.03-1	Water	Water	800mg/L	4	20 mL	1 in 10 (80 mg/L)
Linezolid	SYNkinase	Oxazolidinone	0.25-16	DMSO	Water	3200mg/L	16	5 mL	1 in 10 (320 mg/L)
Ciprofloxacin	Fluka Biochemika	Fluoroquinolone	1-64	DMSO	Water	12800mg/L	32	2.5 mL	1 in 10 (1280 mg/L)
Piperacillin/ Tazobactam (8:1)	Sandoz	Beta-lactam	0.25-64	Ethanol	Water	12800mg/L	64	5 mL	1 in 10 (12800 mg/L)
Ceftriaxone	Sigma-Aldrich	Cephalosporin	4-64	Water	Water	12800mg/L	64	5 mL	1 in 10 (1280 mg/L)
Amoxicillin	Sigma-Aldrich	Beta-lactam	0.125-16	DMSO	Water	3200mg/L	16	5 mL	1 in 10 (320 mg/L)
Tetracycline	Alfa Aesar	Tetracycline	0.015-32	Water	Water	6400mg/L	32	5 mL	1 in 10 (6400 mg/L)
Erythromycin	Cayman Chemical Company	Macrolide	4-256	Ethanol	Water	51200mg/L	128	2.5 mL	1 in 10 (5120 mg/L)

Table 7: Preparation of antimicrobial solutions for agar incorporation, minimum inhibitory concentration determination.

3.3.4 Multi-Locus Variable number tandem repeat Analysis

Multi-locus variable number tandem repeat analysis was carried out using the enhanced fingerprinting service of the CDRN (371). MLVA utilises polymorphisms in the number of tandem repeating sequences across several well characterised loci. PCR amplification of these regions allow charge separation of fragment size/repeat length and subsequent profile comparison. Briefly, 2 µL of DNA extract from the PCR ribotyping process was added to a 96-well PCR plate containing 18 µL amplification mix (10 µL HotstarTaq *Plus* Mastermix, 0.5 µL of two oligonucleotide forward and reverse primer pairs, 1.2 µL $MgCl_2$, 5.8 µL water); (Qiagen). The plate was foil sealed and amplified through 35 PCR cycles with a Veriti™ thermal cycler (Applied Biosystems, USA). Amplimer fragments were separated via capillary electrophoresis (ABI Genetic Analyser 3130x) and peak size identified in GeneMapper software v4.1. Fragment sizes were recalculated to represent numbers of tandem-repeats and compared via dendrogram in the BioNumerics software (701).

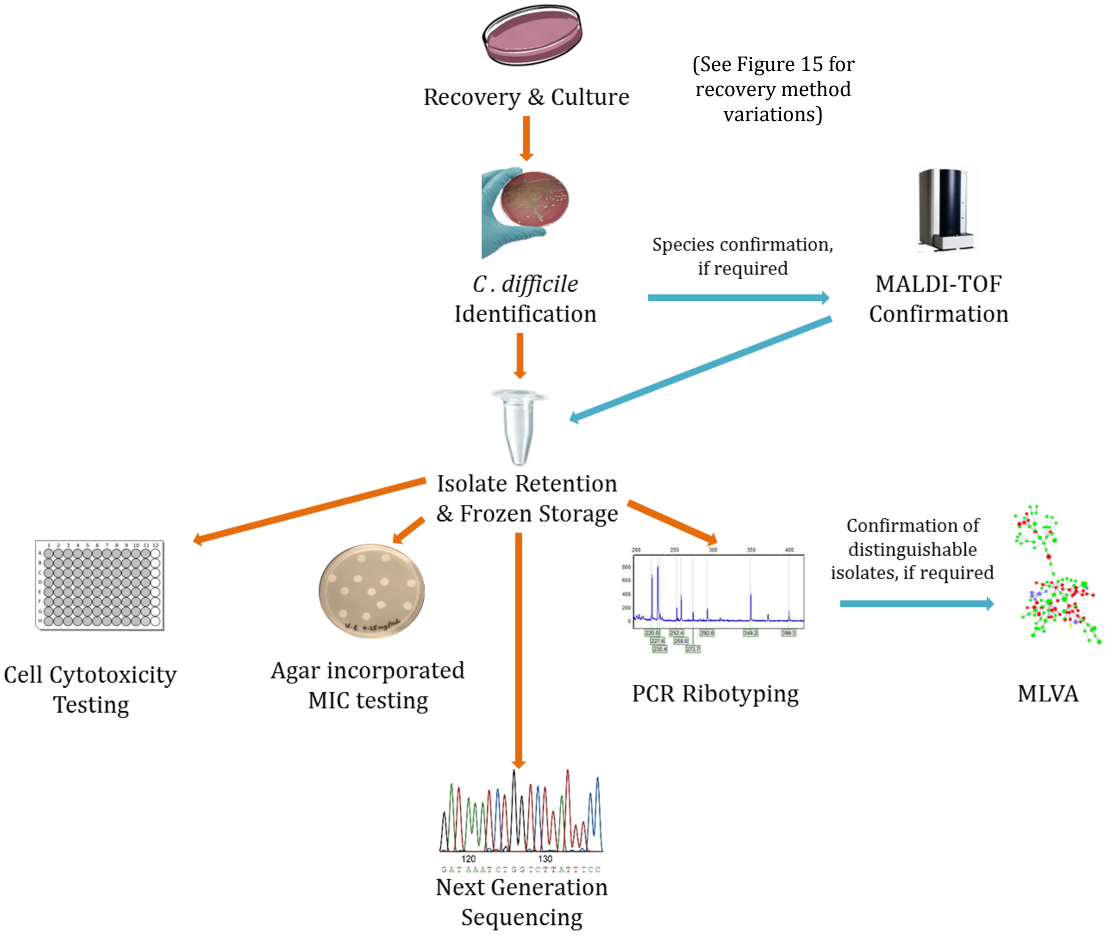


Figure 17: Identification, phenotypic and genotypic characterisation workflow.

Orange arrows represent workflow, blue arrows additional confirmatory steps. MLVA – multi-locus variable number tandem repeat analysis, MIC – minimum inhibitory concentration.

3.3.5 Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics v.21.0.0.1. MICs for each test antimicrobial were compared using a two-tailed Mann-Whitney test. *P* values <0.0002 were classed as extremely significant, whilst *p* values <0.01 were highly significant and *p*<0.015 were defined as significant.

3.4 Results

3.4.1 Isolation and recovery of *C. difficile*

In addition to clinical *C. difficile* isolates, a large proportion of specimens consisted of other clostridial species and multiple replicates from the same patients, together with several zoonotic and environmental isolates. Specimens were received in an array of conditions, with many in a state of putrefaction or overgrown with fungal growth; (Figure 18). Several laboratory note books accompanied the isolates, although correlation of data and sample proved difficult, due to fading and missing labels or an apparent absence of any demographic data. To the best of our knowledge these specimens remained untouched, post isolation and were stored at ambient temperature.

Figure 18: Photographic representation of the variable states of the historical isolates. *From left to right; complete putrefaction, through to visible fungal/bacterial growth and desiccation.*

From 1,253 unique specimens, associated with 476 patients, a total of 89 *C. difficile* isolates were cultured. Fourteen strains were discounted as repeat isolations of the same PCR ribotype from samples connected with the same patients. Therefore, 75 distinct strains were identified from 64 different patients and progressed for phenotypic and molecular characterisation. A total of 117 specimens were not tested due to the recovery of *C. difficile* from previous samples associated with the same patient.

Recovery with direct plating and ethanol shock methods was low, 5% and 1.2% respectively, whilst simple enrichment broth did not increase the yield (5%). Supplementation of broths with germinants in low concentrations generated a slight increase in strain recovery (8%), whilst testing of whole sample volumes offered no enhancement in yield (6.5%). A gradual improvement in recovery numbers was observed with broths containing an elevated taurocholate concentration of 1% (up to 13.5%); (Figure 19).

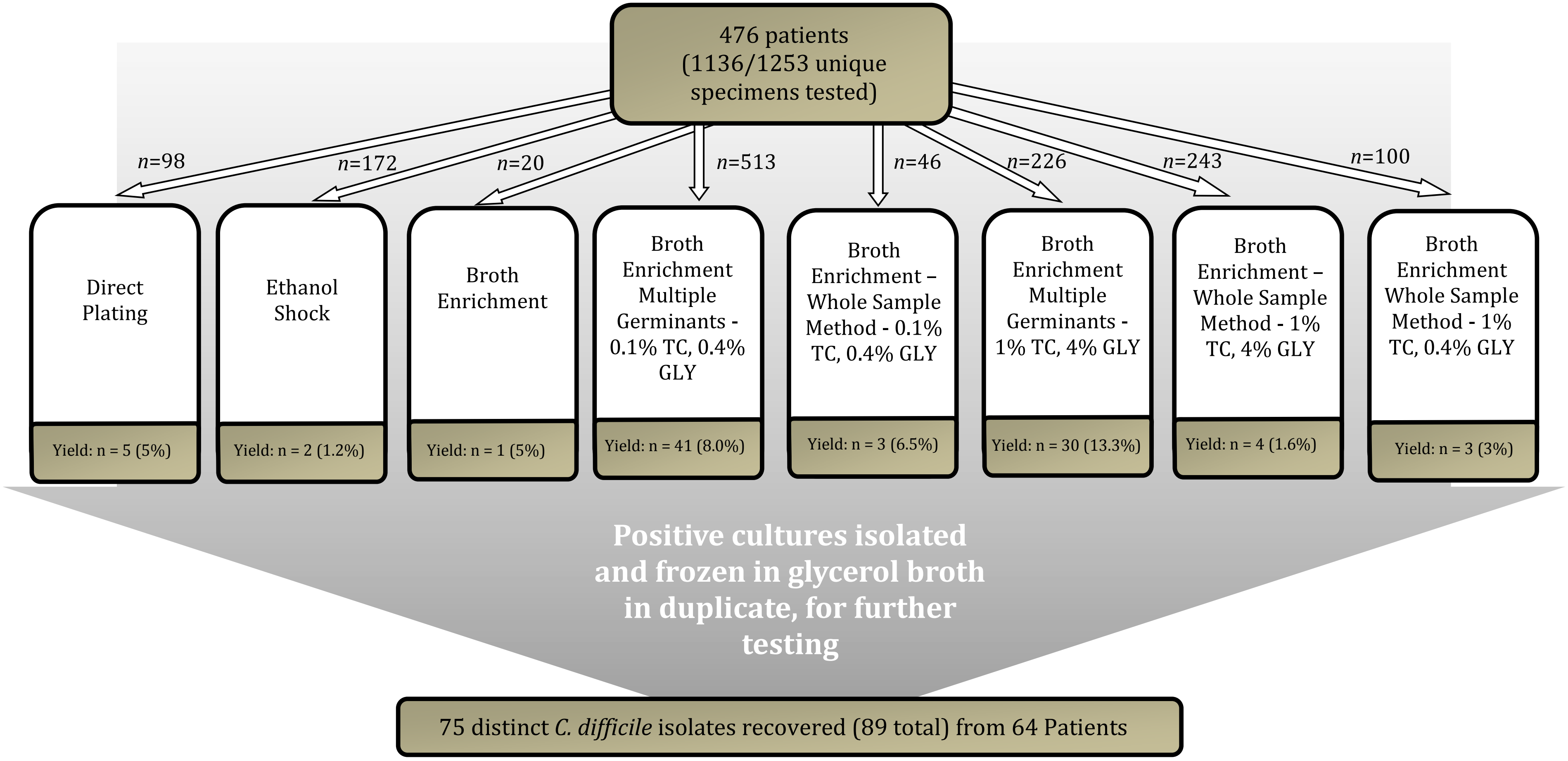


Figure 19: Total yields of historical (1980-86) C. difficile obtained from individual germination and recovery methods. *Individual samples were subjected to multiple recovery methods. Test numbers include isolates recovered in duplicate from the same sample by different isolation methodologies. n sizes for each methodology are totals from multiple test batches. GLY – glycine, TC – taurocholate.*

3.4.2 PCR ribotype and toxin status

Twenty-six known and one previously unobserved PCR ribotype were detected. Ribotype prevalence was dominated by strains 015 and 020, which equated to 21.3% and 17.3% of the total, respectively. Ribotype 001 was isolated on six occasions, whilst a further nine ribotypes were recovered in multiple instances; two (*n=4*), three (*n=3*) and four (*n=2*). Single instances of fifteen other ribotypes were observed; (Table 8).

Hyper-virulent ribotype 027 was recovered on three occasions from specimens dating back to 1981, 1983 and 1986. Two instances of the binary toxin producing 078 strain were observed, in addition to noted MDR ribotypes 012 (*n*=4) and 017 (*n*=3). One strain matched with no previous ribotyping profiles and was subsequently designated the nomenclature, PCR ribotype 862 by the CDRN. Isolates were recovered from specimens dated between 1980 and 1986 with the majority originating from between 1981 and 1983; (95%). *C. difficile* was isolated from a total of 64 patients, with six patients linked to multiple ribotypes; five exhibited two strains, whilst one harboured seven (001, 014, 015, 027, 041, 078 and 200).

The majority of strains demonstrated cell cytotoxicity (96%), with only three ribotypes (4%) identified as toxin negative via CCNA; 010, 033 and novel ribotype 862.

3.4.2.1 Inferred MLST typing

Isolate JV59 (ribotype 862) was defined as multi-locus sequence type 337.

	PCR Ribotype	Isolates (*n*)	% Total	CCNA	Original Specimen Collection Dates
	015	16	21.3	+	1981 - 1984
	020	13	17.3	+	1980 - 1983
	001	6	8.0	+	1981 - 1983
	012	4	5.3	+	1981 - 1982
	014	4	5.3	+	1981 - 1983
	027	3	4.0	+	1981; 1983; 1986
	002	3	4.0	+	1981; 1981; 1983
	017	3	4.0	+	1981; 1982; 1982
	078	2	2.7	+	1981; 1983
	070	2	2.7	+	1980; 1983
	200	2	2.7	+	1981; 1982
	061	2	2.7	+	1983; 1983
	220	1	1.3	+	1982
	056	1	1.3	+	1983
	103	1	1.3	+	1983
	137	1	1.3	+	1983
	041	1	1.3	+	1981
	626	1	1.3	+	1983
	341	1	1.3	+	1983
	032	1	1.3	+	1981
	862	1	1.3	-	1983
	619	1	1.3	+	1983
	033	1	1.3	-	1983
	242	1	1.3	+	1981
	003	1	1.3	+	1986
	023	1	1.3	+	1983
	010	1	1.3	-	1982
Total	**27**	**75**	**100.0**		

Table 8: PCR ribotype prevalence and cell cytotoxicity status amongst UK historical C. difficile isolate collection (1980-86).

CCNA - cell cytotoxicity neutralisation assay.

3.4.3 Antimicrobial susceptibilities of UK *C. difficile* isolates (1980-1986)

The distribution of MICs by compound is shown in Table 10, whilst statistical analyses for each antimicrobial agent are displayed in Table 9. Individual isolate susceptibility patterns are reported in the appendix.

3.4.3.1 Antimicrobial resistance breakpoint analysis

Breakpoints were defined based on the U.S. Clinical & Laboratory Standards Institute (CLSI) (499) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (703) figures; or from previous surveillance reports (201, 225, 444, 480, 704) where no data for *C. difficile* existed. These are outlined in Table 11.

All isolates were sensitive to both FDX and MTZ, with the majority also sensitive to VAN (94.7%). Four isolates (5.3%) demonstrated intermediate resistance to VAN at 4 mg/L, with two of these identified as PCR ribotype 001. MXF susceptibilities were bimodal, seven isolates (9.3%) exhibited resistance (16-32 mg/L) with the remainder susceptible (≤2 mg/L). Less than half of isolates (44.0%) were susceptible to CLI (≤2 mg/L). CIP resistance (>8 mg/L) was universal, and evidence of resistance was established in IPM (1.3%), CHL (5.3%), LZD (5.3%), CRO (12.0%), TET (9.3%) and ERY (16.0%); (Figure 20). No isolates revealed resistance to TGC, AMX, RIF or TZP; (Table 11).

Five (71.4%) of the MXF resistant isolates were PCR ribotype 001 (*n*=2) or 027 (*n*=3), with single instances of ribotype 041 and 200 demonstrating the phenotype (Table 12). PCR ribotypes 012, 015 and 078 were the only strains to demonstrate resistance to TET or LZD. The two most prevalent PCR ribotypes 020 and 015 were susceptible to most agents, only indicating resistance to CIP and CLI, in 100% and 36.0% of isolates respectively.

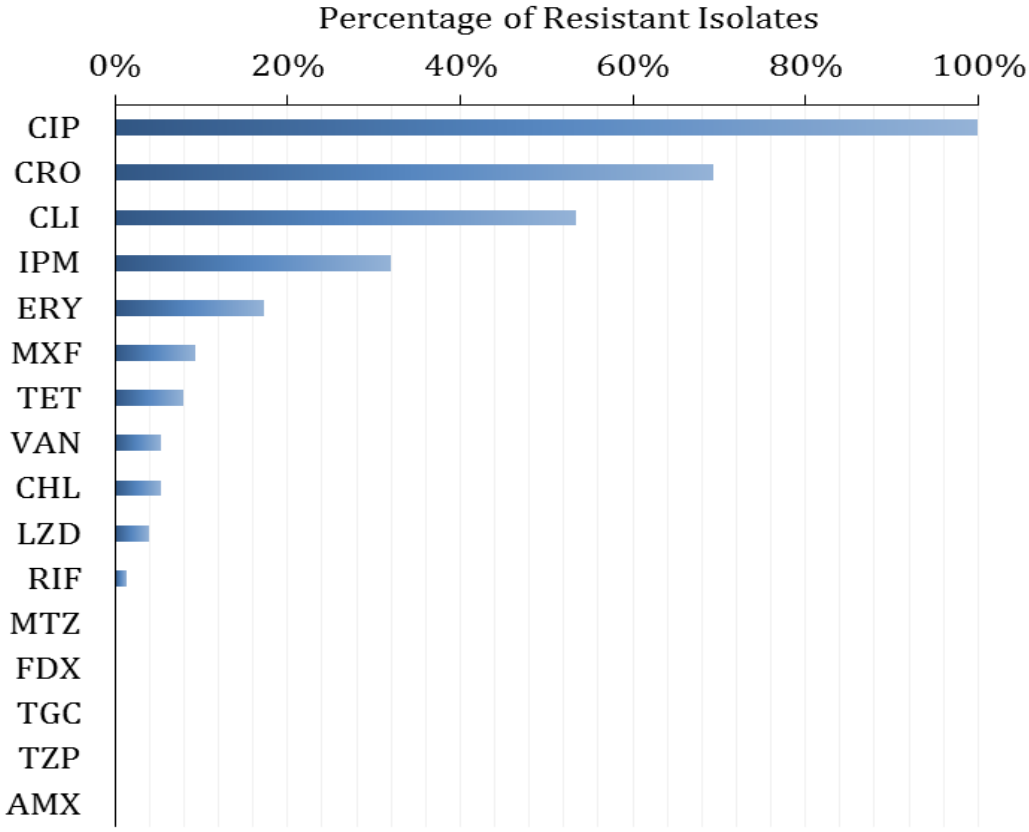


Figure 20: Percentage of historical C. difficile isolates (1980-86) resistant (including intermediate resistance) to a panel of 16 antimicrobials.

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.

Antimicrobial Compound	**Historic UK Isolates (1980-1986)** *n=75 (unless stated)*			
	MIC50 (mg/L)	**MIC90 (mg/L)**	**Geometric $\bar{x}$ (mg/L)**	**Range (mg/L)**
VAN	0.5	1	0.704	0.25 - 4
MTZ	0.125	0.25	0.171	0.06 – 1
RIF	0.001	0.002	0.001	0.001 – 1
FDX	0.03	0.06	0.028	0.004 – 0.125
MXF	1	2	1.617	0.125 – 32
CLI	4	32	4.000	0.125 – >64
IPM	4	8	4.553	2 – 16
CHL	4	8	4.902	2 – 64
TGC	0.03	0.06	0.035	0.03 – 0.125
LZD	2	2	1.725	1 – 16
CIP	16	32	22.00	16 – >64
TZP *(n=42)*	8	8	6.672	4 – 16
CRO	32	64	27.60	16 – 64
AMX	1	2	0.920	0.5 – 2
TET	0.06	0.125	0.104	0.015 – >32
ERY	<4	>256	7.926	<4 – >256

Table 9: Antimicrobial susceptibility data analysis from 75 PCR ribotype UK C. difficile (1980-1986) isolates.

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.

Antimicrobial Compound *n=75* *(unless stated)*		**Number of *C. difficile* isolates with minimum inhibitory concentration (MIC) mg/L**																	
		0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	>256
VAN										2	44	23	2	4					
MTZ								4	45	19	2	5							
RIF		48	26									1							
FDX				3	7	19	20	20	6										
MXF									1	1		40	26			4	3		
CLI									2	2	1	8	22	12	13	6	2	7 *[1]	
IPM													11	40	23	1			
CHL													4	57	9	1	1	3	
TGC							62	10	3										
LZD												27	44		1	3			
CIP															1	44	23	7 *[2]	
TZP (*n=42*)														14	25	3			
CRO														1		22	43	9	
AMX											20	44	11						
TET						6	10	27	25					2	1		4 *[3]		
ERY														62 *[4]		1			12

Table 10: Distribution of historical (1980-86) C. difficile isolate MICs by antimicrobial compound.

*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. *[1] 6 isolates >64, *[2] all isolates >64, *[3] 2 isolates >32, *[4] all isolates <4. Line graphs denote the distribution of isolate MICs, from low (left) to high concentrations (right).*

n=75 (unless stated) **Antimicrobial Compound**	**MIC Interpretive Criteria (mg/L)**			**Susceptible** (*n*)	**Intermediate** (*n*)	**Resistant** (*n*)
	S	**I**	**R**			
VAN^{1}	≤2	4	≥8	71 (94.7%)	4 (5.3%)	0
MTZ^{1}	≤2	4	≥8	75 (100%)	0	0
RIF^{1}	≤0.004	0.008-16	≥16	74 (98.7%)	1 (1.3%)	0
FDX^{1}	≤0.5	1	*	75 (100%)	0	-
MXF^{1}	≤2	4	≥8	68 (90.7%)	0	7 (9.3%)
CLI^{1}	≤2	4	≥8	35 (46.7%)	12 (16.0%)	28 (37.3%)
IPM^{1}	≤4	8	≥16	51 (68.0%)	23 (30.7%)	1 (1.3%)
CHL^{1}	≤8	16	≥32	70 (93.3%)	1 (1.3%)	4 (5.3%)
TGC^{1}	≤0.125	0.25	*	75 (100%)	0	-
LZD^{2}	≤2	4	≥8	71 (94.7%)	0	4 (5.3%)
CIP$^{3, 4}$	≤2	4	≥8	0	0	75 (100%)
TZP$^{3, 4}$ (*n=42*)	≤32	64	≥128	42 (100%)	0	0
CRO^{2}	≤16	32	≥64	23 (30.6%)	43 (57.4%)	9 (12.0%)
AMX^{3}	≤2	4	≥8	75 (100%)	0	0
TET^{5}	≤0.5	1	≥4	68 (90.7%)	0	7 (9.3%)
ERY^{5}	≤0.25	0.5	≥2	63 (84.0%)	0	12 (16.0%)

Table 11: Breakpoint analysis of UK C. difficile isolates (1980-1986) against a panel of 16 antibiotics.

*Breakpoints as defined by [1] Freeman et al. (2015a) based on the U.S. Clinical & Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST); or existing publications: [2] Freeman et al. (2015b); [3] Pirs et al. (2013); [4] Dong et al. (2013); [5] Spigaglia et al. (2011). * No resistant breakpoint defined. 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.*

	PCR Ribotype (*n*=)																					
	015 (16)		020 (13)		001 (6)		012 (4)		014 (4)		027 (3)		002 (3)		017 (3)		078 (2)		200 (2)		Other (19)	
	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
VAN	100	-	100	-	66.7	33.3	100	-	100	-	100	-	100	-	100	-	100	-	50.0	50.0	94.7	5.3
MTZ	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-
RIF	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	94.1	5.9
FDX	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-
MXF	100	-	100	-	67.0	33.0	100	-	100	-	-	100	100	-	100	-	100	-	50.0	50.0	94.7	5.3
CLI	43.7	56.3	38.5	61.5	16.7	83.3	25.0	75.0	75.0	25.0	66.7	33.3	-	100	66.7	33.3	-	100	50.0	50.0	68.4	31.6
IPM	68.8	31.2	92.3	7.7	66.7	33.3	50.0	50.0	100	-	-	100	100	-	-	100	50.0	50.0	-	100	73.7	26.3
CHL	93.7	6.3	100	-	100	-	75.0	25.0	100	-	100	-	100	-	100	-	-	100	100	-	94.7	5.3
TGC	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-
LZD	93.7	6.3	100	-	100	-	75.0	25.0	100	-	100	-	100	-	100	-	-	100	100	-	100	-
CIP	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100
TZP (*n*=42)	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-
CRO	-	100	53.8	46.2	16.7	83.3	-	100	100	-	-	100	-	100	66.7	33.3	100	-	-	100	36.8	63.2
AMX	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-
TET	93.7	6.3	100	-	100	-	-	100	100	-	100	-	100	-	100	-	-	100	100	-	100	-
ERY	100	-	100	-	66.7	33.3	50.0	50.0	75.0	25.0	-	100	100	-	66.7	33.3	100	-	50.0	50.0	84.2	15.8

Table 12: Antimicrobial resistance breakpoints of 75 historical (1980-86) C. difficile isolates by PCR ribotype.

*Breakpoint defined as in **Table 11**. S – Sensitive, R – Resistant (inclusive of intermediate resistance). 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.*

3.4.3.2 Multidrug resistance classification

Seventeen isolates (22.7%) demonstrated resistance to three or more antimicrobial agents, with half of these only classified as MDR due to CIP resistance. Eight isolates (10.7%) were resistant to three antibiotics, four (5.3%) to four and five (6.7%) to five compounds. Ten different combinations of antimicrobial resistance were demonstrated in the cohort, with three different antimicrobial permutations predominating; (TET, CLI, CIP, LZD & CHL; CLI, CIP, ERY, MXF & CRO; CIP and CLI, CIP & ERY); (Table 13). Combined ERY and CLI resistance was apparent in eight (47.1%) MDR isolates (10.7% of total), whilst LZD, CLI and CHL resistance was demonstrated in four (23.5%; 5.3% of total). No ERY resistance was associated with any of the latter isolates.

PCR ribotypes 012, 027 and 078 constituted 23.4% (*n*=4), 17.6% (*n*=3) and 11.8% (*n*=2) of all MDR strains, respectively. This represented all recovery instances of these PCR ribotypes. Single instances of ribotypes 001 and 017 were also resistant to multiple agents. Isolates from these five PCR ribotypes, comprised 64.7% of the total MDR population. Both ribotype 078 isolates displayed the same resistance pattern, demonstrating MICs above the breakpoints of CLI, CHL, LZD, CIP and TET, whilst other ribotypes with multiple isolates exhibited varying resistance profiles.

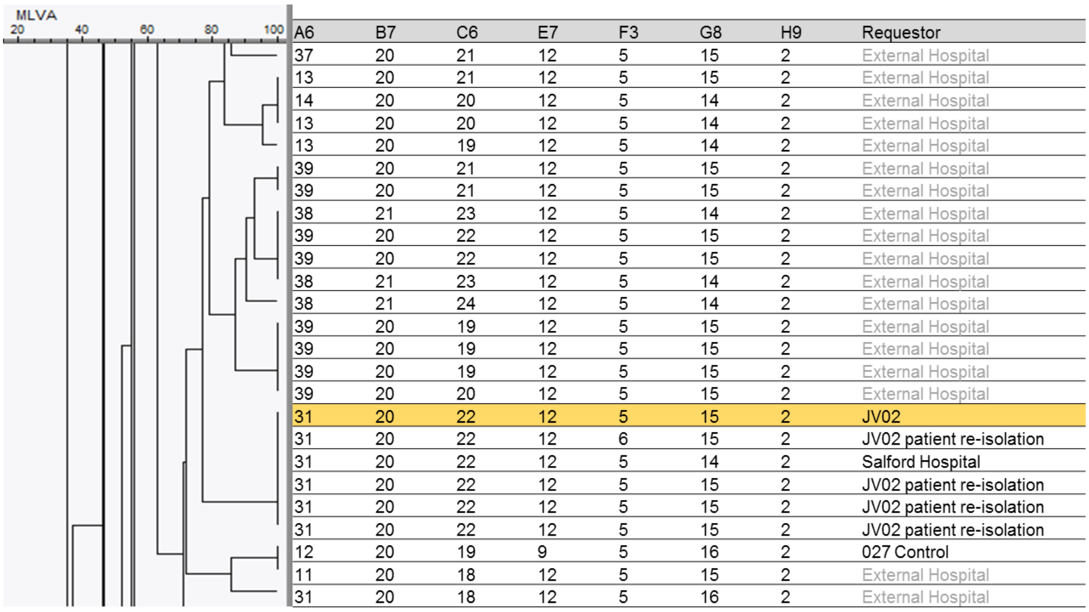
	Number of *C. difficile* isolates resistant to different antimicrobial combinations									
PCR ribotype (*n*=)	CLI, CIP, ERY	CLI, CIP, TET	CIP, TET, ERY	CIP, CRO, ERY	MXF, CLI, CIP, ERY	MXF, CIP, CRO, ERY	CLI, CIP, CRO, TET, ERY	MXF, CLI, CIP, CRO, ERY	CLI, CHL, LZD, CIP, TET	MXF, IPM, CIP, CRO, ERY
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 13: Characteristics of the multi-resistant isolates isolated from (1980-1986).

CLI - clindamycin, CIP – ciprofloxacin, MXF – moxifloxacin, CHL – chloramphenicol, LZD – linezolid, IPM – imipenem, CRO – ceftriaxone, TET – tetracycline, ERY – erythromycin. Breakpoints as defined in ***Table 11****. PCR – polymerase chain reaction.*

3.4.4 Further investigation of PCR ribotype 027 isolates from the historical collection by MLVA

The three recovered ribotype 027 isolates all displayed very similar susceptibility profiles, demonstrating resistance to five antimicrobials (Table 14). However, each isolate proved distinguishable from each other by MLVA, as well as from 633 comparator ribotype 027 strains, dating back to 2010.



A6	B7	C6	E7	F3	G8	H9	Requestor
37	20	21	12	5	15	2	External Hospital
13	20	21	12	5	15	2	External Hospital
14	20	20	12	5	14	2	External Hospital
13	20	20	12	5	14	2	External Hospital
13	20	19	12	5	14	2	External Hospital
39	20	21	12	5	15	2	External Hospital
39	20	21	12	5	15	2	External Hospital
38	21	23	12	5	14	2	External Hospital
39	20	22	12	5	15	2	External Hospital
39	20	22	12	5	15	2	External Hospital
38	21	23	12	5	14	2	External Hospital
38	21	24	12	5	14	2	External Hospital
39	20	19	12	5	15	2	External Hospital
39	20	19	12	5	15	2	External Hospital
39	20	19	12	5	15	2	External Hospital
39	20	20	12	5	15	2	External Hospital
31	20	22	12	5	15	2	JV02
31	20	22	12	6	15	2	JV02 patient re-isolation
31	20	22	12	5	14	2	Salford Hospital
31	20	22	12	5	15	2	JV02 patient re-isolation
31	20	22	12	5	15	2	JV02 patient re-isolation
31	20	22	12	5	15	2	JV02 patient re-isolation
12	20	19	9	5	16	2	027 Control
11	20	18	12	5	15	2	External Hospital
31	20	18	12	5	16	2	External Hospital

Figure 21: BioNumerics output for a MLVA comparison of the historical isolates and the entire CDRN reference database for PCR ribotype 027.

The highlighted data profile denotes isolate JV02. All other "JV02" data refers to strains recovered during re-isolation experiments from different specimens associated with the same patient. A6, B7, C6, E7, F3, G8 & H9 refer to primed, amplified sequences; the number designated refers to the number of tandem repeats at a specific locus. MLVA data is primarily used for cluster analysis, so comparing strains this way can only provide indicative information.

Minimum Inhibitory Concentration (mg/L)

JV ID	RT	VAN	MTZ	RIF	FDX	MXF	CLI	IPM	CHL	TIG	LZD	CIP	TZP	CRO	AMX	TET	ERY
JV02	027	0.5	1	0.002	0.06	32	8	8	4	0.03	1	>64	16	64	1	0.125	>256
JV73	027	0.5	1	0.001	0.06	16	2	8	4	0.06	1	>64	*nt*	64	2	0.125	>256
JV67	027	0.5	1	0.001	0.06	16	2	16	4	0.06	2	>64	*nt*	64	2	0.125	>256
JV14	078	0.5	0.125	0.001	0.004	1	>64	4	64	0.06	16	16	*nt*	16	0.5	4	<4
JV22	078	1	0.125	0.002	0.03	2	>64	8	64	0.06	16	16	*nt*	16	0.5	8	<4

Table 14: Susceptibilities of historical PCR ribotype 027 and 078 isolates against 16 comparator antimicrobials.

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. RT – ribotype, nt – not tested.

3.5 Discussion

3.5.1 Isolation and recovery of *C. difficile*

Due to the absence of improvement in recovery with increased taurocholate concentrations determined by the previous germination efficiency work (Chapter Two), and the high expense of taurocholate, lower concentrations (0.1%) were favoured for the attempted recovery of the majority of specimens. Furthermore, no recovery advantage was observed with their supplementation in solid agar. Therefore, a decision was made to proceed using Schaedlers anaerobic broth with 0.1% taurocholate/0.4% glycine and CCEYL agar plates for the majority of recovery efforts from the historical catalogue. Although CCEY with and without lysozyme addition demonstrated comparable spore recovery, due to its association with improved recovery of stressed, environmental spores (342), it was used here for maximum recovery of potentially damaged, aged spores.

Initial recovery attempts with 1% taurocholate demonstrated slightly elevated yields compared to 0.1% taurocholate broth supplementation (13.3% vs 8.0%). However, this may have been biased by repeat testing of the same specimens, where samples not recovered by one method were retested with another. Therefore, the sample recovery probability of subsequent tests was likely diminished and would impact on an observed recovery rate. It is hypothesized that, since the viability of spores in the specimens was unknown, there is a possibility that many samples tested did not contain any surviving spores. Thus, a reliable comparison of recovery methods for application with this specific collection is not necessarily achievable. Recreating problematic sample recovery is difficult and it is possible that many of these specimens did not contain any recoverable material prior to testing. Pilot testing using actual specimens produced inconsistent results (data not shown), highlighting the potential for low bacterial loads and stochastic variation. Therefore, caution must be taken when considering the findings from these recovery attempts, as it is likely that they do not necessarily translate to isolation from more consistent collections.

Although *C. difficile* was eventually isolated from 15.8% of patient cases, the attrition rate of this collection was high, with overall recovery attempts from each of the multiple patient specimens demonstrating an approximate seven percent success rate. No single approach demonstrated superiority over the rest, but based on the conclusions from Chapter Two, use of enrichment broths supplemented with low-level germinants was considered the most efficient method for the recovery of depleted levels of potentially "super-dormant" spores (645). Assessing all specimens with multiple recovery methodologies would be preferable to determine an optimal protocol, but was not feasible due to cost and time limitations. Since the aim was the recovery of as many strains as possible for further analyses, this was deemed unnecessary for the purposes of this study. Whilst, one research study suggested that pre-heating spores with sub-lethal temperatures facilitated the reactivation of dormant spores (647), a small pilot study performed here, as preliminary work to historical strain recovery (data not shown) demonstrated no advantage of this treatment on recovery from the historical collection. This was later supported by the findings from our group's work on heat exposure and germination (676), which indicated only detrimental effects at temperatures above 70°C. Consequently, heat pre-treatment was excluded from the recovery protocols.

3.5.2 PCR ribotype and toxin status

Strikingly, the prevalence of PCR ribotypes recovered from isolates originating between 1980-1986 corresponded closely with the most prevalent types isolated in 2015 by the CDRN (363). Each of the ten most common ribotypes recovered from the historical collection (Table 8) appeared in the equivalent top 15 list of modern circulating strains, whilst six (027, 001, 014, 002, 020 and 015) feature highly in European prevalence (118, 154, 385, 394). Although the historical collection is clearly dominated by ribotypes 015 and 020, the CDRN data reflects a more even apportioning of the most prevalent strains, with ribotypes 078 and 002 constituting a greater share. The considerable discrepancy in sample size is one explanation for these disparities; (*n*=75 vs *n*=7609). Nonetheless, the recovery of ribotype 002 isolates from the historical collection was not surprising, as this type has demonstrated a transitionary epidemiology over the last 40 years. Ribotype 002 prevalence in the late twentieth century has been reported as high as 15% of CDI cases (381). Decreasing in prominence in the wake of ribotype 027's success (384), 002 is once again a prevalent ribotype (363, 705). Currently reported as the primary cause of community-onset CDI (706, 707), ribotype 002 accounts for 13.5% of all UK cases (275). Interestingly, these correlate less with known CDI risk factors, such as age and antibiotic exposure (708). Emergent ribotypes 005 and 023 are not represented in the historic collection, further indicating their rise in prevalence in modern CDI epidemiological surveillance. Nonetheless, since the original sources of the historical specimens are not specifically known, the collection may be subject to geographic or sampling bias.

Epidemiological comparisons can be made with a study of 69 Scottish isolates from 1979-1986, which demonstrated dominance by ribotypes 002, 014 and 012, constituting 30% of all strains (381). These ribotypes were prevalent in the historical isolates recovered here, representing 15% of the total. Interestingly, in contrast to the present study, only single instances of ribotypes 015 and 001 were identified, whilst no ribotype 020 strains were reported. These ribotypes constituted a high proportion of isolates recovered in this study, which suggests potential bias attributed to localised outbreaks.

While the majority of isolates recovered represent those predominating in modern surveillance, single instances of uncommon PCR ribotypes were observed, including 137, 242, 341, 619 and 626, as well as the novel ribotype 862. These strains may have been superseded by ribotypes with more efficient growth, germination and virulence pathways, promoting their superior aetiological potential. This is demonstrated in CDRN surveillance reports; where particular strains proliferate, the occurrences of others diminish (363).

Further investigation of the previously unassigned ribotype 862, toxin negative isolate, JV59, assigned it to ST-337, part of the obscure clade C-II (2). This strain type is uncommon and generally associated with environmental samples (2), although it has been identified in one clinical report (709). Representing a novel toxinotype (XXXII), this sequence type harbours a unique genetic organisation of the PaLoc, featuring an absence of the *tcdA* gene and several SNPs in the *tcdB* gene (709). This rare finding in a clinical specimen may suggest an inefficiency associated with this atypical genetic assembly. Although this sequence type purportedly exhibits functional clostridial toxin B genes, isolate JV59 repeatedly demonstrated negative cytotoxicity results. This casts doubt as to whether this strain was the aetiology of the patient's symptoms or was purely colonising asymptomatically.

Although separated by approximately 35 years, the historical typing data revealed distinct similarities in populations with modern surveillance studies. While epidemiological investigations showed significant fluctuations in the prevalence of epidemic strains across several years (384, 417), these data suggest that there may be a homeostatic base line of strain distributions that is reverted to, as outbreaks of endemic or epidemic nosocomial strains subside.

Conversely, historical strain recovery could potentially represent the ribotypes that are the “fittest” for long term dormancy and survival, as opposed to the actual epidemiological spread of the time. Previously reported ribotype variability in spore tolerance to external stressors such as heat, pH and organic solvents (710, 711), in addition to differing sporulation and germination efficiencies (712) could potentially explain the recovery of ribotypes that reflect the current epidemiological composition. Nonetheless, since the recovery rate of this historical catalogue was low, an accurate distribution cannot be assumed, making holistic assessment more challenging.

Toxigenic strain dominance was as expected (96%), since specimens originated from patients with suspected CDI. However, the recovery of three non-toxigenic strains suggests that these patients were asymptomatically colonised by the isolated *C. difficile* strains and their diarrhoea was attributable to other undiagnosed aetiologies. Equally they may have been concomitantly colonised by a toxigenic strain, as has been previously described (713, 714). Although the latter explanation cannot be substantiated by the recovery results (i.e. no toxigenic strains were recovered from the patients from whom non-toxigenic strains were isolated), the high attrition rate of this isolate catalogue potentially masks this discovery. Interestingly, two less common, binary toxin gene carrying strains, 023 and 033 were both isolated in single instances.

3.5.2.1 Hyper-virulent PCR ribotype 027 recovery

The hyper-virulent PCR ribotype 027 has been associated with numerous international outbreaks, particularly at the beginning of the twenty-first century (78, 112, 383). Persisting as one of the most prevalent *C. difficile* strain types across Europe (215), ribotype 027 remains of prominent interest to researchers. The earliest example of a ribotype 027 strain recorded in the literature is CD196, an isolate recovered in France in 1985 (289). Two of the ribotype 027 isolates recovered from this historical collection, JV02 and JV73, were originally isolated from clinical specimens dating back to 1981 and 1983, respectively. Therefore, if the findings are true, these potentially represent the earliest PCR ribotype 027 isolates reported to date. As these isolates may be of significance in the understanding of outbreak development associated with this ribotype, further confidence was sought to ensure the findings were accurate.

Firstly, repeat attempts were made to consistently re-isolate the same strains from the original specimen tubes, as well as from other specimens associated with that patient (untouched in these investigations). Ribotype 027 strains were isolated on three occasions and compared to the original finding using MLVA. All subsequent strains recovered proved indistinguishable by this method (data not shown). For additional confidence in the findings, MLVA was also used to ensure that these historical 027 strains were distinct from 633 other 027 isolates analysed by the CDRN enhanced fingerprinting service, including the most commonly used laboratory control strain. No exact profile matches were observed for any of the historical isolates, indicating no direct links to any of the 633 strains processed for MLVA by the reference laboratory over the last eight years. Whilst both isolates JV67 and JV73 demonstrated distinctly different profiles from any other (>10 SNPs), all strains recovered from different specimens associated with the same patient, were indistinguishable (≤2 SNPs). One closely related strain from 2010 demonstrated a profile only 2 SNPs divergent from JV02 (Figure 21). While this would officially be classified as indistinguishable, since one of these polymorphisms was identified in the often highly conserved, G8 loci (376) the relatedness of these isolates maybe further questioned. Equally, when considering the demographic data, since this closest profile was generated in 2010, years before the historical collection even entered our laboratory is strongly suggestive of a distinction between the isolates. Similarly, it was highly unlikely that the related reference isolate had been cultured over the past ten years, remaining only in frozen storage. Whilst sufficient measures were imposed to restrict the possibilities of contamination, we cannot vouch for the period between specimen collection and receipt at our laboratory. Although this does not provide a definitive result, as MLVA is only directly appropriate for cluster analysis, it gives further confidence that the isolates recovered were not contaminants from this laboratory.

Interestingly, all three isolates exhibited MXF resistance (Table 14), where none had been previously reported in isolates dating prior to its introduction (112). Whilst this finding may be unexpected, if we consider the alternative hypothesis of contamination with a modern strain, it may seem more likely that all "questionable" strains would be genetically identical. Contaminating three individual specimens with distinct strains of the same ribotype seems highly unlikely. Since all 027 isolates were demonstrated as distinguishable from each other, this further refutes the theory of contamination.

3.5.2.2 Recovery of multiple ribotypes

Infection by multiple strains of *C. difficile* has been previously described, with rates reported as high as 7-16% of CDI cases (713, 715-718). In accordance with this rate, these data demonstrated that 9.3% of patients from which *C. difficile* was recovered were colonised with multiple strains. These may potentially occur due to recurrent infections and contact with heavily contaminated nosocomial environments.

The recovery of seven different strains from one patient presented a rare and interesting discovery. Nonetheless, one recent study reported a severely ill patient as suffering from eight relapse/reinfection episodes of CDI across a 13 month period (719). In this patient ten phenotypically diverse strains of six PCR ribotypes were recovered, with one instance of a mixed infection with three concurrent types. This case closely reflects the known circumstances of the patient harbouring multiple strains from the historical collection. There is a paucity of clinical data associated with any of the historical strains, but this patient was documented as suffering from chronic diarrhoea for a period of 18 months, treated with several antimicrobials. With this combination of antibiotic therapies and prolonged hospital stay, colonisation with multiple strains and development of multiple symptomatic recurrences is possible. This may be plausible, since the risk of secondary and tertiary reinfections has been demonstrated to become significantly compounded with each recurrent CDI episode (720). However, caution must be taken when directly comparing these rates with previous reports, as recovery issues in this study will have a high impact on the data. Although strict anti-contamination procedures were employed during the recent recoveries, the possibility of contamination events occurring prior to receipt of the specimens to our laboratory cannot be disregarded.

3.5.3 Antimicrobial susceptibilities of UK *C. difficile* isolates (1980-1986)

As expected, there was no evidence in this data set of resistance to the primary treatment drugs, MTZ and VAN. Equally, no resistance to the relatively recently licensed, FDX was observed. Since MTZ resistance was only first reported in 1981 (535) and is still scarce in contemporary surveillance studies (188, 385), the absence of reduced susceptibility to this agent was as anticipated. Similarly, no historical isolates exhibited resistance to VAN, with four strains demonstrating intermediate levels of reduced susceptibility. Nonetheless, VAN resistance remains rare in contemporary studies, with a large scale European surveillance study reporting resistance in only 0.87% of isolates (188).

RIF and FDX were the most active compounds, demonstrating geometric mean MICs of <0.001 and 0.028 mg/L, respectively. Whilst FDX resistance is scarce (118, 721), an estimated 11% of modern *C. difficile* isolates display resistance to RIF (498). However, large proportions of this resistance has been identified as highly concentrated in specific geographical regions, such as Italy or Czech Republic, where high levels of rifamycin consumption are reported (188).

As a fourth generation fluoroquinolone, MXF was not introduced until 1999, with resistance to the compound observed in *C. difficile* soon after (443). Although resistance to this compound was demonstrated in the pre-MXF, historical isolates, resistance to previous generations of quinolones have been demonstrated to impart cross-resistance due to identical target sites (438, 722). Nevertheless, all isolates indicated CIP resistance, but only seven translated this mechanism to a MXF resistant phenotype. Two out of six ribotype 001 isolates were MXF resistant, which was not too dissimilar to the findings of study of Scottish historical isolates by Taori *et al.* (50%) (381), suggesting that even in their infancy fluoroquinolones and associated resistance was potentially contributory to CDI onset. Whilst the striking observation of CIP resistance across all test isolates is not uncommon (198, 723, 724), these strains pre-dated the introduction of this antimicrobial (1987). This could potentially indicate the development of cross-resistance due to exposure to earlier compounds with similar structures, such as nalidixic acid. However, this drug had minimal use as a treatment for urinary tract infections (725) and therefore extensive selection seems improbable. A more plausible explanation would be that the majority of *C. difficile* genomes exhibit an intrinsic resistance to this agent. Further discussion of this finding is presented in Chapter Four, where the data is discussed in the context of the potential genetic mechanisms.

Resistance to ERY, CLI and MXF amongst more recent isolates has been reported as 49%, 65% and 40%, respectively (524). These far surpass the levels in this historical collection, which demonstrated 16%, 37.3% and 9% of isolates as resistant. The historical resistance rates support work by Ackermann *et al.* (521), who determined very similar resistance levels in ERY (18%) and CLI (30%) amongst isolates from 1986-95. They also demonstrated slightly lower findings in MXF (2%), which is probably exaggeratedly elevated in the historical collection by the inclusion of three fluoroquinolone resistant ribotype 027 strains. Nevertheless, Ackermann's research indicated a gradual decrease in susceptibility to each of these agents over three isolate groups, delineated by date.

MIC_{90} data from the Scottish cohort (1979-86) analysed by Taori *et al.* demonstrated comparable results with the historical strains for MTZ, CRO and MXF (381). Conversely, VAN, CLI and TET MICs were elevated above the present study. Adding further confidence to the findings, a study of Australian isolates from between 1979 and 1989 revealed similar susceptibilities to those observed in the present investigation (726). However, the number of isolates independently resistant to CLI and TET were elevated above the present study; (CLI) 100% vs 37.3% and (TET) 22% vs 9.3%. Interestingly they found no evidence of MXF resistance. Nevertheless, the predominance (68%) of toxinotype 0 and the small number of isolates tested for antimicrobial susceptibilities (*n*=9) may have contributed to this variance.

One of the earliest reports of *in vitro* susceptibilities of clinical *C. difficile* strains, described resistance levels of ERY, CLI and TET in pre-1980 strains, as 7%, 14% and 9% respectively (727). These correlate well with the findings of this historical collection, where resistance proportions are slightly higher (Table 11), perhaps due to differences in the age demographics between the cohorts. Furthermore the work by Dzink and Bartlett studied isolates from the USA, and although no ribotyping data was provided, we know from other surveillance that epidemiological differences are apparent. This may further contribute to the variance in antimicrobial susceptibilities. Nonetheless, these resistance rates are greatly reduced in comparison to those of modern collections across the world, where ERY and CLI resistance is reported as high as 100% and 82.7%, respectively (214, 394, 728).

3.5.3.1 Comparison of UK *C. difficile* antimicrobial susceptibility data between (1980-1986) and (2012-2016)

In order to assess the progression of antimicrobial susceptibilities from the early 1980s until the present day, the susceptibility data for 416 UK isolates from the *Clos*ER surveillance study (2012-2016) was used as a modern comparator set. Significant increases in geometric mean MICs between the historical and modern isolate collections were observed. All treatment compounds, MET, VAN and FDX, exhibited significant differences across the two panels ($p<0.0001$, $p<0.0002$ and $p<0.0001$ respectively). CDI pre-disposing agents, MXF and CLI demonstrated highly significant differences ($p=0.0025$ & $p=0.0053$; Table 15). All other test compounds also exhibited statistically significant increases in MIC; (Table 15). These clear statistical differences between the two time periods indicated the development of antimicrobial resistance in *C. difficile* across all classes.

All test compounds demonstrated an MIC_{50} increase of one doubling dilution from the historical (1980-1986) to the modern collections (2012-2016), with the exception of IPM and CHL; (Table 15). Since MIC_{50} is an indicator of the fundamental activity of an antibiotic on the total *C. difficile* population, this reflects a general trend for reduction in susceptibility to most antimicrobial classes. Due to the potential selection pressure exerted by these compounds over long exposure periods (approximately 35 years), these findings were not unexpected.

Although, these data are of value as a whole, the biasing impact of the genotypic distributions of these populations required consideration.

Antimicrobial Compound	**Historic UK Isolates (1980-1986)** *n=75*			***Clos*ER Study UK Isolates (2012-2016)** *n=416*				**Susceptibility Variance (Historic » *Clos*ER)** *(Doubling dilution increase)*		
	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)**	**Signif.***	**MIC$_{50}$ (mg/L)**	**MIC$_{90}$ (mg/L)**	**Geometric $\overline{x}$ (mg/L)**
VAN	0.5	1	0.704	1	2	0.839	$p<0.0002$	+0.5 (1)	+1 (1)	+0.135
MTZ	0.125	0.25	0.171	0.25	0.5	0.283	$p <0.0001$	+0.125 (1)	+0.25 (1)	+0.112
RIF	0.001	0.002	0.001	0.002	0.004	0.002	$p <0.0001$	+0.001 (1)	+0.002 (1)	+0.001
FDX	0.03	0.06	0.028	0.06	0.125	0.050	$p <0.0001$	+0.03 (1)	+0.06 (1)	+0.022
MXF	1	2	1.617	2	8	2.021	$p =0.0025$	+1 (1)	+6 (2)	+0.404
CLI	4	32	4.000	8	16	4.880	$p =0.0053$	+4 (1)	-16 (1)	+0.880
IPM	4	8	4.553	4	8	3.940	$p =0.0133$	0	0	-0.612
CHL	4	8	4.902	4	8	5.462	$p <0.0001$	0	0	+0.560
TGC	0.03	0.06	0.035	0.06	0.06	0.050	$p <0.0001$	+0.03 (1)	0	+0.015
LZD	2	2	1.725	*nt*	*nt*	*nt*	-	-	-	-
CIP	16	32	20.022	*nt*	*nt*	*nt*	-	-	-	-
TZP (*n=42*)	8	8	6.672	*nt*	*nt*	*nt*	-	-	-	-
CRO	32	64	27.601	*nt*	*nt*	*nt*	-	-	-	-
AMX	1	2	0.920	*nt*	*nt*	*nt*	-	-	-	-
TET	0.06	0.125	0.104	*nt*	*nt*	*nt*	-	-	-	-
ERY	<4	>256	16	*nt*	*nt*	*nt*	-	-	-	-

Table 15: Comparison of UK C. difficile antimicrobial susceptibility data from 1980-1986 and 2012-2016. *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. Geometric means are calculated to 3 decimal places. nt - not tested. * Significance based on two-tailed Mann Whitney analysis. – $p<0.0002$ extremely significant, $p<0.006$ very significant, $p<0.02$ significant.*

3.5.3.1.1 Assessing comparator data set similarity

Examination of ribotype distribution across the historical and modern comparator groups demonstrated an imbalance; (Figure 22). Since reduced susceptibility is often strongly correlated with PCR ribotype (188, 444, 729), considerations of bias were essential for the evaluation of the data. In order to eliminate any potential bias, comparator cohorts were matched by ribotype. Ultimately, 59 isolates were selected for analysis, in conjunction with ribotype-matched modern comparators; (Table 16).

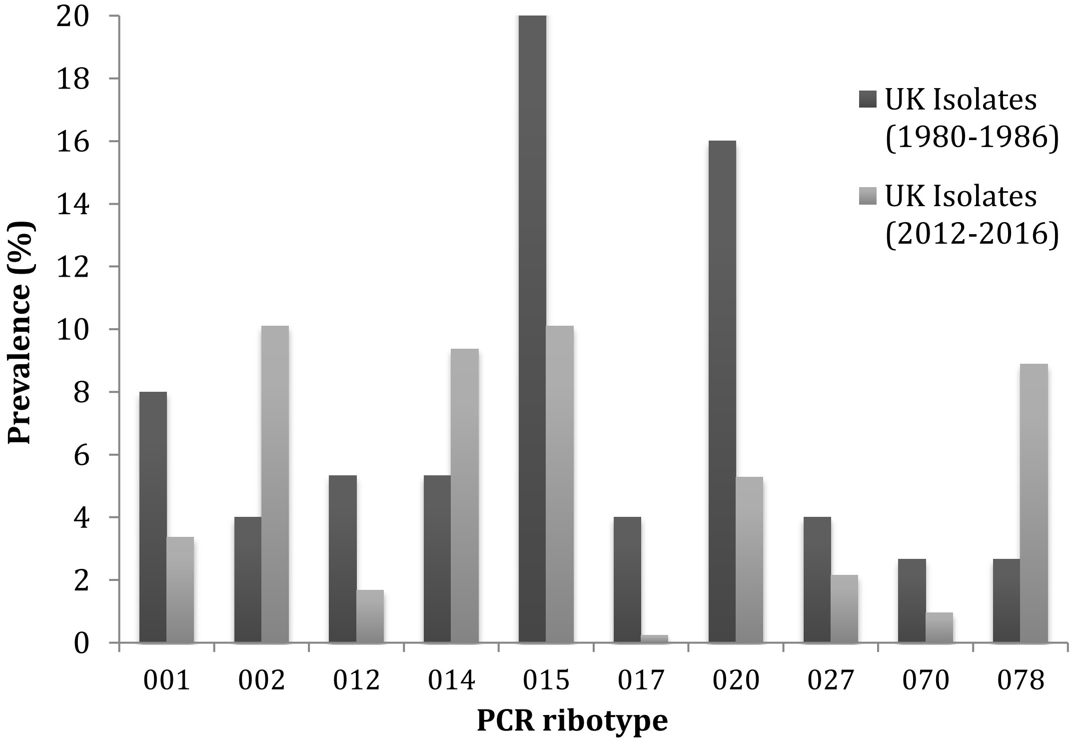


Figure 22: Proportional prevalence of PCR ribotypes UK historical (1980-1986); n=75 and modern (2012-2016); n=416 comparator groups.

3.5.3.1.2 Analysis of PCR ribotype-matched susceptibility data

Elimination of ribotype bias only strengthened the trend seen prior to genotypic balancing, further demonstrating reduced susceptibilities across all antibiotic classes over a 35 year period.

MIC_{90} represents the level of susceptibility of the resistant bacterial population and can therefore be utilised as a valuable tool in describing the potential clinical impact of a population. Increases in MIC_{90} were only associated with agents with direct clinical relevance, i.e. therapeutic and CDI predisposing agents (20, 99, 103). An increased MTZ MIC_{90} of two doubling dilutions potentially demonstrated the impact of the reliance on this nitroimidazole as the primary treatment compound for over 50 years. This perhaps highlights the impact of selective pressure gradually driving a reduction in susceptibility. The largest increase in resistance over time was observed with MXF, revealing a three-fold doubling dilution increase in MIC_{90}. In conjunction with a significant increase in geometric mean MIC, this serves to highlight the relevance of modern fluoroquinolones in *C. difficile* outbreaks and evolution (99, 417, 562). He *et al*. used whole genome and phylogenetic analyses to identify strong links between fluoroquinolone resistance and the dissemination of the hyper-virulent, 027 strain (562), which has elicited several outbreaks since 2003 (34). Rapid clonal expansion of highly fluoroquinolone resistant strains, such as ribotype 027, likely accounts for the major increase in resistance to MXF, a compound that was only introduced into the formulary in 1999.

The temporal differences in *C. difficile* susceptibilities observed in the present study were also identified in the study of Australian isolates by Mackin *et al.* (726). Slight increases in MICs of the primary treatment agents, VAN and MTZ, and a substantial increase in MXF MIC were observed over a period of several decades.

Conversely, with the exception of fluoroquinolone resistance, Hecht *et al.* observed no significant differences between MICs of a collection of 64 isolates from 1983-1998 and those collected from 2000-2004 (478). Unfortunately the study does not delineate the temporal groups any further, as the fifteen year span of the older catalogue may well contribute to this perceived lack of resistance development.

Nevertheless, when assessing variations in susceptibility data, we must reflect upon the methodology. Agar incorporation is considered the gold standard method for MIC determination in *C. difficile* (500, 730, 731), but is subject to inter-experimental variability due to non-exact endpoints. At the higher end of a testing range, a difference in MIC (e.g. 16 to 64 mg/L) is more likely to be a true reflection of reduced susceptibility; in contrast, at the lower end of a testing range it is much more difficult to determine this with confidence. An increase in MIC from 0.002 to 0.008 mg/L is very small in real terms and could be within the error of the method.

	Historic UK Isolates (1980-1986) *n=59*			***Clos*ER Study UK Isolates (2012-2016)** *n=59*			**Susceptibility Variance (Historic » *Clos*ER)** *(Doubling dilution increase)*		
Antimicrobial Compound	**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)**	**MIC$_{50}$ (mg/L)**	**MIC$_{90}$ (mg/L)**	**Geometric $\overline{x}$ (mg/L)**
VAN	0.5	1	0.703	1	1	0.754	+0.5 (1)	0	+0.051
MTZ	0.125	0.25	0.173	0.25	1	0.291	+0.125 (1)	+0.75 (2)	+0.118
RIF	0.001	0.002	0.001	0.002	0.002	0.002	+0.001 (1)	0	0
FDX	0.03	0.0795	0.031	0.03	0.06	0.036	0	-0.0195 (1)	+0.005
MXF	1	2	1.638	2	16	2.845	+1 (1)	+14 (3)	+1.207
CLI	4	16	3.518	8	32	6.034	+4 (1)	+16 (1)	+2.516
IPM	4	8	4.394	4	8	4.606	0	0	+0.211
CHL	4	8	5.000	8	8	5.624	+4 (1)	0	+0.623
TGC	0.03	0.06	0.035	0.06	0.06	0.047	+0.03 (1)	0	+0.012

Table 16: Comparison of antimicrobial susceptibility data from 59 PCR ribotype-paired, UK C. difficile from 1980-1986 and 2012-2016.

VAN-vancomycin, MTZ-metronidazole, RIF-rifampicin, FDX-fidaxomicin, MXF-moxifloxacin, CLI-clindamycin, IPM-imipenem, CHL-chloramphenicol, TGC-tigecycline. Geometric means are calculated to 3 decimal places.

3.5.3.1.3 Temporal comparisons of additional antimicrobial agents

The panel of agents were selected for testing in order to provide an overview of the antimicrobial activity of a broad spectrum of classes. Although there is no direct comparative data from the *Clos*ER study for several of these compounds, the breakpoint analyses offer useful information into the understanding of MDR in the historical collection.

Several contemporary data sets, obtained using the same methodologies, were available for comparison with these additional antibiotic classes. Although the majority of modern surveillance data is generated from international *C. difficile* cohorts, research by Freeman and colleagues indicated MIC data for a selection of UK isolates (732). In their study ceftriaxone MICs were demonstrated to be one doubling dilution higher than those from the historical collection presented here, with resistant proportions reported as five times higher (12% historical and 60% modern). LZD resistance was also reported in higher proportions (5.3% and 13.7%, across the historical and modern strains, respectively) with an increase of three doubling dilutions in MIC_{90}. These may suggest a possible increase in clonal spread or horizontal gene transfer of resistance determinants.

Data for LZD and ERY resistance may link the presence of MDR determinants, such as the *cfr* or *mar* genes, with these phenotypes (528). Similarly, the presence of TET resistance may suggest the existence of mobile genetic elements, such as transposons harbouring TET resistance determinants. TET resistance was observed in 9.3% of historical isolates, far lower than the rate reported by a study of Scottish isolates from 1979-86 by Taori *et al.*, where this rate reduced to comparable levels over the next ten years (381). Interestingly, in the present investigation, resistance to TET did not translate to decreased susceptibility to TGC, a modern tetracycline derivative. This supports the findings in other organisms (733, 734), although one recent study identified the potential for multiple *tet* gene mutations to confer resistance to all tetracycline compounds (735). This was hypothesized to be due to a decreased selectivity of efflux proteins, allowing TGC expulsion. TET resistance levels were as high as 42% in Chinese isolates, compared to only 9.3% in the historical UK isolates (201). Although ribotype prevalence and geographic isolation will affect these comparisons, they remain indicative of potential temporal resistance development.

All isolates from the historical collection demonstrated sensitivity to AMX and TZP. This correlates with several studies indicating total AMX and TZP susceptibility (197, 407, 736). In correlation with the historical isolates, almost all strains in two Japanese studies demonstrated CIP resistance (198, 724).

3.5.3.1.4 Temporal susceptibility comparison by individual PCR ribotype

The relatively small number of isolates recovered from the historical collection meant that an in-depth comparison of antimicrobial susceptibilities by individual ribotype was not feasible and is unlikely to reflect genuine trends. Nonetheless, some evaluations have been proffered for ribotypes 015 and 020, where isolate numbers were sufficient. MIC_{90} figures for the majority of agents, including MTZ and VAN (Appendix), demonstrated a doubling dilution increase in the modern ribotype 020 strains, with CLI levels rising from 16 to 32 mg/L. Since CLI MICs ranged between 2 and 16 mg/L in this ribotype alone, we may be identifying differences between lineages with or without previous exposures to the antibiotic. More notably, MXF MIC_{90} figures increased by two doubling dilutions, reflecting the same trend as analysis of the entire catalogue. The effects are less pronounced in ribotype 015 comparisons, where MTZ and CLI MIC_{90} figures doubled, although this is within the range of error for the agar incorporation susceptibility testing method.

3.5.4 Multidrug resistance investigation

The data from this study demonstrated a MDR rate of 22.7%. This is similar to the MDR rate reported in a previous large scale European surveillance study by Spigaglia *et al.* (26%) (444), but considerably lower than reported recently in a North American study by Peng *et al.* (59.7%) (214). Many factors have the potential to influence the rates of MDR in any given surveillance study. Of these factors it is important to note geographical differences in strain prevalence and antimicrobial prescribing that may have an effect on MDR rates in distinct study populations. The prevalence of particular ribotypes within a collection will influence overall rates of MDR. This may be a feature in the study by Spigaglia *et al.,* where no instances of the commonly reported MDR ribotype 027 were isolated, potentially reducing overall MDR rates. Equally, the selection of antibiotics in the test panel is crucial. In the US surveillance study by Peng and colleagues, the susceptibility testing panel comprised of almost twice as many antibiotics as those in the study by Spigaglia *et al*. Ampicillin and cefoxitin contributed greatly to MDR determinations in the former study; compounds that were not even tested against the European isolates. This factor may have also inflated the MDR rate of the collection tested in the present investigation, as susceptibilities to a large number of antimicrobial agents were assessed.

3.5.4.1 PCR ribotype prevalence of MDR strains

Forty-one percent of the PCR ribotypes recovered displayed a MDR phenotype, largely constituting ribotypes 012, 027 and 078; (23.4%, 17.6% and 11.8%, respectively). These correlated with data reported in the *Clos*ER surveillance study by Freeman *et al.* (188). Interestingly, the *Clos*ER study discovered the high prevalence of MDR in ribotypes 001 and 017, which were also identified in single MDR instances in the historical collection. Nonetheless, the majority of ribotype 001 and 017 isolates in the collection presented in this thesis did not exhibit a multiple resistant phenotype. These may represent distinct lineages of these strain types, isolated prior to their evolution into modern MDR genotypes. As a direct temporal comparison, an historic collection of Scottish isolates demonstrated dominance by ribotypes 012 and 001, representing 43% and 29% of the total MDR strains, respectively (381).

Phylogenetic analysis estimates that the ribotype 017 clone did not reach Europe until 1986 (580). However, the 017 isolates in the historical collection originated from specimens dating 1981 and 1982. Therefore, these isolates may indicate the international dissemination of this ribotype earlier than previously believed. The lack of a multi-resistant phenotype in the majority of these strains, potentially suggests an independent, parallel evolution, where multiple resistance-conferring determinants were yet to be acquired.

3.5.4.2 Multidrug resistance patterns

In the historical collection, nearly half (47.1%, *n*=8) of the MDR strains exhibited a combination of ERY, CLI and fluoroquinolone resistance; (Table 13). This finding was in concordance with a recent large scale review (498), which indicated this combination as the most prevalent (comprising of almost 30% of all MDR isolates). In contrast, this combination was observed in 92% of Korean isolates

(724), further highlighting the potential for geographic variance in resistance element distributions. Resistance to ERY and CLI are often reported in tandem, frequently amongst ribotype 001 isolates (521, 693, 705). One comparative study of strains from 1990 and 2008 by Ilchmann *et al.* demonstrated significant increases in this concomitant phenotype, from 37% to 87% of isolates respectively (p<0.001) (693). Interestingly, European surveillance by Spigaglia *et al.* demonstrated that all modern MDR isolates were characterised by a combined ERY and CLI resistance phenotype (444). The presence of the *ermB* gene has been identified as the most common MLS_B resistance-conferring determinant in *C. difficile* (444, 489, 518), suggesting a proficiency in the dissemination or selection of this gene over time. Where the historical collection demonstrated consistent co-resistance of MXF and ERY, the majority (71%) also exhibited CLI resistance. This pattern is strongly reflective of Ackermann's *C. difficile* collection incorporating strains from 1986-1995 (521).

Research by Spigaglia and colleagues revealed that by far the most dominant MDR phenotype was the combination of ERY, CLI, MXF and RIF; with this permutation dominated by ribotype 001 (444). Although only one historical ribotype 001 isolate was designated as MDR, the pattern of resistance matched the one outlined by Spigaglia *et al*., with the exception of RIF resistance. Large proportions of modern MDR isolates exhibit resistance to RIF (737-740), with the increase in prevalence of this phenotype across Italy and eastern Europe linked to the extensive use of rifamycins in these countries over the last twenty years (498). This offers a potential explanation as to why no evidence of rifamycin resistance was observed amongst the historical collection, whereas it contributes substantially to modern MDR rates. Many of the recently emerging ribotypes, such as 176 and 356 are closely related to pre-existing MDR types such as 027 (739) and 018 respectively (740). They may not be expected in an historical collection and their increasing presence in more recent epidemiological studies could partly explain the increase in MDR over time.

Each of the four ribotype 012 and three 027 isolates exhibited differing combinations of resistance, demonstrating the potential for these ribotypes to develop resistance to varying classes of antibiotics. A similar pattern is reflected in ribotype 012 strains identified by Spigaglia *et al.*, where ten isolates demonstrated five different resistance combinations (444). Differences in fitness cost may impact upon resistance acquisition and survival, as individual isolates respond to specific antimicrobial exposures. Wasels and colleagues demonstrated no detrimental effect of fluoroquinolone resistance mutations on the *in vitro* fitness of *C. difficile* (639), while other transposable elements, such as macrolide-lincosamide-streptogramin B resistance determinants presented a fitness burden (518). Interestingly, a recent observational study of CDI control interventions highlighted a greater decline in CDI cases associated with fluoroquinolone strains when use of the antibiotic class was restricted (387). This notable research by Dingle *et al.* indicated that resistance to key antimicrobial classes may potentially be as important to strain proliferation as multiple resistances.

Four strains displayed phenotypic resistance to CLI, CHL and LZD. This combination of class resistance has been linked to a transposable *cfr* homologue reported in *C. difficile* by Marin and colleagues (528). Whole genome sequencing analysis of all historical strains is presented in Chapter Four, providing a valuable insight into the presence or absence of putative resistance genes such as *cfr*.

3.6 Conclusions

This study highlights some of the difficulties faced when investigating historical culture collections. Whilst recovery was achieved from a proportion of the aged specimens, approximately 85% of patient strains either remained dormant under exposure to conventional germination methods, or more likely, had perished leaving no viable bacterial cells to recover.

The observation of striking similarities between ribotype prevalence amongst the historical isolates and modern epidemiology prompted two potential explanations:

1) Ribotype distribution in the UK has a tendency to revert to a state of equilibrium, when outbreaks subside,

Or

2) The prevailing ribotypes are the fittest for long-term survival and recovery.

Through continued surveillance of this important nosocomial pathogen, emerging ribotypes external to this stable distribution, can be monitored carefully as potential epidemic strains.

This investigation has also potentially identified two of the earliest isolates of PCR ribotype 027 *C. difficile*, dating back to 1981. Due to the originality of these findings, MLVA was utilised to check for contamination events amongst other laboratory strains, ultimately demonstrating that the historical isolates were distinguishable from 633 other ribotype 027 lab strains. Phylogenetic analyses of the historical collection is further discussed in Chapter Four.

Significant increases in antimicrobial resistance were observed between the historical collection (1980-86) and *Clos*ER isolates (2012-2016). Geometric mean MICs were increased in the modern strains for all but one of the antimicrobial comparators (IPM). Susceptibility to the therapeutic agents (MET, VAN, FDX) demonstrated marginal decreases, but resistance was not observed. Reductions in high-level CLI resistance may indicate the success of the management of this known risk antibiotic, but increased resistance in other classes remains a concern. On average lower rates of MDR were observed in the historical collection in comparison to modern comparator studies, with commonly reported MDR ribotypes 001, 012, 027 and 078 detected (118, 381, 444). A combination of ERY, CLI and fluoroquinolone resistance was the most frequently observed resistance pattern. This is reflective of previous studies, indicating the involvement of MDR conferring genes, such as *ermB*. This serves to emphasise the extent of MDR development and highlights the need for antimicrobial stewardship.

MXF resistance was observed in the historical strains, long before the introduction of the compound, further strengthening the argument for co-selection of resistance mutations with earlier generation compounds (741, 742). As expected, due to the current widespread resistance to fluoroquinolones (118, 498), MXF resistance increased considerably, with MIC_{90} data revealing a three-fold doubling increase. Intriguingly, the three ribotype 027 isolates recovered from 1981-86 demonstrated MXF resistance. These strains represent evidence of resistance in this hyper-virulent ribotype, prior to existing reports in the literature (34, 112, 289, 562), and may contribute to the understanding of the evolution of this epidemic strain type. Nonetheless, caution must be taken, with further investigations essential to ascertain the validity of this finding.

Chapter 4 Genomic Interrogation and Phylogenetic Analysis of Historical *Clostridioides difficile* (1980-86)

4.1 Introduction

The acquisition of an historical collection of *C. difficile* isolates has provided an opportunity to generate valuable baseline data, from which we can assess strain dissemination and antimicrobial resistance development. Whilst phenotypic investigations present important information on bacterial susceptibility, they cannot provide information on the mechanistic foundation of resistance. Modern molecular techniques offer the opportunity to elucidate the genetic machineries behind antibiotic resistance and enables epidemiologists to monitor how determinants disseminate.

The advent of NGS has enabled the rapid, inexpensive acquisition of large quantities of genomic data. Unlike Sanger sequencing (743), where DNA is synthesised with chain-terminating di-dNTPs and sequenced in a separate stage of the process, NGS enables sequencing by synthesis on a massively parallel scale (744). Here adaptor-ligated DNA is indexed and hybridised to oligonucleotides fixed to a glass flow cell. Clustering occurs through a process of bridge amplification, where fixed template strands are complemented by DNA polymerase before being washed away, allowing the nascent strand to form a bridge by binding to a second oligo on the flow cell. Polymerase then generates a copy of the original template bound to the flow cell, before the process is repeated to generate millions of copies through clonal amplification. Sequencing of these clusters occurs with the addition of fluorescently labelled nucleotides, which are excited after each nucleotide addition. The differences in signal emission determines the nucleotide incorporation at each step (745). This method produces millions of read sequences, which require complex *in silico* assembly.

Sequencers will produce terabytes of output data for individual base calls. In order to assess the accuracy of each of these determinations the base calls are each associated with a quality score, known as a Phred score. This data is essential to downstream quality assessment and is calculated based on the sequencer peak parameters, such as resolution and shape, and represent the probability of a correct call (746).

Reliable genome assembly and variant calling from NGS data requires a complex pipeline of computational processes for quality control, adapter trimming, sequence alignment, mapping to a reference genome, indel re-alignment, PCR duplicate removal and variant analysis (747). Adding to the challenge of computational assembly, there are multiple options for analysis tool selection at any given stage of the algorithm (748). The majority of practices involve executable, UNIX command line based frameworks without a graphical user interface (GUI), making initial access for beginners particularly difficult. Nonetheless, some GUI based frameworks are available (749). Taverna (750) and Galaxy (751) are two such web-based interfaces, which can be utilised in conjunction with cloud processing, negating the requirement for vast amounts of local processing power. Standalone NGS handling software such as CLC genomics workbench are available and provide a more user friendly, guided analysis, although licenses are highly expensive, limiting their availability (752).

There are many assembly and alignment tools available that can be categorised into two main approaches, string and de Bruijn graph based methods (748). Burrows-Wheeler transformation is an example of the former, where tools such as BWA (Burrows-Wheeler Aligner) (753) and Stampy (754) rely on a reversible, text-based transformation of reads, using cyclical rotation and subsequent sorting to dramatically reduce required processing power. The de Bruijn graph method is based on linking overlapping *k*-mers to generate all potential pathways through a sequence. Software tools such as Velvet (755) enable the simplification of these pathways, reducing computational memory requirements. Whilst no single tool represents the gold standard across all metrics, Hatem and colleagues identified BWA as the best application for longer read lengths, as produced with Illumina NGS chemistry (756).

Variant calling is typically the fundamental goal for NGS, with different tools available for this task, including the Genome Analysis Tool Kit (GATK) (757) and SAM tools (758). Differentiating true SNPs from sequencing artefacts is the most challenging part of the process, therefore the greater the read depth (number of read sequences covering a particular base) the more reliable a variant call (759).

Whilst the GATK attempts to define optimal pipelines for sequence data analysis (757), each research group implements pipelines tailored to their specific needs. Analysis of the *C. difficile* genome is no different, with the use of Stampy for mapping, Velvet for *de novo* assembly and SAM tools for SNP calling seemingly preferred in recent, large scale studies (257, 565, 760, 761). Nonetheless, bioinformatics expertise is required to access these pipelines, hence, the most accessible platform, CLC genomics workbench, was utilised here.

In the pursuit of resistance elements, searchable resistance determinant databases (762, 763) were interrogated with the historical genomes and loci of widely reported resistance-conferring chromosomal mutations were aligned (764). Polymorphisms in the DNA gyrase genes, *gyrA* and *gyrB* have been extensively described in *C. difficile* (438, 443-445), with the substitutions in the QRDR commonly associated with fluoroquinolone resistance (439, 442, 765). The most common amino acid substitution in *gyrA* is that of Thr82>Ile (438, 443-445) and has been strongly associated with the epidemic ribotype 027 (112, 766). Conferring high levels of resistance to modern generation fluoroquinolones, this mutation reduces the antibiotic binding capacity of the gyrase enzyme, resulting in a continuation of unhindered DNA synthesis (440). These genetic regions were examined to ascertain the levels of these polymorphisms in isolates from the early 1980s.

The dissemination of antimicrobial resistance determinants often occurs through the horizontal transfer of mobile elements. Common MDR genes involved in methyltransferase activity, ribosomal protection and efflux have all been reported in *C. difficile*, as associated with transposable elements (444, 488, 489, 528). The main TET resistance encoding element, *tetM*, has been observed primarily on Tn*916* transposons (487, 488), whilst carriage on a Tn*5397*-like determinant has also been reported (489). Methylation of key residues of ribosomal subunits can sterically block antibiotic binding, enabling DNA synthesis to proceed as usual. ErmB and Cfr are two such enzymes with this capability and have been implicated in concurrent resistance in *C. difficile* to ERY, CLI and the PhLOPS$_A$ group of antimicrobials (97, 489, 511, 528). Seventeen different genetic arrangements of the *ermB* gene have been described in *C. difficile* (444, 489, 511), with variation in resistant phenotypes observed (489). An increase in the frequency of *ermB* detection amongst *C. difficile* isolates was observed between 1991-98, correlating with increased use of CLI in the experimental setting (510). Rates among modern strains have been reported as high as 28% amongst European isolates, with confluent ERY and CLI resistance observed (444). Frequencies of *cfr* determinants have been reported as around 10% (530), with Marin *et al.* identifying the gene in seven out of nine LZD resistant isolates (528).

Determination of the prevalence of resistance determinants amongst historical isolates may help to reveal the involvement of these elements in resistance progression over the last 35 years. Identification of the frequencies of resistance genes could further allude to reasons for proliferation amongst specific ribotypes. In this chapter, NGS was performed on all historical isolates, with genomic data interrogated for resistance-conferring chromosomal mutations and transposable determinants. Findings were correlated with susceptibility phenotypes and evaluated for potential clinical impact. Furthermore, phylogenetic analysis of the historical isolates and modern comparators was performed to assess the evolution of common ribotypes.

4.2 Rationale

The determination of genetic aetiology for antimicrobial resistance phenotypes is essential to the understanding of MDR progression. NGS technologies, combined with a plethora of genomic analysis tools were utilised to facilitate a greater awareness of resistance mechanisms and prevalence in the historical collection. By enabling comparisons to modern studies, this data would allow epidemiological differences between two temporally distinct bacterial collections to be identified and evaluated for their influence on MDR development. Phylogenetic analyses were performed to further contribute to the understanding of *C. difficile* evolution and how the presence of resistant determinants progress over time.

4.3 Methodology

4.3.1 Antimicrobial resistance determinant detection

4.3.1.1 Genomic sequencing

4.3.1.1.1 Culture and DNA extraction

Growth from 24 hour *C. difficile* CBA culture was emulsified in pharmacy grade water, achieving a 0.5 McFarland suspension. Emulsifications were transferred to a deep-well extraction block and DNA extracted with QIAamp Fast DNA Kit chemistry (Qiagen) 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). This was followed by a 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.

Quality control and sample transposition were assured by PCR ribotyping of four DNA extracts from disparate plate locations and cross referenced back to the original determination.

4.3.1.1.2 Library preparation and next generation sequencing

Library preparation and sequencing was performed by the University of Leeds Next Generation Sequencing Facility using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina®; (New England Biolabs, USA).

Briefly, dDNA was quantified with the Quant-iT™ High-Sensitivity dsDNA Assay Kit (Thermo Fisher Scientific) and a FLUOstar Omega Microplate Reader (BMG Labtech, UK) to enable an optimal 200 ng of gDNA to be sheared via sonication in a Covaris E220 Focused Ultrasonicator (Covaris, USA). Optimal DNA fragmentation was assessed with an Agilent Technologies 2200 Tapestation (Agilent Technologies, USA), prior to end-repair and A-tailing of fragments using a two stage incubation with end prep enzyme mix; 30 minutes at 20°C, followed by 30 minutes at 65°C. NEBNext adapters were ligated to the end-repaired fragments during a 15 minute incubation at 20°C, followed by cleavage of the adapter hairpin at the uracil base by incubation with the USER® (Uracil-Specific Excision Reagent) Enzyme (New England Biolabs) at 37°C for a further 15 minutes. This process created the Y-shaped adapters necessary for primer annealing and binding to the complementary oligonucleotide sequences on the flow cell. A magnetic bead clean up (AxyPrep Mag PCR clean-up; Axygen, USA) with two 80% ethanol washes removed excess reagents and unbound adapters. Adapter-ligated fragments were indexed and enriched with unique 6bp primer indexes, through eight PCR cycles. Post-PCR samples were subjected to a further magnetic clean up step to remove any primer dimers and an additional Tapestation check to assess the enriched fragment spread. Indexed libraries were quantified as previously (4.3.1.1.1), allowing equal concentrations of sample DNA to be pooled for cluster generation on the Illumina cBot Cluster Generation System (Illumina, USA). Clonally amplified DNA, hybridised to the flow cell, was sequenced on a HiSeq 3000 Sequencing System (Illumina, USA) through sequencing by synthesis.

4.3.1.1.3 Bioinformatic assembly

Raw read data files were processed through a bioinformatics pipeline, using CLC Genomics Workbench (Qiagen) (752). Briefly, forward and reverse reads were compiled into single files by the software, before processed through a trimming protocol. Here poor quality reads (cut-off 0.05) were annotated to be ignored in downstream analyses. *De novo* assembly was achieved via a de Bruijn graph method (755) with minimum contig length set at 200. Multiple contig files were generated and output in FASTA format.

4.3.1.2 Genome annotation and resistance gene identification

Identification of resistance determinants was accomplished with a multiple step algorithm; (Figure 23). Coding DNA sequences (CDS) of all sets of contig assemblies were annotated using the Rapid Annotation using Subsystem Technology (RAST) web server based service, accessible at http://rast.nmpdr.org (767-769). Sequence data was uploaded in FASTA format and annotations were output in both GenBank (an annotated sequence format for use with the National Center for Biotechnology Information (NCBI)) and a fully searchable, tab delimited format (*.csv*).

RAST outputs were interrogated for resistance genes, putatively implicated in *C. difficile*; (Table 17). Nucleotide sequences of known resistance-conferring, mutable genes; DNA-directed RNA polymerase beta subunit (*rpoB*), Large ribosomal subunit proteins (L3, L4 and L22), DNA gyrase subunit A (*gyrA*) and B (*gyrB*) were extracted from RAST output files and converted into a FASTA format using a programmable script coded by the author in the *R* programming language; (Appendix). Sequences were then compared with CD630 genes (314) through multiple sequence alignment, using Clustal Omega 1.2.4 (764).

Assembled contig sequences were interrogated for antibiotic resistance genes using both the Comprehensive Antibiotic Research Database's (CARD) Resistance Gene Identifier (RGI) (762) and ResFinder 3.0 (763). RGI search parameters were set to include perfect, strict and loose hits, with the latter allowing for novel resistance determinant and distant homologue searches. All "perfect" and "strict" hits were further investigated for their relevance to *C. difficile*. Results from CARD and ResFinder were cross referenced to ensure maximal identifying coverage, any discrepancies were re-run as confirmation. Contigs were also searched by PlasmidFinder 1.3 (Center for genomic epidemiology) (770) to identify plasmid-borne genes originating from the Enterobacteriaceae family and enterococcus, streptococcus and staphylococcus genera.

Where the aforementioned software did not identify certain targeted resistance determinants, amino acid sequences of selected putative MDR genes (Table 17) were obtained from the necessary publications. These were then BLAST (v.2.2.26) (771) searched against the individual genomes using the RAST SEED Viewer (v.2.0). The *cfr*, *CD2068 and qnr*-like sequences used for comparisons were obtained from accession numbers KM359438.1, YP_001088582.1 and CAJ69589.1; respectively. The *marR* gene from CD630 was compared to all historical genomes using Clustal Omega alignments.

Determinant	**Mechanism and Putative Phenotype**	**Reference**
lmrA	Bacterial homologue of human MDR efflux pump, MDR1, functional in *E. coli*.	Van Veen *et al*. (1996) (772)
vgaB	ATP-binding cassette (ABC) transporter involved in macrolide-lincosamide-streptogramin resistance.	Chesneau *et al.* (2005) (773)
msrA	ABC-F subfamily involved in ribosomal protection from macrolide and streptogramin resistance.	Reynolds *et al.* (2003) (774)
efrA	ABC transporter involved in MDR efflux, including fluoroquinolones.	Lee *et al.* (2003) (775)
cfr	rRNA methyltransferase encoded by *cfr* causes methylation of position A2503 of the 23S rRNA gene. CHL, LZD, CLI and ERY resistance.	Marin *et al.* (2015) (528), Candela *et al.* (2017) (530)
qnr-like	Pentapeptide repeat proteins protect DNA gyrase from quinolone activity.	Rodríguez-Martínez *et al.* (2008) (776)
CD2068	Homologue of *cmpA*, an ABC transporter involved in MDR efflux, including fluoroquinolones.	Ngernsombat *et al.* (2009) (777)
cme	Efflux pump homologue of *norA.* Resistance to ethidium bromide, safranin O, and ERY.	Lebel *et al.* (2004) (546)
marR	Repressor gene for efflux. Resistance to CHL, cephalosporins, rifamycins, tetracyclines, fluoroquinolones and penicillins.	Leeds *et al.* (2014) (213)
cdeA	MATE family Na^+-coupled efflux pump. Responsible for ethidium bromide and acriflavin resistance, as well as putative fluoroquinolone resistance.	Dridi *et al.* (2004) (509)

Table 17: List of putative resistance genes with potential involvement in C. difficile resistance, used for historical genome interrogation. *MDR – multidrug resistance, MATE - multidrug and toxic compound extrusion, CHL – chloramphenicol, LZD – linezolid, CLI – clindamycin, ERY – erythromycin.*

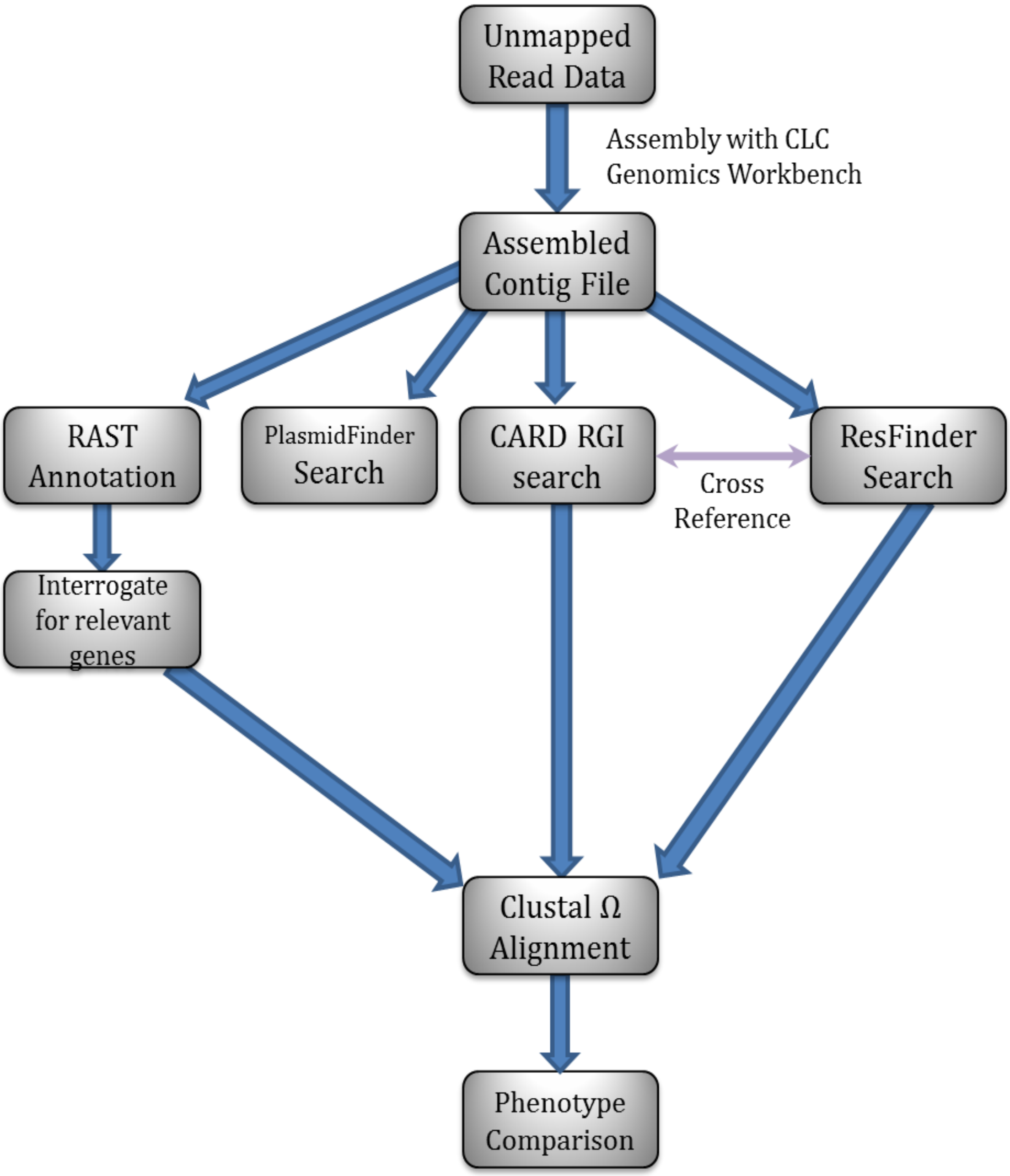


Figure 23: Work flow for sequence analysis and interrogation. *CARD RGI – the Comprehensive Antibiotic Research Database and Resistance Gene Identifier software (762), RAST - Rapid Annotation using Subsystem Technology (767), ResFinder 3.0 (763) (Center for Genomic Epidemiology), Clustal Omega multiple alignment software 1.2.4 (764), PlasmidFinder v1.3 (Center for Genomic Epidemiology) (770).*

4.3.1.3 Mobile element determination

Transposon identification was sought in order to correlate resistance determinant configurations with published data. The sequences of Tn*5397* and Tn*916*-like transposons from *C. difficile* genomes CD630 and M120; accession numbers AM180355.1 and FN665653.1; respectively, were obtained from the NCBI website. The *cfr* gene encoding element, Tn*6218* was acquired from accession number KM359438.1. Sequences were aligned to the genomes of the historical collection using NCBI BLAST.

4.3.1.4 Investigative responses to genotyping data

4.3.1.4.1 Efflux pump inhibition

The impact of efflux mechanisms on antimicrobial susceptibility was investigated for all isolates demonstrating ERY resistance in the absence of *ermB* genes, as well as those resistant to MXF. The agar incorporation method used previously (Chapter Three), was adapted to allow integration of known efflux inhibitors; the plant alkaloid, reserpine (Sigma, UK) (778) and proton motive force inhibitor, Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP); (Sigma, UK) (779). Briefly, both efflux inhibitors were dissolved in 5 mL dimethyl sulfoxide and further diluted in sterile water to create 250 mg/L and 800 mg/L solutions, for CCCP and reserpine respectively. CIP, MXF and ERY dilution series were each tested independently and in the presence of reserpine and CCCP to compare the effect of these compounds on antimicrobial susceptibilities. Wilkins Chalgren anaerobe agar was used with reserpine assays and Brazier's (supplemented with 2% lysed horse blood) with CCCP. Two millilitres of antimicrobial solution, plus equal volumes of efflux pump inhibitor were added to 16 mL of agar base (prepared with a 20% reduction in water content, to accommodate the addition of efflux pump inhibitors). For the control series, where no inhibitor solution was added, agar was supplemented with 2 mL sterile water. As previously, plates were dried and multipoint inoculated with MXF resistant and ERY resistant, *ermB* negative isolates. In line with other research, a four-fold change in MIC was considered as efflux related impairment (444).

4.3.1.4.2 Further antimicrobial susceptibility testing

Streptomycin susceptibility was assessed with the agar incorporation method, as previously described; (Chapter Three). Streptomycin (Sigma, UK) was dissolved in sterile water and tested in doubling concentrations, ranging between 256 and 2,048 mg/L. Isolates harbouring the putative streptomycin resistance gene, *aadE* (JV73 and JV74) were tested alongside nine *aadE* negative isolates.

Isolate JV60, encompassing an EF-Tu homologue of a mutated elongation factor relating to resistance to the kirromycin class of antibiotics, was not tested for phenotypic resistance to this class. Equally, JV73 was not tested for a kanamycin resistant phenotype, related to the presence of the *AAC(6')-Ie-APH(2'')* gene, since it has been demonstrated to have minimal Gram positive and anaerobic activity (780, 781).

4.3.2 Phylogenetic analysis

NGS data from the historical isolates of seven common PCR ribotypes; 001, 002, 014, 015, 020, 027 and 078, encompassing three different clades, was further processed through a phylogenetic analysis pipeline. Sequences were analysed, grouped by PCR ribotype. Additional comparator sequences from these ribotypes were included from the EUCLID study (154), (552 sequences from a pan-European survey [2012-2013]) and He *et al.* (562) (149 ribotype 027 sequences [1985-2010]).

Sequence data was processed by Dr David Eyre (Nuffield Department of Medicine, University of Oxford, John Radcliffe Hospital, Oxford) using a bioinformatics pipeline established for the analysis of bacterial genomic data (257, 782). Briefly, Illumina HiSeq sequencing data was mapped to the *C. difficile* 630 reference genome (314), with the exception of ribotype 027 (clade 2) isolates, which were mapped to CD196 (579) to enable comparisons of the novel genetic elements exhibited by this ribotype. Mapping was performed with Stampy (754), variants were identified using SAMtools mpileup (758) and filtered requiring a read consensus of >75% and a minimum coverage of five reads. Maximum likelihood phylogenetic trees were generated with PhyML (783), from mapped data adapted with ClonalFrameML (784) to exclude regions of recombination. The author analysed the resulting trees using the Interactive Tree of Life (v.4.2) (785).

Prediction intervals were calculated based on SNP and year differences to nearest neighbouring taxa on the maximum likelihood trees. RStudio v.1.1.383 (786) was used to calculate prediction intervals based on Poisson distribution of the estimated molecular clock (0.74 per genome, per year), using the formula:

***ppois (SNP difference, years difference*0.74)*.**

E.g. for 25 actual SNP differences between two genomes 31 years divergent, the calculation was: ***ppois (25, 31*0.74)*** **= 0.71**

4.4 Results

4.4.1 Antimicrobial resistance determinant detection

4.4.1.1 Bioinformatic assembly

All historical isolate NGS read data assembled into multiple contig files successfully. The average assembly size was 4,371,170bp, consisting of 494 contigs with N50 and L50 figures of 128,249 and 26, respectively. Individual genome assembly statistics are reported in the Appendix.

4.4.1.2 Transferable genetic elements

Twenty different antibiotic resistance encoding genetic elements were identified amongst the genomes of the historical *C. difficile* isolate collection. Sixteen of these were detected by a combination of the CARD-RGI and ResFinder 3.0 databases. Eighteen (24.0%) historical strains revealed mobile genetic elements, by this algorithm. Four additional resistance gene homologues were identified via BLAST comparisons.

TET resistance gene, *tetM* was identified in seven (9.3%) strains, all of which demonstrated a TET resistant phenotype. These strains represented three PCR ribotypes; 012 (*n*=4), 078 (*n*=2) and 015 (*n*=1); (Figure 24). All *tetM* genes clustered into two phylogenetically distinct groups; (Figure 25). In all ribotype 012 genomes, *tetM* was contained on the Tn*5397* transposon, matched with 100% coverage and sequence identity. All other genomes harbouring the TET resistance determinant carried it on a Tn*916*-like mobile element, demonstrating 100% coverage and 97% identity with that contained on the *C. difficile* M120 genome. One isolate, JV32, harboured an additional *tetA(P)* gene in conjunction with *tetM*, displaying a TET MIC of >32 mg/L. No other strains exhibited reduced susceptibility to TET; (Table 18).

MLS$_B$ resistance determinant, *ermB*, was detected in six (8.0%) genomes, reflecting ribotypes 014 (*n*=3), 012 (*n*=2) and 010 (*n*=1); (Figure 24). All *ermB* gene sequences clustered by ribotype; (Figure 25). Three isolates (50.0%) demonstrated a combined CLI and ERY resistant phenotype, whilst one (16.7%) exhibited resistance to CLI only and two (33.3%) were fully susceptible to members of the MLS$_B$ class. Eight (10.7%) strains displayed an ERY resistant phenotype, with no evidence of the *ermB* gene, whilst thirty-six (48.0%) showed reduced susceptibility to CLI in the absence of the *ermB* determinant; (Table 18; Figure 26).

Four genomes (5.3%) revealed the presence of a collection of VAN resistance determinants (*vanRG*, *vanSG*, *vanUG*, *vanYG*, *vanG*, *vanXYG*, *vanWG* and *vanTG*), correlating precisely with all intermediate VAN resistant (4 mg/L) phenotypes. Although representing three ribotype variants, all four of these strains were co-recovered from a single patient at multiple time points. Sixty-four remaining strains exhibited CARD-RGI matches of 77.9% similarity to VAN resistance regulator gene, *vanRG*, with single instances of *vanSD* and *vanSG* genes also discovered. None of these strains conferred a reduced susceptibility to VAN; (Table 18).

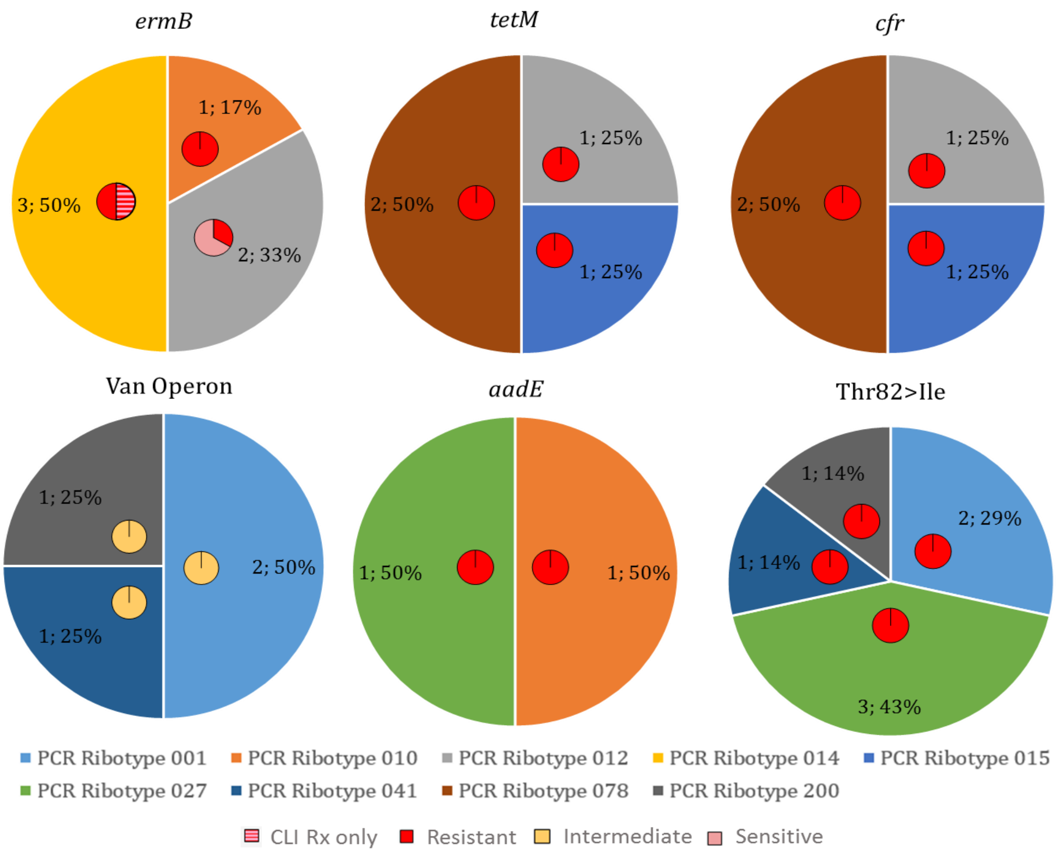


Figure 24: Distribution of resistance determinants and phenotypes identified in historical C. difficile genomes (1980-86) by PCR ribotype. *Inner circles refer to susceptibility phenotype proportions to relevant agents; ermB – erythromycin and clindamycin, tetM – tetracycline, cfr – clindamycin, chloramphenicol and linezolid, Van operon – vancomycin, aadE – streptomycin, Thr82>Ile in gyrA – moxifloxacin. Numbers refer to n size.*

All four LZD resistant isolates, JV14, JV17, JV22 and JV30 demonstrated coding sequences with 100% identity to the *cfr* gene, previously identified in *C. difficile* (528). All *cfr* genes were located on Tn*6218* transposons, correlating with concurrent CHL and CLI resistance.

The genomes of strains JV73 and JV74 demonstrated perfect matches to streptomycin resistance determinant, *aadE*, with the former also revealing an *AAC(6')-Ie-APH(2'')* complex associated with kanamycin resistance. Upon further testing both of these isolates exhibited streptomycin resistance (>2,048 mg/L), whilst all but one *aadE* negative strain indicated MICs of <512 mg/L. Isolate JV47 revealed a streptomycin MIC of >2,048 mg/L in the absence of the *aadE* determinant; (Appendix).

The putative ABC transporter, *CD2068* was detected in 85.3% of historical isolates, with BLAST comparisons revealing identities >98%. The remaining eleven isolates only demonstrated 30-31% homology and consisted of ribotypes 015 (*n=6*), 020 (*n=3*), 341 (*n=1*) and 862 (*n=1*). All isolates revealed >99% identity with *qnr*-like putative pentapeptide repeat-containing protein, with the exception of JV59 which revealed 91% homology. Isolate JV60, ribotype 619, exhibited a sequence similarity (80.6%) with a streptomyces derived elongation factor, EF-Tu, implicated in natural kirromycin resistance.

All genomes revealed 100% identity with the *cme* gene from *C. difficile* strain H3, whether demonstrating an ERY resistant phenotype or not. The multidrug efflux gene, *cdeA* was universally observed in all historical genomes. Conversely, MDR determinants *msrA, lmrA, vgaB* and *efrA*, present in other organisms were not detected in this *C. difficile* collection.

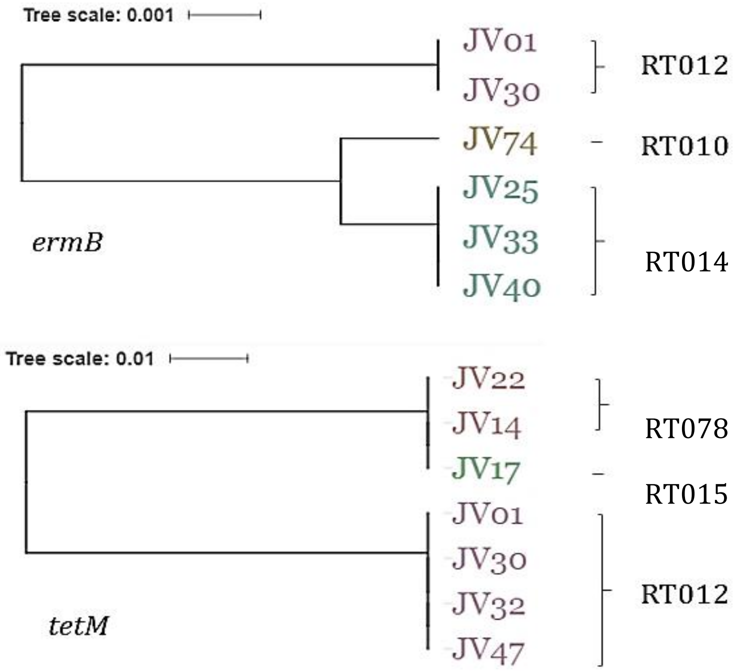


Figure 25: Phylogenetic analysis of ribotype (RT) clustering of ermB (top) and tetM genes (bottom) identified in the historical (1980-86) C. difficile collection. *Genes were aligned with Clustal Omega and visualised by the Interactive Tree of Life.*

4.4.1.3 Mutational resistance

Fifty-six (74.7%) genomes revealed amino acid substitutions in known mutable resistance-conferring genes (*gyrA, gyrB* and *rpoB*); (Table 18).

Seven strains (9.3%), including all three ribotype 027 isolates exhibited the common Thr82>Ile polymorphism in the *gyrA* gene. All MXF resistant phenotypes (16-32 mg/L) correlated with this mutation. Two of six ribotype 001 strains shared these characteristics, along with each ribotype 041 and 200 isolate; (Figure 24). Epidemic ribotype 027 strains also exhibited two further *gyrA* mutations; Leu406>Ile and Asp468>Asn, which have not previously been reported as linked with fluoroquinolone resistance. Across the entire collection, only two other non-synonymous mutations were determined in *gyrA*; Lys413>Asn (*n*=3) and Asp205>Glu (*n*=1), although only the ubiquitous CIP resistant phenotype was displayed in these strains; (Table 18).

Eight variant *gyrB* polymorphisms were identified amongst the strain collection, with fifty (66.7%) genomes revealing at least one non-synonymous mutation in the *gyrB* gene. Whilst, no previously reported mutations related to fluoroquinolone resistance were discovered, five mutant variants were determined. The two most prevalent were Val130>Ile and Ile139>Arg, revealed in 29.3% and 25.3% of historical genomes, respectively. Ribotype 078 strains all exhibited three *gyrB* mutations; Gln160>His, Ser366>Val and Phe375>Leu, whilst all ribotype 017 isolates displayed the Ser366>Ala mutation alone. Single instances of Ile266>Val and Glu523>Gly substitutions were identified in ribotypes 061 and 137, respectively; (Table 18). None of these substitutions correlated with reduced susceptibility to MXF.

Analysis of the RNA polymerase β sub unit encoding gene, *rpoB*, revealed six non-synonymous mutant genotypes; Ile750>Met (10.7%), Ile750>Val (8.0%), Ile750>Glu (1.3%), Pro1113>Ser (1.3%), Val1033>Gly (1.3%) and concomitant Glu1037>Gln, Asn1207>Ala, Ala1208>Thr and Asp1232>Glu mutations, representing all ribotype 027 strains. Newly assigned ribotype 862 strain, JV59, the only isolate exhibiting reduced susceptibility to rifampicin, demonstrated 16 non-synonymous mutations in the *rpoB* gene; (Table 19), however, none had been previously linked to a resistant phenotype.

No non-synonymous mutations were observed in the majority of historical genomes when compared to the *marR* transcriptional regulator sequence of CD630. Sequence identity was 99% for all isolates except JV59, which had previously demonstrated distinct phylogeny.

Strain	PCR Ribotype	Resistance Genes Identified*	Non-synonymous mutations			Phenotypic Resistance Ω
			gyrA	*gyrB*	*rpoB*	
JV01	012	*tetM, ermB, cdeA*				CLI, CIP, CRO, TET, ERY
JV02	027	*cdeA*	Thr82Ile, Leu406Ile, Asp468Asn		Glu1037Gln, Asn1207Ala, Ala1208Thr, Asp1232Glu	MXF, CLI, IPM(I), CIP, CRO, ERY
JV03	015	*cdeA*		Val130Ile		CLI, CIP, CRO(I)
JV04	001	*cdeA*				CLI, CIP, CRO(I)
JV05	020	*cdeA*		Ile139Arg		CLI(I), CIP, CRO(I)
JV06	020	*cdeA*		Ile139Arg		CIP, CRO(I)
JV07	015	*cdeA*		Val130Ile		CLI(I), CIP, CRO(I)
JV08	220	*cdeA*		Ile139Arg	Ile750Val, Pro1133Ser	CLI, CIP, CRO(I)
JV09	015	*cdeA*		Val130Ile		CLI(I), CIP, CRO(I)
JV10	015	*cdeA*		Val130Ile		CLI(I), CIP, CRO(I)
JV11	015	*cdeA*		Val130Ile		CLI, CIP, CRO(I)
JV12	020	*cdeA*		Ile139Arg		CLI, CIP
JV13	020	*cdeA*		Ile139Arg		CLI(I), CIP, CRO(I)
JV14	078	*cfr, tetM, cdeA*	Lys413Asn	Gln160His, Ser366Val, Phe375Leu	Ile750Met	CLI, CHL, LZD, CIP, TET
JV15	020	*cdeA*		Ile139Arg		CLI, CIP, CRO(I)
JV16	056	*cdeA*				CIP
JV17	015	*cfr, tetM, cdeA*	Lys413Asn	Gln160His, Ser366Val, Phe375Leu	Ile750Met	CHL, CLI, CIP, LZD, CRO(I), TET
JV18	103	*cdeA*				CLI, CIP, CRO(I)
JV19	137	*cdeA*		Val130Ile, Glu523Gly		CLI, CIP, CRO(I)
JV20	041	*vanRG, vanSG, vanYG, vanG, vanXYG, vanTG, vanWG, vanUG, cdeA,*	Thr82Ile			VAN(I), MXF, CLI, CIP, CRO(I), ERY
JV22	078	*cfr, tetM, cdeA*	Lys413Asn	Gln160His, Ser366Val, Phe375Leu	Ile750Met	CLI, IPM(I), CHL, LZD, CIP, TET
JV23	001	*vanRG, vanSG, vanYG, vanG, vanXYG, vanTG, vanWG, vanUG, cdeA*	Thr82Ile			VAN(I), MXF, CLI, CIP, CRO, ERY
JV24	200	*vanRG, vanSG, vanYG, vanG, vanXYG, vanTG, vanWG, vanUG, cdeA*	Thr82Ile, Leu406Ile, Asp468Asn			VAN(I), MXF, CLI, IPM(I), CIP, CRO, ERY
JV25	014	*ermB, cdeA*		Ile139Arg		CLI, CIP, ERY

Strain	PCR Ribotype	Resistance Genes Identified*	Non-synonymous mutations			Phenotypic Resistance Ω
			gyrA	*gyrB*	*rpoB*	
JV26	015	*cdeA*		Val130Ile		CLI, IPM(I), CIP, CRO(I)
JV27	020	*cdeA*		Ile139Arg		CLI, CIP
JV28	001	*cdeA*				CLI, CIP
JV29	002	*cdeA*				CLI(I), CIP, CRO(I)
JV30	012	*cfr, tetM, ermB, cdeA*				CLI, CHL, LZD, CIP, CRO(I), TET
JV31	002	*cdeA*				CLI, CIP, CRO(I)
JV32	012	*tetM, tetAP, cdeA*				CLI, IPM(I), CIP, CRO, TET
JV33	014	*ermB, cdeA*		Ile139Arg		CIP
JV34	001	*vanRG, vanSG, vanYG, vanG, vanXYG, vanTG, vanWG, vanUG, cdeA*	Thr82Ile			VAN(I), MXF, CLI (I), IPM(I), CIP, CRO(I), ERY
JV35	020	*cdeA*		Ile139Arg	Ile750Val	CLI, CIP
JV36	015	*cdeA*		Val130Ile		CLI, CIP, CRO(I)
JV37	001	*cdeA*		Val130Ile		CLI, CIP, CRO(I)
JV38	626	*cdeA*		Val130Ile		CLI(I), CIP, CRO(I)
JV39	341	*vanYG, cdeA*		Val130Ile		CIP
JV40	014	*ermB, cdeA*		Ile139Arg		CIP
JV41	002	*cdeA*				CLI, CIP, CRO(I)
JV42	015	*cdeA*		Val130Ile		CLI(I), CIP, CRO(I)
JV43	015	*cdeA*		Val130Ile		CIP, CRO(I)
JV44	020	*cdeA*		Ile139Arg		CIP
JV45	015	*cdeA*		Val130Ile		CIP, CRO(I)
JV46	061	*cdeA*		Val130Ile, Ile266Val	Val1033Gly	IPM(I), CIP, CRO
JV47	012	*tetM, cdeA*				IPM(I), CIP, CRO(I), TET, ERY
JV48	020	*cdeA*		Ile139Arg		CIP
JV49	032	*cdeA*				CIP
JV50	017	*cdeA*		Ser366Ala	Ile750Met	CLI(I), IPM(I), CIP, CRO, ERY
JV51	020	*cdeA*		Ile139Arg	Ile750Val	IPM(I), CIP, CRO(I)
JV52	020	*cdeA*		Ile139Arg	Ile750Val	CLI(I), CIP
JV53	020	*cdeA*		Ile139Arg	Ile750Val	CLI(I), CIP
JV54	015	*cdeA*		Val130Ile		CIP, CRO(I)
JV55	015	*cdeA*		Val130Ile		IPM(I), CIP, CRO(I)
JV56	017	*cdeA*		Ser366Ala	Ile750Met	IPM(I), CIP
JV57	200	*cdeA*		Val130Ile		IPM(I), CIP, CRO(I)

Strain	PCR Ribotype	Resistance Genes Identified*	Non-synonymous mutations			Phenotypic Resistance Ω
			gyrA	*gyrB*	*rpoB*	
JV58	017	*cdeA*		Ser366Ala	Ile750Met	IPM(I), CIP
JV59	862	*cdeA*		See Table 19		RIF(I), IPM(I), CIP, CRO(I)
JV60	619	*S. cinnamoneus EF-Tu, cdeA*				CIP, CRO(I)
JV61	015	*cdeA*		Val130Ile		IPM(I), CIP, CRO(I)
JV62	015	*cdeA*		Val130Ile		IPM(I), CIP, CRO(I)
JV63	001	*cdeA*		Val130Ile		IPM(I), CIP, CRO(I)
JV64	020	*cdeA*		Ile139Arg		CIP, CRO(I)
JV65	033	*cdeA*	Lys413Asn	Gln160His, Ser366Val, Phe375Leu	Ile750Met	CIP
JV66	015	*cdeA*		Val130Ile		IPM(I), CIP, CRO(I)
JV67	027	*cdeA*	Thr82Ile, Leu406Ile, Asp468Asn		Glu1037Gln, Asn1207Ala, Ala1208Thr, Asp1232Glu	MXF, IPM, CIP, ERY
JV68	242	*cdeA*		Ser366Ala	Ile750Met	CIP
JV69	014	*cdeA*		Ile139Arg		CIP
JV70	003	*cdeA*		Ile139Arg	Ile750Val	CIP, CRO(I)
JV71	070	*cdeA*				CIP, CRO(I)
JV72	023	*cdeA*	Asp205Glu			CIP, CRO(I)
JV73	027	*aadE, AAC(6')-Ie-APH(2''), cdeA, ant(6)-Ia*	Thr82Ile, Leu406Ile, Asp468Asn		Glu1037Gln, Asn1207Ala, Ala1208Thr, Asp1232Glu	MXF, IPM(I), CIP, CRO, ERY
JV74	010	*ermB, aadE, cdeA*				CLI, IPM(I), CHL(I), CIP, ERY
JV75	061	*cdeA*				IPM(I), CIP, CRO
JV76	070	*cdeA*				IPM(I), CIP

Table 18: Summary of resistant determinants and phenotypes for 75 historical C. difficile strains (1980-1986). ** Resistance genes were identified either by the Comprehensive Antibiotic Research Database (CARD) Resistance Gene Identifier (RGI) (762) and/or ResFinder 3.0 (763). The cfr gene was identified through BLAST comparisons. Ω Breakpoints as defined previously, based on the U.S. Clinical & Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST); or existing publications. VAN-vancomycin, RIF-rifampicin, MXF-moxifloxacin, CLI-clindamycin, IPM-imipenem, CHL-chloramphenicol, CIP-ciprofloxacin, CRO-ceftriaxone, TET-tetracycline, ERY-erythromycin. (I) indicates intermediate resistance.*

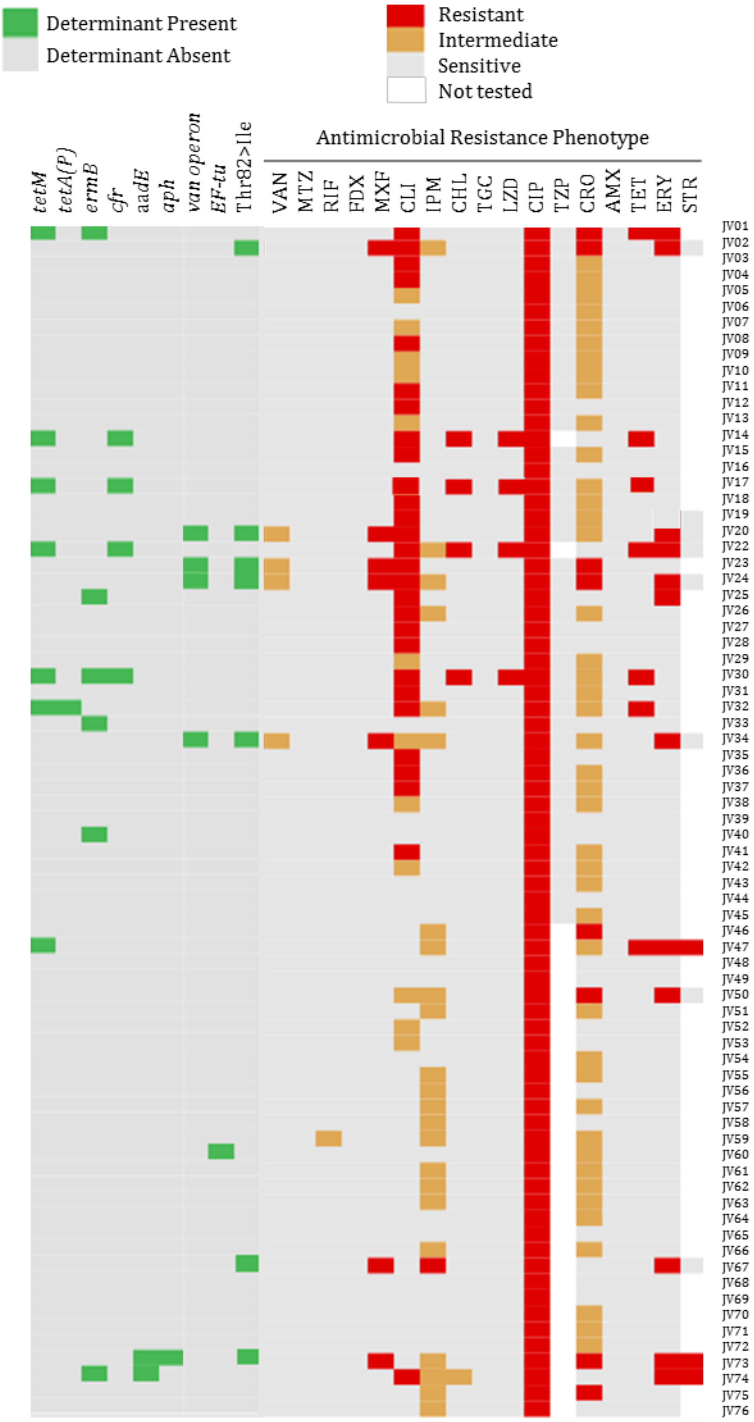


Figure 26: Heat map of antimicrobial resistance genes and phenotypes observed in the historical collection. *Breakpoints as defined previously, based on CLSI and EUCAST definitions, or existing publications. VAN-vancomycin, MTZ-metronidazole, RIF-rifampicin, FDX-fidaxomicin, MXF-moxifloxacin, CLI-clindamycin, IPM-imipenem, CHL-chloramphenicol, TGC-tigecycline, LZD-linezolid, CIP-ciprofloxacin, TZP-piperacillin/tazobactam, CRO-ceftriaxone, AMX-amoxicillin, TET-tetracycline, ERY-erythromycin, STR – streptomycin.*

4.4.1.4 Distinctive genome, JV59

Isolate JV59 demonstrated a high number of non-synonymous mutations in the phenotypically important *gyrA* (*n=12*), *gyrB* (*n*=8) and *rpoB* (*n=16*) genes; (Table 19). Whilst intermediate resistance to RIF was identified in this strain, the gyrase alterations conferred no reduction of susceptibility to MXF.

Non-synonymous mutations		
gyrA	*gyrB*	*rpoB*
Asn4>Lys	Val130>Leu	Thr227>Ser
Val194>Ile	Ile139>Val	Glu291>Gln
Leu406>Gln	Ile348>Leu	Asp312>Glu
Glu410>Asp	Ser366>Ala*	Ala316>Asp
Lys413>Asn	Ser416>Ala	Asp350>Asn
Asp444>Glu	Val563>Ala	Ser575>Ala
Ser478>Ala	Glu581>Asp	Glu603>Asp
Val546>Ile	Glu587>Asp	Asn744>Ser
Ala613>Thr		Asp747>Glu
Lys628>Arg		Gln748>Lys
Glu664>Asp		Ile750>Glu
Glu693>Asp		Lys751>Arg
		Val951>Ile
		Glu1019>Asp
		Ser1038>Thr
		Asp1232>Glu

Table 19: Table of non-synonymous substitutions identified in the JV59 genome in genes known for conferring resistant phenotypes. ** Previously reported in a moxifloxacin sensitive C. difficile strain* (198).

4.4.1.5 Resistance gene identification software comparison

Resistance genes (>90% identity) were identified by both CARD-RGI and ResFinder 3.0 in 92% (46/51) of cases. Both identification systems overlooked determinants recognised by the other. ResFinder failed to identify resistance elements in three instances (5.9%), including failing to detect the presence of an *ermB* gene in JV01, even though it was highlighted with 99.15% identity by the CARD algorithm. Individual VAN resistance element, *vanYG* was not reported by ResFinder in isolate JV39, *vanWG* was missed in JV34. CARD-RGI could not detect resistance determinants on two occasions (3.9%), with the lack of identification of *aadE* genes in JV73 and JV74; (Appendix). ResFinder did not report the highly abundant *cdeA* gene or any *vanRG*, *vanSG* or *vanSD* genes with lesser similarity (77.9%).

Neither software was able to identify the presence of the *cfr* gene, whilst the RAST service assigned the annotation "*Ribosomal RNA Large Subunit Methyltransferase N*" to coding DNA sequences in all isolates regardless of whether they encoded for Cfr or RlmN proteins. No plasmids were identified by the PlasmidFinder 1.3 algorithm.

4.4.2 Phylogenetic analysis

Isolates recovered from the historical catalogue generally formed clusters with at least one other strain from the same collection (Figure 27 - Figure 33), reflecting the original sampling frame. The estimated number of SNPs from the closest "modern" isolate (2012-13) was commonly identified as >13. In keeping with the age differences between the historical isolates and comparator sequences (~30 years) and the estimated rate of *C. difficile* evolution (~0.74 SNPs per genome, per year), the majority of sequences (69%) fit within a 95% prediction interval of Poisson distributions.

Analyses of two PCR ribotypes provided exceptions to these findings. The three ribotype 027 isolates did not cluster together and the historical isolates, JV73 and JV67 were only eight and two SNPs different from genomes recovered in 2008 and 2006; respectively; (Figure 27). The Poisson probability distribution of these events were calculated as 4.51×10^{-5} and 3.93×10^{-3}, for JV73 and JV67 respectively. Isolate JV02 demonstrated closest relatedness to another UK isolate from 2008, 13 SNP differences, but within the prediction interval; (p=0.07). Furthermore, the three ribotype 027 genomes demonstrated multiple non-conserved regions between each isolate. Isolates JV14 and JV22, representing PCR ribotype 078, demonstrated identical genomes, representing six SNP differences from an Irish strain from 2013; (Figure 29). Prediction intervals for six SNPs in isolates of this presumed age were outside of the 95% probability projection, based on current molecular clock estimations; p=0.0003.

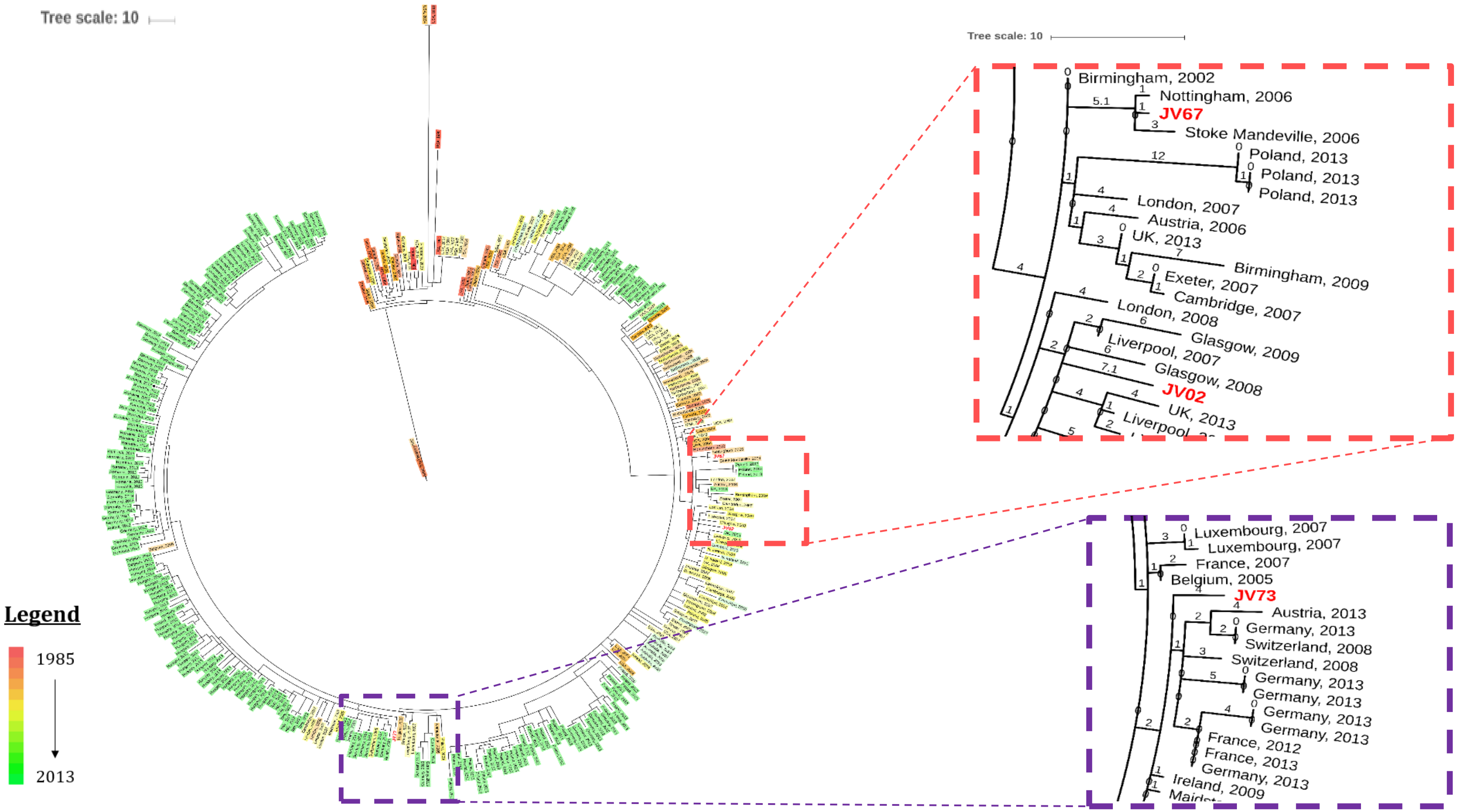


Figure 27: Phylogenetic tree representation of "historical" ribotype 027 isolates amongst >350 comparator 027 strains from 1985-2013, acquired from the published He et al. (562) and the EUCLID studies (154). *Maximum likelihood trees were estimated as previously (257), and generated with the Interactive Tree of Life (785). Colour gradient represents isolate age.*

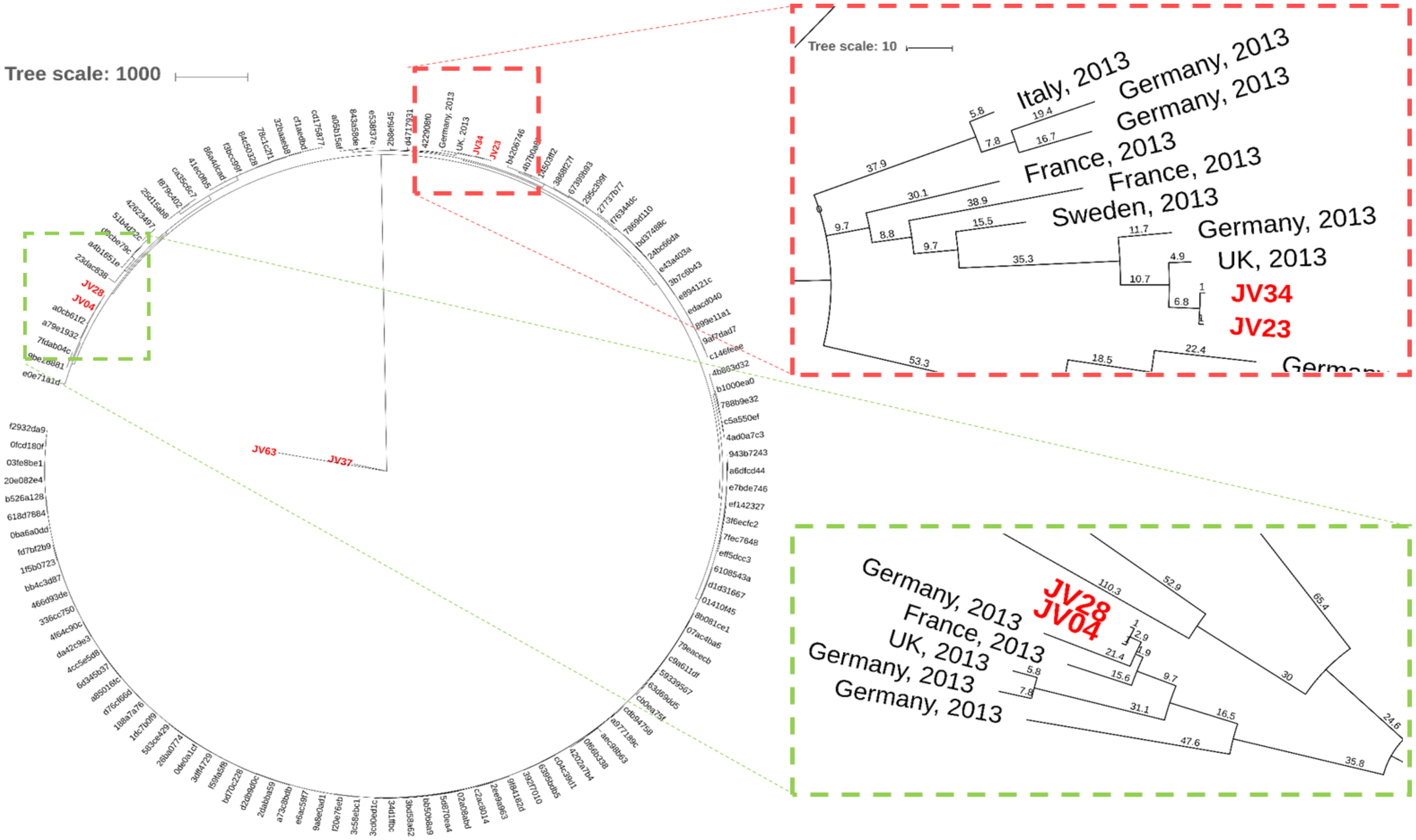


Figure 28: Phylogenetic tree representation of "historical" ribotype 001 isolates amongst comparator 001 strains from 2013, acquired from the EUCLID study (154).*Maximum likelihood trees were estimated as previously (257), and generated using the Interactive Tree of Life (785).*

Figure 29: Phylogenetic tree representation of "historical" ribotype 078 isolates amongst comparator 078 strains from 2013, acquired from the EUCLID study (154). *Maximum likelihood trees were estimated as previously (257), and generated using the Interactive Tree of Life (785).*

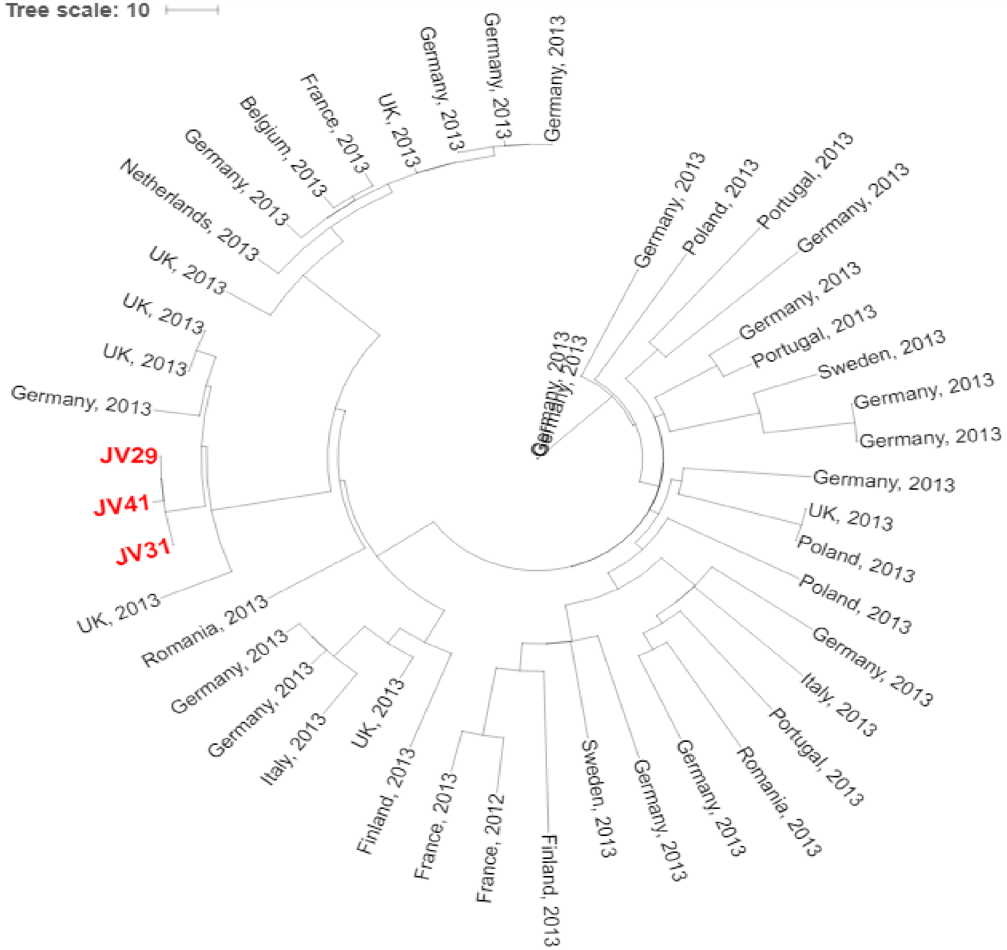


Figure 30: Phylogenetic tree representation of "historical" ribotype 002 isolates amongst comparator 002 strains from 2013, acquired from the EUCLID study (154). *Maximum likelihood trees were estimated as previously (257), and generated using the Interactive Tree of Life (785)*.

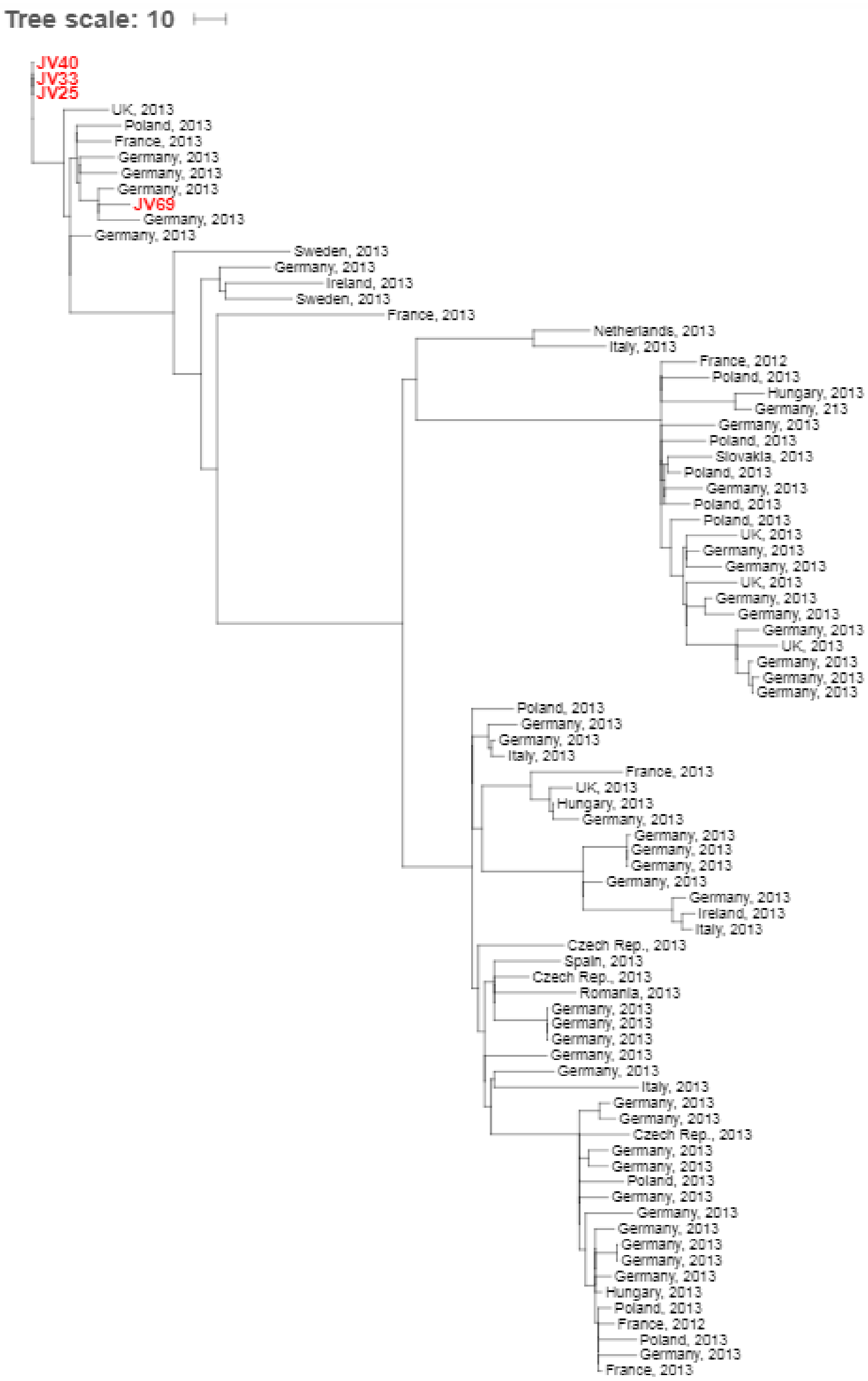


Figure 31: Phylogenetic tree representation of "historical" ribotype 014 isolates amongst comparator 014 strains from 2013, acquired from the EUCLID study* (154)*. *Maximum likelihood trees were estimated as previously* (257)*, and generated using the Interactive Tree of Life* (785)*.*

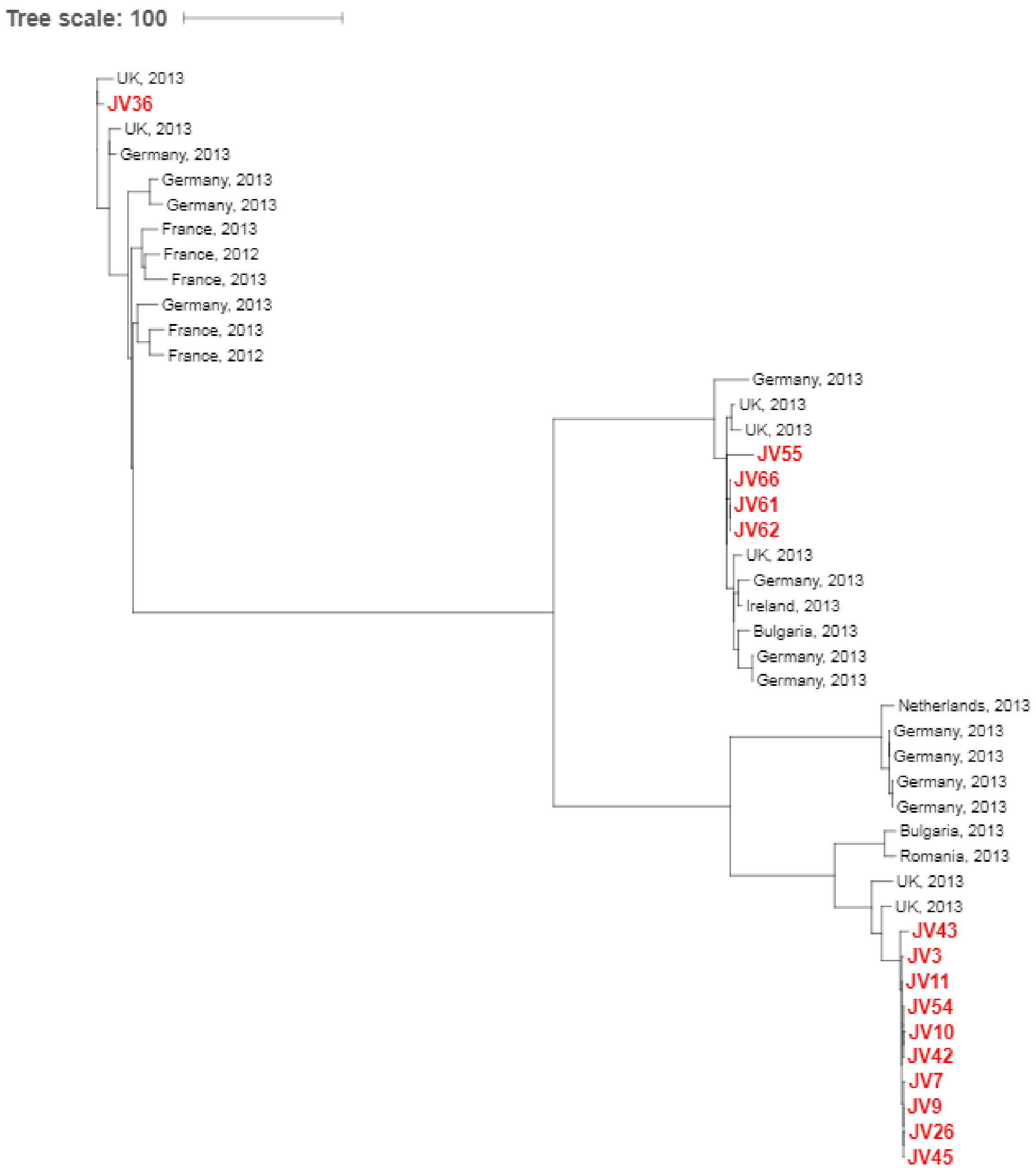


Figure 32: Phylogenetic tree representation of "historical" ribotype 015 isolates amongst comparator 015 strains from 2013, acquired from the EUCLID study (154). *Maximum likelihood trees were estimated as previously (257), and generated using the Interactive Tree of Life (785).*

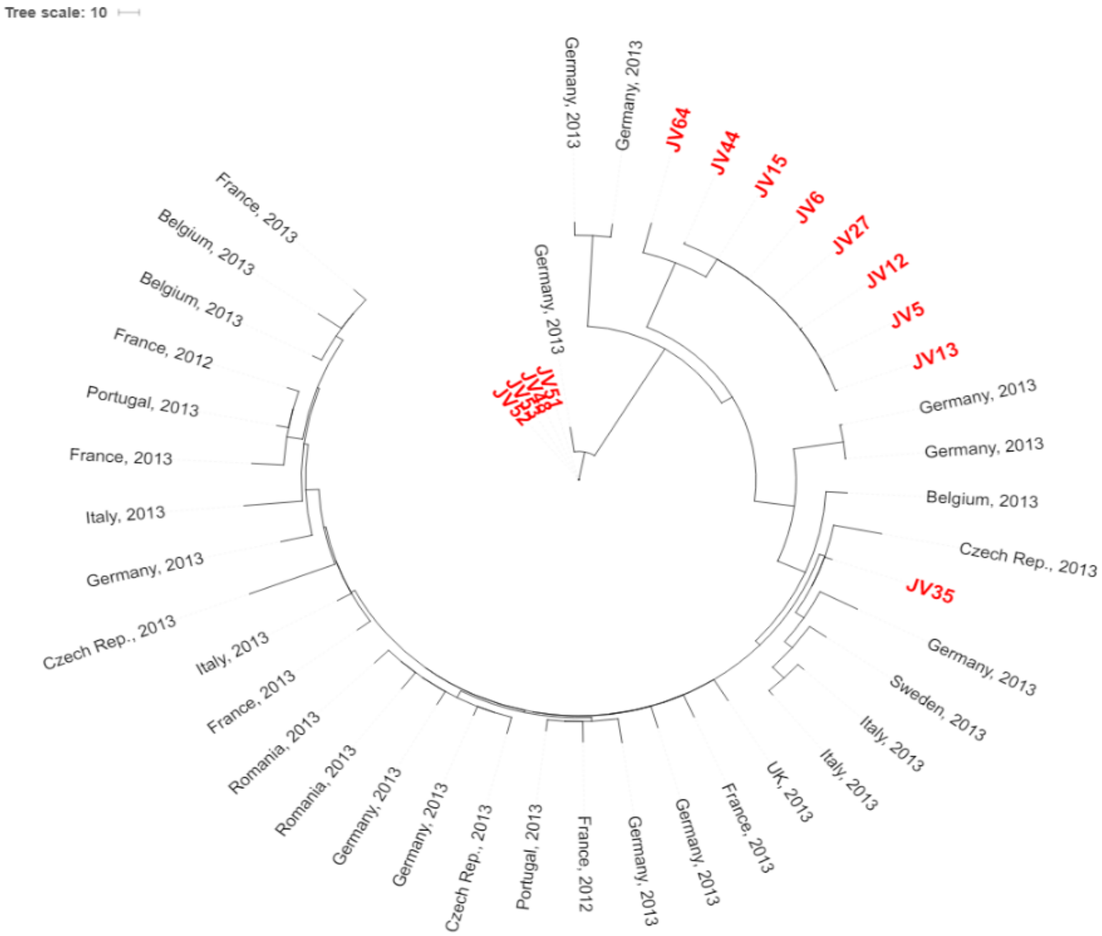


Figure 33: Phylogenetic tree representation of "historical" ribotype 020 isolates amongst comparator 020 strains from 2013, acquired from the EUCLID study [154]. *Maximum likelihood trees were estimated as previously [257], and generated using the Interactive Tree of Life [785].*

4.5 Discussion

4.5.1 Antimicrobial resistance determinant detection

Whilst the *C. difficile* core genome is comprised of approximately 1,000 genes, the pan genome consists of nearly 10,000 (573). This demonstration of genomic plasticity highlights the potential for resistant determinant acquisition through mobile genetic elements. The historical collection harboured twenty resistance conferring genes with the potential to spread amongst the gut microbiome, along with multiple mutational elements. As these strains are exposed to antibiotics, a selection process may result in concentration of MDR *C. difficile,* causing greater clinical challenges.

4.5.1.1 Macrolide-lincosamide-streptogramin-B resistance

Methylation of ribosomal RNA is widely accepted as the most prevalent mechanism of resistance to the MLS_B classes of antimicrobials, with the ERY resistance methylation genes predominant in resistant *C. difficile* (444, 498). Whilst up to 86.7% of modern isolates have demonstrated the presence of the *ermB* gene (521, 728), only eight percent of this historical collection harboured the determinant. This disparity is potentially attributable to the temporal differences between the strain catalogues, since more comparable frequencies (15%) were reported in *C. difficile* from 1987-1998 (510). This North American study by Tang-Feldman *et al.* demonstrating an elevated occurrence rate was observed over a period spanning a decade and was primarily associated with increased CLI use and subsequent selection (510). This increase in resistant genotype prevalence over time may be as expected, since the frequency and heterogeneity of genetic arrangements previously reported in *ermB* determinants suggests high levels of transposition and genetic exchange amongst *C. difficile* and other species (489, 496, 511, 512).

Reflective of previous publications (510, 521, 787), resistance to both ERY and CLI in the *ermB* positive isolates was not absolute, with a minority of strains containing the determinant, but displaying CLI sensitivity. Here, two strains remained lincosamide sensitive, whilst harbouring this resistance-conferring element. This could potentially be explained in terms of an inducible MLS_B phenotype, often reported in Gram positive bacteria (788, 789). Isolates demonstrating contrasting phenotypes (ERY resistant / CLI sensitive) arise because CLI is known as a poor inducer. This inducible phenotype could have important clinical implications in instances where MLSB therapy is used.

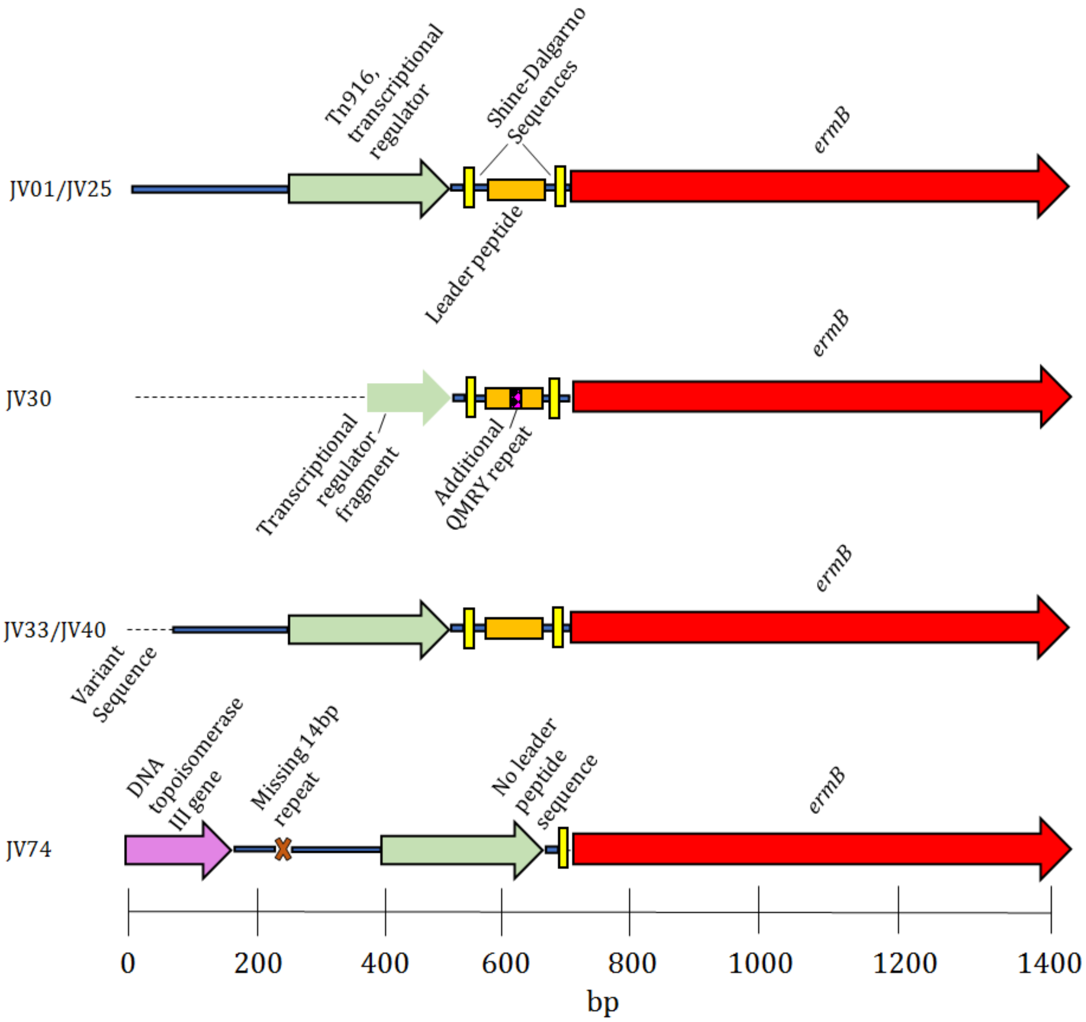


Figure 34: Schematic representation of the configuration of regulatory sequences upstream from the ermB gene of historical C. difficile genomes. *Thick blue lines represent identical nucleotides to the consensus sequence, whilst dashed lines represent sequence variation.*

Analysis of the upstream regions of the *ermB* gene was performed to establish any sequence differences that may be inhibiting methyltransferase expression (Figure 34). ERY sensitive isolate JV30 exhibited an additional repeating region on the leader peptide sequence. Since mutations in *erm* leader peptide regions have been reported to reduce the efficacy of expression induction (790), this sequence polymorphism has the potential to cause the sensitive phenotype observed here. During an un-induced state the Shine-Dalgarno sequence of the *ermB* gene is concealed within a hairpin structure in the mRNA, resulting in inhibited translation. In the presence of an ERY inducer molecule the ribosome stalls during leader peptide translation, exposing the *ermB* Shine-Dalgarno sequence for translation (791). Therefore, sequence mutation in this region may impact on RNA secondary structure and expression inducibility, ultimately affecting susceptibility phenotype. Furthermore, the transcriptional regulator is truncated in this genome and may impact the transcription of the methyltransferase.

These data cannot explain the exact mechanisms behind the apparent lack of *ermB* expression in isolates JV33 and JV40. Since these sequences are comparable with those of ERY resistant isolates (JV01/JV25), they may require a pre-incubation with sub-MIC ERY in order to induce expression. Alternatively, there could be a potentially distant, trans-acting element silencing the gene. Whilst the sequences 237bp upstream of the transcriptional regulator are different between resistant and sensitive isolates, these regions may be too distant to impact *ermB* expression.

Interestingly, isolate JV74 demonstrated an absence of leader peptide or Shine-Dalgarno sequence, but still an ERY resistant phenotype. This may be explained in terms of a substantial deletion of upstream sequence resulting in a lack of *ermB* Shine-Dalgarno obstruction and subsequent over expression of the *ermB* gene (791). This would suggest that resistance in this strain was constitutive as opposed to inducible.

Additional determinants have been demonstrated to confer this susceptibility pattern in other genera, with an *msrA* gene responsible for resistance in streptococci (789). The mechanism of *msrA*-like proteins has recently been attributed to one of ribosomal protection, as opposed to efflux, clarifying long standing controversy in this area (792). Although not previously reported in *C. difficile*, this protection protein has selective activity for 14 and 15-membered ring macrolides, and is therefore incapable of inhibiting lincosamide activity, thus generating phenotypic differentiation. Nonetheless, this study was unable to identify any close *msrA* homologues amongst the historical collection. Further unidentified determinants responsible for this phenotype could potentially demonstrate similar ribosomal protection mechanisms.

Since *ermB* presence usually confers ERY resistance (444, 510, 521), the unexpected finding of two strains (JV33 and JV40) harbouring the gene, but not indicating the resistant phenotype (even after secondary testing) was intriguing. This may be due to expression complexities associated with promotor or regulator sequences or even a mutation within the *ermB* gene, leading to loss of functionality. The latter notion has been previously reported in an *ermB* positive, ERY sensitive *C. difficile* strain (793). Nonetheless, although three different *ermB* gene sequences were observed (Appendix), these did not correlate with the resistant phenotypes and cannot explain the differences detected. Further investigations would be required to determine the mechanism behind these discrepancies.

Similarly, since ten strains tested in this study exhibited an ERY resistant phenotype without the presence of the classic *ermB* gene, further elements conferring a reduced susceptibility must be conveying influence. Similar to one large study of MDR *C. difficile* strains, where 17% of isolates exhibited ERY resistance in the absence of an *ermB* determinant (444), this collection revealed 12% with the same genotype/phenotype combination. Additional efflux transporters (794, 795) or target modifications in rRNA domains (513, 796) could provide ulterior mechanisms for macrolide resistance, although no non-synonymous mutations were discovered in the L22 or 23S large subunits of rRNA in either this or Spigaglia's study (444). The latter research study also concluded that all nine *ermB* negative strains referred for supplementary MIC testing were unaffected by efflux inhibitors, CCCP and reserpine. Correspondingly, CCCP and reserpine testing revealed no demonstrable effect on ERY MICs in these historical strains, suggesting the presence of further, undetermined elements relevant to the macrolide resistance mechanism. No proximal homologues to *mefA* or *mefE* determinants were detected amongst the historical collection, with all CARD-RGI outputs revealing <35% similarity.

4.5.1.2 Oxazolidinone resistance determinants

LZD resistance amongst *C. difficile* is uncommon (797-799), although higher prevalence has been demonstrated in ribotype 001 and 027 isolates (225). Whilst it is not a typical treatment option for CDI, as with many broad spectrum antimicrobials, resistance to this oxazolidinone has the potential to induce disease. This protein synthesis inhibitor targets the 23S ribosomal RNA unit, with resistance conferred via alteration of the central loop domain V (800). In *C. difficile* the predominant resistance aetiology is the presence of the *cfr* gene and the subsequent methylation at position A2503 (528, 530, 532). The discovery of this gene in all four LZD resistant isolates doubtlessly conferred the phenotype in these strains, all of which revealed concurrent CHL and CLI resistance, as previously associated with this multiple resistance determinant (225, 528). Interestingly, two of these isolates represented the 078 ribotype, in support of the work by Marin *et al.* who identified the *cfr* gene on a Tn*6218* transposon amongst ribotypes 017 and 078 (528). In support of this data the same transposable element harboured the *cfr* gene in all LZD historical genomes. Curiously, the RAST annotation service proved unable to distinguish between the Cfr and RlmN methyltransferases, since both methylate the A2503 position, assigning CDSs in all isolates as a generic "*rRNA methyltransferase*". However, the latter methylates at C-2, as opposed to the C-8 position directed by the *cfr* gene, and crucially is not associated with reduced antimicrobial susceptibility (801).

Whilst, the presence of the *cfr* gene is the primary source of LZD resistance in *C. difficile*, point mutations in ribosomal proteins have also been linked to oxazolidinone resistance in its absence (528, 802, 803). Here, investigations of three previously implicated ribosomal proteins, L3, L4 and the 23S subunit revealed several non-synonymous substitutions. Though mutations in the 23S ribosomal subunit are the most prevalently reported resistance-conferring element in other species (800, 804), with the Gly2576>Thr substitution strongly implicated, none were discovered during an *in vitro* passage experiment with *C. difficile* (805). Nonetheless, further regions of the central loop domain, crucial to drug-ribosome interactions have been discovered, including the substitutions Gly2032>Ala and Gly2447>Thr (*E. coli* numbering) (806). In the genomes investigated in this thesis, 12 isolates revealed three variant amino acid substitutions at the Ile750 codon (Table 18), in the proximity of the aforementioned point mutations. Intriguingly, all LZD resistant isolates exhibited a methionine replacement at this position, providing further credence to the notion that these point substitutions may effect the tertiary structure of the LZD active site. Interestingly, replacement of the aliphatic isoleucine at the same codon with either valine or glutamic acid did not correlate with a resistant phenotype. Since valine has very similar properties to isoleucine, this exchange may not convey as significant structural changes as the sulphur-containing methionine. Nevertheless, five isolates demonstrated the Ile750>Met point mutation in the absence of a resistant phenotype, potentially suggesting that this polymorphism is not independently responsible for the phenotype.

Examination of the L3 ribosomal protein gene revealed only one non-synonymous substitution amongst all historical genomes. Identified in the highly distinct genome of isolate JV59, an Ile205>Val mutation did not correlate with LZD resistance. However, SNPs were detected in the L4 protein in concurrence with reduced susceptibility; (Appendix). Resistant isolates JV14, JV17 and JV22 exhibited Gly71>Asp and Val163>Ile point mutations, which although is distal to the drug binding site, has been linked to reduced susceptibility to LZD (805). The former substitution has been associated with resistance in *C. perfringens* (807), whilst a proximal Lys68>Asn mutation has been implicated in *C. difficile* (805). Although the finding of this substitution correlated with LZD resistance in these isolates, JV30 exhibited a lower level of resistance (8 mg/L), in the absence of any L4 gene variations. Whilst it seems more plausible that the presence of the *cfr* gene is likely the primary aetiology of the phenotype, these substitutions may be contributory. Non-synonymous amino acid replacement at the Val-163 codon was deemed as less plausible as an influential factor in the reduction of LZD susceptibility, as JV65 harboured modification at this position, independent of resistance.

The rate of *cfr* prevalence amongst this historical collection was lower than reported in modern collections, 5.3% vs 10% (530). Although CLI use is currently subject to greater restriction (68, 103), this increase may be attributed to the introduction of LZD in the interim period (808), resulting in increased selection of this gene. The identification of the *cfr* gene amongst an isolate collection from the 1980's suggests that this determinant may have contributed to *C. difficile* outbreaks in a period of elevated CLI use.

4.5.1.3 Tetracycline resistance determinants

All TET resistance discovered in this historical catalogue was correlated with the primary determinant in *C. difficile*, *tetM* (486, 488). The importance of this element was supported by the absence of any TET sensitive isolates harbouring the gene. The 9.3% prevalence rate of *tetM* in this collection corresponds closely with the 13.0% resistance rates amongst UK isolates from 1979-86 (381). Although TET resistance rates in the UK have reduced since this period (381, 394), European frequencies were reported as high as 17.1% in MDR isolates (444). In support of previous work (444, 488), this TET resistant group encompassed all instances of both ribotypes 012 and 078. These two ribotypes have historically contained resistance determining transposable elements, with the Tn*5397* and Tn*916*-like transposons found in 012 and 078, respectively (488). These findings are reflected unerringly in the historical genomes interrogated in this thesis. This complete coverage of particular strain types is indicative of resistant elements proving advantageous, leading to retention and subsequent clonal expansion. Interestingly, a recent report from Dingle *et al.* revealed the presence of the *tetM* gene in 76.9% of ribotype 078 isolates (809), 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.

The presence of an additional TET resistance element, *tetA(P)*, in conjunction with *tetM* in isolate JV32, correlated with a >32 mg/L TET MIC. This MFS efflux determinant featuring 12 transmembrane domains, is ubiquitous amongst *C. perfringens* (810) and has previously been reported in 19% of zoonotic *C. difficile* isolates (576). The minimal clinical data associated with this specimen documents "*profuse diarrhoea following tetracycline*". Coupled with the susceptibility findings this potentially indicates that, whilst *tetA(P)* imparts no independent resistant phenotype (576), these two elements combined may demonstrate an exacerbating effect. Nonetheless, *tetM* mediated TET resistance in *C. difficile* has been linked to a diverse range of resistant phenotypes (811), with gene configuration contributory to this.

4.5.1.4 Fluoroquinolone resistance determinants

The QRDR has been outlined as a region of mutational hotspots, relevant to fluoroquinolone resistance development (438). The majority of substitutional determinants are reported in these short regions of the *gyrA* and *gyrB* genes (765, 812-814) and the examination of historical genomes from this study revealed comparable findings. All MXF resistance detected in the historical collection correlated with the common Thr82>Ile substitution. Associated with high-level resistance (16-32 mg/L), the majority of strains carrying this genotype represented ribotypes 001 and 027. In addition to this mutation, all three 027 isolates carried the same two, non-synonymous substitutions, Leu406>Ile and Asp468>Asn. Whilst neither of these two extra-QRDR alterations have been independently associated with a resistant phenotype, they cannot be dismissed as contributory factors based on this data set. Similarly, the two further non-synonymous substitutions identified in *gyrA* of the collection did not convey MXF resistance.

While the isolates displaying MXF resistance are well characterised with the Thr82>Ile mutation, the rationalisation for the ubiquitous CIP resistance was not clear. While there are several known mechanisms of fluoroquinolone resistance in bacteria, unfortunately, the data presented in this study cannot provide sufficient information to identify a mechanism for the putatively intrinsic CIP resistance observed here. Whilst evidence for the involvement of putative efflux or gyrase protective homologues, such as *CD2068* (777) or *qnr*-like genes (815, 816) is undermined by lack of absolute correlation between genotype and phenotype, all extra-QRDR mutations revealed a non-existent relationship with CIP resistance. Therefore, further unidentified mechanisms are likely connected, whether that be efflux or mutational inhibition. Although *C. difficile* does not contain any close *parC* homologues, the role of the topoisomerase IV must be performed by an alternative enzyme, as it is crucial to DNA synthesis. Therefore, there may be an unidentified enzyme of similar function, with a lower affinity to CIP, which potentially inhibits its action. Discussion of the evidence available from this investigation, including the reasoning why they are unlikely to be the cause of CIP resistance, is presented below.

Multiple sequence alignments of two putative fluoroquinolone resistance determinants, a *qnr*-like, pentapeptide protein and an ABC transporter, *CD2068* was performed to further investigate a potential mechanism for the apparent, intrinsic CIP resistance observed here. *CD2068* has been associated with upregulation in the presence of CIP and the impact on fluoroquinolone susceptibility has been reported in *C. difficile* (777). Demonstrating 63% identity with the CpmA transporter observed in other clostridia (817), this putative MDR efflux determinant exhibited >98% identity in the majority of historical genomes. Since this ATP-activated element has demonstrated membrane translocation of several antimicrobial classes, including fluoroquinolones (777), it has the potential to be associated with the CIP resistance observed in these strains. However, it cannot explain the resistance in those isolates exhibiting low homology (30%) to this gene and therefore, it is likely that the CIP resistance cannot be attributable to this determinant. Interestingly, the presence of this *CD2068*-like gene with low homology seemed confined to specific ribotypes (015 and 020, particularly). Coupled with its low homology determination in single instances of two rare, distinctive ribotypes (341 and 862), it appears to represent an element associated with clonal divergence.

Further genomic analysis revealed the presence of homologues (>99%) to the DNA gyrase protecting, *qnr*-like element (815, 816). However, this *C. difficile* element only demonstrates a low homology to the *E. coli* gene responsible for a pentapeptide repeating protein, which inhibits the quinolone molecule from binding to the gyrase active site. Whilst close homologues to the *C. difficile* element discovered in other clostridia conferred a reduction in susceptibility to CIP, when transformed in to an *E. coli* strain, the CD *qnr*-like protein demonstrated minimal effect on CIP MIC (776) and is therefore unlikely to be the cause of intrinsic resistance.

Corroborating other findings (814), 74.7% of all test isolate genomes demonstrated no QRDR mutations. Two QRDR-independent polymorphisms in the *gyrB* gene, Val130>Ile and Ile139>Arg, were highly prevalent amongst MXF sensitive isolates, signifying that they convey no resistant phenotype. This finding is in support of previous work indicating these mutations in sensitive isolates (814). Nonetheless, it cannot be dismissed that they contribute to the steric hindrance of antimicrobial binding, as codon 139 represents a side chain area of the protein, likely imparting a greater conformational change (814). Many of these silent mutations in the *gyrB* gene were observed as highly ribotype-specific, with Val-130 intrinsically linked to ribotypes 001 and 015, and Ile-139 to the closely related 014 and 020 strains. Equally, the previously reported Ser366>Ala was localised to ribotype 017 and 242 genomes (198). These findings reflect the study by MacAogain *et al.* who identified similar ribotype traits, suggesting homoplasic variations (814). This apparent parallel evolution of analogous substitutions highlighted the potential for consistent, high-level mutability in this important resistance determining region. Due to the lack of correlation between these polymorphisms located outside of the QRDR and CIP resistance these are unlikely to impact on the phenotype.

Assessment of the highly distinct genome of isolate JV59, revealed a plethora of amino acid substitutions in the DNA gyrase genes; (Table 19). All *gyrA* substitutions were located outside of the QRDR, whilst only the Ser-366 and Ser-416 codons of *gyrB* demonstrated SNPs inside the central region. Both of these non-synonymous mutations have been reported to confer no resistance to fluoroquinolones (198, 814), as observed in the phenotypic analysis of this isolate. Therefore, although harbouring a highly variant set of gyrase genes from the rest of the collection, these variations appear to represent the distinct phylogeny of this ribotype 862 isolate, rather than an adaptive response to antimicrobial pressure. While they do not appear deleterious in nature, this ribotype has not previously been reported and it is therefore likely that elements of its genetic organisation may have contributed to its inability to compete with other, more successful ribotypes in clinical environments.

4.5.1.5 Vancomycin resistance determinants

Whilst VAN resistance is common in other genera, such as staphylococci and enterococci, it is relatively rare in *C. difficile* (108, 188, 818, 819). Nonetheless, reduced susceptibility has been reported amongst clinical isolates, particularly in the context of outbreak situations (199, 200, 820). Interestingly, whilst no resistance was detected in this historical collection, reduced susceptibility was observed in four strains isolated from temporally distinct specimens, associated with the same patient. The potential for horizontal gene transfer between these concomitant strains may well have been high, and as one of these isolates represented ribotype 001, a type occasionally associated with reduced susceptibility to VAN (188), this may have been the source of transmission.

The identification of eight *van* genes in each intermediate resistant isolate supported the suggestions that this operon only encodes for low-level resistance (467, 821). The *vanG*-like cluster identified by Sebaihia (314), consists of five open reading frames, *vanR, vanS, vanG, vanXY* and *vanT*, whilst regulatory genes *vanUG, vanRG* and *vanSG* complete the operon (821). In this data set, the presence of all eight genes correlated with an intermediate resistant phenotype, whilst the independent detection of homologues appeared to confer no influence on VAN resistance. Interestingly, it has been reported that although the *vanG* complex was often present in *C. difficile*, it did not correlate with a resistant phenotype (468).

Ammam *et al.* were able to confirm the functionality of the genes, with identifiable modified peptidoglycan precursors, but could not explain the lack of cell wall uptake. One explanation was proffered, suggesting a lack of elimination of unmodified peptidoglycan building blocks and subsequent competitive inhibition. Whilst this seems plausible, the revelation that the *C. difficile* cell wall is synthesised in a novel configuration may contribute to the deficient cell wall modification (686). Nevertheless, the clinical implications of these genes appears minimal, as the faecal concentrations of VAN are reportedly far superior to the resistant breakpoint (520-2,200 mg/L) (822).

4.5.1.6 Further resistance determinants

The identification of an EF-Tu, elongation factor homologue in isolate JV60 may be indicative of resistance to the kirromycin class, as demonstrated in streptomyces (823). This class of protein synthesis inhibitors act to block the disassociation of the elongation factor and ribosome, preventing formation of essential peptide bonding (824). Minimal data is available regarding *C. difficile* susceptibilities to this class, although Clabots *et al.* were able to demonstrate MICs in the range of 0.06-0.5 mg/L for a panel of strains exposed to efrotomycin, a member of the kirromycin class (825). However, the resistance phenotype of isolate JV60 was not able to be tested for this class, as the compounds are highly expensive and the clinical relevance deemed minimal. Equally, the determination of isolate JV73, possessing the kanamycin resistance determining complex, *AAC(6')-Ie-APH(2'')*, was not further investigated, as kanamycin has been demonstrated to have minimal activity in anaerobic bacteria (780, 781). Nonetheless, this transposable resistance element (826), further demonstrates the ability of *C. difficile* to assimilate a range of resistance conferring elements. The *AAC(6')-Ie-APH(2'')* gene may also contribute to multiple resistances; by encoding for a multifunctional enzyme, combining sequential acetylation and phosphorylation (827). The enzyme can reduce electrostatic and steric interactions, decreasing the binding affinity of several aminoglycosides to the rRNA target (828).

Both strains JV73 and JV74 harboured the *aadE* gene, a resistance determinant for streptomycin (829). Generally, there is an absence of activity of aminoglycosides against anaerobic bacteria; however Pirs *et al.* demonstrated only 81% streptomycin susceptibility in *C. difficile* isolates of human origin (704). Nonetheless, breakpoints were defined as high as 1,000 mg/L, elevated considerably above potential gut concentrations achieved by the drug (830, 831). Here, both isolates harbouring the resistance determinant demonstrated high levels of resistance to streptomycin, whilst the majority of *aadE* negative comparators were sensitive to the drug (<512 mg/L). Nevertheless, since one isolate demonstrated resistance in the absence of the gene, it suggests that additional determinants are involved. Whilst streptomycin resistance in *C. difficile* is of lesser importance, as it is not deemed as a classic risk factor (832), in particular patient cohorts such as those with paediatric cancers, aminoglycoside administration has been correlated with an increased CDI risk (833). The presence of these determinants also further highlighted the potential for horizontal gene transfer across the bacterial kingdom.

The presence of chromosomal efflux gene, *cdeA*, in all isolates supported previous findings of high prevalence amongst *C. difficile* (834). Whilst this MATE protein encoding homologue was initially considered a putative cause for the ubiquitous CIP resistance, in this study the presence of sodium ion gradient based expulsion inhibitor, reserpine, revealed no effect on susceptibilities. Equally in the primary study by Dridi *et al.*, native *C. difficile* promotors did not sufficiently respond to CIP exposure to elicit resistance (509). These outcomes corroborated to suggest further unidentified elements as the source of the underlying CIP resistance, for instance modified promotors or additional efflux determinants.

4.5.1.7 Multidrug resistant strains

Of the MDR strains identified in the historical collection, 76.5% exhibited transposable resistance-conferring elements, predominantly *tetM, ermB* and *cfr*. Chromosomal mutations in the *gyrA* gene were contributory in 41.2% of isolates demonstrating an MDR phenotype. Whilst fluoroquinolone resistance substitutions have been reported as highly influential in the success of ribotype 027, this data suggests that mobile elements may offer a substantial contribution to the spread of MDR strains. Since for genetic transposition to occur, no antimicrobial pressure is necessary, the opportunity for this event to transpire is more widespread. Nonetheless, evidence eluded to in Chapter Six of this thesis indicates that once acquired, resistance to fluoroquinolones seemingly causes no detriment to *C. difficile* fitness and would therefore likely be retained through subsequent generations. The retention of such elements, combined with transposable multi-agent resistance determinants, may ultimately result in an untenable position, where many antibiotic classes become ineffective against this prevalent pathogen.

Interestingly, two MDR isolates did not demonstrate any of the well-defined resistance conferring determinants identified in this investigation. This suggests the contributions of unknown or yet to be fully understood factors, such as the multi-compound extrusion system, CdeA, ubiquitous in this collection. Equally, whilst genes encoding DNA gyrase and RNA polymerase elements were examined for amino acid substitutions, alterations in other chromosomal regions, yet to be implicated in antimicrobial resistance, may well be responsible.

4.5.2 Phylogenetic analysis

Phylogenetic analyses were utilised to corroborate the notion that the isolates recovered from the historical collection genuinely originated from the early 1980s. Harnessing the knowledge of the *C. difficile* molecular clock (257, 569), the majority of these genomes were assessed for age authenticity, based on their relatedness to modern genomes in evolutionary tree analyses. Since this type of analysis presents no absolute answer, only probability of relatedness, the investigation proved complex. Where distant relationships were ascertained, with divisions of thousands of SNPs separating genomes (Figure 28), an evolutionary distinction could be assumed. However, the majority of instances were not so definitive.

The unusual discovery of MXF resistant ribotype 027 isolates, pre-dating the addition of the agent to the formulary, raised questions regarding the accuracy and reliability of the isolates' presumed ages. Whilst this may have developed due to exposure to earlier quinolones, such as nalidixic acid, phylogenetic analysis was performed to elucidate ancestral relationships of "historical" isolates, in order to identify how these strains related to modern *C. difficile* populations. Seven ribotypes were investigated to establish evolutionary relationships in distinct clades. These genomes consisted of many of the most prevalent ribotypes, both in the historical collection and also amongst modern epidemiology. The comparator database for ribotype 027 was the most extensive, including isolates from over 30 years ago. Unfortunately, such historical comparators were not available for other ribotypes at the time of analysis. Nonetheless, in some instances considerable genomic distinction from modern genomes provided convincing conclusions; (Figure 28 and Figure 33).

Whilst spatial clustering and root proximity on phylogenetic trees offer valuable evidence into genomic ancestry, the magnitude of SNP deviations from the closest modern relation may provide an additional measure of confidence in the true age of an isolate. Prediction intervals were calculated to represent a probabilistic lower limit of evolution, based on current *C. difficile* molecular clock estimations (0.74 SNPs per genome, per year) (257, 569). In this context they refer to the probability of an observed number of SNPs or fewer occurring in the number of years separating a genome from its nearest neighbour on the phylogenetic tree. It was observed that 69% of genomes from the historical collection correlated with a 95% prediction interval; (Figure 35). This inspired confidence in the age demographics of these historical isolates, potentially supporting the accuracy of proposed evolutionary rates. Nonetheless, this analysis also casts some doubt on the validity of the genomes observed outside of the expected range. Since molecular clock data only provide estimations, it may be conceivable that a small proportion of ancestral genomes may deviate from this projected range.

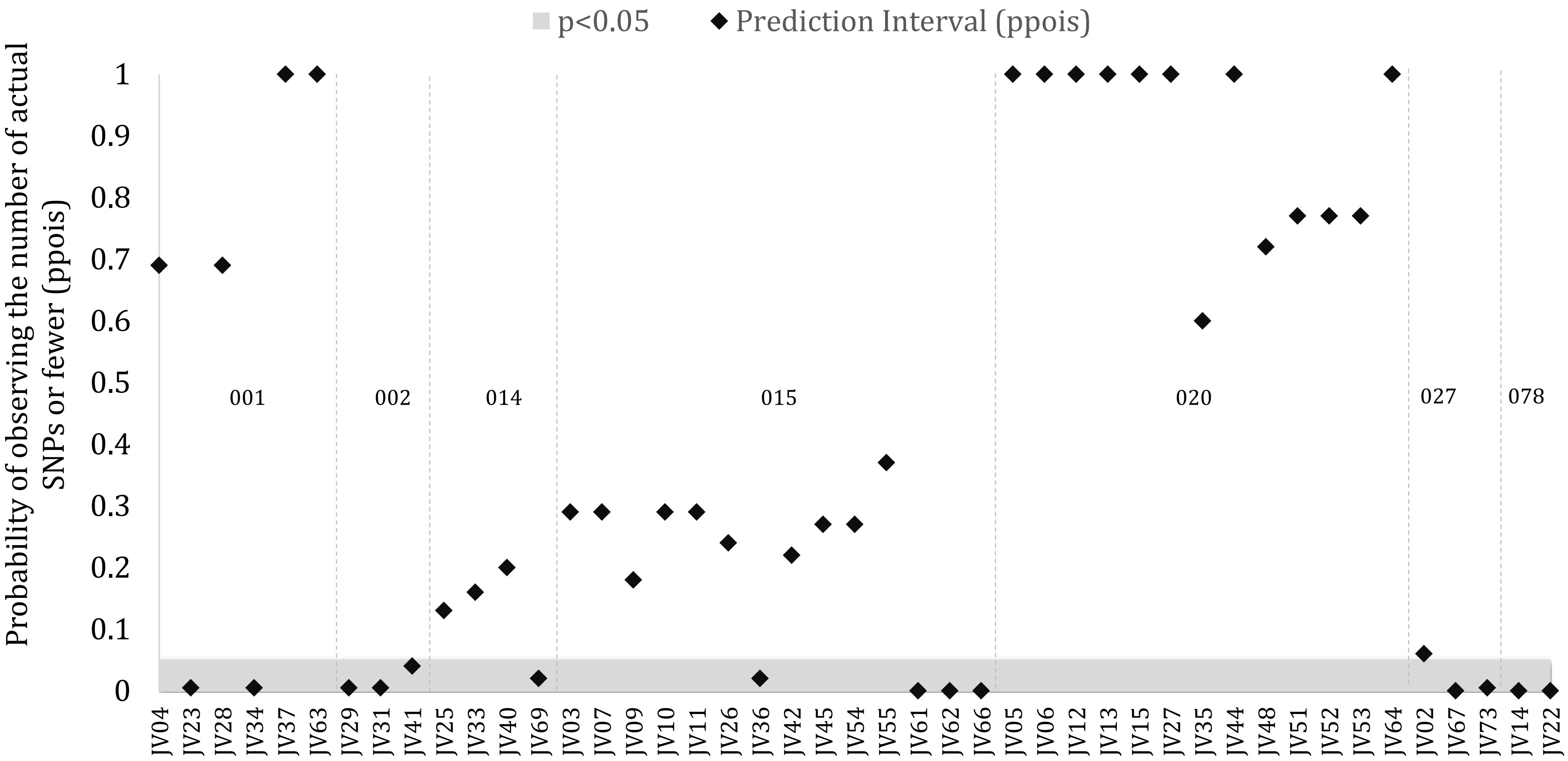
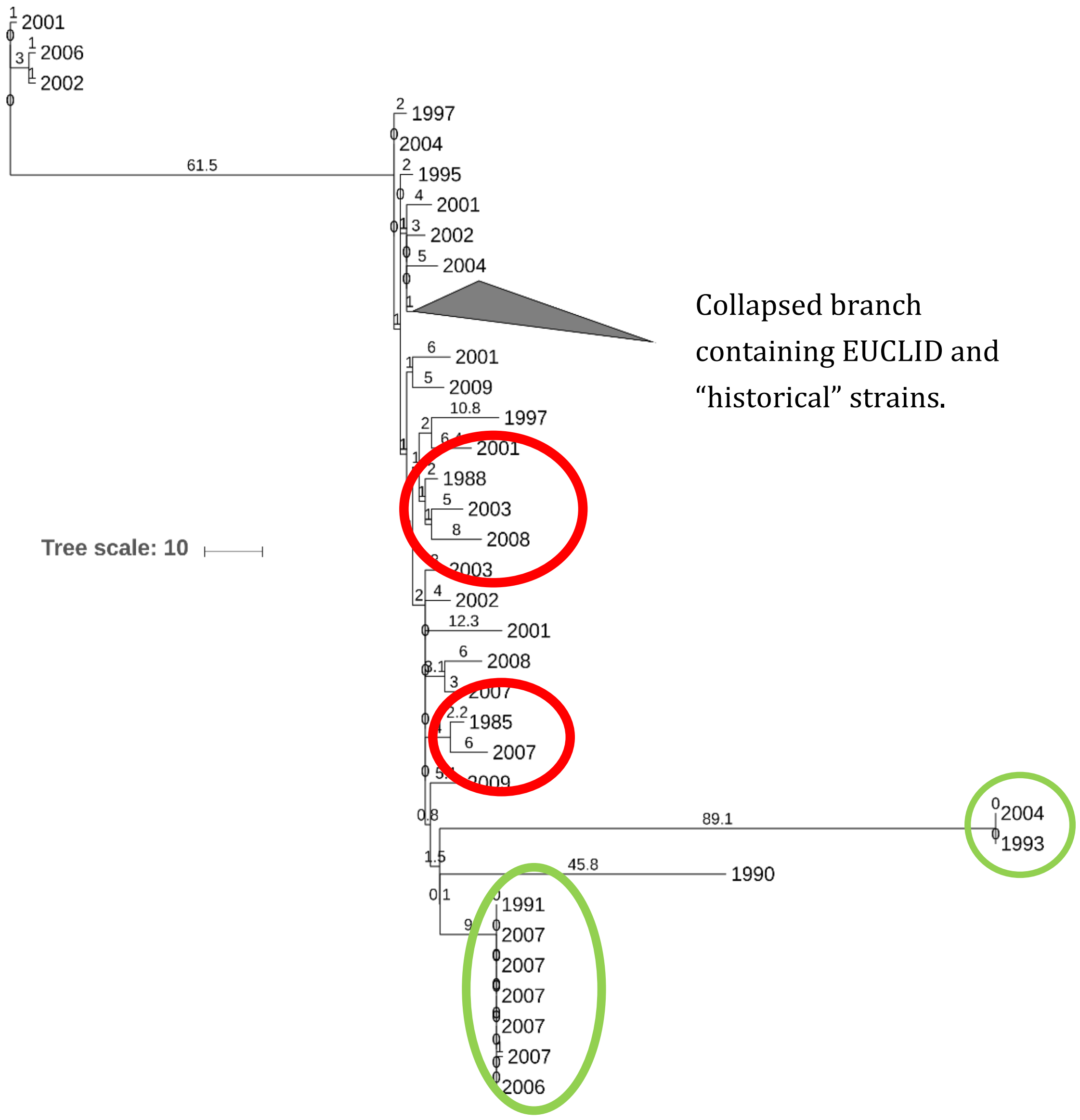


Figure 35: Prediction intervals representing the probabilities of the observed number of single nucleotide polymorphisms (SNPs) or fewer occurring in the time period separating neighbouring taxa, based on Poisson distribution of C. difficile molecular clock estimations (0.74 SNPs per genome, per year) ***[257]****. Numeric labels refer to PCR ribotype groups.*

Unexpectedly, the three "historical" ribotype 027 isolates clustered amongst the modern genomes of recent EUCLID strains. Combined with the presence of fluoroquinolone resistance, prior to previously reported acquisition time scales (562), these isolates did not necessarily fit the anticipated characteristics of early ribotype 027 strains. Whilst the closest relative of isolate JV02 (putatively from 1981) was an isolate from Glasgow from 2008, the prediction interval probability (based on 0.74 SNPs per genome, per year (257)) for 13 or fewer SNPs occurring over 27 years was p=0.07. This observed SNP difference would fall into the expected range (95% prediction interval) of the Poisson distribution of the molecular clock (Figure 35), alluding to the finding of a true historical isolate. Nonetheless, the other two isolates JV67 and JV73 were only eight and two SNPs divergent, respectively. Based on the current understanding of the molecular clock, the latter finding would conflict with the notion that it was greater than 25 years older than comparator genomes. However, further analysis of branches comprising older isolates (back to 1985) indicated the assimilation of some modern strains, denoting close ancestries. If we consider the branches close to the root of the phylogram (Figure 36), it can be observed that isolates >20 years apart reveal only ~8 SNPs difference. Equally, isolates separated even further by time have shown zero SNP differences between genomes from 1991 to 2007 and 1993 to 2004. These findings would also lie outside of the 95% prediction interval estimations. This evidence is highly suggestive of a greater complexity to temporal analyses based on phylogeny. Assuming that this sequence data (acquired from highly cited publications) is reliable, a relationship is portrayed where additional considerations may be necessary when investigating the dating of *C. difficile* genomes, other than clustering and SNP differentiation. The extent of evolutionary dormancy experienced by a strain, whilst existing in a quiescent spore form is one potential explanation.

The striking observation of two clusters of identical genomes, spanning 11 and 16 years, appeared to contradict the current understanding of *C. difficile* evolution. Further investigation into these isolates revealed another possible intriguing explanation for these seemingly erroneous findings. All isolates clustered with zero SNP differences (highlighted in green in Figure 36) originated from Arizona, with one isolate putatively deriving from 1991 clustered amongst strains from 2006/2007 (563). These genomes were sequenced in a study by Songer *et al.*, which revealed the presence of ribotype 027 in retail meats purchased in Arizona (835). However, a follow up publication from the same research group (836), used MLVA to demonstrate that many of these isolates were indistinguishable from each other, with all data strongly suggestive of specimen contamination with a laboratory strain. This seems increasingly likely to have been with the identical 1991 isolate, prior to whole genome sequencing. These data serve to highlight the caution required during interpretation of external data sets.

The convoluted nature of the individual *C. difficile* PCR ribotype evolutionary trees may be partially explained by the sporulation of this bacteria (837). Since genetic replication does not occur when bacteria exist in the quiescent spore form, evolution has been reported as far slower in spore-forming organisms (838, 839). With no way of knowing how long a particular strain has existed in spore form, it proves difficult to confidently establish temporal, evolutionary links. Whilst it may be assumed that the isolates from the historical collection existed in spore form for over 30 years, the proportion of time, so-called “modern” strains have remained in this state is practically incalculable. We know *C. difficile* is harboured as spores, asymptomatically in both humans and animals (8) and can potentially exist for prolonged periods in the environment (840). This unknown period of quiescence contributes to the difficulty of aging through phylogenetic analysis. While on the face of it, the “historical” ribotype 027 strains demonstrated <10 SNPs difference to genomes from 2013, we cannot be certain as to the number of generations the “closely related” strains have passed through in comparison to others. Longitudinal studies could provide further information as to the impact of spore state on molecular clock estimations.



Collapsed branch containing EUCLID and "historical" strains.

Figure 36: Expanded phylogenetic tree of ribotype 027 genomes from 1985-2009, acquired from the published He et al. study *(562). Red circles highlight branches of closely related strains originating between up to 20 years apart. Green circles highlight taxa clusters consisting of temporally distant genomes, identified as zero SNPs different. Maximum likelihood trees were estimated as previously (257), and generated with the Interactive Tree of Life (785).*

The close relatedness to a neighbouring isolate from 2013 of the two ribotype 078 genomes isolated from the historical collection does not fit within even the lowest ranges of molecular clock estimations for this distinct ribotype (575). It is still reasonable to consider the potential involvement of evolutionary dormancy during unknown periods of existence in spore form. Whilst it must only be considered as conjecture, there may be an exacerbated evolutionary stasis effect linked with ribotype 078, due to its association with zoonotic carriage. This could potentially result in an extended existence in spore form, whilst carried asymptomatically amongst animals.

Analysis of the phylogenetic tress for all ribotypes, with the exception of 027, demonstrated clustering of the genomes from this collection, as maybe expected. However, the 027 phylogram presented a different picture, with isolates spread out amongst genomes of all ages. This finding is supported by the identification of groups of older isolates amongst more modern genomes in the existing data. Where older strains are branched closely with more modern genomes, the *in silico* fluoroquinolone susceptibility statuses correlated; sensitive with sensitive and resistant with resistant. This was also true for the three 027 strains recovered from the historical collection. However, the resistance identified in these isolates potentially exhibits a deviation from the expected molecular clock behaviour, indicating the possible complicating role of spore quiescence. Furthermore, these findings may suggest the earlier emergence of fluoroquinolone resistance in regions outside of North America, as previously documented (562). One potentially occurring in the UK (relating to JV02/JV67) and one emerging from Western Europe (relating to JV73). Whilst gyrase genes are highly mutable, it is plausible that resistant mutants may have arisen from multiple locations. The phylogenetic analyses performed here potentially support this hypothesis.

The analysis of PCR ribotype 027 isolates provided a substantial insight as to the relatedness and common ancestry of the strains isolated from the historical collection, due to the considerable temporal span of the genomes available. However, this is not the case for other ribotypes. Due to a distinct lack of pre-twenty-first century genomes for non-027 ribotypes, the database inadequacies pose difficulties to the accurate estimations of chronology. Nonetheless, where vast genomic deviations are apparent more confidence can be ascertained.

Interestingly, all ribotype 014 isolates clustered towards the root tree, sufficiently distant from modern genomes. Three distinct phylogenetic tree branches were observed, this was in support of previous findings indicating three different sequence types within this ribotype (576).

The outcome of these phylogenetic analyses potentially casts some doubt on the reliability of aged isolate collections. Whilst appropriately controlled collections with suitable restrictions may be considered dependable, without the knowledge of a collection's access and handling history, no matter how well anti-contamination procedures are obeyed, queries regarding the age of isolates cannot be answered with absolute certainty. Though measures were implemented, such as the use of negative controls and bench top settle plates, the risk of single cell contamination cannot be eradicated entirely. Conversely, the recovery of rare ribotypes, highly unlikely to be circulating in the laboratory environment and the recovery of identical strains in previously untouched specimens, all contributed to confidence in the findings.

4.6 Conclusions

NGS provides an excess of data for subsequent analysis. While genomic interrogations enable the identification of genotypic resistance determinants, a multitude of tools are available to generate further genetic links including those regarding ancestry and evolution. Here, we observed a diverse range of bacterial resistance determinants, with reduced prevalence from contemporary isolates. Whilst not all resistant phenotypes can be explained by the current level of understanding, the acquisition of NGS data will allow future retrospective analyses for the further elucidation of resistance mechanisms. The data presented here indicated that resistance progression has developed over the past 35 years, with horizontal gene transfer and antibiotic exposure the probable driving forces.

Whilst the majority of phylogenetic analyses conform to current molecular clock estimations, several isolates were observed outside this range. Though we may question the reliability of isolations from historical collections, there remains a possibility of spore dormancy impacting heavily on evolutionary rates.

Chapter 5 The Impact of *Clostridioides difficile* Mutation Frequencies on Fluoroquinolone Resistance

5.1 Introduction

Fluoroquinolone resistance is often considered as an important feature of major *C. difficile* outbreaks, particularly those associated with ribotype 027 (34, 112). Differing strain propensities for spontaneous mutation may contribute to evolutionary survival and a subsequent favoured clonal spread of certain ribotypes, particularly those harbouring mutational resistance determinants.

The process of DNA replication is not infallible, on occasion DNA polymerase enzymes make mistakes when pairing nucleotide bases (582). Whilst DNA repair systems have evolved to correct mismatches, errors result in spontaneous mutations that become permanent additions to the daughter cell's genome (583). Evolutionary principles assume that spontaneous bacterial mutations generally occur at frequencies between 10^{-10} and 10^{-9} per genome, per generation (584), whilst precise frequencies are highly species dependant (585). Where a consistent genome is favoured in a stable environment, the stochastic nature of spontaneous mutations benefit the bacteria when exposed to external stressors (613, 841). Elevated potential for mutagenesis offers possible survival opportunities through the acquisition of favourable, resistance conferring genotypes. A comparatively increased capability of one strain type to mutate at important antimicrobial resistance determining regions more readily than another, potentially contributes to successful dissemination of the more mutable type.

Mutation frequencies have been demonstrated to vary amongst organisms, ranging between $1x10^{-9}$ and $7x10^{-3}$ (232, 590, 608, 611, 842, 843). Equally, varying fluoroquinolone mutation frequencies have been observed between individual compounds within the class (610, 611) and under differing selection concentrations (591, 612). In *C. difficile*, fluoroquinolone resistant mutants are reportedly generated at rates as high as 2.4×10^{-5} for levofloxacin and 6.6×10^{-5} for MXF (812). Nonetheless, there is a paucity of data separating *C. difficile* mutation frequencies by ribotype. The determination of any variance may proffer a further contributory factor to the distribution of ribotypes, particularly the widespread success of ribotype 027 in clinical environments.

C. difficile genomes reveal highly conserved methyl-directed mismatch repair genes (573, 600), and whilst there is minimal data available regarding the *C. difficile* specific mechanism, *mutS* and *mutL* knockouts in *Clostridium acetobutylicum* resulted in elevated mutagenesis (599). An assessment of the influence of these genes, in relation to *C. difficile* mutation frequency, may help to identify possible explanations for variable mutability and link to any association with ribotype prevalence.

Ultimately, an elevated capacity to mutate crucial fluoroquinolone resistance genes potentially confers an evolutionary advantage, allowing progeny to survive exposure to such agents. Subsequent capacity for clonal expansion of resistant strains may contribute to endemicity and epidemic potential. Here, bacterial mutagenesis of fluoroquinolone resistance determining regions is investigated in a range of *C. difficile* ribotypes to examine the influence of mutability differences on *C. difficile* propagation.

5.2 Rationale

In this chapter, the mutability potential of seven prominent PCR ribotypes was explored through increasing mutational assays and fluoroquinolone selection. *C. difficile* mutation frequency assays were implemented to ascertain whether particular PCR ribotypes or individual isolates demonstrated differing propensities to development fluoroquinolone resistance-conferring mutations.

5.3 Methods

5.3.1 *C. difficile* mutation frequency determination

5.3.1.1 Test isolates

C. difficile isolates (*n*=44) from seven of the most prevalent disease-causing PCR ribotypes (001, 012, 015, 017, 020, 027, and 078) (118, 385) were acquired from the historical collection (1981-83) investigated in Chapter Three; (*n*=18) and the modern *Clos*ER; (*n*=26). All isolates selected for mutability testing demonstrated susceptibility to MXF (≤2 mg/L), with the exception of resistant strain CD4362, which displayed an MIC of 32 mg/L. MICs of all original test isolates were confirmed via retesting through an agar incorporation susceptibility testing method, as outline in Chapter Three. *Clos*ER strain identifiers were generated with a prefix of *CD* to the last four digits of original reference number (e.g. CD9609). Strain demographics and MXF MICs are reported in the Appendix.

Isolate Reference Number	Isolation Date	Origin	Original MXF MIC (mg/L)
CD3904	2015	Örebro, Sweden	1
CD3891	2015	Örebro, Sweden	2
CD9609	2016	St Antoine, France	1
CD9946	2016	Glasgow, UK	1
CD3809	2012	Dublin, Rep. Ireland	1
CD3051	2013	AGES, Austria	1
CD3079	2013	Örebro, Sweden	1

Table 20: Demographics of seven moxifloxacin susceptible PCR ribotype 027 isolates investigated in the mutation frequency assays. *MXF – moxifloxacin, MIC – minimum inhibitory concentration.*

5.3.1.2 Mutation frequency calculations

Mutation frequencies were calculated with an adaptation of the method described by O'Neill *et al.* (619); (Figure 37). Briefly, Brazier's media enhanced with 2% lysed, defibrinated horse blood was supplemented with MXF to create 4, 8 and 16 mg/L incorporated agars. Plates were dried at 37°C for 25 minutes and pre-reduced overnight. Strains were cultured for 18 hours on CBA to isolate distinct colonies. Ten to fifteen colonies of overnight growth were emulsified into 5 mL pre-reduced BHI broths and cultured for six hours. Total *C. difficile* populations were enumerated via serial dilution in pre-reduced PBS and plated onto Brazier's agar. Mutations were selected through inoculation of 100 μL of neat broth culture onto each of ten MXF-incorporated (4x MIC) agar plates and spread. All plates were incubated anaerobically at 37°C for 48 hours, prior to enumeration by direct colony counting. For each ribotype 027 isolate test, three colonies were picked off from the MXF selective plates and transferred into Schaedlers anaerobic broth for MXF MIC determination. Mutant isolate pickoffs of other ribotypes were tested intermittently to ensure the antibiotic concentration in the agar was accurate. An agar incorporation testing methodology was used as described in Chapter Three.

Individual assays were controlled using *C. difficile* isolates (obtained through pilot investigations) with MXF MICs of 0.5x and 2x the antibiotic concentration of the test agars. Control strains were streaked onto the same MXF supplemented Brazier's agar used in the assays. Test data was confirmed only if the 0.5x MIC, positive control demonstrated visible growth, whilst growth of the 2x MIC, negative control was inhibited after 48 hour culture.

Mutation frequencies were determined as:

$$\frac{\mathbf{TVC^{mut}\ (CFU/mL)}}{\mathbf{TVC\ (CFU/mL)}}$$

Where TVC^{mut} was the total colony counts from all ten MXF-incorporated agars, TVC is the total viable count from non-selective Brazier's agar, per millilitre. Mutation frequencies were reported as a mean average of a minimum of three biological replicates.

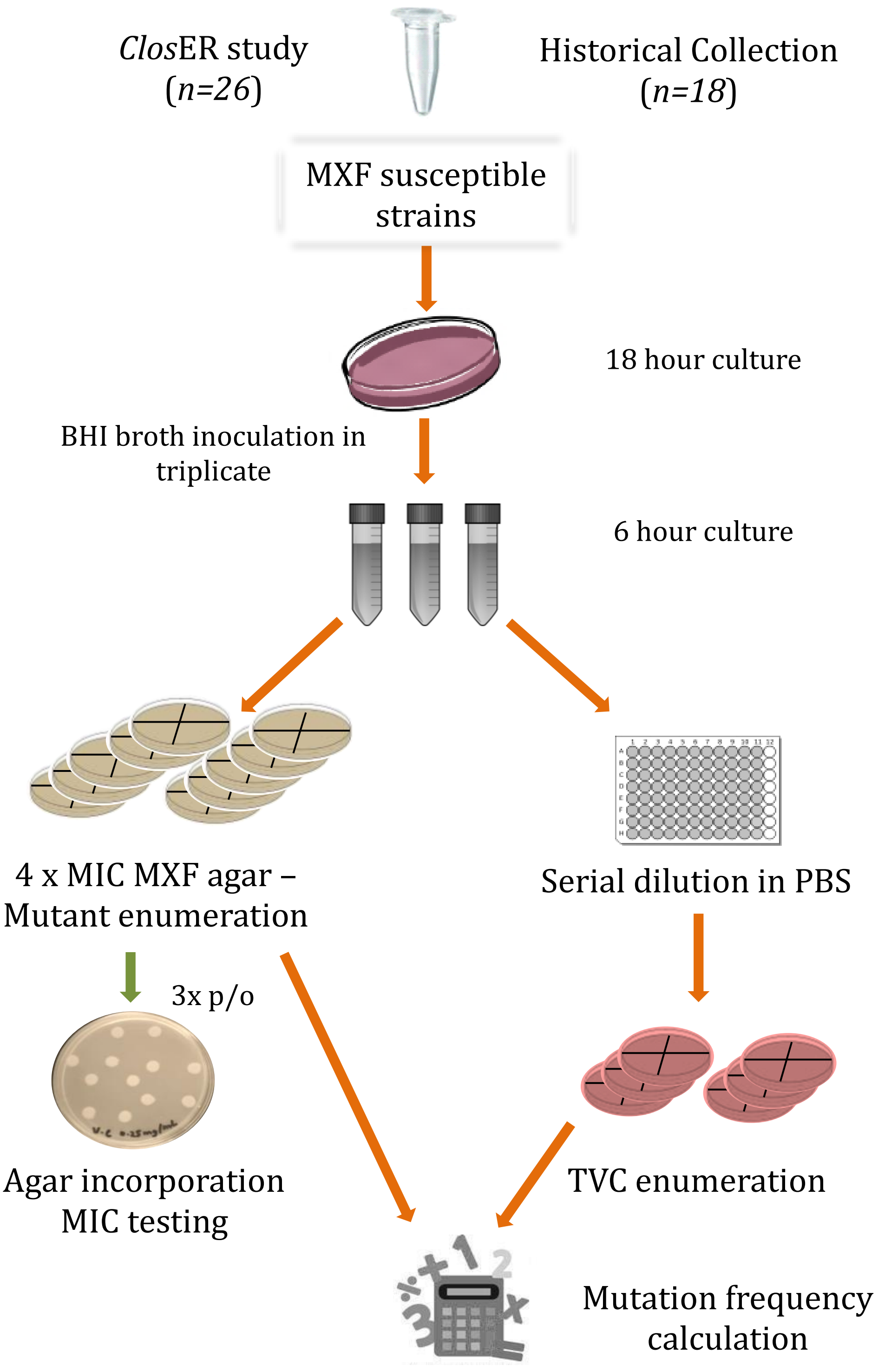


Figure 37: Flow diagram of mutation frequency determination methodologies. *TVC – total viable count, MIC – minimum inhibitory concentration, BHI – brain heart infusion, MXF – moxifloxacin, p/o – pickoff.*

5.3.1.3 Experimental design

Mutation frequencies were determined at 4x MIC for the entire panel of isolates. An expanded selection of ribotype 027 strains were subjected to repeat assays at 4x MIC, in addition to further testing at 4, 8 and 16 mg/L MXF concentrations. Mutant isolates were assessed for MXF MIC by agar incorporation and retained, frozen (-20°C) in glycerol broth for further molecular analysis. Mutation frequency assays were repeated in triplicate for all primary experiments, whilst ribotype 027 isolates were subjected to a minimum of six replicates.

Mutant strains generated from the primary experiments were further exposed to frequency testing under increased MXF concentrations. Six 4 mg/L MXF MIC ribotype 027 mutants were tested with exposures of 8 and 16 mg/L MXF, and three 32 mg/L MXF MIC 027 mutants (including one MXF resistant 027 isolate, CD4362) were subjected to 32 and 64 mg/L testing; (Figure 38).

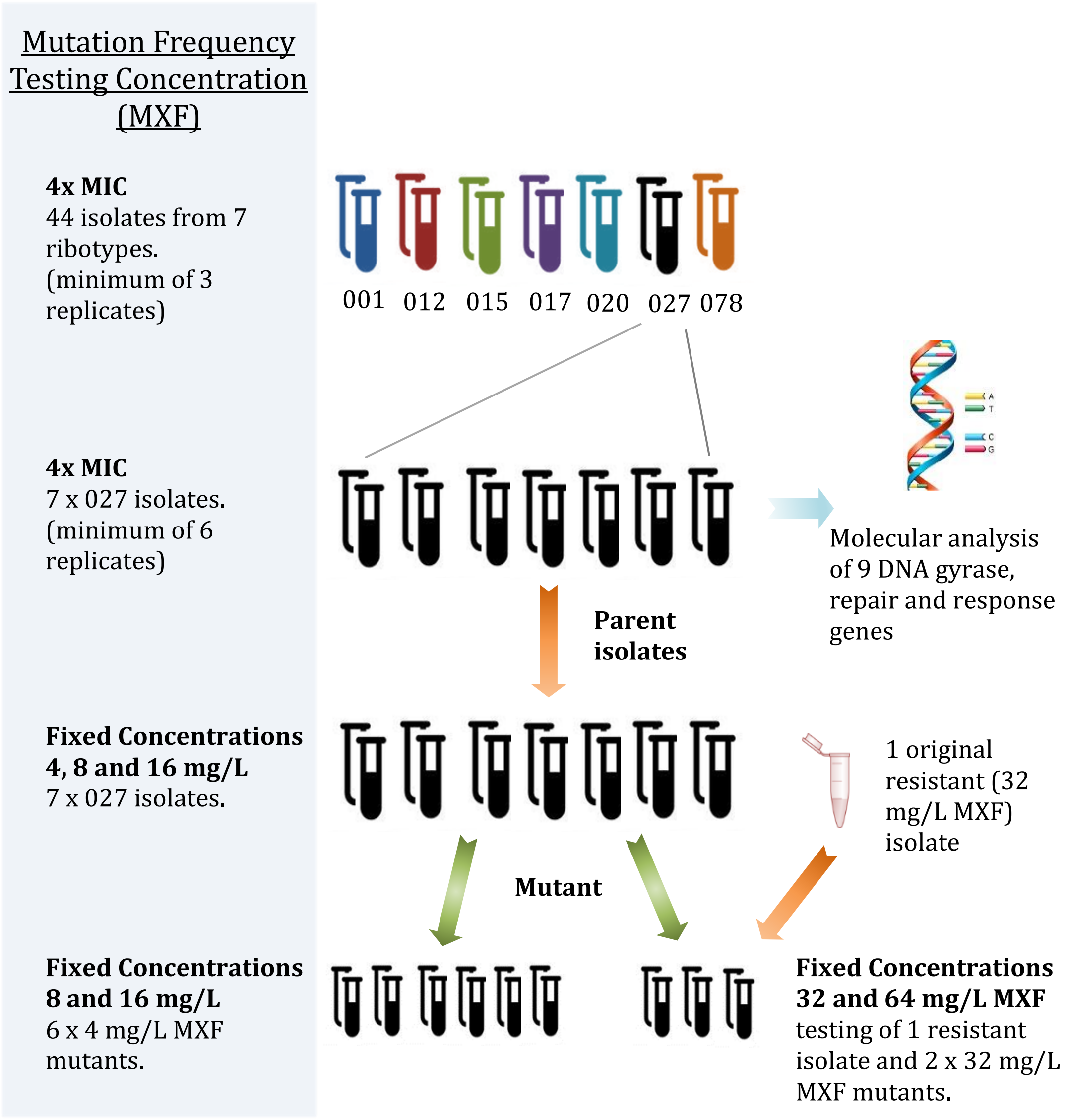


Figure 38: Experimental design of mutation frequency investigations. *Orange arrows refer to parent isolate testing, green arrows refer to reduced moxifloxacin (MXF) susceptibility mutant testing. MIC – minimum inhibitory concentration.*

5.3.1.4 Next generation sequencing and single nucleotide polymorphism identification

All seven PCR ribotype 027 parent isolates, one strain with pre-existing MXF resistance (CD4362) were processed for NGS as previously; (Chapter Four). Sequencing was performed by the University of Leeds, Next Generation Sequencing facility, whilst *de novo* assembly was performed as before.

Genomes were annotated using RAST (767) and SNP differences in DNA gyrase genes, as well as in seven genes related the DNA repair and SOS response (Table 21) were identified through multiple sequence alignment using Clustal Omega (764).

Gene of Interest	Function
mutS (601)	DNA mismatch repair
MutS related protein gene	DNA mismatch repair
mutL(601)	DNA mismatch repair
lexA (598)	SOS-response repressor protease
recA (598)	DNA recombination and repair
recX (844)	RecA Regulator
hfq (602, 845)	RNA-binding protein

Table 21: List of C. difficile genes putatively related to mutability, compared for sequence homology.

5.3.1.5 Statistical analyses

All statistical tests were performed using IBM SPSS Statistics v.21.0.0.1. Mutation frequency data was analysed using Kruskal-Wallis and the Dunn-Bonferroni multiple comparison *post hoc* test. Differences were deemed significant at $p<0.05$ and highly significant at $p<0.001$.

5.4 Results

5.4.1 *C. difficile* mutation frequency determination

5.4.1.1 Mutation frequencies with 4x MXF MIC selection

Considerable inter and intra ribotype variation of mutation frequencies associated with fluoroquinolone resistance were observed ($3.77 \times 10^{-9} – 5.91 \times 10^{-6}$, $\bar{x} = 4.15 \times 10^{-7}$); (Figure 39). The highest frequency was detected in ribotype 027 isolate, CD3904. Five isolates (CD0128, JV05, CD3904, CD9609 and CD0222) constituting four out of the seven ribotypes tested (012, 020, 027 (*n=2*) and 078), exhibited markedly elevated mutation frequencies ($5.83 \times 10^{-7} – 5.91 \times 10^{-6}$). Ribotype 027 demonstrated two isolates with mutation frequencies greater than 1×10^{-6} (CD3904 and CD9609). Highly polarised frequencies were observed in these four ribotypes, with 020 and 027 strains demonstrating the largest divergence; $3.38 \times 10^{-9} – 4.58 \times 10^{-6}$ and $7.23 \times 10^{-9} – 5.91 \times 10^{-6}$, respectively.

Mutation frequencies for ten isolates were assigned as "*below the lower limit of detection*", as no resistant colonies were detected. These included all but one ribotype 017 isolate (CD3771) and were designated low average frequencies of $\bar{x} = 5.10 \times 10^{-9}$. Mean average mutation frequencies indicated 027 as the most mutable ribotype when exposed to four-fold MXF MIC pressure ($\bar{x} = 1.48 \times 10^{-6}$); (Table 22). No direct correlation was observed between mutation frequency and isolate age, averaging 2.71×10^{-7} and 5.28×10^{-7} for historical and modern isolates, respectively.

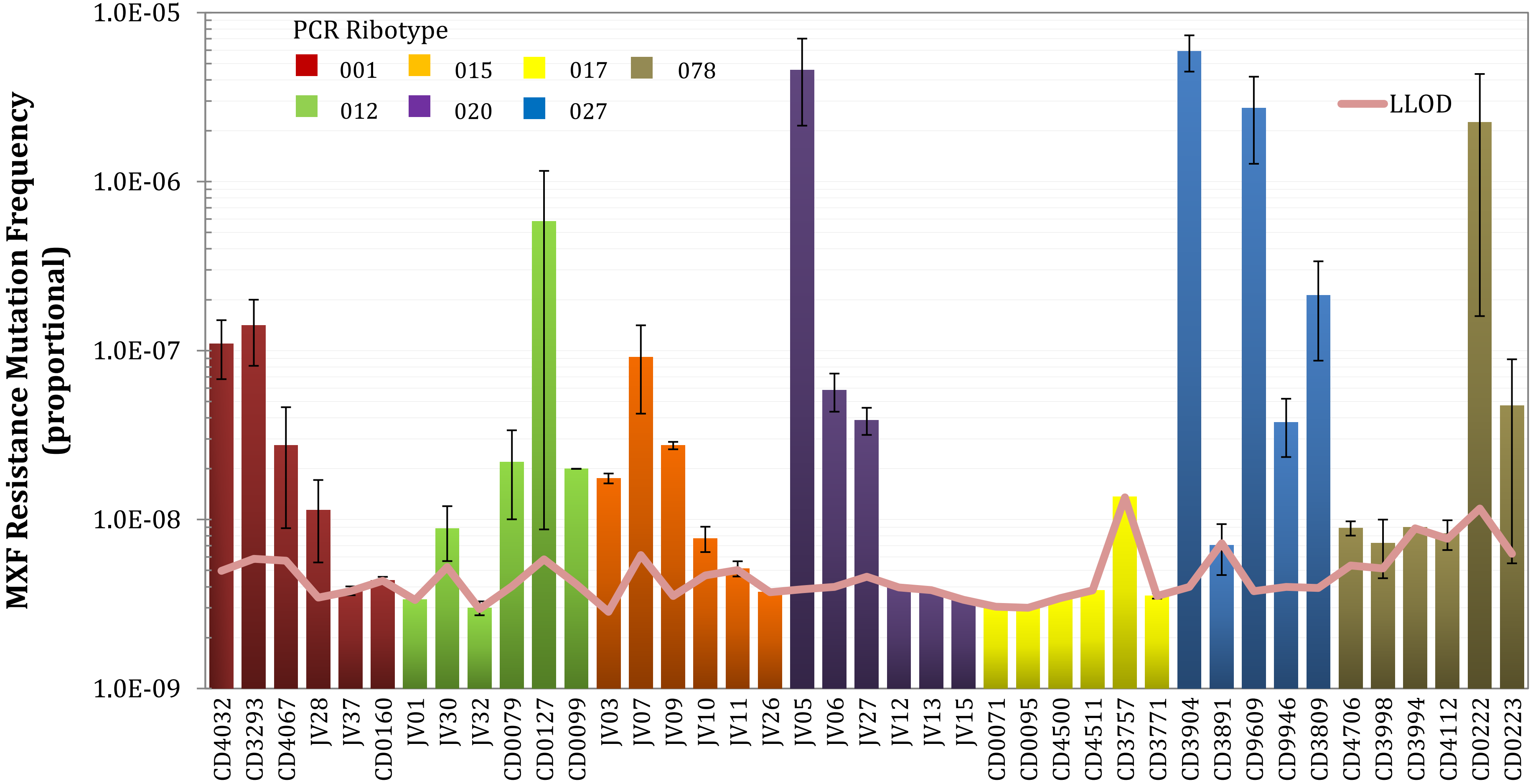


Figure 39: Mean (±SE) mutation frequencies of C. difficile isolates from seven PCR ribotypes exposed to 4x MIC moxifloxacin (MXF) pressure. *Lower limit of detection (LLOD) calculated as 1 / (mean total viable counts/mL). No error bars are displayed where the frequency was below the LLOD.*

PCR Ribotype	Mutation Frequency - Moxifloxacin Selection (4 x MIC)		
	Mean ($\bar{x}$)	*Minimum*	*Maximum*
001	4.95×10^{-8}	3.79×10^{-9}	1.41×10^{-7}
012	1.07×10^{-7}	3.00×10^{-9}	5.83×10^{-7}
015	2.55×10^{-8}	$<3.72 \times 10^{-9}$ *	9.17×10^{-8}
017	5.10×10^{-9}	$<3.05 \times 10^{-9}$ *	$<1.37 \times 10^{-8}$ *
020	7.82×10^{-7}	$<3.38 \times 10^{-9}$ *	4.58×10^{-6}
027	1.48×10^{-6}	7.04×10^{-9}	5.91×10^{-6}
078	3.88×10^{-7}	7.24×10^{-9}	2.25×10^{-6}

Table 22: Mutation frequency determinations by PCR ribotype. *All ribotype data consisted of six isolates each, except 027 (n=5). * frequency allocated as the lower limit of detection, where no resistant colonies were identified.*

Categorising mutation frequency data by PCR ribotype further demonstrated the high levels of variation; (Figure 40). Multiple comparison analyses using the Dunn-Bonferroni test revealed significant differences between ribotypes 017 and 001 (*p*=0.008), 015 (*p*=0.038) and 078 (*p*=0.025), whilst highly significant differences were observed in comparison to ribotype 027; (*p*<0.001). Further differences were identified between ribotype 027 and 012, (*p*=0.007); (Table 23).

		PCR Ribotype (*p*=)						
		001	**012**	**015**	**017**	**020**	**027**	**078**
PCR Ribotype (*p*=)	**001**		1.000	1.000	***0.008**	1.000	0.869	1.000
	012	1.000		1.000	1.000	1.000	***0.007**	1.000
	015	1.000	1.000		***0.038**	1.000	0.885	1.000
	017	***0.008**	1.000	***0.038**		0.079	*****<0.001**	***0.025**
	020	1.000	1.000	1.000	0.079		0.495	1.000
	027	0.869	***0.007**	0.885	*****<0.001**	0.495		1.000
	078	1.000	1.000	1.000	***0.025**	1.000	1.000	

Table 23: Chequerboard of P values for PCR ribotype mutation frequency pairwise comparisons. *Values were determined by the Kruskal-Wallis and by Dunn-Bonferroni tests. * - significant, *** - highly significant.*

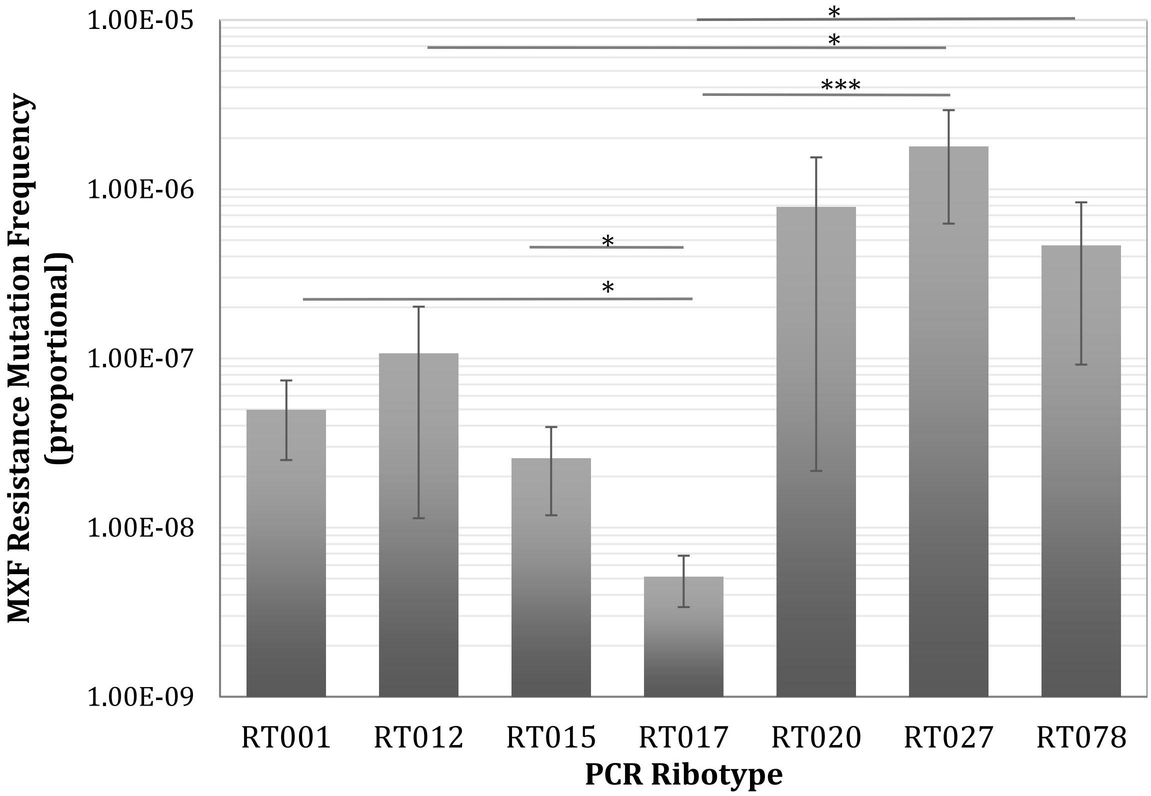


Figure 40: Mean average (±SE) moxifloxacin (MXF) selected mutation frequencies by PCR ribotype (RT). *All RT groups n=6, except RT027 (n=5). * - significant, *** - extremely significant by Dunn-Bonferroni test.*

Fluoroquinolone pressure selected a diverse range of mutant, MXF resistant phenotypes, with MICs ranging between 4 mg/L and 64 mg/L; (Table 24). Isolates from ribotypes 001 and 078 generated only highly resistant mutants (>32 mg/L), while the remainder, including ribotype 027 strains, produced a series of susceptibility phenotypes from 4 mg/L upwards. Not all ribotype 027 isolates generated resistant mutants >8 mg/L, with one (CD9946) adapting minimally to survive (4 mg/L MXF MIC). This isolate demonstrated the median mutation frequency.

PCR Ribotype	Strain	Original MXF MIC (mg/L)	Mutant MXF MICs (mg/L)		
			P/O 1	P/O 2	P/O 3
001	JV28	1	32	32	64
	CD0160	1	32	32	32
	CD4032	1	4	4	4
012	CD0099	1	4	8	32
	CD0079	1	8	16	32
	JV30	2	32	32	32
	JV32	2	32	32	32
	CD0127	1	8	8	16
015	JV03	1	32	32	32
	JV07	1	8	8	8
	JV09	1	16	16	16
	JV10	2	4	8	8
	JV11	2	8	8	8
	JV26	1	8	8	8
020	JV05	1	32	32	32
	JV06	1	8	8	8
	JV27	1	4	4	4
027	CD3904	1	4	32	32
	CD3891	2	4	32	32
	CD9609	1	4	8	32
	CD3051	1	4	8	8
	CD9946	1	4	4	4
	CD3809	1	4	16	16
	CD3079	1	8	16	32
078	CD0223	1	32	32	32
	CD0222	1	32	32	64
	CD4112	2	32	32	32

Table 24: Cross section of minimum inhibitory concentrations of C. difficile mutants generated with 4 mg/L MXF pressure. *The minimum inhibitory concentrations (MIC) for three pick-offs (P/O) represent colonies from different biological replicates. MXF – moxifloxacin.*

5.4.2 PCR ribotype 027 mutability

5.4.2.1 PCR ribotype 027 mutation frequencies at 4x MIC

The observation of elevated mutation frequencies, combined with the broad distribution of mutational propensities detected in ribotype 027 isolates (Table 22), advocated a more in depth focus on this clinically important strain type. Two additional susceptible strains were identified from the *Clos*ER study (188), allowing further analysis of a total of seven European, clinical isolates.

Additional ribotype 027 isolates, CD3051 and CD3079 demonstrated comparably low mutation frequencies of 3.02 x 10^{-8} and 2.93 x 10^{-8}, respectively; (Figure 41). Frequencies appear to be of almost bimodal distribution, with delineation into two approximate groups of either high (>10^{-7}) and low (<10^{-9}) mutability. Again, no apparent relationship was observed between fluoroquinolone mutation response and isolate age or origin. The three isolates originating from the same institution, Örebro University Hospital in Sweden, revealed demonstrably different responses, particularly those from the same year of isolation (CD3904 and CD3891).

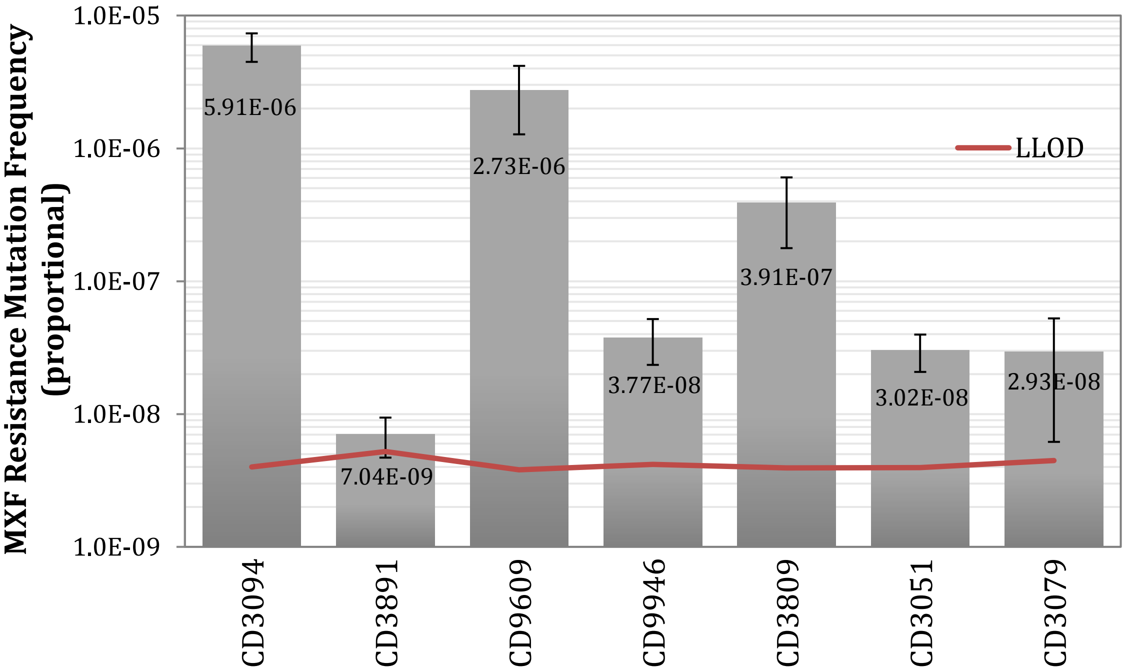


Figure 41: Mean (±SE) mutation frequencies of seven PCR ribotype 027 isolates with 4x MIC moxifloxacin (MXF) selection. *Data represent a minimum of three replicate tests. Lower limit of detection (LLOD) calculated as 1 / (mean total viable counts/mL). No error bars are displayed where frequency was below the LLOD.*

5.4.2.2 PCR ribotype 027 mutation frequencies at fixed MXF concentrations

Since the lowest frequency determined was associated with that of the only isolate with an initial MXF MIC of 2 mg/L (CD3891), all 027 strains were examined for their response to a fixed MXF concentration of 4 mg/L. To further ensure robust findings, six biological replicates were tested for each isolate, with minimal effect observed on the majority of the data. Reducing the selectivity of the agar for isolate CD3891 from 8 mg/L to 4 mg/L dramatically altered the mutation frequency, from 7.04 x 10^{-9} to 2.11 x 10^{-6}; (Figure 42). Isolate CD3904 demonstrated a mutation frequency that was significantly elevated above CD3051 (*p*=0.003), CD9946 (*p*=0.038) and CD3079 (*p*=0.050).

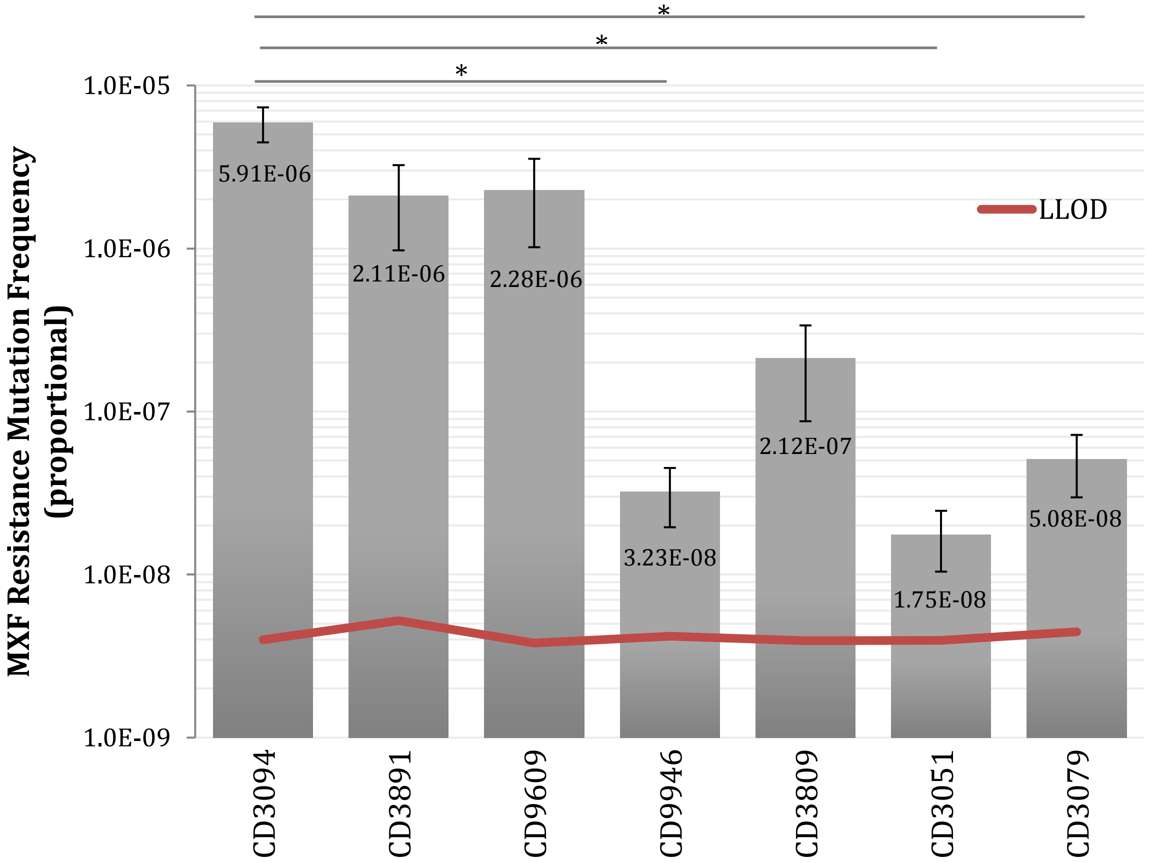


Figure 42: Mean (±SE) mutation frequencies of seven PCR ribotype 027 isolates selected under 4 mg/L moxifloxacin (MXF) pressure. *Lower limit of detection (LLOD) calculated as 1 / (mean total viable counts/mL). No error bars are displayed where frequency was below the LLOD. * p<0.05.*

Mutation frequencies determined at elevated MXF concentrations (8 mg/L and 16 mg/L) demonstrated a substantially reduced capability for ribotype 027 isolates to adapt and generate resistant colonies; (Table 25). Mean mutability decreased from 2.12×10^{-6} (4 mg/L) to 4.25×10^{-9} (8 mg/L), with no colony growth detected under 16 mg/L MXF pressure. This resulted in a frequency designation of below the lower limit of detection; $<3.69 \times 10^{-9}$. Only four isolates exhibited colony formation on 8 mg/L MXF agar, albeit in a vastly reduced capacity; (Table 25). No correlation was observed between mutation frequency in the 4 and 8 mg/L assays. CD3891 demonstrated the highest mutation frequencies at 8 mg/L (7.04×10^{-9}), whilst the two other isolates exhibiting high frequencies to 4 mg/L ($<10^{-6}$), CD3904 and CD9609, revealed no recordable growth.

	Mutation Frequency Moxifloxacin Selection (*n*=)		
Strain	4 mg/L	8 mg/L	16 mg/L
CD3904	5.91×10^{-6} (*6*)	$<3.35 \times 10^{-9*}$	$<3.35 \times 10^{-9*}$
CD3891	2.11×10^{-6}	7.04×10^{-9} (*6*)	$<3.14 \times 10^{-9*}$
CD9609	2.73×10^{-6} (*6*)	$<4.01 \times 10^{-9*}$	$<4.01 \times 10^{-9*}$
CD9946	3.77×10^{-8} (*6*)	$<3.24 \times 10^{-9*}$	$<3.24 \times 10^{-9*}$
CD3809	3.91×10^{-7} (*6*)	4.14×10^{-9}	$<4.14 \times 10^{-9*}$
CD3051	3.02×10^{-8} (*6*)	4.17×10^{-9}	$<4.17 \times 10^{-9*}$
CD3079	2.93×10^{-8} (*6*)	3.77×10^{-9}	$<3.77 \times 10^{-9*}$

Table 25: Comparison of mutation frequencies of seven PCR ribotype 027 strains selected under 4, 8 and 16 mg/L moxifloxacin pressure. *n = 3 unless stated. Where no resistant colonies were identified frequencies are defined as <, with values referring to the lower limit of detection.*

5.4.2.3 Molecular analysis of PCR ribotype 027 isolates investigated in mutation frequency assays

With the exception of one isolate, all MXF sensitive ribotype 027 strains investigated in this chapter demonstrated one hundred percent homology for all DNA gyrase, mismatch repair and SOS response genes. The exception was CD9946, demonstrating substantial SNP divergence from the other isolates of the same ribotype, exhibiting a host of synonymous and non-synonymous mutations; (Table 27). Two non-synonymous amino acid substitutions were observed in *gyrA*, Ile406>Leu and Asn468>Asp, with no impact on the fluoroquinolone susceptibility phenotype. Both of these variants were exclusively present in the genomes of the ribotype 027 isolates (*n*=3) from the historical collection; (Chapter Four). One *gyrB* modification, Val130>Ile, represented a commonly observed polymorphism amongst the historical strains (*n*=29/75), with no perceived consequence to resistance phenotype. Resistant isolate (32 mg/L) CD4362 harboured the common Thr82>Ile substitution. Sequencing of single mutant colonies for each isolate revealed individual instances of Thr82>Ile and Asp71>Tyr mutations in *gyrA* and Gly429>Val and Gln434>Lys substitutions in *gyrB*; (Table 26).

Parent Isolate	**Parent MXF MIC (mg/L)**	**Mutant MXF MIC (mg/L)**	**Mutant Strain Amino Acid Substitution**	
			gyrA	***gyrB***
CD3904	1	32	Thr82>Ile	-
CD3891	2	32	Thr82>Ile	-
CD9609	1	32	Thr82>Ile	-
CD9946	1	4	-	Gly429>Val
CD3809	1	16	Asp71>Tyr	-
CD3051	1	4	-	Gln434>Lys
CD3079	1	32	Thr82>Ile	-

Table 26: Characteristics of C. difficile ribotype 027 mutant strains generated during mutation frequency investigations (4x MIC). *Parent and mutant progeny moxifloxacin (MXF) minimum inhibitory concentrations (MICs) and mutant strain amino acid substitutions are displayed.*

Single non-synonymous mutations in each of the *mutS*, *mutL* and *lexA* genes were observed in the CD9946 genome, whilst five were identified in the *mutS* related protein; (Table 27). This isolate did not demonstrate an extreme level of mutation compared to the other isolates with no deviance in these genes, therefore no correlation could be determined between these SNPs and mutation frequency. No nucleotide modifications were detected in any of the *recA*, *recX* or *hfq* genes.

Isolate CD9946	***gyrA***	***gyrB***	***mutS***	***mutS* related protein, family 1**	***mutL***	***lexA***	***recA***	***recX***	***hfq***
Non-synonymous substitutions	Ile406 >Leu, Asn468 >Asp	Val130 >Ile	Met5>Ile	Phe42>Leu, Asn191>Asp, Ser266>Ala, Ile377>Val377, Val556>Ile	Asp379>Gly	Thr9>Ile	None	None	None
Number of synonymous substitutions	3	5	7	4	11	2	0	0	0

Table 27: Single nucleotide polymorphisms in the DNA gyrase, mismatch repair and SOS response genes of PCR ribotype 027 isolate CD9946. *SNPs were identified in comparison to the homologous genes of all other 027 test isolates.*

5.4.2.4 Mutation frequencies of primary mutant strains under exposure to fixed MXF concentrations

Secondary culture and selection with increased fluoroquinolone concentrations (8 mg/L and 16 mg/L) resulted in all primary mutants (MXF MIC 4 mg/L) producing subsequent populations exhibiting higher levels of MXF resistance; (Figure 43). Mean average mutation frequencies were 1.24 x 10^{-5} and 1.71 x 10^{-8}, for 8 mg/L and 16 mg/L respectively. Isolates CD3809 and CD3051 demonstrated strikingly raised frequencies under 8 mg/L exposure (3.61 x 10^{-5} and 3.81 x 10^{-5}; respectively). Isolates CD3904 and CD3891 expressed a slightly elevated proclivity for mutability when exposed to higher MXF concentrations (16 mg/L); (4.73 x 10^{-8} vs 2.50 x 10^{-8} and 2.76 x 10^{-8} vs 1.47 x 10^{-8}; respectively). No 4 mg/L mutants were generated from isolate CD3079 to enable further testing.

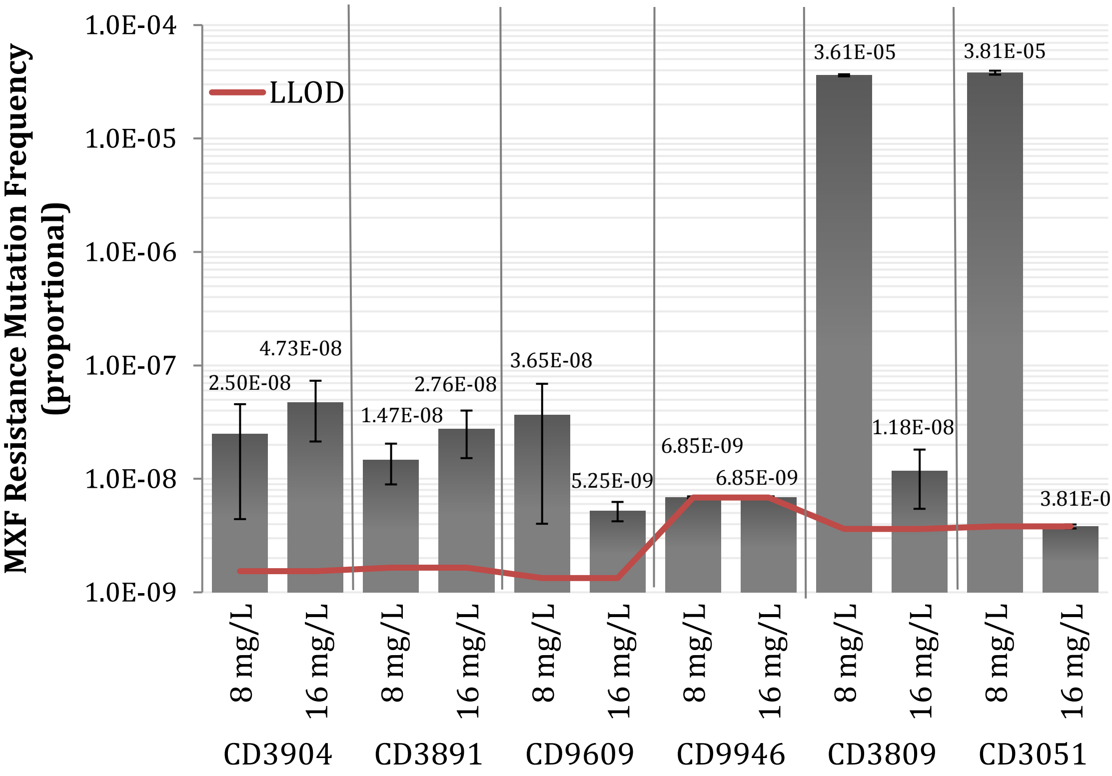


Figure 43: Mean (±SE) mutation frequencies of six PCR ribotype 027 mutants (4 mg/L MXF MIC) selected under 8 and 16 mg/L moxifloxacin (MXF) pressure. *Lower limit of detection (LLOD) calculated as 1 / (mean total viable counts/mL). No error bars are displayed where the frequency was below the LLOD.*

Further testing of two mutant strains with 32 mg/L MICs (from CD9609 and CD3079) generated during initial 027 mutation frequency assays demonstrated distinctly different responses when assessed with further elevated MXF concentrations; (Appendix). No colonies were generated by CD9609 mutant at either raised concentration, therefore, mutation frequencies were determined as <2.81 x 10^{-9}. Conversely, the CD3079 mutant responded at a frequency of 2.65 x 10^{-5} under 32 mg/L pressure and 6.08 x 10^{-9} at 64 mg/L. Isolate CD4362, exhibiting pre-existing MXF resistance (32 mg/L MIC), was observed to mutate at 4.67 x 10^{-5} and 5.71 x 10^{-8}, under 32 and 64 mg/L MXF pressure; respectively. MXF MICs for all subsequent mutant colonies were determined as 128 mg/L.

5.4.2.5 Minimum inhibitory concentrations of secondary mutant strains produced during stepped exposure investigations

All but one 4 mg/L MXF MIC mutant generated in mutation frequency experiments demonstrated further increases in MIC after secondary assay and selection with elevated MXF pressure; (Table 28). A mutant isolate generated from CD9946 did not produce any further stepwise when selected with increased fluoroquinolone concentrations.

In four isolates, 8 mg/L MXF exposure selected for mutants with MICs of up to 16 mg/L, however, one strain (CD3891 4 mg/L mutant) was able to generate mutant colonies with MICs of 32 mg/L. Three out of six 4 mg/L mutants tested produced colonies with MICs of 32 mg/L, under 8 or 16 mg/L selection. Two mutant strains (CD3904 and CD3051) generated a homogeneous response under 8 mg/L selection, with all progeny revealing consistent MICs. The remainder produced a heterogeneous reaction, with differing MICs observed between colonies; (Table 28). A CD3051 mutant was the only strain unable to grow on 16 mg/L MXF agar, while able to produce colonies at 8 mg/L. Of the two mutants demonstrating initial step mutant MICs of 32 mg/L, only one (CD3079) was able to withstand up to 64 mg/L MXF pressure, generating mutants with a corresponding MIC.

Concurrent testing of an original MXF resistant strain, CD4362, highlighted a potential to produce further resistant phenotypes, capable of surviving up to 128 mg/L. Sequencing revealed a further Ala118>Ser substitution in the *gyrA* gene; (Appendix).

Strain	Primary Mutant MXF MIC (mg/L)	MXF Pressure (mg/L)	Secondary Mutant MXF MIC (mg/L)		
			P/O 1	P/O 2	P/O 3
CD3904	4	8	16	16	16
		16	16	32	-
CD3891	4	8	32	-	-
		16	32	16	-
CD9609	4	8	16	8	-
		16	32	16	16
CD9946	4	8	No mutants		
		16	No mutants		
CD3809	4	8	8	16	-
		16	16	16	-
CD3051	4	8	8	8	8
		16	No mutants		
CD3051	32	32	No mutants		
		64	No mutants		
CD3079	32	32	32	32	32
		64	64	64	64
CD4362	32	32	32	32	64
		64	128	128	128

Table 28: Minimum inhibitory concentrations of second-step mutants of C. difficile selected with further exposure of first-step mutant 027 isolates to 8, 16, 32 or 64 mg/L moxifloxacin (MXF) pressure. *Where available, three colonies from each strain and each MXF concentration were tested for MXF MIC. "-" refers to no colony available for testing. MIC – minimum inhibitory concentration, P/O – pick off.*

5.5 Discussion

5.5.1 *C. difficile* mutation frequency determination

The importance of fluoroquinolone resistance and its impact on CDI rates has recently been highlighted (387). Therefore, investigating the propensities for individual *C. difficile* isolates to develop MXF resistance *in vitro* may be crucial to the further understanding of the proliferation of the organism. Here, an interrogation of a panel of isolates consisting of historical and modern strains highlighted potential strain and ribotype specific variance in fluoroquinolone resistance conferring mutation frequencies. Whilst these data indicated interesting findings, further exposure of this strain collection to mutability assays with additional antibiotics is necessary to corroborate the results. The use of a compound such as rifampicin, where mutability could be assessed independently from DNA gyrase gene mutations, is essential to confirm the observed mutational differences. By establishing comparable levels of mutability in other areas of the genome, this data could potentially validate the hypothesis of variable mutation frequencies, as opposed to alternative explanations such as hetero-resistance. Therefore, these findings are discussed in provisional context.

The most striking observation from the mutation frequency screening experiment was the variation of both inter and intra ribotype mutability demonstrated; (Figure 39). While the mean average spontaneous mutation frequency at four times MIC selection, a common approach successfully applied by others (232, 608, 610, 611, 619), was determined as 4.15 x 10^{-7}, the range was expansive: 3.77 x 10^{-9} – 5.91 x 10^{-6}. This range reflects the results of fluoroquinolone resistance mutability research in other organisms (590, 608, 610, 611, 842, 843), although much of this relates to earlier generations of quinolones, such as CIP. One such study identified CIP-induced mutations in *E. coli*, with a closely comparable range of 1 x10^{-9} to 1.5 x 10^{-6} (608).

Although there is a paucity of data for MXF-resistance mutation frequencies in *C. difficile*, Locher *et al.* demonstrated this agent as generating higher levels of spontaneous mutation compared to other antimicrobials, including LZD and VAN (232). Nevertheless, the average observed frequency here is greater than that reported in *C. difficile* by Locher *et al.* (~2 x 10^{-8}) (232), but lower than previously described by Spigaglia and colleagues, where levels of mutation amongst five fluoroquinolone sensitive isolates were reported as between 3.8 x 10^{-6} and 6.6 x 10^{-5}

(812). Nonetheless, the disparities in variance between the findings of the present study and other published data may be explained by the greater number of strains tested in this investigation. Equally, in selected instances Spigaglia's findings represented colony formation from only two-fold MIC selection, as opposed to four-fold exposure tested here. Though findings of the present study determined mutation frequencies based on the ability of a colony to demonstrate an increase in MIC, the study by Spigaglia *et al.* searched for *gyrA* or *gyrB* mutations in three colony pick offs before allocating a mutation frequency (812). Whilst this may seem more appropriate, this approach neglects to consider alternative resistance aetiologies. DNA gyrase alterations are often associated with high-level resistance (443, 812) and ignoring slight reductions in susceptibility could overlook important intermediatory steps, potentially caused by alternative mechanisms, such as efflux. The slight decreases in susceptibility may act as a precursor step to high-level resistance mutations and consequently may be considered as potentially significant to the dissemination of fluoroquinolone resistance. Hence, the methodology for measuring mutation frequency selected here, of using a defined MIC multiple concentration with secondary susceptibility confirmation, may produce a more comparable and clinically representative data set. Although gyrase substitutions were identified for ribotype 027 mutant isolates, the majority of other strains were unable to be genotyped due to the high expense of sequencing several colonies of multiple isolates. Interestingly, Spigaglia only observed the classic Thr82>Ile substitution in spontaneous mutants generated by levofloxacin pressure and not MXF (812). However, since only five isolates were tested, this finding may denote an unrepresentative test panel.

Whilst mutation frequencies for ten isolates were determined as below the lower limit of detection, further bacterial concentration steps via centrifugation would have potentially enabled the recovery and enumeration of resistant mutants with greater precision. However, since the inter strain divergence between mutation frequencies was clearly apparent and an excessive amount of work would have been required to repeat ten tests, in triplicate, this was not performed.

A major factor to consider when assessing mutation frequencies is the amount of antimicrobial selection pressure to expose test isolates to, as spontaneous mutation has been demonstrated as selection concentration dependant (591, 608, 842). Since susceptible isolates will vary in MIC, standardisation of experimental approach is critical. Consequently, testing at multiples of original MICs is often implemented as best practice (232, 608, 610, 611, 619). Studies have revealed elevated mutation frequencies at four times MIC (608, 610, 611), whilst imposing sufficient selection pressure on a population to identify genuine mutational response. Greater fold increases appear to reduce bacterial capacity to mutate, since large step increases in concentration prove more efficient in inhibiting growth (608, 842). A study by Spence and Towner observed marked reductions in mutation frequency of *Acinetobacter baumannii* exposed to MXF at concentrations between four and eight-fold MIC. This is equally reflected here, where data from focussed experimentation with ribotype 027 isolates showed distinct reductions at 8 mg/L and 16 mg/L; (Table 25).

Although all the primary test strains were MXF susceptible (< 2 mg/L), the initial MICs were inconsistent, defined as either 1 mg/L or 2 mg/L; (Appendix). Therefore the use of a multiple of MIC concentration resulted in antimicrobial pressures infused in the agars of 4 mg/L and 8 mg/L. This disparity in testing protocol potentially resulted in some of the inter strain variation observed, with discrepant conclusions determined for those strains tested at higher concentrations. Seventeen isolates (42%) demonstrated an initial 2 mg/L susceptibility phenotype, with a noticeably reduced mean mutation frequency of 6.40×10^{-9} compared to 7.05×10^{-7} for all 1 mg/L MIC isolates. Nonetheless, in order to include a larger number of isolates in frequency testing, a stringent parameter was necessary to enable cross comparisons of all isolates. During focussed ribotype 027 investigations, CD3891 demonstrated conspicuously low mutation frequencies at four times MIC (Figure 41), but when tested at the same concentrations as comparator strains (4 mg/L MXF pressure), mutability was considerably raised; (Figure 42). These approaches generated highly divergent results. Nonetheless, the latter approach may have resulted in test concentrations too close (only one doubling dilution increase) to the original MIC for a requirement of mutation to survive the fluoroquinolone pressure. Since MIC data is generally regarded as somewhat approximated, this may have been the case.

Interestingly, mutant colonies produced by isolates within all ribotype groups; (with the exception of ribotype 078), exhibited a broad spectrum of susceptibilities (from 4 mg/L to 32 or 64 mg/L). Resistant progeny of all three 078 isolates investigated for further susceptibility testing, had MICs greater than 32 mg/L. Whilst this ribotype is not frequently associated with MXF resistance, reduced susceptibility is not uncommon (188, 480, 846), and MDR does occur (188, 846). This was observed in both 078 isolates from this historical collection; (Chapter Three).

Whilst the data from this mutation frequency investigation demonstrated putative mutability variance between individual bacterial strains, one alternative explanation to the differences observed could be the existence of hetero-resistance amongst strain populations. The presence of minority sub-populations of highly resistant bacteria, concealed in standard MIC determination methodologies (847), may have contributed to the variance observed in this data set. Evidence of hetero-resistance had been reported in *C. difficile*, primarily associated with MTZ (452), but has also been demonstrated with fluoroquinolones in other species (848, 849). The epidemiological and clinical relevance of this somewhat ambiguous phenomenom has the potential to be vast. If ribotype 027 has a greater propensity to demonstrate hetero-resistance to MXF than other strains, this could prove contributory to the progression of fluoroquinolone resistance in this epidemic strain type.

5.5.2 PCR ribotype 027 mutability

Large differences in mutation frequencies were observed across the examined isolates, but the significantly higher rates observed in general for ribotype 027 strains (compared with those for ribotypes 012 and 017 [Figure 40]) were of particular interest. Given these results and the clinical importance of *C. difficile* ribotype 027, focussed investigation of these strains was undertaken. Since ribotype 027 is a prominent, outbreak causing ribotype (34, 112), and is commonly associated with fluoroquinolone resistance (562), this ribotype was of particular interest for further examination. Observations of two potentially hypermutable strains (CD3904 and CD9609), the highest mean average mutation frequency (Table 25) and a broad diversity of mutation frequencies between the isolates (7.23×10^{-9} - 5.91×10^{-6}), further underlined ribotype 027 as being of particular of interest.

5.5.2.1.1 Phenotypic analyses

The variability of MXF-selected mutation frequency observed across all ribotypes, remained apparent in the focussed analyses of ribotype 027. This variation may be reflective of the potential for strain survival and population maintenance through clonal expansion. As it may be reasonably assumed that highly mutable strains are more likely to mutate in clinical situations, survival and onward transmission of these strains may prove advantageous. Dong *et al.* reported correlations between high fluoroquinolone mutation frequencies in *S. aureus* and greater progeny resistance (850). The data set acquired here largely supports this notion, with the three isolates indicating the highest mutational ability all producing mutants with ≥32 mg/L MIC. In contrast, three out of four ribotype 027 isolates demonstrating the lowest mutability (CD9946, CD3809 and CD3051) all failed to generate resistant mutants with MICs >16 mg/L conferred by the Thr82>Ile substitution. This may be reflective of an amino acid substitution dependent bacterial fitness impact (639), with a potential for bacteria containing intermediate resistance conferring SNPs to be less fit than others. This hypothesis is partially supported by further investigations into bacterial fitness in Chapter Six.

Fluoroquinolone resistance is an almost universal characteristic of ribotype 027, with very few sensitive isolates reported (118, 851). This was reflected in the difficulty in obtaining such strains for this investigation. Since sensitive isolates are rare, that would suggest that although mutability may vary, these strains may either develop and retain resistance to this class of antimicrobials or be quickly superseded by other more clinically adapted bacteria. The successful dissemination of this ribotype may have originated from a propensity for high-level mutagenesis under pressure from these antibiotics. Interestingly, three of the seven strains originated from Swedish institutions, which are reportedly amongst the lowest consumers of fluoroquinolones in Europe (852). Equally, one further sensitive strain was isolated from the United Kingdom, where antibiotic stewardship programs are more established than other countries (853, 854). This goes to further the argument for regulated prescribing regimens as a measure to reduce resistance.

Based on the data produced in this study, the four-fold MIC approach to mutability testing appeared to generate optimal findings. Colony forming units were readily countable (<200 CFU) and a diverse, measurable response was observed across all strains. Increased MXF pressure, particularly at 16 mg/L often inhibited all vegetative growth, necessitating frequencies to be reported as “less than the lower limit of detection". This may therefore be considered as the mutation prevention concentration for these isolates (855, 856).

Secondary mutation investigations were undertaken in order to assess the subsequent mutability of progeny with reduced susceptibilities. Whilst no clear trend was observed, all isolates, with the exception of CD9946 revealed the propensity to generate highly MXF resistant (>32 mg/L) mutants; (Table 28). Resistance acquisition through incremental steps has been previously reported (812), and is deemed crucial for bacteria to reach a level of reduced fluoroquinolone susceptibility necessary to gain an adaptive advantage in clinical environments. Although clinical levels are generally likely to surpass these test concentrations (857), the tapering effect of antimicrobial concentrations post treatment may allow for mutational response to survive antimicrobial pressure. This may confer *C. difficile* strains a temporal advantage over commensal gut flora and therefore a proliferation opportunity. Interestingly, pre-existent MXF resistant isolate CD4362 demonstrated the propensity to develop further resistance up to 128 mg/L. The *gyrA* sequencing of this highly resistant mutant revealed the common Thr82>Ile mutations in addition to an Ala118>Ser substitution, reported in other strains with MXF MICs >64 mg/L (766). This elevated level of resistance becomes of greater clinical concern when considered in relation to the faecal concentrations achieved by MXF treatment. Edlund *et al.* reported concentrations in the faeces to reach a peak of 65.7 mg/L (857). Although faecal concentrations may be lower than true intestinal levels, this figure remains well below the tolerable level of these high-level mutants. Resistance to this extent could potentially result in increased CDI cases, where fluoroquinolone use is putatively involved as a predisposing factor. Since no fitness cost has been associated with this phenotype (639), resistant substitutions can be retained for clonal expansion.

5.5.2.1.2 Genomic analyses

In order to ascertain the source of the extensive variation in mutation frequencies amongst isolates of the same PCR ribotype, several mechanisms with demonstrable effects on mutability in other organisms were investigated (583, 593, 594, 606, 627). Unfortunately, since all but one strain demonstrated full homology of mismatch repair and SOS response genes, no correlations could be identified. The variation in mutation frequencies could not be connected to any identifiable differences in the repair and response genes tested here. As there are likely to be many more ancillary genes involved in these processes (593, 841), differences in alternative loci may be relevant to mutation frequency variance.

Intruigingly, isolate CD9946 represented a highly distincitive genome in comparison to the other strains examined, with multiple synonymous and non-synonymous mutations exhibited in these genes. Interestingly, the single amino acid substitution detected in the *mutS* gene, Met5>Ile, represented a start codon alteration. Modification of start codons have previously been reported to impact on the efficiency of translation, with a reduced binding affinity of tRNA hypothesised as the primary cause (858-860). Nevertheless, the substitution in the *mutS* start codon did not appear to have any detrimental effect on the strain's mutability in response to fluoroquinolone stress and may not have affected translation of this gene. Further protein expression analyses would help to elucidate the validity of this theory (861). Also, the threonine substitution at codon nine of the *lexA* gene does not occur near the binding site of the LexA protein (598), and it is therefore improbable that this would impact the efficacy of the translated protein; reflected in the relatively low mutation frequency (3.23 x 10^{-8}) observed in this isolate. Similar observations were made with the *mutL* gene, as the only non-synonymous alteration occurred at Asp-379, outside of the distinct domains recently associated with *mutS* interactions in *in silico* docking simulations (862). The eighteen silent mutations detected across the *mutS* and *mutL* genes of CD9946 are more likely to represent the evolutionary distinction between this relatively distant genome, than convey any discernible influence on the mismatch repair system of this isolate.

Nonetheless, isolate CD9946 was conspicuous by its muted response to resistance development (no resistant mutants were generated in step-wise experiments) and genomic analysis indicated a distinctly distant genotype in comparison to the other ribotype 027 strains examined; (Table 27). It is not unreasonable to suggest that a plethora of SNP differences in this genome may contribute to influence the efficacy of DNA repair response.

Molecular analysis of the DNA gyrase genes was performed to establish any variance in baseline characteristics at these loci, potentially influencing antimicrobial mutagenesis. As expected, no *gyrA* or *gyrB* polymorphisms were observed in the majority of fluoroquinolone sensitive ribotype 027 isolates. However, three SNPs were detected in strains CD9946, two in *gyrA* and one in *gyrB*; (Table 27). These amino acid substitutions, although variant from the majority of genomes examined, were detected in other fluoroquinolone susceptible isolates; (Chapter Four). Interestingly, the Ile406>Leu and Asn468>Asp modifications observed were evident in all of the ribotype 027 genomes investigated from the historical collection. Although the historical strains demonstrated fluoroquinolone resistance, this was associated with the characteristic Thr82>Ile mutation. Therefore, it is conceivable that the gyrase polymorphisms possessed by this MXF sensitive isolate are artefacts of archaic horizontal gene transfer, characterising this PCR ribotype (563). The valine substitution at codon 130 of the *gyrB* gene also appears to transmit minimal influence on fluoroquinolone resistance, as it has been detected in the genomes of 22 MXF sensitive *C. difficile* isolates from the historical collection (Chapter Four) and in no resistant strains. Therefore, this difference appears to be of evolutionary origin, as opposed to driven by quinolone exposures.

These findings further verify the reports of a high-level of gene conservation in *C. difficile mutS*, and *mutL* homologues (573, 600), thus indicating the presence of an alternative source of mutation variability in these strains. The mechanisms relevant to the diverse mutability observed here, are likely to be more complex, potentially epigenetic in nature.

5.6 Conclusions

The high proportion of CDI cases associated with ribotype 027 strains resistant to modern fluoroquinolones (34, 118) is a major clinical concern. Provisional evidence reported in this chapter suggests that a greater propensity for mutagenesis, effecting resistance to these antimicrobials, may contribute to the wide distribution of these polymorphisms. Increased mutability potential may have provided an advantage to these strains with regards to fluoroquinolone survival and resistance acquisition (863). The rarity of fluoroquinolone sensitive ribotype 027 isolates (118) suggests that resistance may develop more readily in these strains and be retained in the absence of any bacterial fitness cost (639). Whilst, subsequent clonal expansion has enabled this ribotype to reach epidemic levels, other lineages demonstrating lower mutability may have been lost to evolution. The mechanism driving this mutability variance in these isolates does not appear to be directly related to the common DNA SOS and mismatch repair genes, *mutS*, *mutL*, *recA* or *lexA*, and further investigation is necessary to establish the cause.

Chapter 6 The Impact of Fluoroquinolone Resistance-Conferring Mutations on *In Vitro* Bacterial Fitness

6.1 Introduction

Fluoroquinolones have been associated with CDI as a predisposing risk factor (92, 99) and have been strongly implicated in major international outbreaks of PCR ribotype 027 (34, 92). A recent retrospective analysis reported correlations between a reduction in fluoroquinolone prescribing and CDI caused by fluoroquinolone resistant strains (387). However, the potential for *C. difficile* strains to retain the resistant phenotype in the absence of antimicrobial pressure remains unclear. The fitness associated with these resistant determinants may be a factor in the maintenance of fluoroquinolone resistance in this important nosocomial pathogen.

The impact of resistance determinants may not always be constrained to an antibiotic susceptibility phenotype. Several reports have linked resistance-conferring amino acid substitutions to a demonstrable burden on bacterial fitness (518, 585, 629, 864). Whilst typically related to a reduction in growth rates, the effects on the functionality of crucial pathways, such as sporulation and toxin production in *C. difficile* can also be appropriate measures of fitness. Investigations of fluoroquinolone resistance mutations have demonstrated diverse responses across a host of bacterial species (630, 633, 634), with some more detrimental to fitness than others. Both transferable elements and chromosomal mutations have been correlated with fitness disadvantages in *C. difficile*, with *ermB* (518) and *rpoB (637)*, respectively. However, the relationship is not always straightforward; rifamycin resistance-conferring mutations in different locations on the *rpoB* gene have demonstrated minimal effect on *C. difficile* fitness (638). Similarly, Wasels *et al.* observed no impairment with the common fluoroquinolone resistance, Thr82>Ile mutation in isogenic mutants created via allelic exchange, whilst the rarer valine substitution at the same codon imposed a significant fitness burden (639).

We investigated the impact of *gyrA* and *gyrB* mutations on *C. difficile* PCR ribotype 027 fitness, through growth rate analysis and, for the first time, using a competitive co-culture assay.

6.2 Rationale

In this chapter, the bacterial fitness impact of common fluoroquinolone resistance-conferring substitutions was determined *in vitro*. A collection of MXF susceptible 027 parent strains and their resistant progeny were investigated for differences in growth rate, toxin production and competitive co-culture response. Continuous co-culture modelling was performed with one strain pairing, to further test the effects of the Thr82>Ile mutation in a bacterial turnover environment, more reflective of clinical situations. Whilst previous findings in ribotype 012 have indicated an absence of a fitness burden associated with the majority of resistance mutations (639), equivalent discoveries in ribotype 027 may suggest a contributory factor to the success of this epidemic ribotype.

6.3 Methods

6.3.1 Test isolates

Seven MXF susceptible (1-2 mg/L) PCR ribotype 027 strains (parent) and seven corresponding MXF resistant mutants (Table 26), generated through previous mutation frequency assays (Chapter Five), were subjected to *in vitro* fitness investigations. Bacterial responses were evaluated for both parent and mutant comparators (Figure 44) to determine the impact of fluoroquinolone resistance substitutions.

Parent Isolate	Parent MXF MIC (mg/L)	Mutant Strain Identifier	Mutant MXF MIC (mg/L)	Mutant Strain Amino Acid Substitution	
				gyrA	***gyrB***
CD3904	1	CD3904 Mut	32	Thr82>Ile	-
CD3891	2	CD3891 Mut	32	Thr82>Ile	-
CD9609	1	CD9609 Mut	32	Thr82>Ile	-
CD9946	1	CD9946 Mut	4	-	Gly429>Val
CD3809	1	CD3809 Mut	16	Asp71>Tyr	-
CD3051	1	CD3051 Mut	4	-	Gln434>Lys
CD3079	1	CD3079 Mut	32	Thr82>Ile	-

Table 29: Characteristics of C. difficile ribotype 027 strains tested during bacterial fitness investigations. *Parent and mutant progeny moxifloxacin (MXF) minimum inhibitory concentrations (MICs) and mutant strain amino acid substitutions are indicated.*

6.3.1.1 Test isolate variant detection

Genomes were sequenced, annotated and interrogated for fluoroquinolone related resistance mechanisms, using RAST (767), CARD RGI (762) and ResFinder 3.0 (763), as previously; (Chapter Four). Variant detection was performed between parent and mutant genomes using CLC Genomics Workbench (752); (Appendix). Briefly, sequence reads were mapped to the *C. difficile* R20291 reference genome and realigned for indel and structural variants, prior to basic variant detection and amino acid change determination. SNP differences in fluoroquinolone resistance determining genes, *gyrA* and *gyrB* were confirmed through multiple sequence alignment using Clustal Omega (764).

6.3.1.1.1 Sanger sequencing

Sanger sequencing of the QRDRs of both *gyrA* and *gyrB* genes was used to confirm SNP identification. As previously described (Chapter Four), DNA was extracted from an overnight, single colony emulsion using a QIAxtractor and the QIAamp Fast DNA Kit.

6.3.1.1.1.1 Amplification of *gyrA* and *gyrB* regions

Primer sequences were obtained from previously published literature (438, 812), and synthesised by metabion international AG; (Germany). The QRDR sections of the DNA gyrase genes were amplified using the primer pairs: *gyrA*F (5´-AATGAGTGTTATAGCTGGACG-3´), *gyrA*R (5´-TCTTTTAACGACTCATCAAAGTT-3´) and *gyrB*F (5´-AGTTGATGAACTGGGGTCTT-3´), *gyrB*R (5´-TCAAAATCTTCTCCAATACCA-3´), generating 390bp regions of *gyrA* and *gyrB*, respectively.

The PCR reactions consisted of 12.5 µL Dreamtaq green PCR master mix (Thermo Fisher Scientific), 0.3 µL forward primer, 0.3 µL reverse primer, 10.9 µL PCR water and 1 µL DNA target. PCR amplification was performed using a 2720 thermal cycler (Applied Biosystems) with an initial denaturation stage of 94°C for 15 minutes, followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 58°C (*gyrA*) and 54°C (*gyrB*) and a 30 second extension phase at 72°C. A final extension stage of five minutes at 72°C was implemented. PCR clean-up was performed using the QIAquick PCR Purification Kit; (Qiagen), as per the manufacturer's instructions.

6.3.1.1.1.2 Sanger sequencing of QRDR regions

Purified PCR product was quantified using a Nanodrop 2000C (Thermo Fisher Scientific) and subsequently diluted by 100-fold in sterile water. Sequencing was performed by the Leeds Teaching Hospitals Trust, Molecular Microbiology Department, using BigDye™ Terminator Kit v.3.1 (Thermo Fisher Scientific) and an ABI 3130xl genetic analyser. Sequence data was analysed in comparison with the *gyrA* and *gyrB* sequences of the CD630 reference genome (314), using CLC Genomics Workbench.

6.3.2 *In vitro* fitness determination for fluoroquinolone resistance mutations

6.3.2.1 Maximal growth rate determination

Bacterial growth curves were generated for all strains, in order to determine the beginning and end of the exponential growth phases. Based on the assay outlined by Wasels *et al.* (518), ten colonies of overnight CBA culture were emulsified into 25 mL BHI broth in soda glass universals. Broths were cultured anaerobically, whilst shaking on an Orbital Shaker PSU-10i (Grant-Bio, UK) at 150 rpm, for 18 hours. Optical densities of the bacterial cultures were measured with a Genesys 20 spectrophotometer (Thermo Fisher Scientific) and diluted to 0.5 (±0.05) OD_{600} with sterile BHI broth. Dilution ratios were recorded and recreated under anaerobic conditions, to limit the exposure of the culture to O_2. Fresh 25 mL BHI broths were inoculated with 1 mL of standardised culture and cultured for a further 11 hours, under the same conditions as previously. At hourly time points (including zero hour) broths were mixed via multiple inversions and 200 µL was transferred to a 96-well microtitre tray. Absorbance measurements were taken at 595nm using a Tecan Infinite F200 pro plate reader. All absorbance measurements were calculated as the mean averages of three independent culture replicates. Growth curves were plotted and the log phase was determined as between the initiation and cessation of exponential growth. Maximal growth rates were calculated using the following formula:

$$\ln\frac{\boldsymbol{Nt}}{\boldsymbol{N}0} = \boldsymbol{\alpha}\,(\boldsymbol{t} - \boldsymbol{t0})$$

Where, *ln* is natural logarithm, *Nt* is the absorbance at log phase end point, N_0 is absorbance at the beginning of the log phase, α is the growth rate constant and t is time at log phase end (865, 866).

6.3.2.2 Assessment of toxin production by cytotoxigenic culture

Clostridial toxin production was measured via toxigenic culture and cell-cytotoxicity titre assay. Strains were cultured in BHI broth for 48 hours, before centrifugation for 10 minutes at 12,000 g. Twenty microlitres of supernatant was inoculated into microtitre tray wells containing 180 µL of confluent Vero cell culture. Serial dilution to 10^{-6} was performed and cell cultures were incubated in 5% CO_2 for 48 hours. Toxin action was confirmed by neutralisation of additional wells with a *C. sordellii* antitoxin. Toxin titres were recorded, with positivity determined at >50% cell rounding, as previously; (Chapter Three).

6.3.2.3 Competitive batch culture

Independent overnight BHI cultures of parent and mutant strains were standardised as previously (6.3.2.1) and combined in equal bacterial concentrations (0.5 mL:0.5 mL) into 25 mL BHI broths. Co-cultures were incubated, whilst shaking for 24 hours. Population distributions were quantified at both zero and 24 hour time points by serial dilution to 10^{-7} (20 µL:180 µL) in peptone water (Oxoid, UK), before plating onto selective and non-selective Brazier's agar. Mutant populations were assessed with MXF-incorporated agar (0.25x MIC). After 48 hour anaerobic incubation, colonies were counted and mutant populations subtracted from the TVC counts to determine parent populations. All strains were tested in both biological and technical triplicate. *In vitro* fitness was calculated as:

$$s = \ln(CI) / [t \times \ln(2)]$$

Where, s is selection coefficient, CI is competition index and t is number of generations (518).

Number of generations is $$\frac{\log_{10} N_t - \log_{10} N_0}{\log_{10} 2}$$

Where, N_t is total population at time point 24 hours and N_0 is total population at time point zero hours (631).

Competition index is $$\frac{R_{(t1)} / S_{(t1)}}{R_{(t0)} / S_{(t0)}}$$

Where $R_{(t0)}$ is the resistant (mutant) population (CFU) at time point zero, $R_{(t1)}$ is the resistant population at 24 hours and S is the sensitive (progenitor) population (CFU).

Fitness of the parent strains were set at 1 and the relative fitness of the mutant in competition (w) was defined as; $w = 1 + s$, per generation.

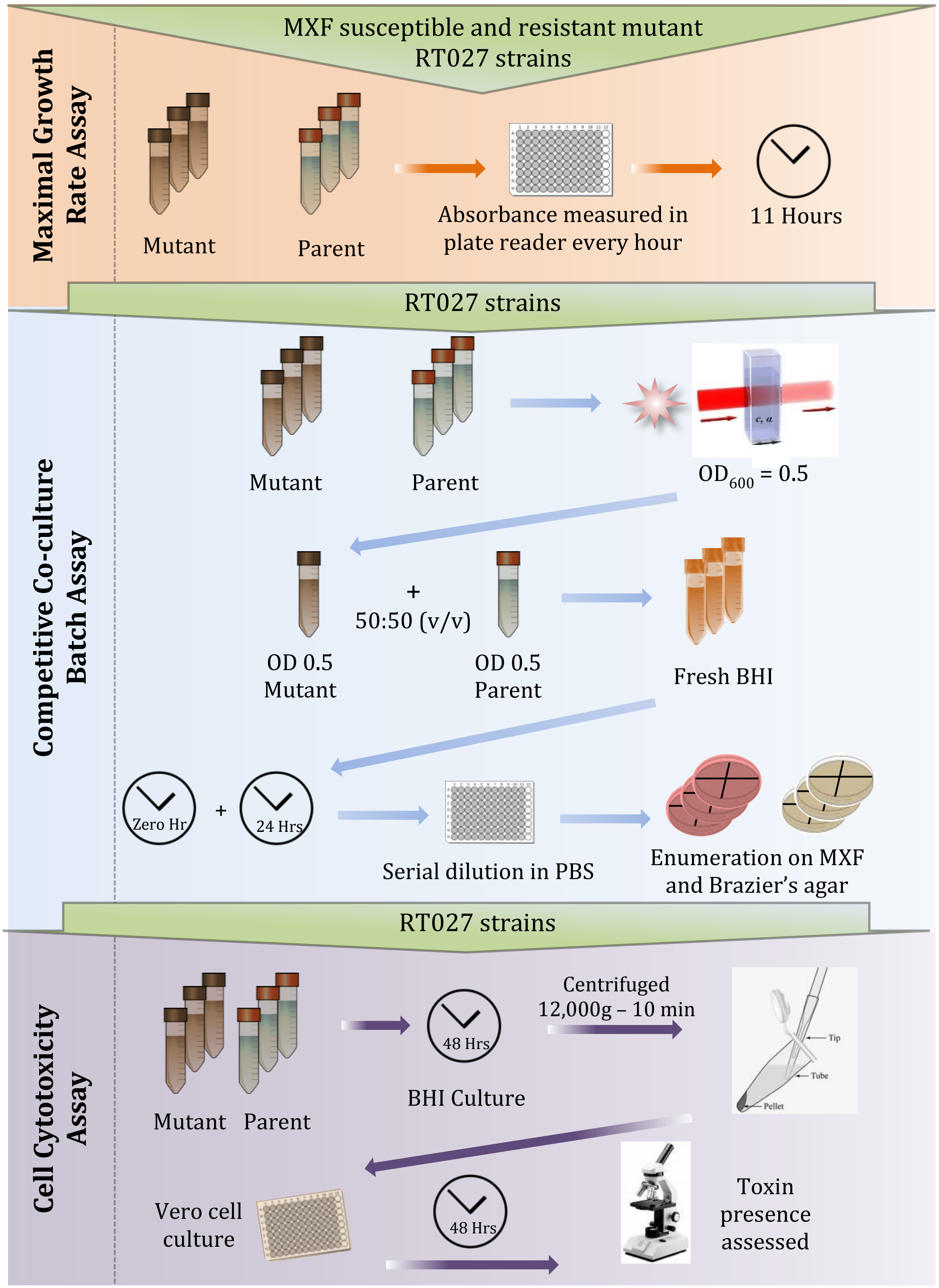


Figure 44: Flow diagram of bacterial fitness determination methodologies. *Seven PCR ribotype moxifloxacin (MXF) susceptible parent strains (MIC < 2mg/L) and resistant progeny were assessed by three fitness determining assays. BHI – brain heart infusion, OD – optical density, RT – ribotype.*

6.3.2.4 Competitive co-culture in a continuous chemostat model

A continuous competitive co-culture chemostat model was implemented to further investigate any associated effects of fluoroquinolone mutation on *C. difficile* fitness.

6.3.2.4.1 Chemostat model configuration

A one litre sealed, glass chemostat vessel was autoclave sterilised with BHI media *in situ*. The chemostat was connected to an additional growth media supply (BHI) by sterile tubing, fed through a peristaltic pump; (Watson-Marlow 101 U/R peristaltic pump; Watson-Marlow, UK). BHI was continuously fed into the chemostat over the span of the model, at a rate of 42 mL h^{-1}. Expended growth media sources were aseptically replaced daily, to ensure a consistent supply of nutrients. The pH of the vessel was measured and maintained at 6.8 (±0.2) using a P200 ChemoTrode (Hamilton, USA), in conjunction with an Anglicon Bio Solo 3 pH controller; (Anglicon, UK). Duran bottles containing acid (1M HCl) and alkali (1M NaOH) solutions were connected to the chemostat vessel through the peristaltic pumps of the controller unit with sterile tubing. The chemostat was positioned on an AGE magnetic stirrer (VELP Scientifica, Italy), with an integrated magnetic flea continuously mixing the culture at approximately 150 rpm. Temperature was maintained at 37°C (±2°C) using a silicone beaker heater and the BriskHeat SDS Benchtop Digital Temperature Controller; (BriskHeat, USA). To ensure anaerobicity, the vessel was continually sparged with N_2 from a generator (Parker Balston, USA). The full continuous culture model apparatus is presented in Figure 45, whilst the chemostat configuration is displayed in Figure 46. Additional details are shown in the Appendices.

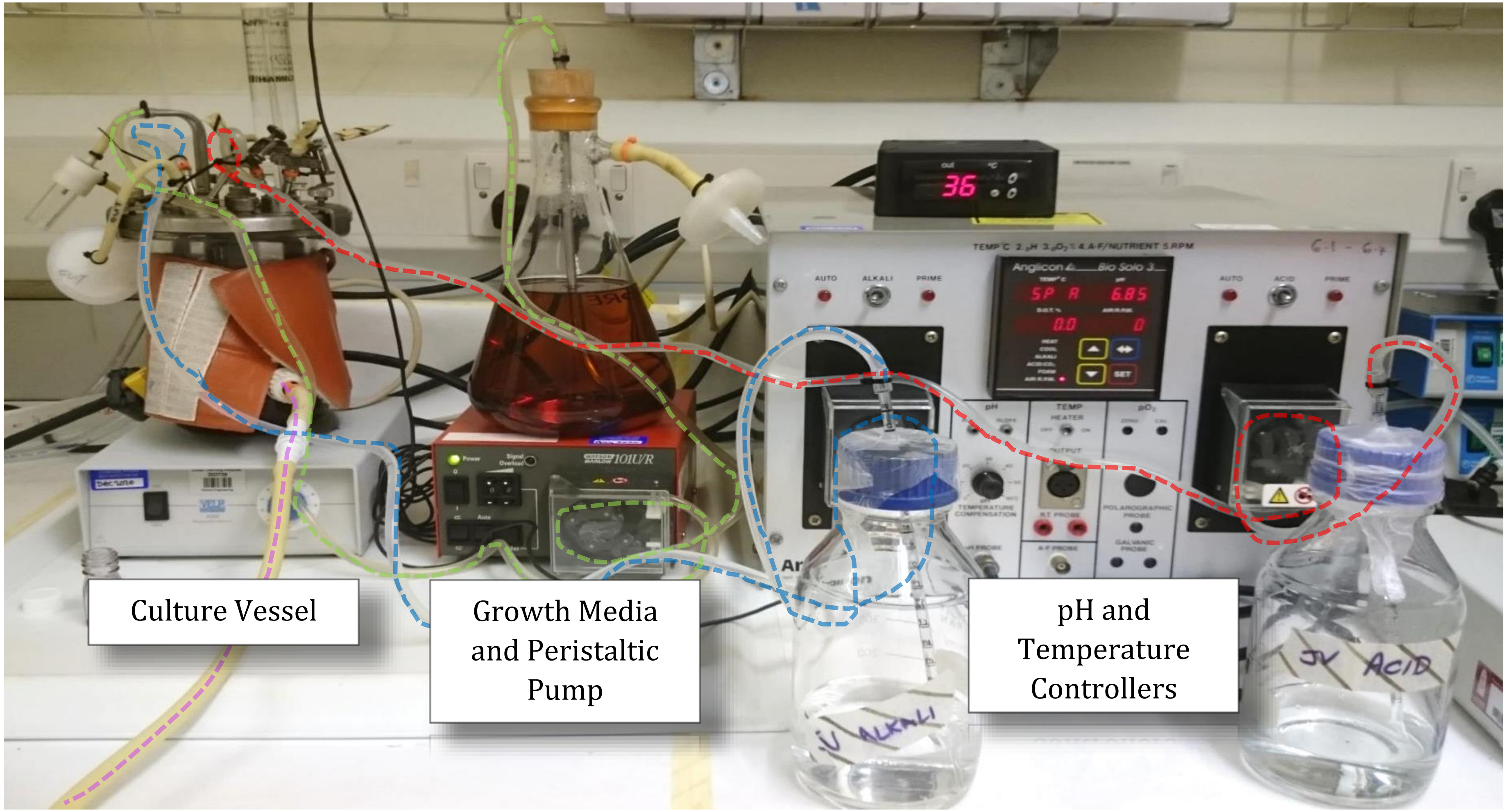


Figure 45: Continuous competitive co-culture chemostat configuration. *Red line represents acid flow, blue represents alkali, green represents nutrient media flow (BHI) and purple represents waste output.*

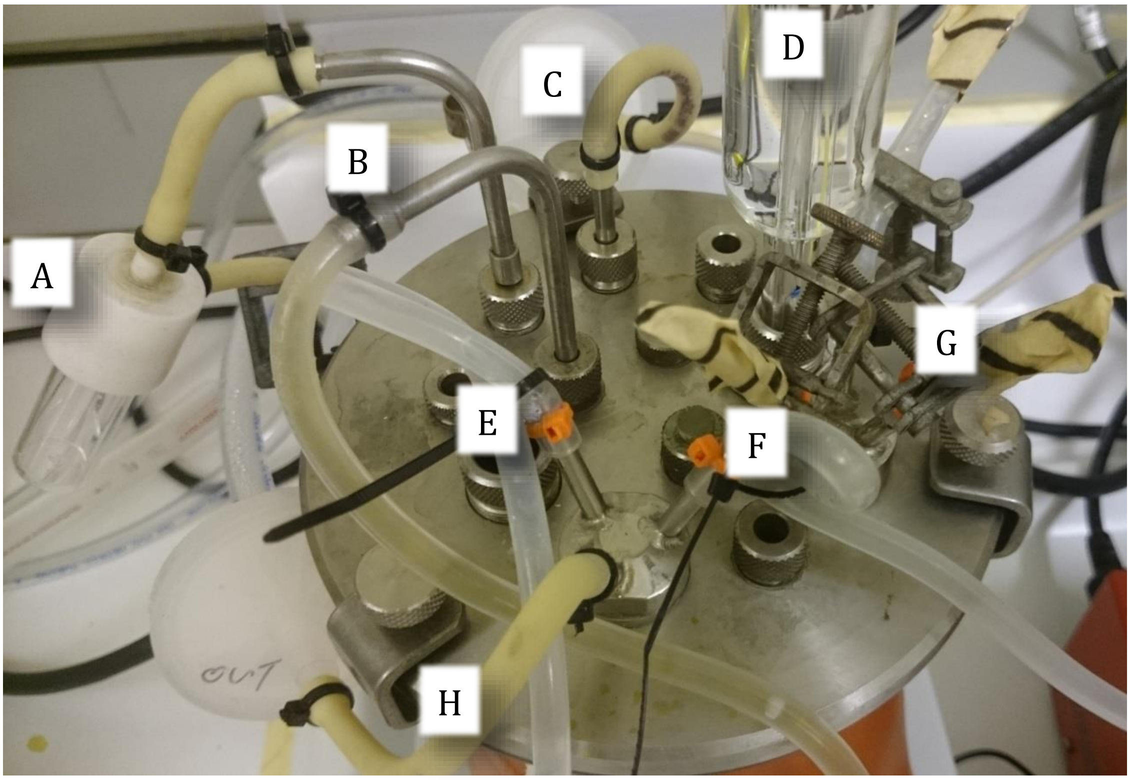


Figure 46: Continuous co-culture vessel port configuration. *A – Sample port, B – Growth media input, C – Nitrogen input, D – pH probe, E – Alkali input, F – Acid input, G – Inocula input, H – Gas outlet.*

6.3.2.4.2 Experimental design

Equal proportions of the CD3079 parent (1 mg/L MXF MIC) and CD3079 Mut (32 mg/L MXF MIC) strains were syringe inoculated into the continuous culture model. Prior to inoculation, these initial cultures were standardised as previously (6.3.2.3). The population balance of the inoculum was measured to ensure that the parent to mutant colony ratio was sufficiently balanced (±0.2 OD), before proceeding with the experimental run. Population dynamics were tested at the zero time point using the previously described viable count method (6.3.2.3). This enabled baseline proportions to be established for the assessment of population progression. Sampling was achieved aseptically by using a 20 mL syringe to draw a vacuum through a sterile glass bijou connected to a sampling tube; (Appendix). Each model replicate was run for a period of eight days (192 hours) with samples tested every 24 hours for population dynamics and further resistance mutations (>32 mg/L and >64 mg/L MXF MICs). Fitness of the mutant strain was calculated as previously (6.3.2.3). Three independent model replicates were run and the data was compiled.

6.3.2.4.3 Population dynamics testing

Parent and mutant populations were assessed by comparing TVCs and MXF breakpoint plate colony counts to differentiate between fluoroquinolone sensitive parent and resistant, mutant bacteria. Samples were serially diluted to 10^{-7} in sterile peptone water with 20 µL aliquots inoculated onto both non-selective Brazier's agar (supplemented with 2% lysed horse blood) and breakpoint, Brazier's agar containing 8 mg/L MXF. Colony counts from breakpoint plates were subtracted from TVC counts, obtained from the Brazier's agar, to acquire sensitive parent counts. These were compared to resistant mutant counts to assess the population ratios. Spore populations were also determined with a one hour ethanol shock (50% v/v) prior to dilution and plating on Brazier's agar, as previously. Additionally, samples were inoculated onto further breakpoint plates, containing 32 and 64 mg/L MXF, in order to investigate elevated mutational response. All tests were carried out in triplicate with mean averages reported. Several colonies from both 32 and 64 mg/L MXF plates had their MICs determined, via the agar incorporation method; (previously described in Chapter Three).

6.3.3 Statistical analyses

All statistical tests were carried out using IBM SPSS Statistics v.21.0.0.1. Due to the skewed data in one of the fitness assay variables, Thr82>Ile containing isolates were analysed using a Wilcoxon signed rank test. P values <0.05 were identified as significant, whilst $p<0.001$ as highly significant. Holistic batch co-culture data was compared using the same statistical test.

For continuous competitive co-culture model data, mutant to parent population ratios were assessed after normality testing using the Shapiro Wilk test statistic. The paired t-test was utilised to compare ratios at the zero and peak difference time points.

6.4 Results

6.4.1 *In vitro* fitness determination for fluoroquinolone resistance mutations

6.4.1.1 Parent and mutant isolate characteristics

Mutant colonies representing the highest MXF MICs generated for each strain tested in mutability assays (Chapter Five) were selected for further investigation into the effect of fluoroquinolone resistance adaptations on strain fitness; (Table 30). All *gyrA* and *gyrB* substitutions were confirmed by Sanger sequencing (Appendix), with the detected mutations all demonstrating close proximity to the fluoroquinolone target region of DNA gyrase; (Figure 47).

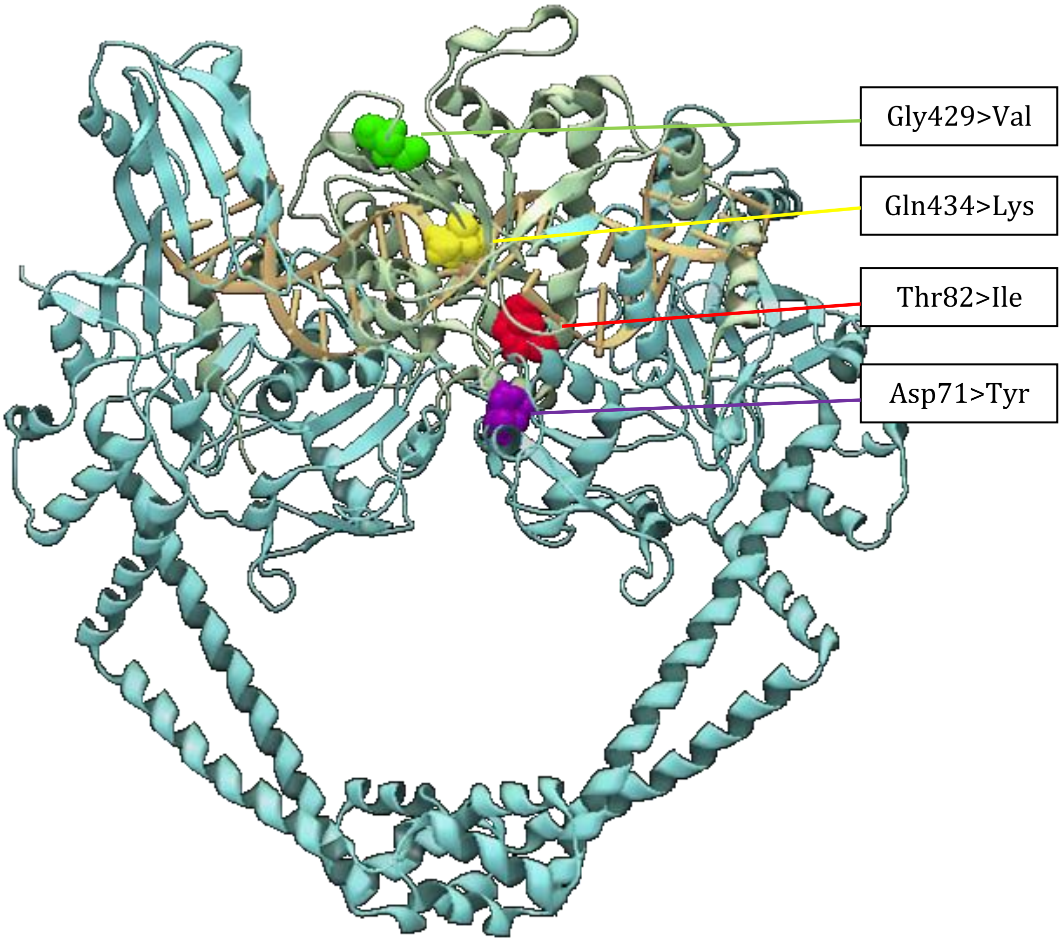


Figure 47: Visualisation of the non-synonymous mutation locations on the protein structure on the C. difficile 630 DNA gyrase complex interacting nucleic acid (orange). *Light blue structures represent gyrase subunit A, light green represents gyrase subunit B. Atomic spatial depictions denote amino acid substitutions; GyrA: red – Thr82>Ile and purple – Asp71>Tyr. GyrB: green – Gly429>Val and yellow – Gln434>Lys. Image created using CLC Genomics Workbench.*

6.4.1.2 Maximal growth rate determination

Based on bacterial growth curves (Figure 48), log phase was estimated to begin at the three hour time point, where absorption increased by an average of 176% from the previous time point; (in comparison to 137% between to prior two readings). The end of log phase was determined as the nine hour time point, since no further increase in absorbance was recorded after this point. For the purpose of maximal growth rate calculations, the three and nine hour measurements were applied.

Maximal growth rates of all parent and fluoroquinolone resistant isolates remained comparable, ranging between 0.0050-0.0065 OD_{595} min^{-1} ($\bar{x}$ = 0.0060) and 0.0053-0.0070 OD_{595} min^{-1} ($\bar{x}$ = 0.0061), respectively; (Table 30). Individual parent to mutant growth rate comparisons demonstrated no substantial differences, ranging between -0.0006 and 0.0007 OD_{600} min^{-1} ($\bar{x}$ = 0.00007). Three mutant strains (CD3904, CD9609 and CD9946) exhibited slightly reduced maximal growth rates than their progenitor isolates, whilst three demonstrated marginally elevated rates (CD3809, CD3051 and CD3079). One parent and mutant strain pairing revealed almost the exact same growth rate calculation (CD3891); (Figure 49).

Analysis of maximal growth rate and mutation frequency revealed a positive correlation. Parent isolates demonstrating mutation frequencies greater than $1x10^{-7}$ (Chapter Five) revealed a correlation with marginally elevated maximal growth rates (0.0062-0.0065 OD_{595} min^{-1}), compared to those with lower mutation frequencies (0.0050-0.0059 OD_{595} min^{-1}); (Table 30).

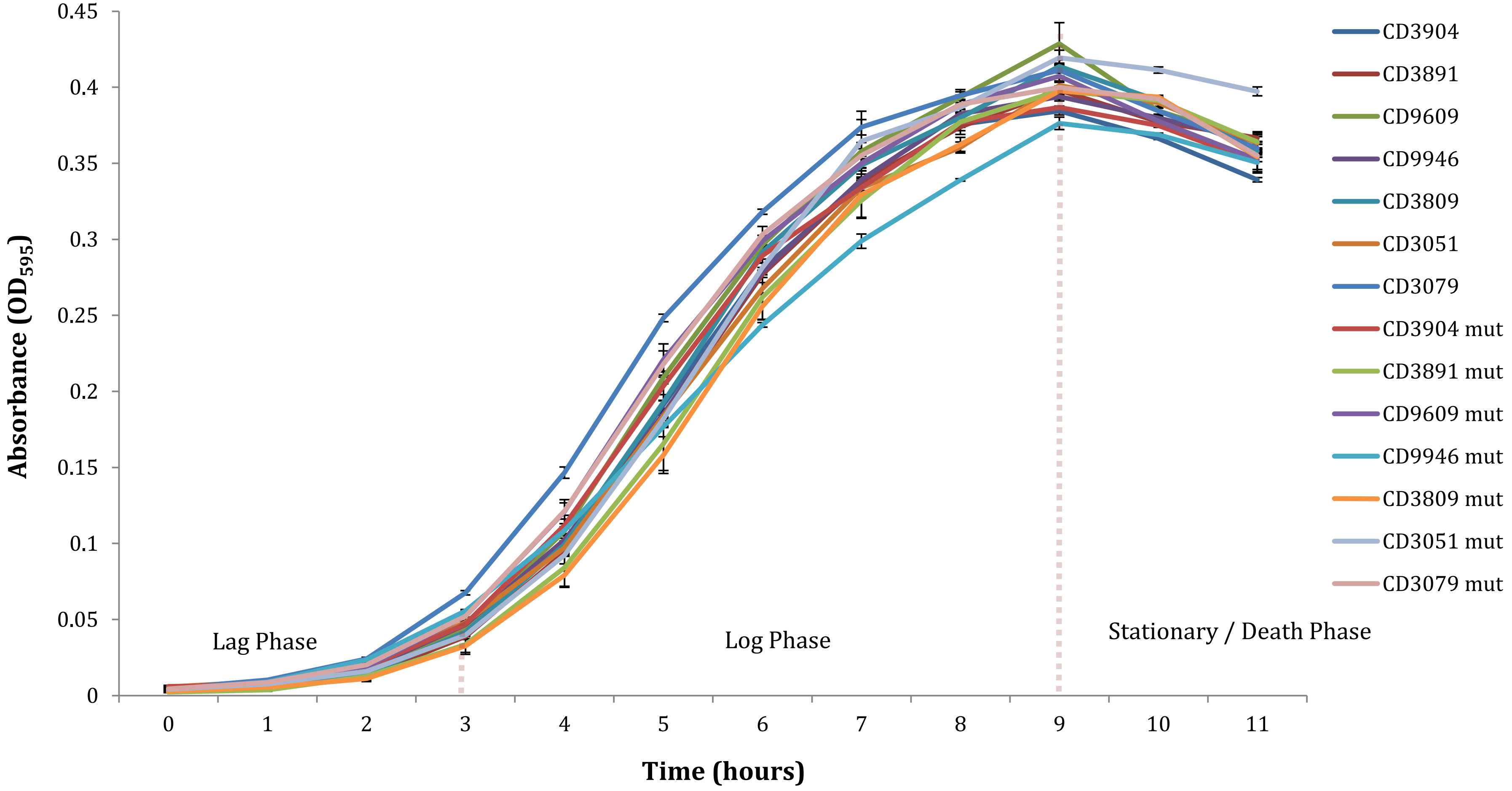


Figure 48: Growth curves of seven PCR ribotype 027 strains and their fluoroquinolone resistant progeny. *Vertical dotted lines represent the beginning and end of log phase, used to calculate the maximal growth rates. Absorbance measurements represent the mean of triplicate values (±SE). mut refers to fluoroquinolone resistant mutant progeny.*

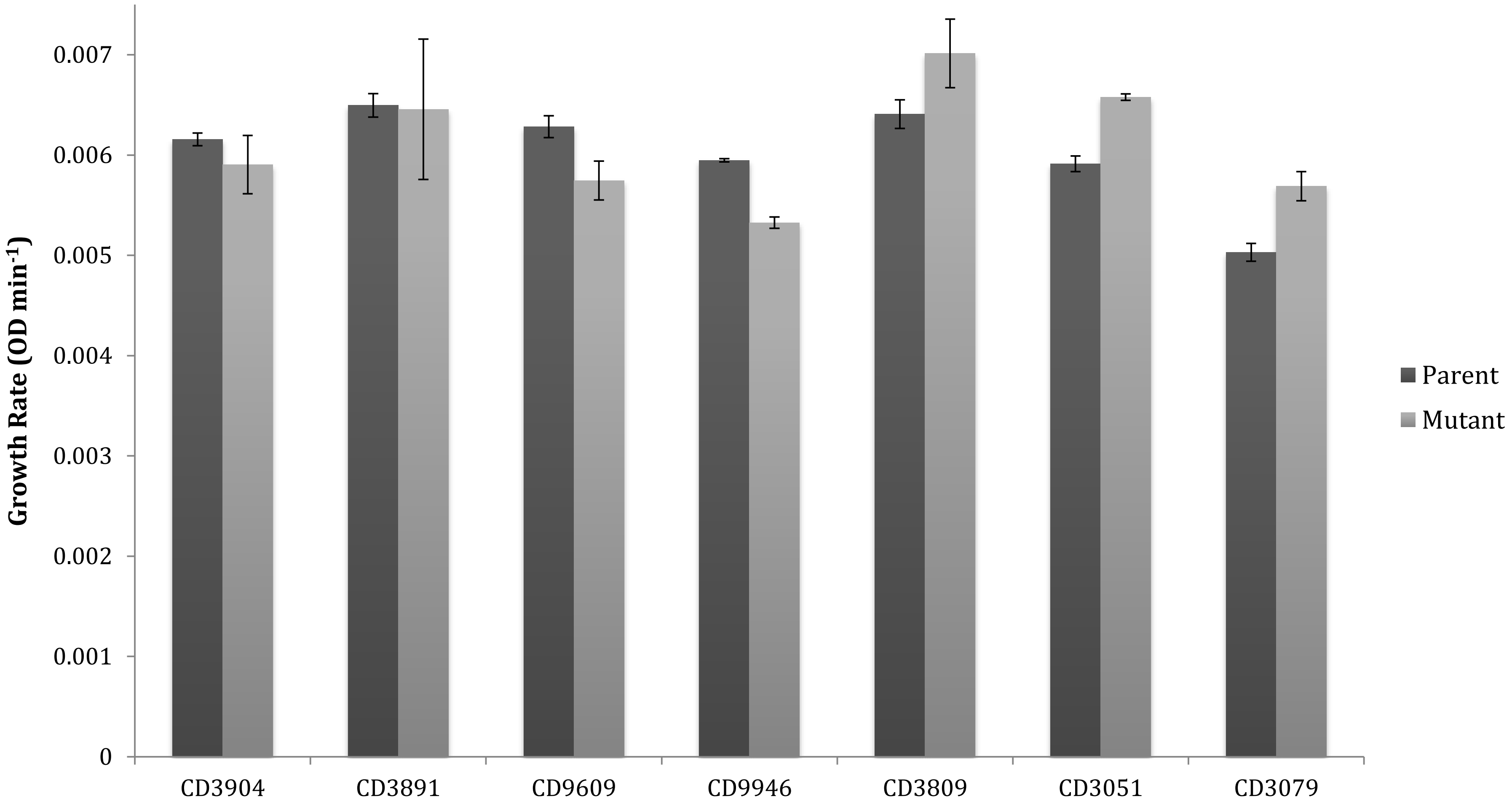


Figure 49: Mean maximal growth rates (±SE) of seven PCR ribotype 027 parent strains and their fluoroquinolone resistant, mutant progeny. *Rates were calculated from triplicate values, based on absorbance (595nm) at beginning (3 hours) and end of log phase (9 hours).*

6.4.1.3 Assessment of toxin production by cytotoxigenic culture

All parent and mutant strains produced clostridial toxin, negated by *C. sordellii* anti-toxin, effective to a titre of 10^{-4}; (Table 30). No differences were observed between parent and mutant isolates.

6.4.1.4 Competitive batch culture

A variation of fitness (*w*) responses to fluoroquinolone resistance conferring mutations were observed, ranging between *w*=0.80 and 1.24 ($\bar{x}$ = 1.13), relative to parent fitness set at 1; (Figure 50). In six isolates, fluoroquinolone resistant, mutant progeny exhibited a fitness advantage when cultured in direct competition with parent strains. All mutants containing a Thr82>Ile substitution displayed a considerably elevated fitness level, with collective 24 hour mutant to parent ratio scores significantly higher than the zero time point scores; *p*=0.002. Isolate CD9946, containing a Gly429>Val substitution, demonstrated the largest fitness benefit (*w*=1.24). Notably, the single fluoroquinolone resistant strain exhibiting the Asp71>Tyr mutation (CD3809) demonstrated a distinct burden to fitness, with relative fitness defined as *w*= 0.80. All strain comparisons identified a statistically significant variation between the ratios of fluoroquinolone sensitive parents and resistant mutants at the zero hour and twenty-four hour time points; (t(20) = 4.307, *p*<0.001).

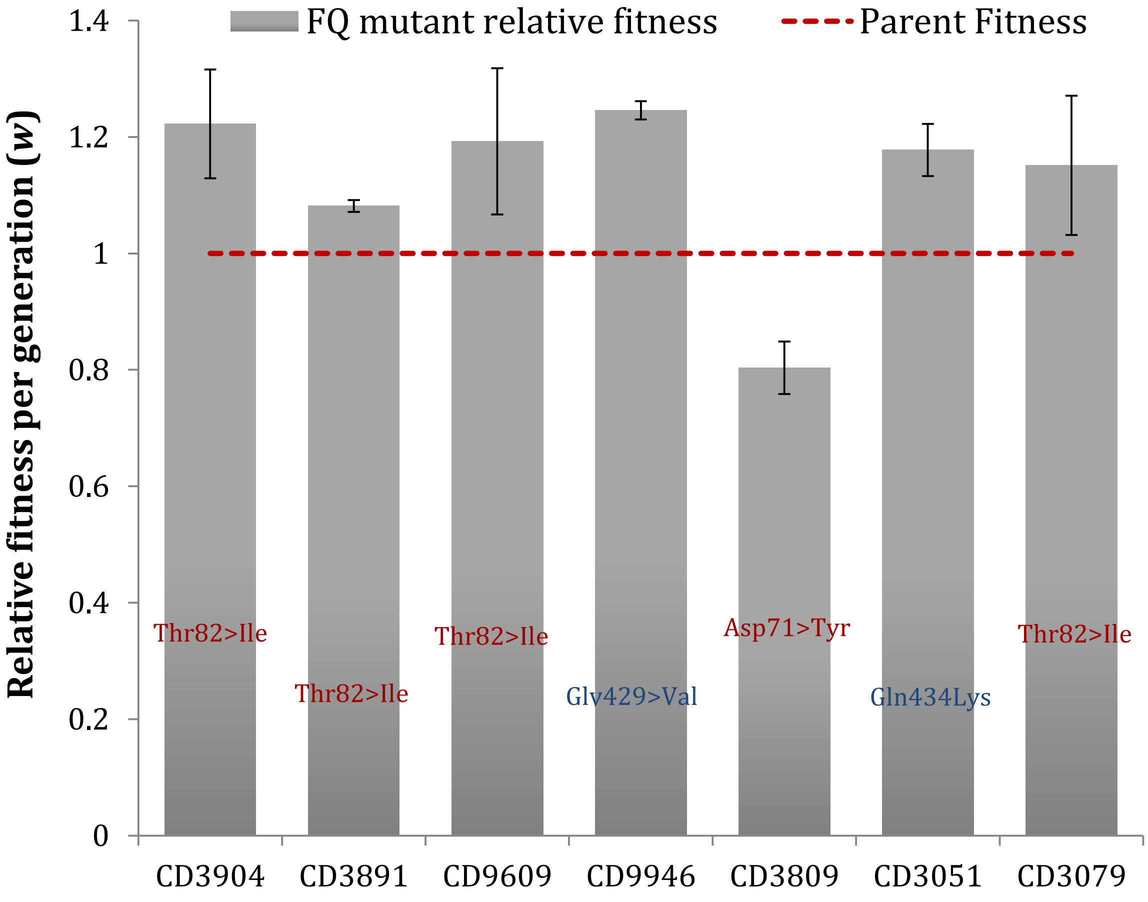


Figure 50: Mean relative fitness (±SE) of fluoroquinolone (FQ) resistant progeny compared to parent fitness (set to 1) in competitive co-culture assays. *Values are based on three biological replicates. Red represents gyrA substitutions, blue represents gyrB substitutions.*

Strain Identifier	MXF (4 mg/L) induced Mutation Frequency (proportional)	Maximal Growth Rate (OD_{595} min^{-1})		Competition Assay Parent:Mutant Ratio		Co-culture Relative Fitness (*w*)		Cell Cytotoxicity Assay Status (+ve titre)	
		Parent	**FQR Mutant**	**Zero Hour**	**24 Hours**	**Parent**	**FQR Mutant**	**Parent**	**FQR Mutant**
CD3904	5.91 x 10^{-6}	0.0062	0.0059	1.17	0.46	1	1.2224	+ (10^{-4})	+ (10^{-4})
CD3891	2.11 x 10^{-6}	0.0065	0.0065	0.92	0.68	1	1.0815	+ (10^{-4})	+ (10^{-4})
CD9609	2.73 x 10^{-6}	0.0063	0.0057	0.94	0.58	1	1.1925	+ (10^{-4})	+ (10^{-4})
CD9946	3.77 x 10^{-8}	0.0059	0.0053	1.03	0.40	1	1.2459	+ (10^{-4})	+ (10^{-4})
CD3809	3.91 x 10^{-7}	0.0064	0.0070	0.95	1.20	1	0.8036	+ (10^{-4})	+ (10^{-4})
CD3051	3.02 x 10^{-8}	0.0059	0.0066	0.97	0.74	1	1.1777	+ (10^{-4})	+ (10^{-4})
CD3079	2.93 x 10^{-8}	0.0050	0.0057	1.19	0.69	1	1.1512	+ (10^{-4})	+ (10^{-4})

Table 30: Fitness testing of seven PCR ribotype 027 strains and fluoroquinolone resistant (FQR) progeny determined by batch culture. *Parent strain moxifloxacin (MXF) mutation frequencies are shown to enable growth rate comparisons.*

6.4.1.5 Competitive co-culture in a continuous chemostat model

Fluoroquinolone resistant mutant to parent ratios demonstrated a gradual increase across the duration of the model, averaging at $\bar{x}$ = 1.43, with a peak of 1.80 at 96 hours; (Figure 51). Mutant to parent population ratios were significantly different between the zero ($\bar{x}$ = 1.16, SD=0.38) and 96 hour time points ($\bar{x}$ = 2.7, SD=1.9); t(8)=-2.294, p=0.025. Proportional disparity was briefly reduced between 120 and 144 hours, before rising again towards peak levels. Populations of highly MXF resistant bacteria peaked after 24 hours, with counts on 32 mg/L and 64 mg/L MXF-containing agars observed at 7.34 x 10^4 and 4.44 x 10^1 $\log_{10}$ CFU/mL, respectively. By 72 hours, >32 mg/L mutant counts decreased to stable populations for the remainder of the model duration, ranging between 7.89 x 10^3 and 1.17 x 10^4 $\log_{10}$ CFU/mL; ($\bar{x}$ = 9.54 x 10^3). Greater than 64 mg/L MXF mutants were undetectable between 48 and 96 hours, with sporadic low-level detection identified towards the end of the culture model. All colonies obtained from 64 mg/L MXF agars were confirmed to have MXF MICs of 128 mg/L. Colonies present on 32 mg/L MXF agars from early (days 1-2) and late (days 6-8) stages of each model had MXF MICs of 64 mg/L. MXF resistant mutant fitness remained relatively consistent throughout the duration of the continuous co-culture model (w= 1.11–1.45, $\bar{x}$ = 1.25); (Appendix).

Total *C. difficile* counts peaked after 24 hours (1.23 x 10^8 $\log_{10}$ CFU/mL), with the population levelling out by 48 hours, remaining stable through to the conclusion of the model ($\bar{x}$ = 7.93 x 10^7 $\log_{10}$ CFU/mL); (Figure 52). Spore counts demonstrated an exponential increase between 24 and 48 hours (4.07 x 10^3 – 6.32 x 10^6 $\log_{10}$ CFU/mL), corresponding to the initial reduction and subsequent stabilisation of total *C. difficile* counts.

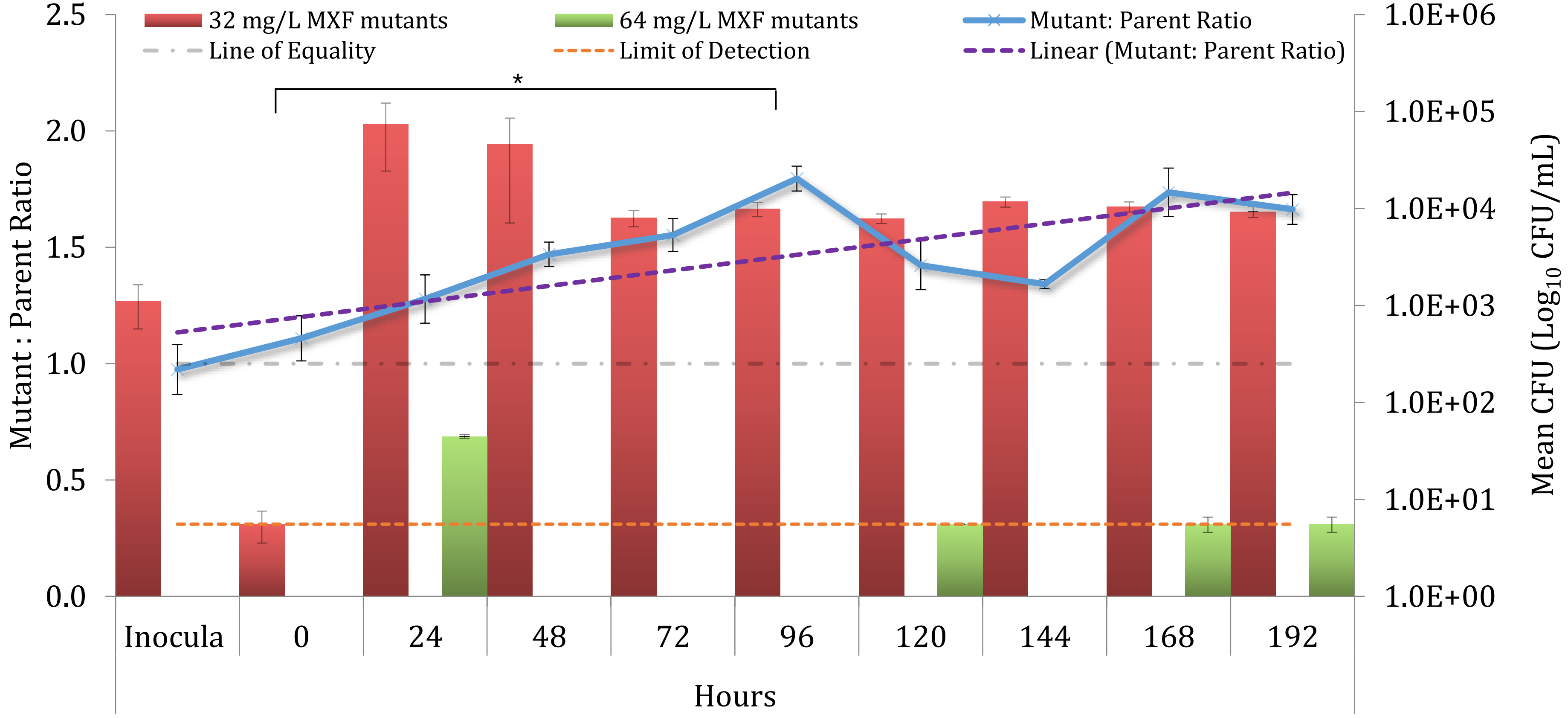


Figure 51: Progression of mutant (CD3079 Mut) and parent (CD3079) ribotype 027 C. difficile ratios in a continuous co-culture chemostat model. *The linear trend line (purple) demonstrates the increasing divergence between mutant and parent strain populations. Highly resistant populations >32 mg/L and >64 mg/L MXF were documented to track further resistance development. The line of equality represents the baseline of equivalent mutant and parent strain proportions, data above this signifies a fitness advantage for the mutant strain. Data are presented as mean averages (±SE) of three independent model replicates. * p=0.025, significantly different mutant : parent ratios. Line series relate to the left-hand y-axis and bar charts relate to the right-hand y-axis.*

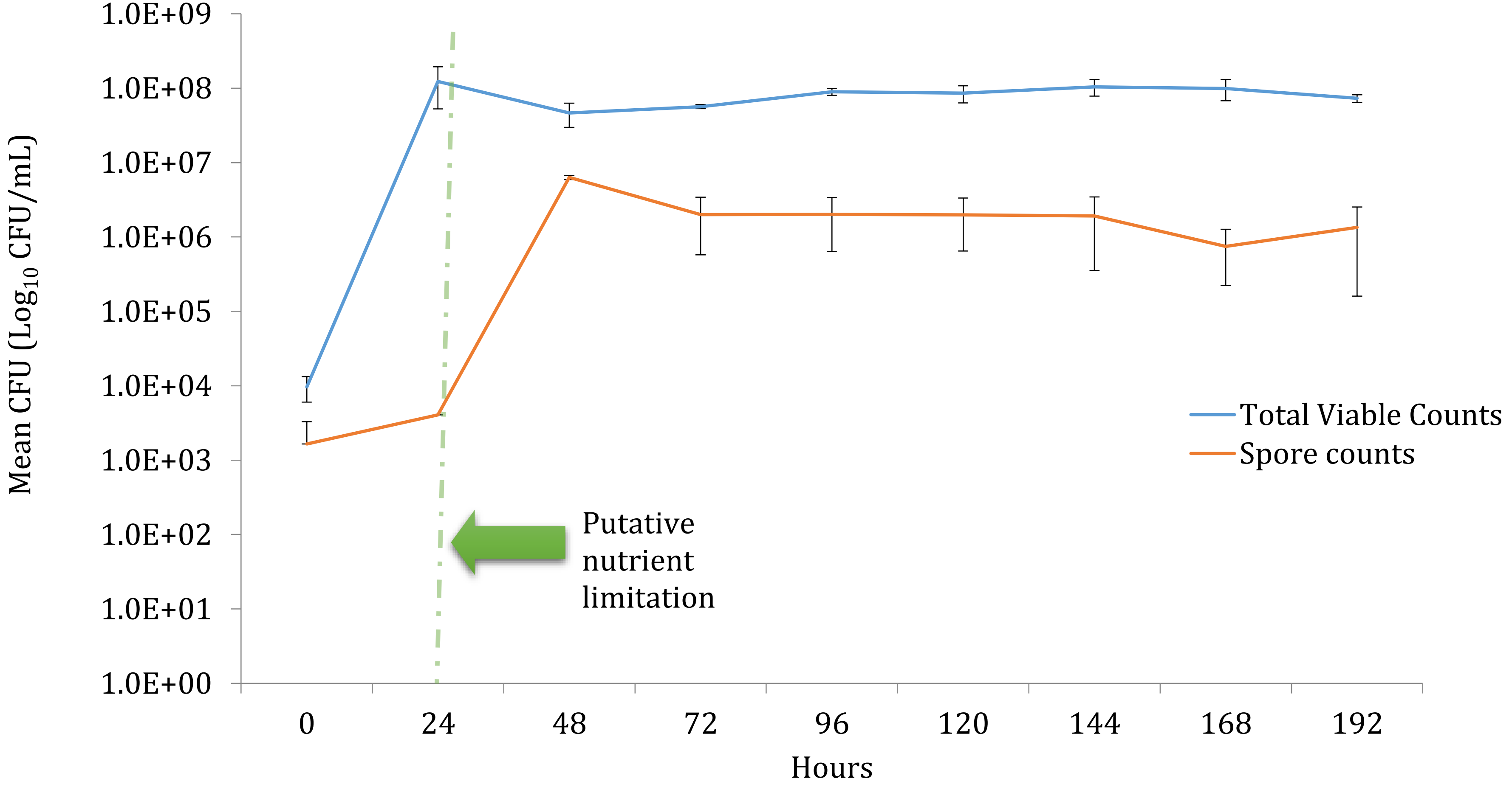


Figure 52: The progression of total C. difficile and spore populations observed in a continuous co-culture chemostat model. *The point of perceived nutrient limitation is indicated by the green dashed line. Data represents mean averages (±SE) of three independent model replicates.*

6.5 Discussion

6.5.1 *In vitro* fitness determination for fluoroquinolone resistance mutations

The effect of fluoroquinolone resistance determinants on an organism's fitness has been widely investigated in a host of bacterial species (587, 629, 630, 633, 635). While fitness burdens are regularly reported, commonly associated with a decreased growth rate (585, 629, 864), beneficial effects of gyrase and topoisomerase mutations have also been observed (630, 633, 634). However, there is a dearth of information regarding the fitness effect of fluoroquinolone mutations in *C. difficile*, particularly ribotype 027. Fluoroquinolone resistance was a notable characteristic of PCR ribotype 027 strains involved in large outbreaks of severe disease around the world and fitness impacts may have been a factor in the predominance of this ribotype (99, 387, 562). *In vitro* batch culture experiments with *C. difficile* may yield indicative data, whilst chemostat models have been used successfully to predict clinical CDI risk and treatment response (117, 125, 867-869).

6.5.1.1 Competitive batch culture

The competitive batch culture findings of this investigation demonstrated evidence of both benefit and burden to bacterial fitness, with amino acid substitution dependent responses observed. Whilst the majority of mutations exhibited a significant advantageous effect ($p<0.001$), intriguingly, an Asp71>Tyr mutation in isolate CD3809 demonstrated a distinct fitness burden; (Figure 50). Therefore, these data present a complex picture, with mutational site impacting heavily on bacterial fitness.

In this study Thr82>Ile mutations demonstrated no fitness burden on *C. difficile*, in agreement with previous findings in this organism (639) as well as in others (870-872). Furthermore, a significant advantage was observed in these mutant isolates in co-culture experiments. This common fluoroquinolone resistance substitution has been identified in isolates associated with major ribotype 027 outbreaks (34, 92), suggesting that resistance acquisition without fundamental hindrance to fitness may offer a substantial contribution to clonal expansion. A recent retrospective analysis of local and national data provided further insight into the influence of fluoroquinolones on CDI rates (387). Dingle *et al.* identified major and significant reductions in CDI caused by fluoroquinolone resistant strains, but no changes in case rates due to susceptible strains, in the context of a fluoroquinolone restricted environment. Whilst the aforementioned study highlighted the important role of antibiotics in selection and maintenance of certain populations, it does not necessarily explain the whole story. In a three-year pan-European study of *C. difficile* PCR ribotypes and resistance, Freeman *et al.* described the continued prevalence of MXF-resistant PCR ribotype 027 in Cyprus, against a background of decreasing fluoroquinolone use. In contrast, the PCR ribotype 027 isolates from Cyprus became progressively less resistant to other antimicrobials tested, inferring no detriment to fitness from the presence of fluoroquinolone resistance mutations in these isolates (118). This suggests the involvement of additional factors. Infection control interventions in the Netherlands, without fluoroquinolone restriction, resulted in substantial decreases in ribotype 027 prevalence in the aftermath of a series of outbreaks, whilst other types flourished in its absence (873). The data outlined in this chapter suggest that, for Thr82>Ile mutants in particular, retention of a resistant genotype incurs no detectable fitness cost, and so may be retained in the absence of antibiotic pressure. This may help to explain the continued presence of fluoroquinolone resistant *C. difficile* PCR ribotype 027 in some locations.

The only available data for *C. difficile* comes from a study by Wasels *et al.,* who observed no fitness cost to the organism, associated with the majority of amino acid substitutions investigated (639). However, one *in vitro* mutant variant of the CD630 strain (ribotype 012) did demonstrate a statistically significant impairment in co-culture investigations. Interestingly, the amino acid substitution present was the less common Thr82>Val mutation. This could explain the rarity of the mutant variant, as strains acquiring the mutation could incur the reduced growth rate and suffer from competitive inhibition in clinical environments. Lee *et al.* reported epidemiological shift in Korean hospitals, aligned with this fitness dynamic *(724)*. They described a shift from a predominance of ribotype 001 to a broad distribution of other ribotypes, representing a change from Thr82>Val to Thr82>Ile. This followed the fitness model, since the ousted strain type harboured the substitution variant demonstrating the greater fitness burden (639). Nonetheless, there are other factors that potentially had a major impact on strain prevalence, including fluoroquinolone MICs and additional antibiotic class resistances of the emerging strains.

In batch culture, one Asp71>Tyr containing mutant demonstrated a burden on fitness. Although proximally located to the advantageous mutation, Thr82>Ile, a contrasting fitness response was observed. Whilst the mechanisms of these polarised responses are not yet understood, there may be a considerable impact on the tertiary structure of the gyrase molecule, affecting replication efficacy. Although the fitness cost of CD3809 Mut alterations may be attributed to the *gyrA* Asp71>Tyr mutation, as this amino acid substitution was only present in a single instance, further isolates featuring the same modifications are required to substantiate this hypothesis.

The mutations in *gyrB*, conveying low-level resistance, also displayed no fitness burden. This correlated with previous findings (639), where mutations at Asp-426, generating equally moderate MIC increases (4 mg/L MXF), produced very slight (non-significant) fitness advantages. Since these intermediary steps are an important stage in evolutionary resistance development, it may be that the absence of a fitness cost/slight fitness advantage may shift the population baseline and allow further advantegeous mutations to achieve higher levels of resistance. Whilst *gyrB* mutations may appear of less importance, producing only marginal decreases in fluoroquinolone susceptibility, they can act as a transitional step up the resistance hierarchy. The capacity to incur fluoroquinolone resistance mutations without detriment, as shown in the present study, may be a contributory factor to widespread dissemination in this ribotype. Research in other genera support this notion, with resistance mutations generating the least burden, identified as the most clinically prevalent (636).

Although a definitive cause for this fitness advantage is unknown, Marcusson *et al.* suggested that modifications to the genes involved in the supercoiling process may ultimately affect gene expression (635). By eliciting increased gyrase promotor activity and the upregulation of other core processes, beneficial adaptations may be acquired. Furthermore, D'Ambrozia discovered altered transcriptomes between parent and *gyrA* mutant, *Neisseria gonorrhoeae* isolates (874), indicating a potential for differential expression associated with fluoroquinolone resistance mutation in other bacteria. Whilst it remains unclear as to why sterically proximal mutations can impart such opposing effects, one hypothesis may be that the change in amino acid chemistry may affect the enzyme-nucleic acid binding affinity.

Though it might be expected that advantageous chromosomal mutations would be optimised and preserved through Darwinian evolution, we cannot discount the impact of these substitutions on other factors, crucial to the bacterium. Interestingly, Hiramatsu *et al*. suggested that the *S. aureus* wildtype *gyrA* gene may have offered resistance to natural antibacterials, such as nybomycin, as the emergence of mutations conferring resistance to modern fluoroquinolones resulted in nybomycin susceptibility (875). This suggests that the retention of a survival element (natural antimicrobial resistance), was perhaps more important in archaic lineages than the slight fitness advantage arising from an additional Thr82>Ile susbstitution.

The ability to outcompete other strains may have been a factor in the successful expansion and maintenance of epidemic ribotype 027 strains. Robinson *et al.* demonstrated a competitive advantage for ribotype 027 versus other non-027 strains in an *in vivo* murine model (430), although the precise mechanisms are not clear. The authors proffered an explanation of an elevated ability to outcompete others for limited nutrients, postulating an increased replication rate of the *thyA* gene, encoding for thymidylate synthase enzyme, as a potential factor in ribotype 027 fitness (430). Combined with such physiological factors, additional benefits conferred by gyrase mutations, however slight, have the potential to compound any ecological advantages held by this ribotype. Nonetheless, as no mention of fluoroquinolone resistotype was made in the study by Robinson *et al.*, differing susceptibilities may have been a contributory factor.

Since the discovery of both fitness advantages and burdens, amongst a similar cohort of isolates is of great interest, an additional presentation of the data was produced to further outline the findings; (Figure 53). Inoculation of co-culture experiments with carefully balanced proportions of test populations is vital to any fitness investigation. However, although optical densities of the individual cultures were meticulously measured, with a tolerance of ±0.05 OD_{600} variability, a consistent, precise balance of populations is practically impossible to achieve. Thus, in two strains of equal growth rate, a slight imbalance in initial populations would only be exacerbated with exponential growth, potentially skewing findings. Nonetheless, in this investigation a repeatable fitness effect was observed across the replicates, whether or not the mutant strain population outweighed its competitor strain at the zero time point. This pattern was also reflected whether or not the mutation was advantageous or detrimental. The majority of the seven batch competition experiments generated atleast one replicate representing a slight imbalance favouring parent and one weighted towards mutant populations. For example, CD3904 replicates A and C were imbalanced in the favour of the parent strain, whilst replicate B exhibited the opposite imbalance. Nevertheless, after 24 hours growth, all cultures demonstrated the same predominance of mutant isolate populations; (Figure 53). For the majority of parent and mutant test pairings, each replicate generated comparable final ratios. This observation adds to the robustness of the findings, although the result of intentionally varied inocula proportions would provide interesting insight towards the ultimate assessment of this hypothesis.

Whilst these findings are stark, it is not inconceivable that compensatory mutations may have occurred during fitness investigations (876). Since genomic analysis was not implemented for the end-stage resistant mutants, there is a possiblity that those reflecting no fitness burden may have generated compensatory mutations in order to redress a fitness imbalance.

Maximal growth rate calculations revealed minimal differences between parent and mutant strains, demonstrating a lack of correlation with co-culture data. This suggests that fitness variability may only become apparent in direct competitive culture. Equally, no effect was observed on strain cytotoxicity. However, the possibility cannot be excluded that a competitive growth advantage could result in increased toxin load.

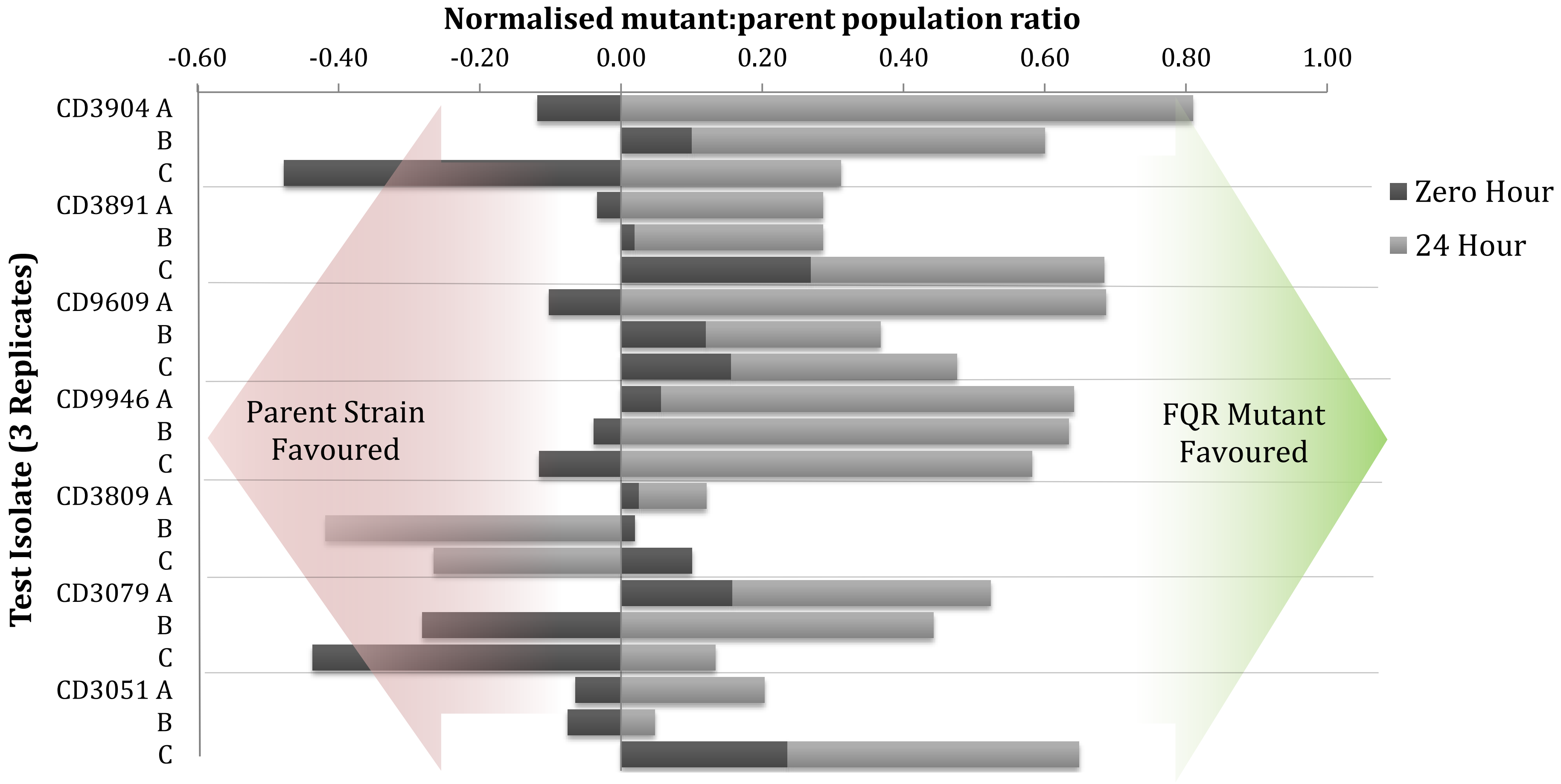


Figure 53: Representation of mutant to parent ratios within starting inocula (zero hours) and at 24 hours, for three replicate competitive co-culture assays. *Values >0 represent greater numbers of FQR mutants within the population, whilst <0 represents greater numbers of parent strain. FQR – fluoroquinolone resistant. A, B & C represent biological replicates.*

6.5.1.2 Bacterial fitness of the *gyrA* Thr82>Ile mutation in a continuous co-culture chemostat model

Animal modelling has been extensively used to test *in vivo* CDI response (877-881), however this methodology requires specialist skills and environments to achieve reliable results. Whilst batch culture experiments can provide easily obtainable insights into bacterial responses, it is not particularly reflective of the bacterial turnover occurring in gastrointestinal environments. Continuous culture models provide more representative data regarding the dynamics within clinical bacterial populations (882). The concept of continual nutrient replenishment and population dilution permits a highly controlled environment for adaptive evolutionary analyses (882), where strain dynamics and serial adaption under selective pressures can be assessed. Onderdonk *et al.* utilised this method to successfully investigate the impact of environmental pressures on *C. difficile* toxin production (883).

6.5.1.2.1 Relative fitness of MXF resistant *C. difficile* isolate CD3079 Mut in a continuous co-culture model.

The balance of mutant and parent population ratios in co-culture represents the ideal foundation to measure the effect of genetic mutations, whether advantageous or detrimental, on the fitness of an organism. Here the proportion of mutant to parent strain populations in the initial inocula were remarkably concordant (0.98 ratio). Based on this close proximity, the continuous co-culture model demonstrated that isolate CD3079 Mut, encompassing amino acid substitution Thr82>Ile in the important *gyrA* gene, revealed a gradual increase in divergence in population from its progenitor strain. In support of batch culture findings, these data demonstrated a lack of detrimental effect of the aforementioned DNA gyrase mutation on *C. difficile* fitness. Intriguingly, this mutant progeny demonstrated an advantage over its parent strain, CD3079. The fitness calculations for this strain generated consistent findings throughout the experimental model, regardless of at which time point the determination was made; (Appendix). The mean average fitness of the mutant was established as w=1.25, which supports the results of the batch culture investigation of this strain; (w=1.16). This advantage surpasses the fitness effects observed in *C. difficile* by Wasels *et al.* (w=0.90-1.09) *(639)*, as well as those reported across a range of organisms (w=0.80-1.05) (884). It also correlates closely with the highest relative fitness report in a single instance of a *C. difficile* isolate containing an *rpoB* mutation

(w=1.26) (638), suggesting that this may be a substantial benefit, in the context of other findings. Further confidence in this finding could be attained, since each independent experimental model replicate exhibited consistent trends with regards to the population ratios; (Appendix). These data all indicate how a slight bacterial fitness advantage can quickly become exacerbated in continuous culture, resulting in significant growth benefits.

Nutrient limitation may impact the behaviour and fitness of an organism. When assessing the total vegetative and spore populations of each co-culture (Figure 52), it is plausible that after 24 hours the availability of nutrients lessened. Total viable populations peaked at this stage, prior to a reduction in numbers, whilst spore populations expanded extensively just after. This is suggestive of an inhospitable environment, triggering sporulation as a survival mechanism. Thereafter, the gradual supply of fresh nutrients correlates with population stabilisation. Since populations of high-level MXF resistant strains (>32 mg/L) diminished soon after the point of putative nutrient limitation, it may also suggest that these bacteria become burdened with additional mutations and cannot compete efficiently at high levels. The sporadic instances of 128 mg/L MXF resistant mutant detection may be explained by the same rationalisation, which would reflect observations of high-level resistance reversion in previous *C. difficile* models (117). Intriguingly, the models described by Saxton *et al.* also demonstrated low-level toxin production prior to detectable germination, during investigations with MXF and ribotype 027. The presence of a minority, resistant sub-population, able to germinate in the presence of high MXF concentrations may explain this phenomenon. This concept may also be evident in the continuous culture model described here, where early detection of highly resistant colonies was observed.

One interesting observation, apparent in all independent model replicates, was the reduction in mutant to parent ratio occurring between 96 and 120 hours, prior to returning to a gradual increase after 144 hours; (Figure 51 and Appendix). Since this time point correlated with the creation of fresh breakpoint media, one explanation for this could be the deterioration of the antibiotic efficacy in the fluoroquinolone-incorporated agar used to quantify mutant populations. Nonetheless, this does not explain the rapid increase observed beyond 144 hours, where relatively fresh culture plates were utilised, or the absence of any effect on the 32 mg/L MXF viable counts. However, differences at higher concentrations may prove less discernible. This period may also align with the formation of biofilm, temporarily impacting the planktonic population dynamics, resulting in a decreased mutant to parent ratio.

6.5.2 Study limitations

Whilst multiple stage chemostat models using faecal emulsions have proven effective in modelling CDI responses to a plethora of environmental pressures (117, 125, 350, 869, 885), they require a complexity of expertise to operate successfully. While these may be more reflective of a human intestinal environment, they cannot replicate the interactions with an immune response (867). Therefore, whilst this model does not reflect the complexity of interactions *in vivo*, the pure continuous *C. difficile* culture system implemented here, enabled an extension of the batch culture investigations into the fitness of fluoroquinolone resistant strains; (6.4.1.3). Further development of this chemostat system to accommodate culture in a faecal emulsion would closer imitate the clinical environment and the microbial interactions between colonic flora, as utilised by Saxton *et al.* (117). One study has suggested that the fitness behaviour of an organism maybe different between *in vitro* and *in vivo* experiments, suggesting there may be a culture medium specific element to the adaption to a fitness burden (886).

It is also possible that compensatory mutations may have occurred during fitness investigations (876). Since genomic analysis was not implemented on the resistant mutants detected towards the end of the continuous co-culture model, there is a chance that those demonstrating no fitness burden may have acquired compensatory mutations in order to redress a fitness imbalance. Instances of *gyrB* mutation reversion have been previously observed in *in vitro C. difficile* models *(117).*

Although the mutants in this investigation were not developed via controlled gene insertion (allele exchange), the mutations represent a natural adaptive response and may be more clinically representative. Nonetheless, *in vivo* modelling would provide additional insight, as variable outcomes have been observed (886).

6.6 Conclusions

In summary, fluoroquinolone resistance was a feature of PCR ribotype 027-associated outbreaks of severe disease. This PCR ribotype continues to persist and is highly prevalent in some locations (34, 112). This study demonstrates a fitness advantage associated with the common Thr82>Ile *gyrA* mutation, which may have afforded this strain an epidemiological advantage. Without hindrance of an associated fitness impairment, the potential for clonal expansion is increased. Notably, a lack of fitness detriment associated with this phenotype, theoretically allows for the retention of fluoroquinolone resistance in the absence of antimicrobial pressure.

Chapter 7 Concluding Discussion and Further Work

7.1 Discussion

This thesis presents evidence of antimicrobial resistance progression in *C. difficile*, through comparisons of historical and modern isolate collections. Potential contributory factors have also been suggested with the demonstration of variable mutation frequencies and a fitness benefit associated with common fluoroquinolone resistance mutations observed.

The discovery of MXF resistant isolates from the early 1980s demonstrated the potential for the development of cross-resistance between generations of fluoroquinolones, as previously reported (438). The isolation of three ribotype 027 strains from the historical collection, potentially provided two of the earliest instances of this epidemic, hyper-virulent ribotype. Originating from 1981 and 1983, these two isolates pre-dated the CD196 strain recovered from a French patient in 1985 (289). While it is likely that this ribotype existed prior to this isolation, CDI cases caused by 027 strains may have been rarer due to the scarcity of a resistance advantage. The detection of fluoroquinolone resistance in this important ribotype, prior to the major clonal expansion of the early 2000s, may suggest the presence of genomic differences hampering these strains from flourishing where others did. Although outside the scope of this work, comparative analysis of SNP locations between these historical isolates and modern, resistant strains may reveal distinct regions with the potential to affect epidemiological success.

Whilst it is known that resistance often rapidly follows the introduction of a new antibiotic (535), there is a paucity of data comparing *C. difficile* epidemiology across decades, using modern molecular techniques (381, 726). Here we compared phenotypic resistance prevalence amongst an historical collection of *C. difficile* isolates originating from the UK between 1980-1986 and modern UK strains from a large-scale surveillance study (2012-2016) (118). Comparisons revealed significant increases in the geometric mean MICs of all comparator compounds (p=0.01-p<0.0001), with the exception of imipenem. Whilst no evidence of resistance to the primary treatment drugs (vancomycin, metronidazole and fidaxomicin) was detected in the historical isolates, all demonstrated slightly lower geometric mean MIC, when compared to modern strains; (VAN 0.135, MTZ 0.112 and FDX 0.001 mg/L). Interestingly, the MXF MIC_{90} was two doubling dilutions lower in the historical isolates, suggesting a substantial expansion of high-level resistance amongst modern strains. This development of high-level resistance may substantially increase the risk of CDI onset in colonised patients with fluoroquinolone exposures and therefore, generate considerable concern for clinicians.

Ribotype prevalence in the historical catalogue reflected modern epidemiology, with the collection dominated by ribotypes 015 and 020. Whilst it cannot be ignored that the recovered strains may represent those ribotypes fittest for long term survival, distributions potentially indicate a baseline of strain types that is reverted to as outbreaks (e.g. ribotype 027 or long-term periods of endemicity, such as ribotype 001 in the UK during the 1990s) subside. The presence of the rare ribotypes 242, 341, 619, 626 and 862 may indicate that these were more common decades ago, but have subsequently become more or less extinct, due to competition inhibition by more successful genotypes. Importantly, these data cannot account for the interim period between the two collections, as they only inform us about two disparate time points. Nonetheless, taken as a whole, the data indicate increasing resistance to multiple antimicrobials in *C. difficile*.

Next generation sequence data revealed the presence of many resistance determining elements in the historical genomes. Although not as prevalent as indicated in modern surveillance studies, resistance determinants, such as *ermB, tetM* and *cfr* were detected in genomes from the 1980s. The discrepancy in prevalence between the temporally distinct isolate collections highlighted the progression of these elements, potentially through clonal expansion and horizontal gene transfer. This escalation in the presence of resistance determinants emphasises the concerning progression towards increased incidence of multidrug resistance in *C. difficile*. The situation becomes self-perpetuating, as the greater the abundance of transposable genes in the gut microbial pan-genome, the more opportunities for gene acquisition.

The phylogenetic investigations performed on these strains highlighted the necessity for caution, both when interrogating historical isolate collections and in the assessment of molecular clock data. Whilst contamination of historical specimens with modern isolates cannot wholly be dismissed, rigorous measures were imposed during the recovery protocols to ensure the risk was minimal. Though the phylograms indicated that the majority of isolates fitted within the predicted range of SNP differences, molecular clock estimations did not always correlate with age distinctions. The dormancy experienced by *C. difficile* during its existence in spore form conceivably contributes to the evolutionary rate. However, as this is an almost indeterminable measure, it should be reasonable to consider that estimations of molecular clock rates are only guideline approximations and outlying findings may be observed. The impact of spore quiescence on evolutionary estimations is therefore an area requiring further examination.

Investigations of *C. difficile* mutability under fluoroquinolone selection revealed a diverse range of responses, with ribotype 027 isolates demonstrating the highest rates ($\bar{x}$=1.48 x 10^{-6}). This discovery revealed the high plasticity of the QRDR, particularly amongst the hyper-virulent types and may contribute to the success of these strains. Though the mechanism driving these different responses is yet to be elucidated, several genes relating to the DNA SOS and mismatch repair systems revealed no correlations with mutability phenotypes. The cause may lie in, yet undiscovered, complex epigenetic mechanisms.

The impact of any genetic alteration on bacterial fitness potentially influences the survival and evolution of a genotype (585). Here the effects of fluoroquinolone resistance mutations on *C. difficile* growth rates, toxin production and co-culture competition were assessed in the epidemic ribotype 027. Three fluoroquinolone resistance conferring mutations, both in the *gyrA* and *gyrB* genes, indicated no burden to fitness, with a significant advantage observed in isolates harbouring the common Thr82>Ile substitution (w=1.16, p=0.002). A continuous co-culture chemostat model was successfully established for the further evaluation of bacterial fitness testing, with repeatable results observed for one Thr82>Ile harbouring isolate. The capacity for *C. difficile* to develop common fluoroquinolone resistance mutations without detriment to the core functions of the bacteria, revealed the potential for resistance spread through unabated clonal expansion. Similarly, where resistant mutants arise they may quickly proliferate and outgrow sensitive populations in the gut, causing additional clinical challenges. From ribotype 027 phylogeny it was observed that the majority of ancestral strains were fluoroquinolone sensitive, whilst recent lineages are almost entirely resistant. The fitness advantages of resistance substitutions, observed in this ribotype, may be a contributing factor in this evolutionary direction. Nonetheless, an absence of fitness burden was not a universal trait with MXF resistance mutations. A single instance of an Asp71>Tyr substitution revealed a substantial detriment in batch culture, highlighting the importance of SNP location to bacterial fitness.

In conjunction with the absence of a fitness burden associated with common fluoroquinolone resistance substitutions, clonal expansion of genetic elements may result in even more problematic, clinical challenges. Whilst resistance to antibiotic treatment options remains uncommon, an assemblage of elements providing protection from other antimicrobial classes may generate increased occurrences of CDI. Current trends are moving away from managing this antibiotic-mediated disease with paradoxical antimicrobial treatments and towards alternative therapeutics, such as monoclonal antibody (255), microbial transplants (216, 887) and probiotic therapies (245). While, in time, research may generate efficacious, novel treatment options, controlled antimicrobial stewardship programs remain essential to reduce resistance development contributing to the onset of disease.

7.2 Further work

Whilst the elucidation of the mechanism behind the vegetative cell replication inhibition effected by high concentrations of glycine would be of interest, further optimisation of additional *C. difficile* culture based media would have greater clinical relevance. A comprehensive assessment of all widely used growth media, both agar and broth, would enable the recommendation of a "gold standard" recovery method, potentially leading to a reduction in false negative determinations of CDI and an improved clinical response.

Beyond the scope of this thesis, further extensive attempts to recover aged *C. difficile* isolates from the historical collection would be valuable. Further investigations of historical *C. difficile* isolates would be worthwhile in their contributions to the knowledge base, however caution must be taken when considering the provenance of isolates and any potential contamination issues.

With regards to the mutation frequency investigations, repeat testing of the same panel of isolates with exposures to an agent with an alternative resistance mechanism, such as RIF, would corroborate the mutability findings. Assessment of *C. difficile* mutability frequencies when exposed to the commonly used ciprofloxacin and levofloxacin, as well as testing of all ribotype groups under elevated concentrations, would further clarify the mutational consequences for *C. difficile*.

In order to confirm the consistency of the fitness responses outlined in this thesis, the assays could be repeated with end stage mutants sequenced to ensure no additional compensatory mutations had occurred. Equally, corroboration of the fitness effects of mutations tested in only single replicates (e.g. Asp71>Tyr) could contribute to the significance of the conclusions.

The introduction of a fluorescent marker, such as green fluorescent protein (GFP), into the mutant populations tested in bacterial fitness assays would enable the use of flow cytometry to assess small variations in growth rates/fitness. Where only slight effects were observed, they could be accurately quantified. Nonetheless, assays would require suitable controls to ensure the addition of fluorescent markers did not contribute a fitness effect.

Both batch and continuous co-culture fitness experiments would benefit from being performed in faecal slurry, as opposed to pure broth. This would facilitate the assessment of fitness response in an environment closer reflecting a clinical setting and the effects of bacterial interactions amongst colonic microbial populations. Repeat model testing with different strains exhibiting the same substitution, as well as others with diverse mutations would all increase the robustness of the hypotheses outlined here. Equally, since the continuous culture chemostat model has produced consistent results across several replicates, this model could be used to test the impact of other resistance elements or even to perform fitness assays in other organisms.

The analyses performed as part of this PhD research are only the tip of the iceberg when considering the use of NGS data. Generation of vast amounts of genomic information provides a whole host of opportunities for genome mining for novel resistance determinants and the identification of genetic relationships to phenotyping data. With the ever increasing availability of sequencing technologies, this collection of genomic information (directly linked to phenotypic resistance determinations) could provide a basis for future antimicrobial resistance prediction algorithms. Applications such as machine learning (888, 889) have the potential to harvest genomic data sets and produce robust links between the pan-genome and antimicrobial resistance.

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Appendix

8.1 Supplementary data for *C. difficile* germination assays

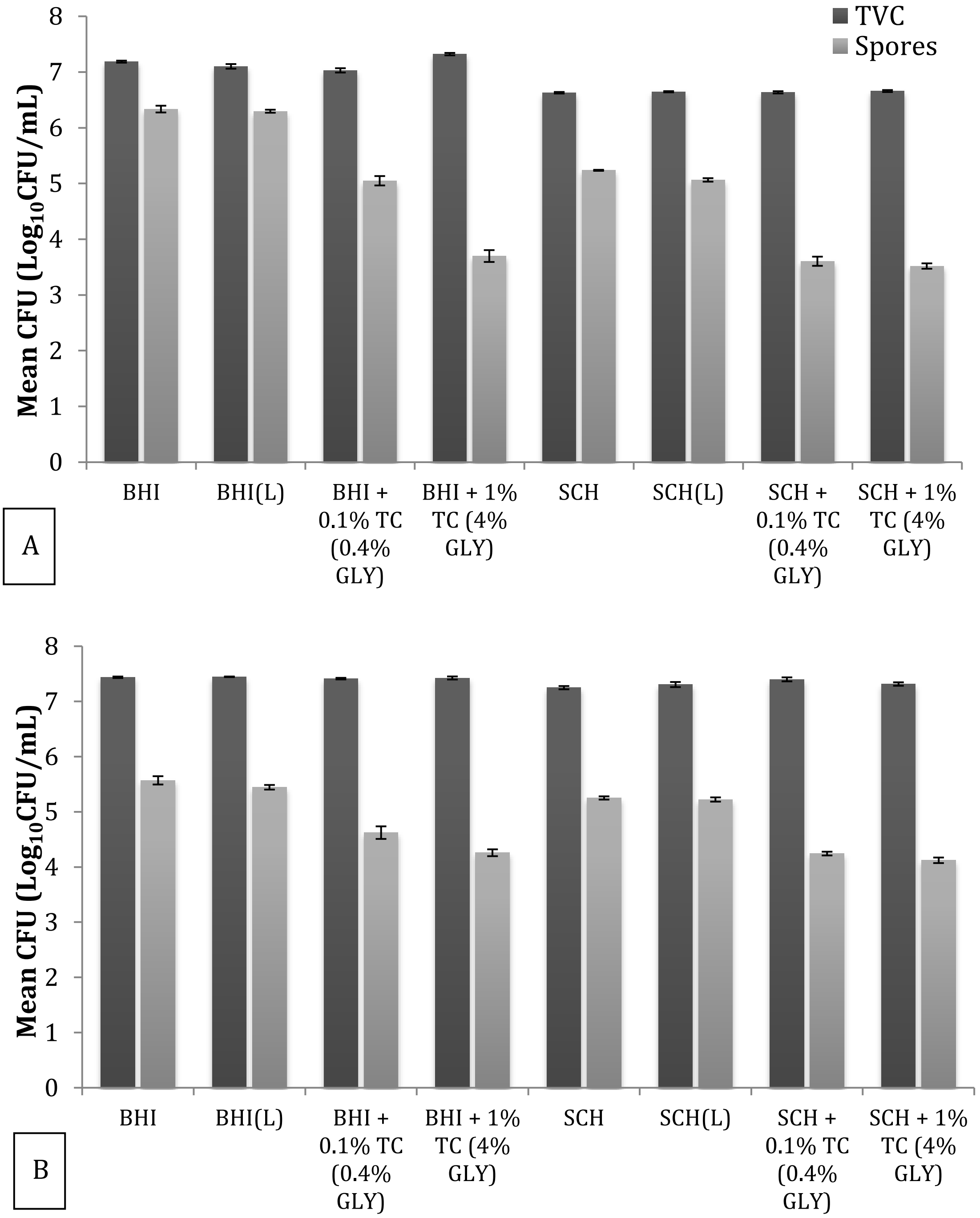

Figure 54: Germination of PCR ribotype 001 (A) and 078 (B) C. difficile aged (six weeks) spores in broths supplemented with various germinant concentrations.

Germination efficiency is represented by differences in mean (±SE) total viable counts (TVC) and spore counts. Broths were exposed to germinants for 90 minutes. BHI – Brain Heart Infusion, SCH – Schaedlers anaerobic broth, L – lysozyme (5mg/L), GLY – glycine, TC – sodium taurocholate. Counts are based on triplicate broth testing.

8.2 Supplementary data for historical *C. difficile* isolate characterisation (1980-86)

				Susceptibility Testing (MIC mg/L)															
Strain	**Specimen Date**	**PCR Ribotype**	**Cell Cyto-toxicity**	**VAN**	**MTZ**	**RIF**	**FDX**	**MXF**	**CLI**	**IPM**	**CHL**	**TGC**	**LZD**	**CIP**	**TZP**	**CRO**	**AMX**	**TET**	**ERY**
JV01	03.12.81	012	+	1	0.125	0.001	0.03	2	>64	4	4	0.06	1	32	16	64	2	32	>256
JV02	31.12.81	027	+	0.5	1	0.002	0.06	32	8	8	4	0.03	1	>64	16	64	1	0.125	>256
JV03	30.03.82	015	+	0.5	0.06	0.001	0.03	1	8	4	4	0.03	2	32	4	32	1	0.125	<4
JV04	06.05.82	001	+	1	0.125	0.002	0.015	2	16	4	4	0.03	1	16	4	32	0.5	0.06	<4
JV05	01.06.82	020	+	0.25	0.06	0.001	0.06	1	4	2	4	0.03	1	32	8	32	0.5	0.06	<4
JV06	23.08.82	020	+	0.25	0.06	0.001	0.015	1	2	4	4	0.03	1	32	8	32	0.5	0.125	<4
JV07	23.09.82	015	+	0.5	0.125	0.001	0.06	1	4	4	4	0.03	2	32	4	32	1	0.125	<4
JV08	13.12.82	220	+	0.5	0.06	0.002	0.06	1	8	2	4	0.03	2	32	8	32	0.5	0.125	<4
JV09	03.12.81	015	+	1	0.125	0.001	0.015	1	4	4	4	0.03	2	32	8	32	1	0.125	<4
JV10	09.12.82	015	+	1	0.125	0.001	0.03	1	4	4	4	0.03	2	32	8	32	1	0.125	<4
JV11	17.11.82	015	+	1	0.25	0.001	0.03	2	16	2	8	0.03	2	32	8	32	1	0.125	<4
JV12	19.04.82	020	+	0.5	0.25	0.002	0.06	2	16	2	8	0.03	2	16	4	16	1	0.06	<4
JV13	01.06.82	020	+	0.5	0.25	0.002	0.06	2	4	2	4	0.03	2	32	4	32	0.5	0.06	<4
JV14	09.12.82	078	+	0.5	0.125	0.001	0.004	1	>64	4	64	0.06	16	16	*nt*	16	0.5	4	<4
JV15	28.03.83	020	+	0.5	0.25	0.002	0.06	2	16	2	8	0.03	2	32	8	32	1	0.125	<4
JV16	03.10.83	056	+	1	0.25	0.002	0.03	2	2	4	8	0.03	2	32	4	16	0.5	0.125	<4
JV17	01.02.82	015	+	1	0.125	0.001	0.03	2	>64	4	64	0.03	16	32	8	32	1	4	<4
JV18	09.05.83	103	+	1	0.25	0.002	0.06	2	32	4	8	0.03	2	32	8	32	1	0.125	<4
JV19	10.09.83	137	+	1	0.25	0.002	0.03	2	16	4	8	0.03	2	32	4	32	0.5	0.125	<4
JV20	04.12.81	041	+	4	1	0.002	0.008	32	8	4	4	0.03	1	>64	8	32	0.5	0.125	>256

				Susceptibility Testing (MIC mg/L)															
Strain	**Specimen Date**	**PCR Ribotype**	**Cell Cyto-toxicity**	**VAN**	**MTZ**	**RIF**	**FDX**	**MXF**	**CLI**	**IPM**	**CHL**	**TGC**	**LZD**	**CIP**	**TZP**	**CRO**	**AMX**	**TET**	**ERY**
JV22	31.12.81	078	+	1	0.125	0.002	0.03	2	>64	8	64	0.06	16	16	*nt*	16	0.5	8	<4
JV23	04.12.81	001	+	4	1	0.002	0.008	32	64	4	8	0.03	2	>64	8	64	0.5	0.125	>256
JV24	04.12.81	200	+	4	0.5	0.001	0.015	16	8	8	4	0.03	1	>64	4	64	1	0.125	>256
JV25	04.12.81	014	+	1	0.25	0.002	0.06	1	32	4	4	0.03	2	16	8	16	0.5	0.06	16
JV26	31.12.81	015	+	1	0.125	0.001	0.06	1	8	8	4	0.03	2	32	8	32	1	0.06	<4
JV27	03.12.81	020	+	0.5	0.25	0.002	0.06	1	8	4	2	0.03	1	32	4	16	0.5	0.03	<4
JV28	15.07.82	001	+	1	0.25	0.002	0.03	1	16	4	4	0.03	2	32	4	16	0.5	0.06	<4
JV29	07.01.81	002	+	1	0.125	0.002	0.125	1	4	4	4	0.03	2	16	8	32	1	0.015	<4
JV30	10.02.81	012	+	2	0.125	0.002	0.125	2	>64	2	32	0.03	8	32	8	32	2	32	<4
JV31	02.01.81	002	+	1	0.125	0.002	0.03	2	8	4	4	0.03	2	16	8	32	1	0.015	<4
JV32	10.03.82	012	+	2	0.25	0.002	0.125	2	8	8	8	0.03	2	32	16	32	2	>32	<4
JV33	10.03.82	014	+	0.5	0.25	0.001	0.06	1	2	2	4	0.03	1	16	4	16	0.5	0.015	<4
JV34	31.12.81	001	+	4	0.5	0.001	0.03	16	4	8	4	0.03	1	>64	4	32	0.5	0.06	>256
JV35	11.04.83	020	+	1	0.25	0.002	0.125	2	8	4	4	0.03	2	32	8	16	1	0.125	<4
JV36	19.04.83	015	+	1	0.25	0.002	0.06	2	8	4	4	0.03	2	16	8	32	1	0.015	<4
JV37	19.04.83	001	+	1	0.25	0.002	0.06	2	8	4	4	0.03	2	16	8	32	1	0.03	<4
JV38	06.05.83	626	+	0.5	0.25	0.001	0.06	2	4	4	4	0.03	2	16	8	32	1	0.03	<4
JV39	15.09.83	341	+	0.5	0.125	0.002	0.06	0.25	1	4	4	0.03	1	32	8	16	1	0.015	<4
JV40	13.09.83	014	+	0.5	0.125	0.001	0.06	0.125	2	2	2	0.03	1	16	4	4	0.5	0.015	<4
JV41	03.10.83	002	+	1	0.125	0.002	0.03	2	8	4	4	0.03	2	16	8	32	1	0.06	<4
JV42	25.11.83	015	+	0.5	0.125	0.001	0.03	2	4	4	4	0.03	2	16	8	32	1	0.06	<4
JV43	22.11.84	015	+	1	0.25	0.001	0.125	2	2	4	4	0.03	2	16	8	32	1	0.06	<4

				Susceptibility Testing (MIC mg/L)															
Strain	**Specimen Date**	**PCR Ribotype**	**Cell Cyto-toxicity**	**VAN**	**MTZ**	**RIF**	**FDX**	**MXF**	**CLI**	**IPM**	**CHL**	**TGC**	**LZD**	**CIP**	**TZP**	**CRO**	**AMX**	**TET**	**ERY**
JV44	11.04.83	020	+	0.5	0.25	0.002	0.125	2	2	4	8	0.03	1	16	4	16	1	0.06	<4
JV45	19.04.83	015	+	1	0.125	0.001	0.03	2	1	4	4	0.03	2	16	8	32	1	0.03	<4
JV46	07.04.83	061	+	0.5	0.125	0.001	0.008	1	1	8	4	0.03	2	16	*nt*	64	0.5	0.03	<4
JV47	22.06.81	012	+	1	0.125	0.001	0.015	1	2	8	2	0.06	1	16	*nt*	32	2	>32	>256
JV48	13.11.80	020	+	0.5	0.125	0.001	0.008	1	2	4	4	0.06	1	16	*nt*	16	1	0.03	<4
JV49	07.02.81	032	+	0.5	0.125	0.001	0.004	1	0.125	4	4	0.03	1	16	*nt*	16	0.5	0.06	<4
JV50	15.02.81	017	+	0.5	0.125	0.001	0.03	1	4	8	4	0.03	2	16	*nt*	64	1	0.06	>256
JV51	02.03.81	020	+	0.5	0.125	0.001	0.015	1	2	8	4	0.03	2	16	*nt*	32	1	0.125	<4
JV52	04.03.81	020	+	0.5	0.125	0.001	0.015	1	4	4	4	0.03	1	16	*nt*	16	1	0.03	<4
JV53	10.03.81	020	+	0.5	0.125	0.001	0.015	1	4	4	4	0.06	2	16	*nt*	16	1	0.06	<4
JV54	01.02.82	015	+	0.5	0.125	0.001	0.015	1	2	4	4	0.06	2	16	*nt*	32	2	0.06	<4
JV55	17.06.82	015	+	0.5	0.125	0.001	0.015	1	0.25	8	4	0.03	1	16	*nt*	32	1	0.06	<4
JV56	15.07.82	017	+	0.5	0.125	0.001	0.015	1	0.25	8	4	0.03	2	16	*nt*	16	1	0.03	<4
JV57	31.08.82	200	+	0.5	0.125	0.001	0.015	1	2	8	4	0.03	1	16	*nt*	32	1	0.125	<4
JV58	23.09.82	017	+	0.5	0.125	0.001	0.015	1	1	8	2	0.03	2	16	*nt*	16	1	0.06	<4
JV59	09.03.83	862	-	1	0.125	1	0.015	1	2	8	4	0.125	1	16	*nt*	32	1	0.03	<4
JV60	21.03.83	619	+	0.5	0.125	0.001	0.03	1	1	4	4	0.06	1	16	*nt*	32	2	0.06	<4
JV61	23.03.83	015	+	0.5	0.125	0.001	0.03	1	2	8	4	0.03	1	16	*nt*	32	1	0.06	<4
JV62	14.08.83	015	+	0.5	0.125	0.001	0.015	1	2	8	4	0.03	1	16	*nt*	32	2	0.06	<4
JV63	02.08.83	001	+	0.5	0.125	0.001	0.015	1	2	8	4	0.03	1	16	*nt*	32	1	0.125	<4
JV64	04.11.83	020	+	0.5	0.125	0.001	0.015	1	2	2	4	0.03	2	16	*nt*	32	1	0.06	<4
JV65	09.11.83	033	-	0.5	0.125	0.001	0.004	1	1	4	4	0.03	2	8	*nt*	16	0.5	0.06	<4

				Susceptibility Testing (MIC mg/L)															
Strain	**Specimen Date**	**PCR Ribotype**	**Cell Cyto-toxicity**	**VAN**	**MTZ**	**RIF**	**FDX**	**MXF**	**CLI**	**IPM**	**CHL**	**TGC**	**LZD**	**CIP**	**TZP**	**CRO**	**AMX**	**TET**	**ERY**
JV66	20.12.82	015	+	0.5	0.125	0.001	0.015	1	1	8	4	0.125	1	16	*nt*	32	1	0.125	<4
JV67	28.01.86	027	+	0.5	1	0.001	0.06	16	2	16	4	0.06	2	>64	*nt*	64	2	0.125	>256
JV68	05.01.81	242	+	0.5	0.125	0.001	0.06	2	2	4	4	0.03	2	16	*nt*	16	1	0.125	<4
JV69	19.10.81	014	+	0.5	0.125	0.001	0.015	1	2	2	4	0.03	2	16	*nt*	16	1	0.06	<4
JV70	06.02.86	003	+	0.5	0.125	0.001	0.03	2	2	4	4	0.03	2	16	*nt*	32	1	0.06	<4
JV71	09.09.83	070	+	0.5	0.125	0.001	0.03	2	2	4	4	0.03	2	16	*nt*	32	1	0.06	<4
JV72	18.10.83	023	+	0.5	0.125	0.001	0.008	1	0.5	4	4	0.125	1	16	*nt*	32	2	0.03	<4
JV73	15.11.83	027	+	0.5	1	0.001	0.06	16	2	8	4	0.06	1	>64	*nt*	64	2	0.125	>256
JV74	21.10.82	010	-	0.5	0.125	0.001	0.03	1	>64	8	16	0.03	2	16	*nt*	16	1	0.125	>256
JV75	21.03.83	061	+	0.5	0.125	0.001	0.008	1	1	8	4	0.03	2	16	*nt*	64	1	0.06	<4
JV76	7.11.80	070	+	0.5	0.25	0.002	0.008	1	0.125	8	4	0.03	2	32	*nt*	16	2	0.125	<4

Table 31: Minimum inhibitory concentrations of 16 antimicrobials against a panel of 75 historical C. difficile isolates (1980-1986).

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. nt – not tested. Red cells indicate resistance, orange cells indicate intermediate resistance based on the U.S. Clinical & Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST); or existing publications. MIC – minimum inhibitory concentration.

PCR Ribotype 015

	Historic UK isolates (*n=16*) (1980-1986)			***Clos*ER Study UK isolates (*n=42*) (2012-2016)**			**Difference**		
	MIC$_{50}$ (mg/L)	**MIC$_{90}$ (mg/L)**	**Geometric Mean MIC (mg/L)**	**MIC$_{50}$ (mg/L)**	**MIC$_{90}$ (mg/L)**	**Geometric Mean MIC (mg/L)**	**MIC$_{50}$ (mg/L)**	**MIC$_{90}$ (mg/L)**	**Geometric Mean MIC (mg/L)**
VAN	0.75	1	0.707	1	1	0.755	0.25	0	0.048
MTZ	0.125	0.25	0.136	0.25	0.5	0.230	0.125	0.25	0.094
RIF	0.001	0.001	0.001	0.002	0.004	0.002	0.001	0.003	0.001
FDX	0.03	0.06	0.030	0.06	0.06	0.045	0.03	0	0.015
MXF	1	2	1.297	2	2	1.614	1	0	0.317
CLI	4	8	2.954	4	17.6	4.579	0	9.6	1.626
IPM	4	8	4.757	4	8	4.416	0	0	-0.340
CHL	4	6.4	4.362	4	8	5.384	0	1.6	1.022
TIG	0.03	0.048	0.034	0.06	0.06	0.052	0.03	0.012	0.018

PCR Ribotype 020

	Historic UK isolates (*n=13*) (1980-1986)			***Clos*ER Study UK isolates (*n=22*) (2012-2016)**			**Difference**		
	MIC$_{50}$ (mg/L)	**MIC$_{90}$ (mg/L)**	**Geometric Mean MIC (mg/L)**	**MIC$_{50}$ (mg/L)**	**MIC$_{90}$ (mg/L)**	**Geometric Mean MIC (mg/L)**	**MIC$_{50}$ (mg/L)**	**MIC$_{90}$ (mg/L)**	**Geometric Mean MIC (mg/L)**
VAN	0.5	0.5	0.474	0.75	1	0.707	0.25	0.5	0.233
MTZ	0.125	0.25	0.154	0.25	0.5	0.322	0.625	0.75	0.553
RIF	0.001	0.002	0.001	0.002	0.004	0.002	0.001	0.002	0.001
FDX	0.06	0.112	0.034	0.06	0.125	0.052	0	0.013	0.018
MXF	1	2	1.306	2	8	2.446	1	6	1.141
CLI	4	14.4	4.219	8	32	10.623	4	17.6	6.404
IPM	4	4	3.232	4	8	4.537	0	4	1.306
CHL	4	8	4.450	8	8	6.025	4	0	1.575
TIG	0.03	0.054	0.033	0.06	0.06	0.055	0.03	0.006	0.021

Table 32: Comparison of antimicrobial susceptibility data by individual PCR ribotype for the two most prevalent types in the historical collection (015 & 020). *VAN-vancomycin, MTZ-metronidazole, RIF-rifampicin, FDX-fidaxomicin, MXF-moxifloxacin, CLI-clindamycin, IPM-imipenem, CHL-chloramphenicol, TGC-tigecycline. Geometric means to 3 d.p. MIC- minimum inhibitory concentration.*

8.3 Supplementary data for genomic resistance determinant identification in historical *C. difficile* isolates (1980-86)

		Genes Identified (% identity)				
		CARD RGI		ResFinder 3.0		PlasmidFinder 1.3
Strain	PCR Ribotype	95-100%	75-94%	95-100%	75-94%	95% threshold
JV01	012	*tetM (100)* *cdeA (100)* *ermB (99.15)*	*vanRG (77.87)*	*tetM (95.68)*	-	-
JV02	027	*cdeA (99.09)*	*vanRG (77.87)*	-	-	-
JV03	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV04	001	*cdeA (99.52)*	*vanRG (77.87)*	-	-	-
JV05	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV06	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV07	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV08	220	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV09	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV10	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV11	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV12	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV13	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV14	078	*cdeA (97.51)* *tetM (90.45)*	-	*tetM (100)*	-	-
JV15	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV16	056	*cdeA (98.87)*	*vanRG (77.87)*	-	-	-
JV17	015	*cdeA (97.51)* *tetM (90.45)*	-	*tetM (100)*	-	-
JV18	103	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV19	137	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV20	041	*vanRG (100)* *vanSG (100)* *vanYG (100)* *vanG (100)* *vanXYG (100)* *vanTG (99.86)* *vanWG (99.64)* *cdeA (99.32)* *vanUG (98.67)*	-	*vanRG (100)* *vanSG (100)* *vanYG (100)* *vanG (100)* *vanXYG (100)* *vanTG (99.95)* *vanWG (99.88)* *vanUG (99.56)*	-	-
JV22	078	*cdeA (97.51)* *tetM (90.45)*	-	*tetM (100)*	-	-
JV23	001	*vanRG (100)* *vanSG (100)* *vanYG (100)* *vanG (100)* *vanXYG (100)* *vanTG (99.86)* *vanWG (99.64)* *cdeA (99.32)* *vanUG (98.67)*	-	*vanRG (100)* *vanSG (100)* *vanYG (100)* *vanG (100)* *vanXYG (100)* *vanTG (99.95)* *vanWG (99.88)* *vanUG (99.56)*	-	-

		Genes Identified (% identity)				
		CARD RGI		ResFinder 3.0		PlasmidFinder 1.3
Strain	PCR Ribotype	95-100%	75-94%	95-100%	75-94%	95% threshold
JV24	200	*vanRG (100)* *vanSG (100)* *vanYG (100)* *vanG (100)* *vanXYG (100)* *vanWG (100)* *vanTG (99.86)* *vanUG (98.67)* *cdeA (98.86)*	-	*vanRG (100)* *vanSG (100)* *vanYG (100)* *vanG (100)* *vanXYG (100)* *vanTG (99.95)* *vanWG (99.88)* *vanUG (99.56)*	-	-
JV25	014	*cdeA (99.77)* *ermB (98.37)*	*vanRG (77.87)*	*ermB (100)*	-	-
JV26	015	-	*vanRG (77.87)*	-	-	-
JV27	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV28	001	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV29	002	*cdeA (99.09)*	*vanRG (77.87)*	-	-	-
JV30	012	*cdeA (100)* *tetM (100)* *ermB (97.55)*	*vanRG (77.87)*	*ermB (100)* *tetM (95.68)*	-	-
JV31	002	*cdeA (99.09)*	*vanRG (77.87)*	-	-	-
JV32	012	*cdeA (100)* *tetM (100)* *tetA(P) (98.28)*	*vanRG (77.87)*	*tetA(P) (98.81)* *tetM (95.68)*	-	-
JV33	014	*cdeA (99.77)* *ermB (98.37)*	*vanRG (77.87)*	*ermB (100)*	-	-
JV34	001	*vanRG (100)* *vanSG (100)* *vanYG (100)* *vanG (100)* *vanXYG (100)* *vanTG (99.86)* *cdeA (99.32)* *vanUG (98.67)*	-	*vanRG (100)* *vanSG (100)* *vanYG (100)* *vanG (100)* *vanXYG (100)* *vanTG (99.95)* *vanWG (99.88)* *vanUG (99.56)*	-	-
JV35	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV36	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV37	001	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV38	626	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV39	341	*vanYG (98.82)*	*cdeA (78.05)* *vanRG (77.87)*	-	-	-
JV40	014	*cdeA (99.77)* *ermB (98.37)*	*vanRG (77.87)*	*ermB (100)*	-	-
JV41	002	*cdeA (99.09)*	*vanRG (77.87)*	-	-	-
JV42	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV43	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV44	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV45	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV46	061	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV47	012	*cdeA (100)* *tetM (100)*	*vanRG (77.87)*	*tetM (95.68)*	-	-
JV48	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV49	032	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-

		Genes Identified (% identity)				
		CARD RGI		ResFinder 3.0		PlasmidFinder 1.3
Strain	PCR Ribotype	95-100%	75-94%	95-100%	75-94%	95% threshold
JV50	017	*cdeA (98.41)*	*vanSG (82.72)*	-	-	-
JV51	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV52	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV53	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV54	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV55	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV56	017	*cdeA (98.41)*	*vanSD (86.76)*	-	-	-
JV57	200	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV58	017	*cdeA (98.41)*	-	-	-	-
JV59	862	*cdeA (93.65)*	-	-	-	-
JV60	619	*cdeA (100)* *Streptomyces cinnamoneus EF-Tu mutant (80.58)*	*vanRG (77.87)*	-	-	-
JV61	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV62	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV63	001	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV64	020	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV65	033	*cdeA (97.51)*	-	-	-	-
JV66	015	*cdeA (99.32)*	*vanRG (77.87)*	-	-	-
JV67	027	*cdeA (99.09)*	*vanRG (77.87)*	-	-	-
JV68	242	*cdeA (98.64)*	-	-	-	-
JV69	014	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV70	003	*cdeA (99.55)*	*vanRG (77.87)*	-	-	-
JV71	070	*cdeA (99.55)*	*vanRG (77.87)*	-	-	-
JV72	023	*cdeA (99.09)*	-	-	-	-
JV73	027	*AAC(6')-Ie-APH(2'')-Ia (99.79)* *APH(2'')-If (87.56)* *cdeA (99.09)*	-	*aac(6')-aph(2'') (99.41)* *aph(2'')-Ih (94.41)* *aadE (100)* *ant(6)-Ia (100)*	-	-
JV74	010	*cdeA (100)* *ermB (98.78)*	*vanRG (77.87)*	*aadE (100)* *ermB (100)*	-	-
JV75	061	*cdeA (99.77)*	*vanRG (77.87)*	-	-	-
JV76	070	*cdeA (99.55)*	*vanRG (77.87)*	-	-	-

Table 33: Comparison of resistance determinants identified by CARD RGI - Comprehensive Antibiotic Research Database – Resistance Gene Identifier, ResFinder 3.0 and PlasmidFinder 1.3.

The cfr gene was not detected by either of the algorithms, only by BLAST comparisons.

Isolate	LZD MIC (mg/L)	Non-synonymous substitution	
		L3 ribosomal protein	**L4 ribosomal protein**
JV14	16	-	Gly71 >Asp, Val163>Ile
JV17	16	-	Gly71 >Asp, Val163>Ile
JV22	16	-	Gly71 >Asp, Val163>Ile
JV59	1	Ile205 >Val	Lys98 >Arg
JV65	2	-	Val163 >Ile

Table 34: List of non-synonymous substitutions in the historical C. difficile collection (1980-86).

LZD – linezolid, MIC – minimum inhibitory concentration. No other non-synonymous mutations were identified in the other historical strains.

Isolate	ERY/CLI Susceptibility Phenotype (R or S)	Non-synonymous substitutions in *ermB* gene*
JV01	R/R	None
JV25	R/R	Ile75>Thr, Arg118>His, Lys152>Gln, Ala191>Val, Tyr217>His, Asp242>Asn
JV30	S/R	None
JV33	S/S	Ile75>Thr, Arg118>His, Lys152>Gln, Ala191>Val, Tyr217>His, Asp242>Asn
JV40	S/S	Ile75>Thr, Arg118>His, Lys152>Gln, Ala191>Val, Tyr217>His, Asp242>Asn
JV74	R/R	Arg118>His, Lys152>Gln, Ala191>Val, Tyr217>His, Asp242>Asn

Table 35: List of non-synonymous substitutions in the ermB gene of six historical C. difficile genomes (1980-86).

** compared to the CD630 gene sequence (Sebaihia et al. (2006)). R - resistant, S - sensitive, based on breakpoints defined by Clinical & Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST); or existing publications.*

Isolate	Assembly size (bp)	Number of Contigs	N50	L50
JV01	4,294,084	339	159,199	10
JV02	4,323,376	326	119,501	12
JV03	4,411,449	236	134,981	10
JV04	4,201,848	301	199,826	8
JV05	4,220,432	361	108,331	14
JV06	4,190,182	257	136,281	10
JV07	4,420,362	240	143,270	10
JV08	4,109,750	348	83,868	14
JV09	4,412,611	244	127,345	11
JV10	4,416,385	237	149,170	9
JV11	4,419,205	250	135,359	10
JV12	4,180,700	254	94,622	13
JV13	4,242,652	448	122,477	13
JV14	4,558,737	1290	108,761	15
JV15	4,173,684	208	133,545	10
JV16	4,186,391	386	118,189	11
JV17	4,498,895	1160	99,064	15
JV18	4,125,058	279	173,858	9
JV19	4,134,833	260	165,737	10
JV20	4,278,197	224	134,691	12
JV22	4,047,675	301	108,712	13
JV23	4,337,571	385	152,058	10
JV24	5,911,314	5177	1,540	764
JV25	4,257,057	240	133,619	11
JV26	4,414,022	280	127,224	11
JV27	4,161,753	178	123,869	12
JV28	4,143,686	162	138,307	9
JV29	4,229,942	189	135,424	11
JV30	4,303,203	238	147,813	7
JV31	4,327,345	448	149,725	10
JV32	4,316,338	414	185,750	8
JV33	4,249,241	214	108,373	12
JV34	4,292,483	230	138,336	10
JV35	4,187,322	270	149,150	10
JV36	4,388,508	302	125,447	12
JV37	4,387,631	314	143,692	10
JV38	4,439,936	319	129,717	12
JV39	5,081,435	2569	3,763	302
JV40	4,273,282	295	95,990	13
JV41	4,323,529	194	149,777	8
JV42	4,417,284	256	127,297	11
JV43	4,681,326	695	23,533	60
JV44	4,176,809	236	122,446	12

Isolate	Assembly size (bp)	Number of Contigs	N50	L50
JV45	4,421,752	276	127,319	11
JV46	4,236,135	324	144,291	10
JV47	4,360,673	500	139,604	10
JV48	4,330,455	243	142,950	11
JV49	4,458,558	400	138,336	8
JV50	4,313,082	530	131,864	11
JV51	4,393,504	434	137,734	10
JV52	4,467,179	636	137,796	9
JV53	4,468,119	663	142,998	11
JV54	4,528,290	608	127,237	10
JV55	4,411,271	508	165,844	9
JV56	4,411,134	946	156,199	10
JV57	4,345,760	303	132,351	11
JV58	4,238,422	473	109,353	14
JV59	4,742,161	391	201,523	8
JV60	4,356,689	499	137,783	9
JV61	4,501,974	730	127,248	12
JV62	4,451,252	633	132,209	11
JV63	4,870,482	473	117,650	13
JV64	4,437,542	423	108,851	13
JV65	4,073,858	380	94,693	14
JV66	4,314,602	208	115,267	14
JV67	4,189,156	240	112,951	12
JV68	4,224,388	344	104,221	15
JV69	4,263,571	327	135,900	10
JV70	4,303,203	238	213,813	7
JV71	4,301,305	837	135,360	9
JV72	4,576,657	699	123,632	13
JV73	4,377,357	750	17,627	78
JV74	4,283,808	495	135,099	9
JV75	4,250,749	337	144,291	10
JV76	4,287,172	671	157,051	10

Table 36: Assembly statistics for historical C. difficile genomes (1980-86).

N50 refers to the length of the contiguous sequence representing the 50th percentile of the genome length, after size ordering. L50 represents the number of contigs making up top 50th percentile of the genome.

Test Isolate	*aadE* gene status	Streptomycin MIC (mg/L)
JV02	-	<256
JV19	-	<256
JV20	-	<256
JV22	-	512
JV24	-	<256
JV34	-	<256
JV47	-	>2048
JV50	-	512
JV67	-	512
JV73	+	>2048
JV74	+	>2048

Table 37: Streptomycin minimum inhibitory concentrations (MICs) of two C. difficile isolates with the aadE gene and nine comparator isolates without.

8.4 Supplementary data for moxifloxacin mutability testing

Strain Identifier	PCR Ribotype	Study Origin	Country of Origin	Date of Isolation	Original MXF MIC (mg/L)
CD4032	001	*Clos*ER	UK	2015	1
CD3293	001	*Clos*ER	UK	2014	1
CD4067	001	*Clos*ER	UK	2015	2
JV28	001	JV	UK	1982	1
JV37	001	JV	UK	1983	2
CD0160	001	*Clos*ER	Czech Rep.	2016	1
JV01	012	JV	UK	1981	2
JV30	012	JV	UK	1982	2
JV32	012	JV	UK	1982	2
CD0079	012	*Clos*ER	UK	2014	1
CD0127	012	*Clos*ER	France	2016	1
CD0099	012	*Clos*ER	France	2016	1
JV03	015	JV	UK	1982	1
JV07	015	JV	UK	1982	1
JV09	015	JV	UK	1981	1
JV10	015	JV	UK	1982	1
JV11	015	JV	UK	1982	2
JV26	015	JV	UK	1981	1
JV05	020	JV	UK	1982	1
JV06	020	JV	UK	1982	1
JV27	020	JV	UK	1981	1
JV12	020	JV	UK	1982	2
JV13	020	JV	UK	1982	2
JV15	020	JV	UK	1983	2
CD0071	017	*Clos*ER	Ireland	2016	2
CD0095	017	*Clos*ER	UK	2015	2
CD4500	017	*Clos*ER	Spain	2015	2
CD4511	017	*Clos*ER	Poland	2015	2
CD3757	017	*Clos*ER	France	2015	2
CD3771	017	*Clos*ER	France	2015	2
CD3904	027	*Clos*ER	Sweden	2015	1
CD3891	027	*Clos*ER	Sweden	2015	2
CD9609	027	*Clos*ER	France	2016	1
CD9946	027	*Clos*ER	UK	2016	1
CD3809	027	*Clos*ER	Rep. Ireland	2012	1
CD3051	027	*Clos*ER	Austria	2013	1
CD3079	027	*Clos*ER	Sweden	2013	1
CD4362	027	*Clos*ER	France	2015	32

Strain Identifier	PCR Ribotype	Study Origin	Country of Origin	Date of Isolation	Original MXF MIC (mg/L)
CD4706	078	*Clos*ER	UK	2015	1
CD3998	078	*Clos*ER	UK	2015	1
CD3994	078	*Clos*ER	UK	2015	1
CD4112	078	*Clos*ER	UK	2015	2
CD0222	078	*Clos*ER	Germany	2016	1
CD0223	078	*Clos*ER	Germany	2016	1

Table 38: Demographic data for all isolates tested during mutation frequency investigations.

JV prefix and study origin refer to isolates recovered from the historical catalogue investigated in this thesis. MXF – moxifloxacin, MIC – minimum inhibitory concentration.

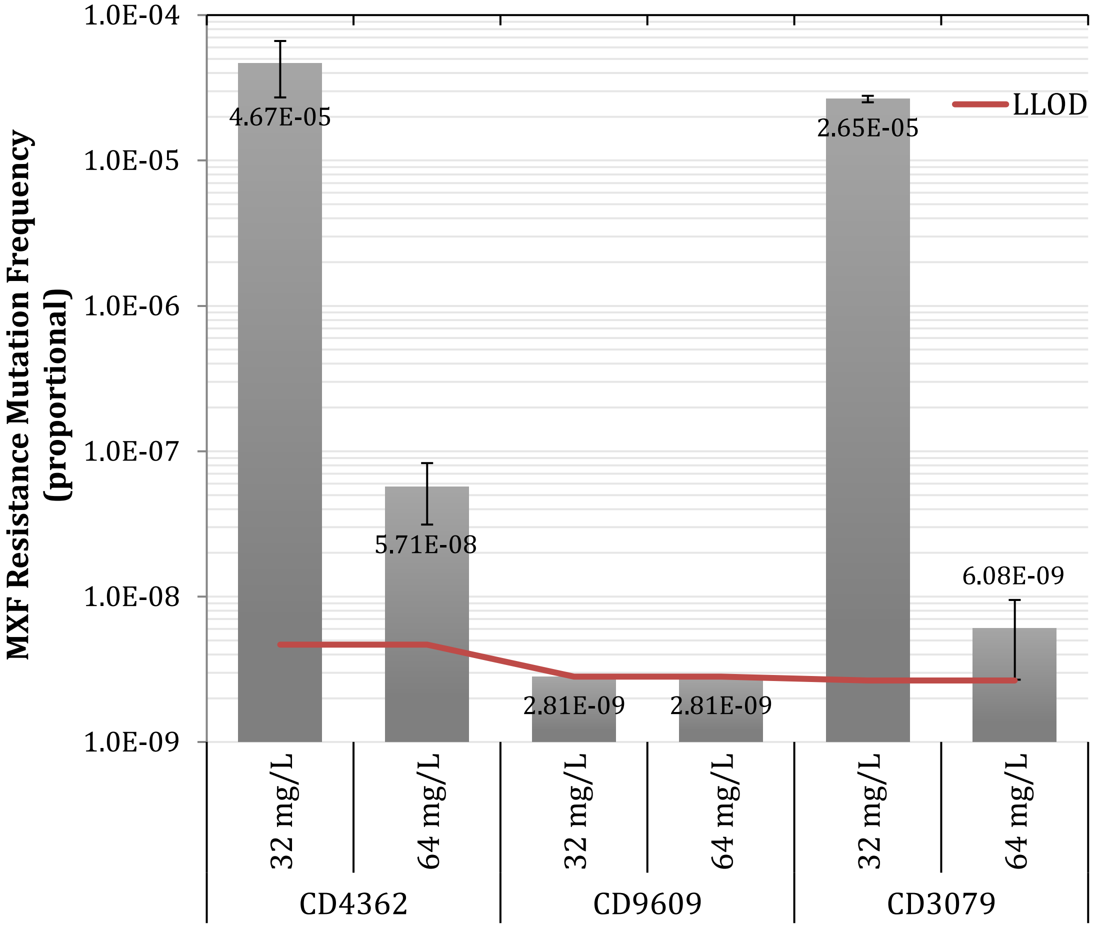

Figure 55: Mean (±SE) mutation frequencies of two PCR ribotype 027 mutants and one resistant isolate (32 mg/L MXF MIC) under 32 and 64 mg/L moxifloxacin (MXF) pressure.

Lower limit of detection (LLOD) calculated as 1 / (mean total viable counts/mL). No error bars are displayed where frequency was below the LLOD.

Parent Isolate	Parent MXF MIC (mg/L)	Mutant Strain Identifier	Mutant MXF MIC (mg/L)	*gyrA* substitution	*gyrB* substitution
CD3051	1	CD3051 Step Mut	4	Asp71>Val	-
CD3079	1	CD3079 Step Mut	4	Asp71>Val	-
CD4362	32	CD4362 Step Mut	128	Thr82>Ile, Ala118>Ser	-

Table 39: Amino acid substitutions in DNA gyrase genes from additional moxifloxacin (MXF) resistant C. difficile isolates.

8.5 Supplementary data for variant detection using CLC genomics workbench

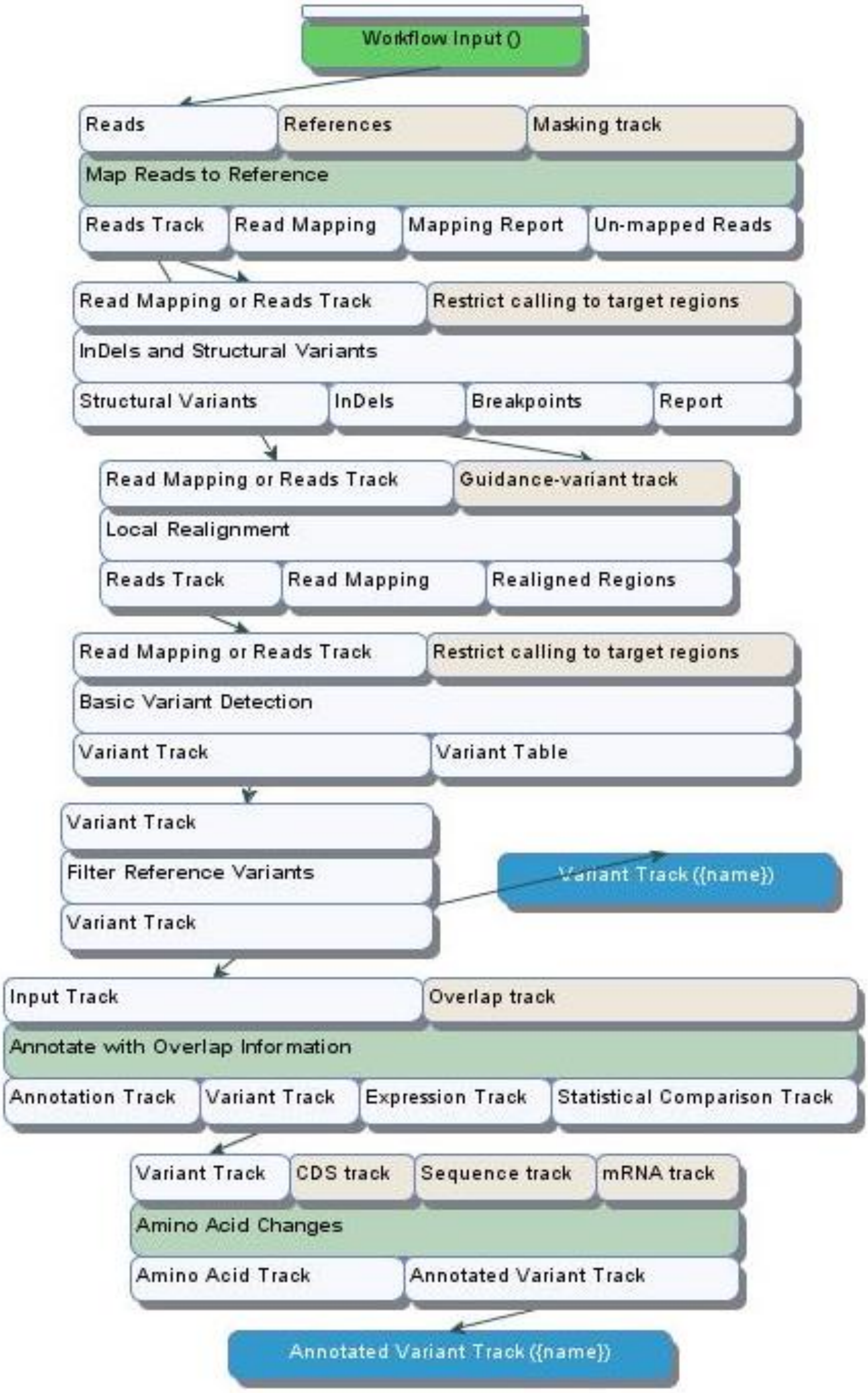

Figure 56: CLC genomics workbench workflow for variant detection and amino acid substitutions. *Parameters were set a default for all processes.*

8.6 Supplementary data for Sanger sequence alignment

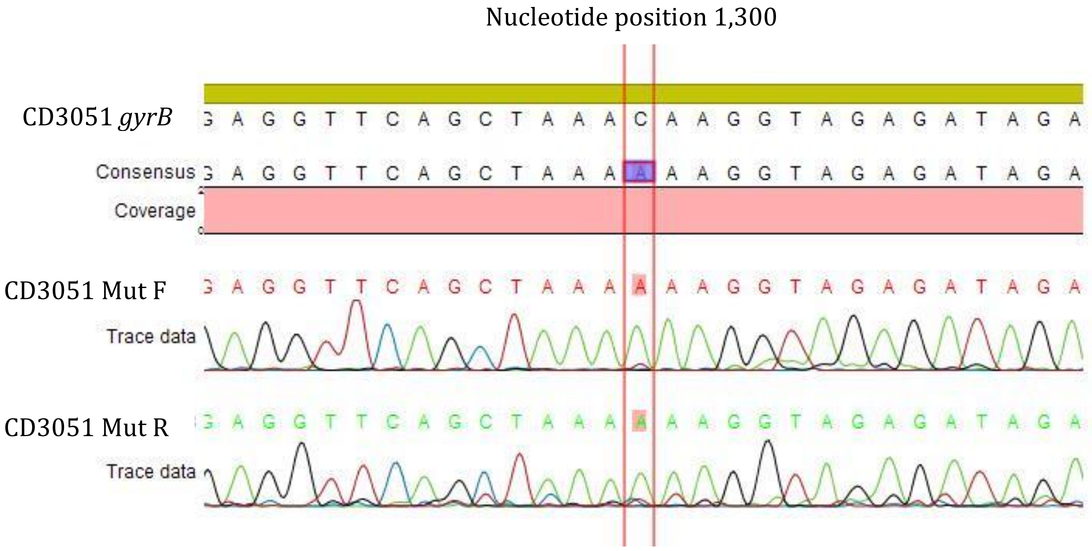

Figure 57: Example chromatogram confirming CD3051 Mut gyrB substitution Gln434>Lys (CAA>AAA) using CLC Genomics Workbench and Sanger sequence trace data to ensure correct nucleotide calling. *F –forward, R – reverse sequences.*

8.7 Supplementary data of *R* programming code for the collation of gene sequences from RAST output *.xls* files into a FASTA text format

This program was written as part of a Biotechnology and Biological Sciences Research Council (BBSRC) - Strategic Training Awards for Research Skills (STARS), Next Generation Biologists 'Essential computing skills for molecular biology' course, with some assistance from the tutors. This simple program in the *R* programming language collates individual DNA sequences of specific genes from RAST annotation output files and prefaces them with “> ” as required for the FASTA file format. One text file is generated with defined gene sequences for all genomes. These output files were used in conjunction with Clustal Omega for multiple sequence alignment and SNP identification. Lines of code beginning with a “#” are in-code comments to assist in the comprehension of the coding.

```
AllFiles <- Sys.glob("C:/Users/Jon/Desktop/All_RAST_CSV_files/JV*.csv")
# Defines file location and accesses all .csv files prefaced with JV under “AllFiles” variable
geneOfInterest<-"DNA gyrase subunit A"
# Defines a variable for gene name that matches the RAST output.
extractGeneFasta<-function(filename, geneOfInterest){
# Defines a function called extractGeneFasta with two inputs
 data<-read.csv(filename, stringsAsFactors = FALSE)
# Import data (not as factor class (default for strings), i.e.  import as character class)
  row<-grep(geneOfInterest, data$"function", fixed = TRUE)
# Extract the row number for the defined gene. Dollar symbol means give a column from "data" file
  sequence<-data[row,"nucleotide_sequence"]
# Extract the sequence
    if (length (sequence)!=1)stop("did not find single sequence")
# This line generates an error in 0 or >1 sequence is found
   label<-sprintf("> %s",filename)
   # Prefaces sequence with label (> ). "%s" is a space holder for further text
  names (sequence)<-label
 return(sequence) }
# return sequence with label concatenation
for (filename in AllFiles){
# Acquire output, with sequences appended after each iteration of the loop.
   result<-extractGeneFasta(filename, geneOfInterest)
   printResult<- paste(names(result), result[1], sep = "\n")
   write(printResult, "genefasta.txt",append=TRUE)
```

8.8 Supplementary data for phylogenetic analyses

Isolate	PCR ribotype	Isolation Date	Isolation Date of Nearest Neighbour	SNPs to Nearest Neighbour	Prediction Interval (ppois)
JV04	001	1982	2013	25.3	0.69
JV23	001	1981	2013	12.7	0.01
JV28	001	1982	2013	25.3	0.69
JV34	001	1981	2013	12.7	0.01
JV37	001	1983	2013	6696.3	1.00
JV63	001	1983	2013	6229.4	1.00
JV29	002	1981	2013	12.9	0.01
JV31	002	1981	2013	12.9	0.01
JV41	002	1983	2013	14.9	0.04
JV25	014	1981	2013	18.2	0.13
JV33	014	1982	2013	18.2	0.16
JV40	014	1983	2013	18.2	0.20
JV69	014	1981	2013	14.5	0.02
JV03	015	1982	2013	20.6	0.29
JV07	015	1982	2013	20.6	0.29
JV09	015	1981	2013	19.6	0.18
JV10	015	1982	2013	20.6	0.29
JV11	015	1982	2013	20.6	0.29
JV26	015	1981	2013	20.6	0.24
JV36	015	1983	2013	13.7	0.02
JV42	015	1982	2013	19.6	0.22
JV45	015	1983	2013	19.6	0.27
JV54	015	1983	2013	19.6	0.27
JV55	015	1982	2013	21.5	0.37
JV61	015	1983	2013	4.9	2.17×10^{-06}
JV62	015	1983	2013	4.9	2.00×10^{-06}
JV66	015	1982	2013	4.9	1.00×10^{-06}
JV05	020	1982	2013	91.7	1.00
JV06	020	1982	2013	91.7	1.00
JV12	020	1982	2013	92.7	1.00
JV13	020	1982	2013	92.7	1.00
JV15	020	1983	2013	91.7	1.00
JV27	020	1981	2013	91.7	1.00
JV35	020	1983	2013	23.5	0.60
JV44	020	1983	2013	92.7	1.00

Isolate	PCR ribotype	Isolation Date	Isolation Date of Nearest Neighbour	SNPs to Nearest Neighbour	Prediction Interval (ppois)
JV48	020	1980	2013	27.8	0.72
JV51	020	1981	2013	27.8	0.77
JV52	020	1981	2013	27.8	0.77
JV53	020	1981	2013	27.8	0.77
JV64	020	1983	2013	37.7	1.00
JV02	027	1981	2008	13.1	0.06
JV67	027	1986	2006	2	3.93×10^{-05}
JV73	027	1983	2008	8	0.01
JV14	078	1982	2013	5.8	5.69×10^{-06}
JV22	078	1981	2013	5.8	3.13×10^{-06}

Table 40: Comparison of single nucleotide polymorphism (SNP) differences between historical isolates analysed phylogenetically and prediction intervals based on Poisson distributions of previous C. difficile molecular clock estimations (0.74); (Eyre et al. 2013).

8.9 Supplementary data for continuous competitive co-culture modelling of bacterial fitness

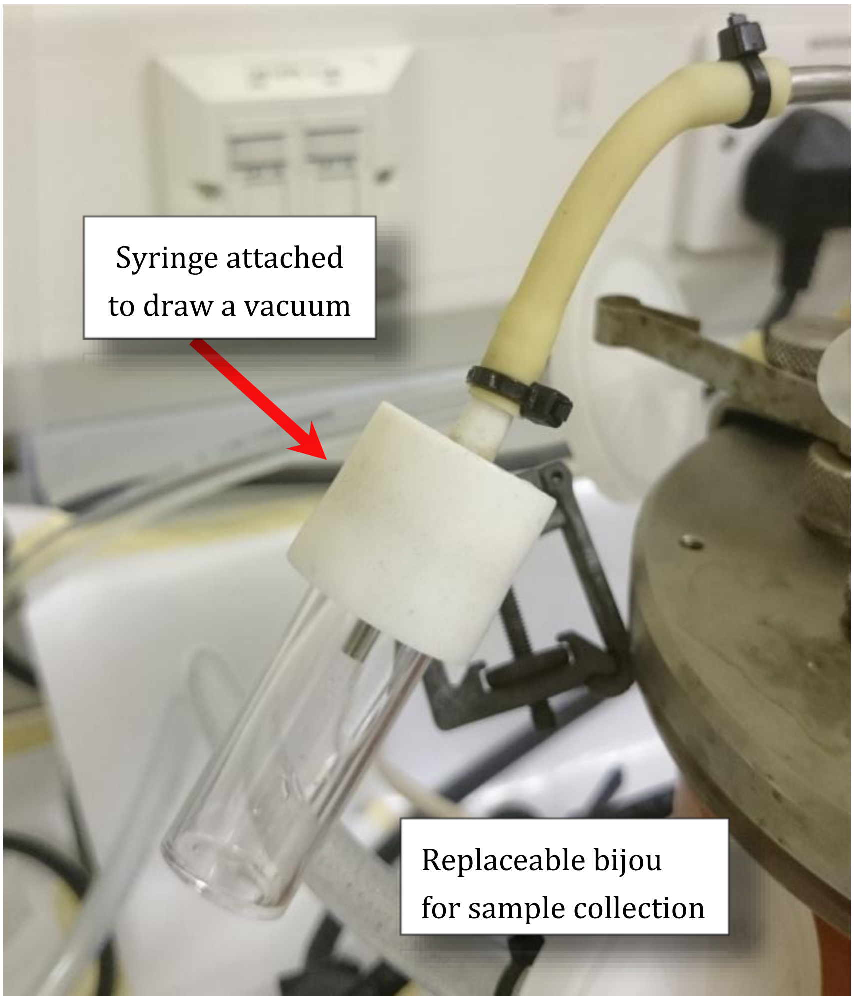

Figure 58: Continuous culture chemostat model sample port configuration.

A syringe draws a vacuum, pulling culture into the sample bijou. The tubing was clamped and the bijou replaced. Twenty millilitres of sample was run off before sample collection, to account for culture accumulation in the tubing.

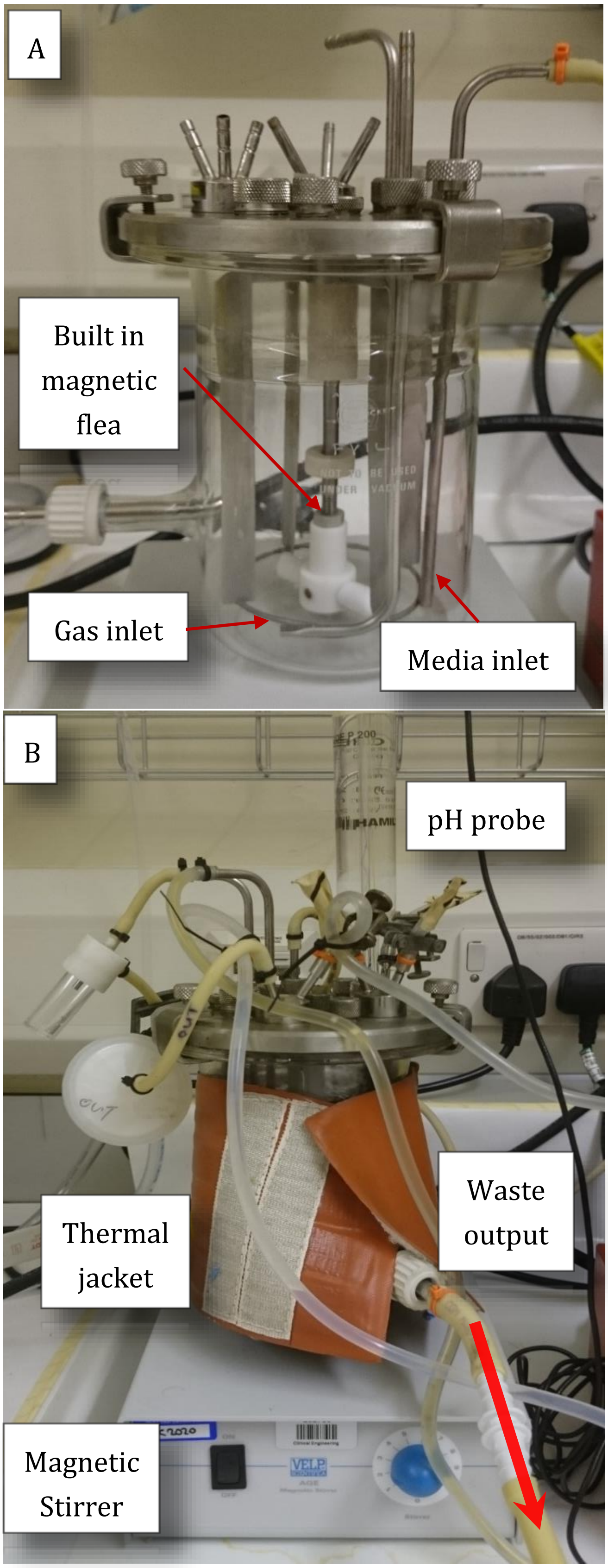

Figure 59: Continuous co-culture vessel configuration. *A – Internal configuration of chemostat, prior to experimental use. B – Visualisation of the fully connected, operative chemostat with thermal jacket.*

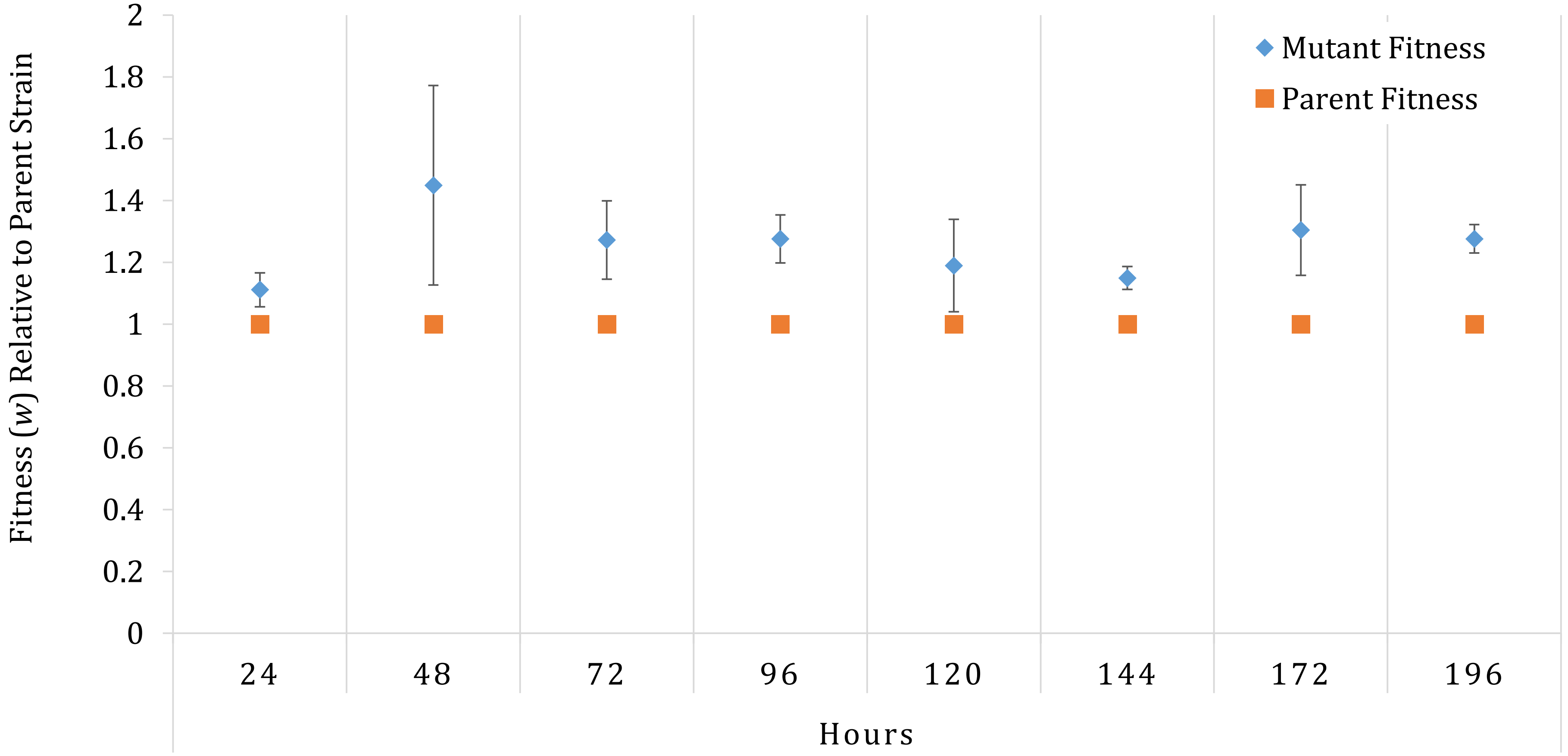

Figure 60: Relative fitness of moxifloxacin (MXF) resistant ribotype 027 C. difficile mutant compared to sensitive parent strain, tracked over the continuous culture model duration.

Parent fitness was set at a baseline of w=1. Data represents the mean averages (±SE) of three independent chemostat models.

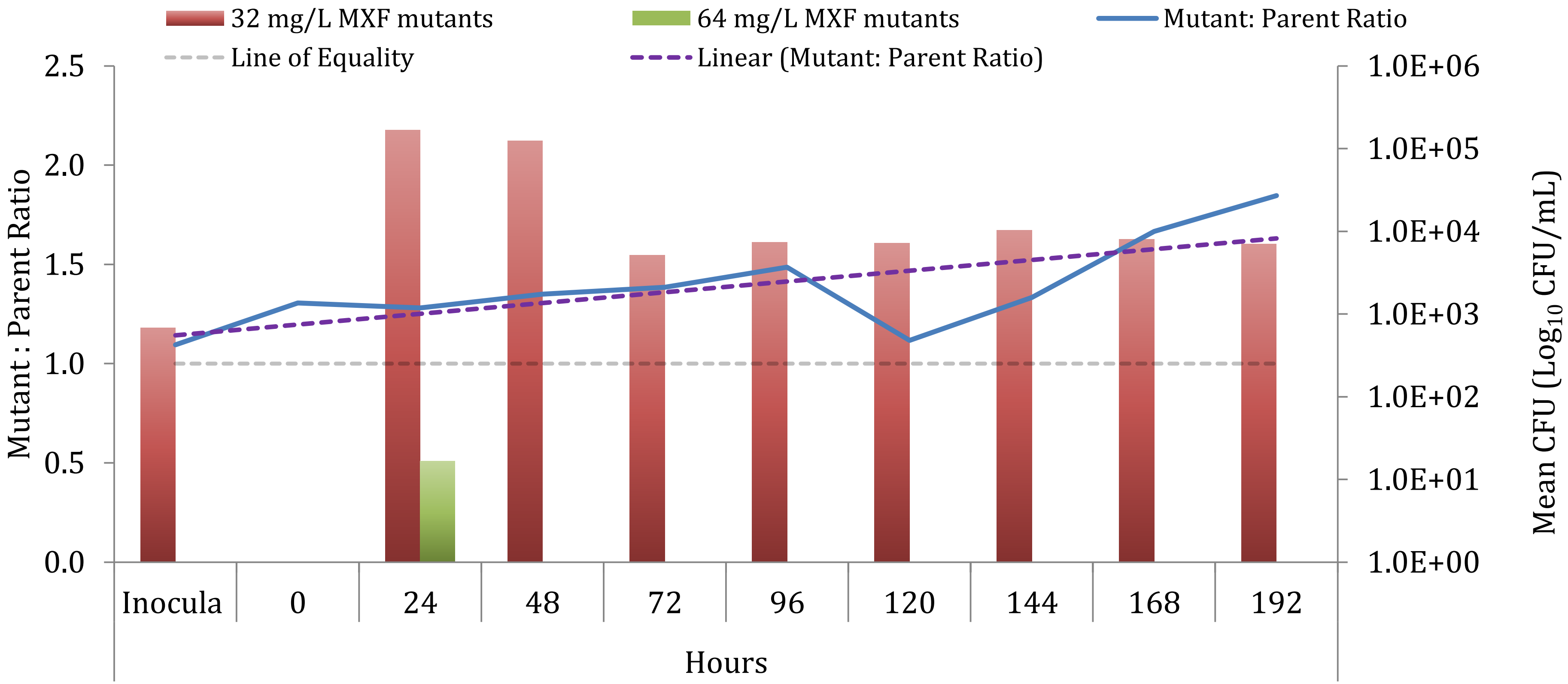

Figure 61: Progression of mutant and parent ribotype 027 C. difficile ratios in a continuous co-culture chemostat model – Run 1.

The linear trend line (purple) demonstrates the increasing divergence between mutant and parent strain populations. Highly resistant populations >32 mg/L and >64 mg/L moxifloxacin (MXF) were documented to track further resistance development. The line of equality represents the baseline of equivalent mutant and parent strain proportions, data above this signifies a fitness advantage for the mutant strain. Data represents mean averages (±SE) of triplicate plate counts.

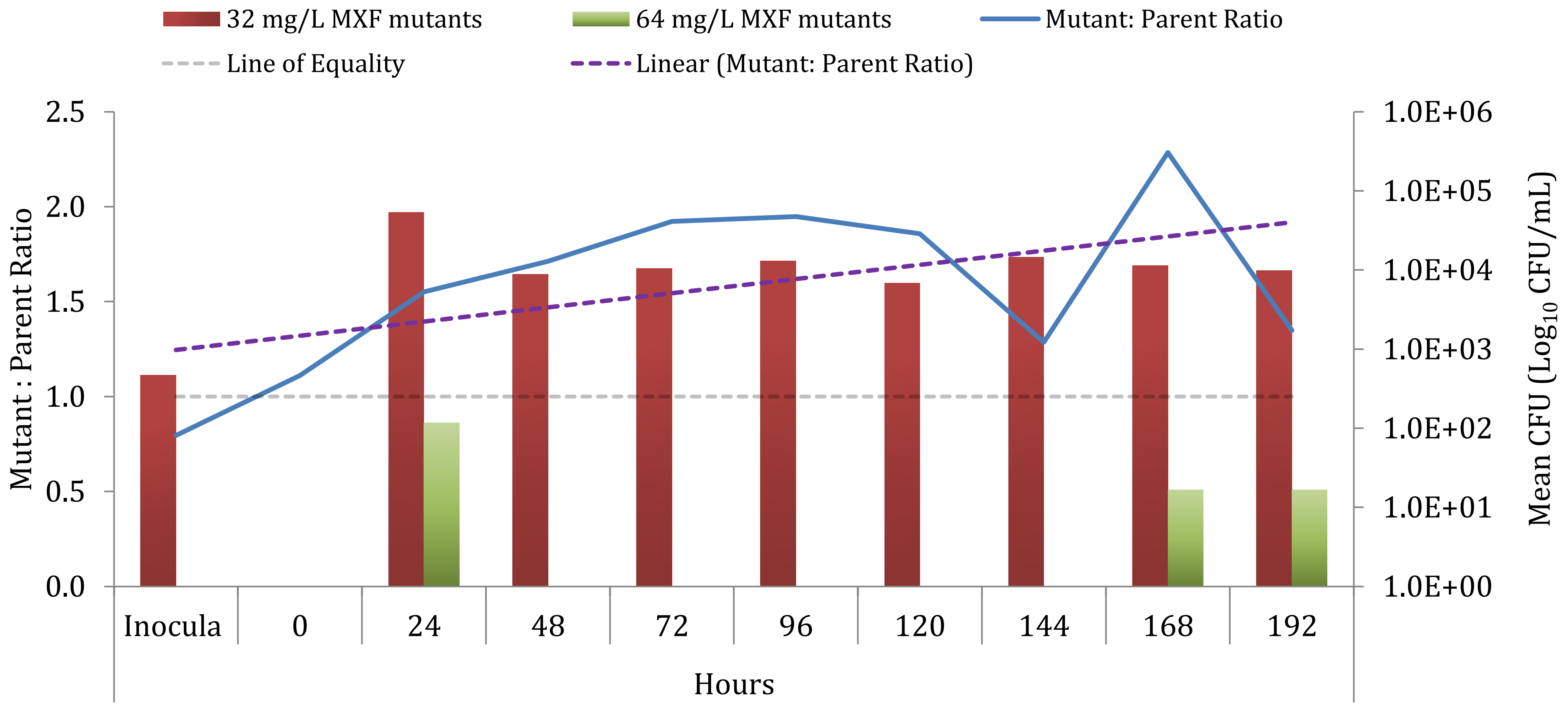

Figure 62: Progression of mutant and parent ribotype 027 C. difficile ratios in a continuous co-culture chemostat model – Run 2.

The linear trend line (purple) demonstrates the increasing divergence between mutant and parent strain populations. Highly resistant populations >32 mg/L and >64 mg/L moxifloxacin (MXF) were documented to track further resistance development. The line of equality represents the baseline of equivalent mutant and parent strain proportions, data above this signifies a fitness advantage for the mutant strain. Data represents mean averages (±SE) of triplicate plate counts.

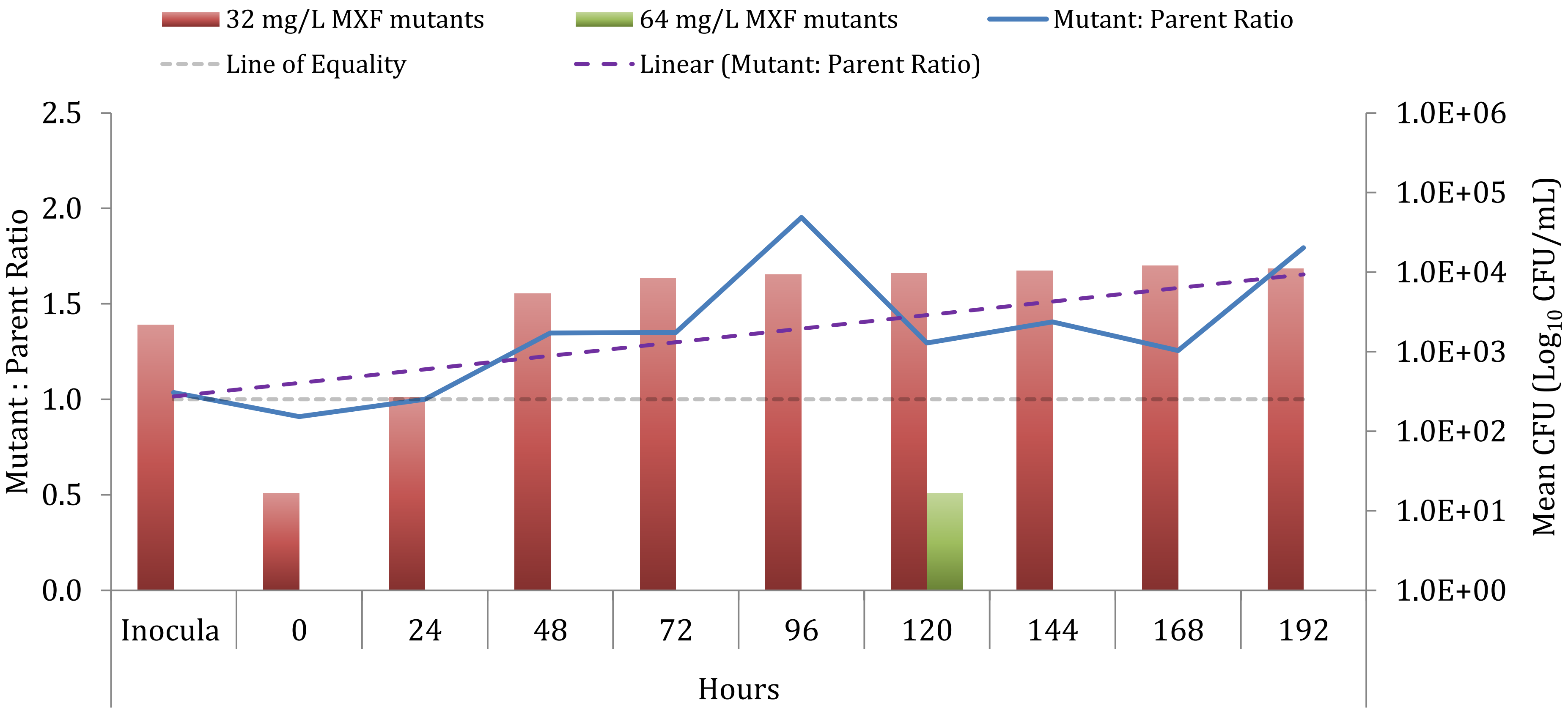

Figure 63: Progression of mutant and parent ribotype 027 C. difficile ratios in a continuous co-culture chemostat model – Run 3.

The linear trend line (purple) demonstrates the increasing divergence between mutant and parent strain populations. Highly resistant populations >32 mg/L and >64 mg/L moxifloxacin (MXF) were documented to track further resistance development. The line of equality represents the baseline of equivalent mutant and parent strain proportions, data above this signifies a fitness advantage for the mutant strain. Data represents mean averages (±SE) of triplicate plate counts.

Model Replicate	Experimental Time point (Hours)	MXF plate concentration (mg/L)	MXF MIC (mg/L)
1	24	64	128
2	24	64	128
2	24	64	128
2	168	64	128
2	192	64	128
3	120	64	128
1	48	32	64
1	144	32	64
2	48	32	64
2	192	32	64
3	48	32	64
3	144	32	64

Table 41: Moxifloxacin (MXF) minimum inhibitory concentrations (MICs) of colonies cultured on MXF breakpoint plates from the continuous co-culture experiments.

8.10 List of Suppliers

Acros Organics, Fisher Scientific, Bishop Meadows Road, LE115RG, Loughborough, Leicestershire, UK

Agilent Technologies, 5301 Stevens Creek Blvd, Santa Clara, CA 95051, USA

Alfa Aesar, Shore Rd, Port of Heysham Industrial Park, Heysham LA3 2XY, UK

Anglicon, Brighton-Systems, Unit K, Quarry Road Industrial Estate, Newhaven, East Sussex, BN9 9DG, UK

Applied Biosystems Inc, 850 Lincoln Centre Drive Foster City, CA 94404. USA

Applied-Maths, Inc. 11940 Jollyville Rd., Suite 115N, Austin, TX 78759, USA

Astellas Pharma Europe Ltd, 2000 Hillswood Dr, Lyne, Chertsey KT16 0RS, UK

Axygen, Corning Inc. One Riverfront Plaza, Corning, NY 14831, USA

BMG Labtech Ltd, 8 Bell Business Park, Aylesbury HP19 8JR, UK

BriskHeat Corporation, 4800 Hilton Corporate Dr, Columbus, OH 43232, USA

Bruker Daltonik GmbH Life Sciences, Fahrenheitstr. 4, D-28359 Bremen, Germany

Cayman Chemical Company, 1180 E Ellsworth Rd, Ann Arbor, MI 48108, USA

Covaris, Inc. 14 Gill St unit h, Woburn, MA 01801, USA

Denley Hydraulics Ltd, Spen Vale Street, Heckmondwike WF16 0NQ, UK

Don Whitley Scientific, Victoria Works, Victoria St, Bingley BD16 2NH, UK

E&O Laboratories Ltd, Burnhouse, Bonnybridge FK4 2HH, UK

Fluka Biochemika, Fluka Chemie AG, Industriestrasse 25, CH-9470 Buchs/Switzerland

Gibco, Life Technologies, 8717 Grovemont Cir, Gaithersburg, MD 20877, USA

Grant–Bio, Grant Instruments, 29 Station Road, Shepreth, Cambridge, SG8 6GB, UK

Hamilton Laboratory Solutions, 825 E Albert Dr, Manitowoc, WI 54220, USA

Holten, Thermo Fisher Scientific, 168 Third Avenue Waltham, MA 02451, USA

Illumina, Inc. 5200 Illumina Way, San Diego, CA 92122, USA

LabM Ltd, Moss Hall Rd, Heywood, Bury BL9 7JJ, UK

Leica DM IL, 35578 Wetzlar, Germany

Merck Sharp & Dohme Ltd, Hertford Road, Hoddesdon, Hertfordshire, EN11 9BU, UK

metabion international AG, semmelweisstrasse 3, 82152 planegg/steinkirchen, Germany

MP Biochemicals, 3 Hutton Centre Dr #100, Santa Ana, CA 92707, USA

New England Biolabs, 240 County Rd, Ipswich, MA 01938, USA

Oxoid Ltd, Wade Rd, Basingstoke RG24 8PW, UK

Panasonic, Tokyo, Shinagawa, Higashishinagawa, 1 Chome−39−9, Japan

Parker Balston, Parker Hannifin Corporation, Gas Separation and Filtration Division, 4087 Walden Ave. Lancaster, NY 14086, USA

Pfizer, Tadworth KT20 7NT, UK

Qiagen, Qiagen Str. 1, 40724 Hilden, Germany

Sandoz International GmbH, Industriestrasse 25, 83607 Holzkirchen, Germany

Santa Cruz Biotechnology, Inc. 10410 Finnell Street, Dallas, Texas 75220, USA

Sigma-Aldrich Corp., St. Louis, MO, USA

SYNkinase, Manning Bdg, 381 Royal Parade, Parkville VIC 3052, Australia

Tecan Group AG, Seestrasse 103, 8708 Männedorf, Switzerland

Thermo Fisher Scientific,168 Third Avenue Waltham, MA USA 02451

VELP Scientifica, Via Stazione, 16, 20865 Usmate Velate MB, Italy

Watson-Marlow Ltd. Bickland Water Road, Falmouth, Cornwall, United Kingdom TR11 4RU, UK