CHARACTERISATION OF CLINICAL *CLOSTRIDIUM DIFFICILE* PCR RIBOTYPE 002 ISOLATES FROM DIFFERENT TIME LINEAGES

By

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DECLARATION OF CONTENT

I, Iye Linda Ameh confirm that thesis is the result of my work except where specifically stated. Where Information has been derived from other sources, I confirm that this has been indicated in this thesis.

Iye Linda Ameh Date

ABSTRACT

Clostridium difficile (*C. difficile*) is a leading cause of healthcare-associated infections (HAIs) and an important public health threat. Recently, the prevalence *C. difficile* PCR ribotype 002 in the UK has been noted, yet the drivers for this increased prevalence remain unclear. The aim of this study was to characterise *C. difficile* PCR ribotype 002 (CD002) isolates from different time lineages and assess any phenotypic and genotypic traits that may help explain the emergence of this ribotype.

A total of 60 clinical isolates of CD002 (isolated between 2007-2014 in the UK and across Europe) were used in this study. Antimicrobial susceptibilities to a range of antimicrobial agents were assessed using the agar dilution method. Maximum specific growth rate (μ_{max}) was measured by batch culture growth curves, and cytotoxin production ($\log_{10}$ relative units (RU)) was evaluated in a Vero cell cytotoxicity assay. Factors associated with *C. difficile* persistence: sporulation capacity, spore adherence capability, and biofilm formation capacity were characterised using standard techniques. For selected strains, phenotypic microarrays (PMs) were used to elucidate nutrient utilisation profiles, and competition for glucose between isolates from different CD002 lineages was investigated in a single-stage fermenter. Using 1D SDS gel electrophoresis, followed by Liquid Chromatography-Tandem Mass spectrometry (LC-MS/MS), the whole cell proteomes of three CD002 isolates were compared.

All CD002 were susceptible to metronidazole, vancomycin, fidaxomicin, chloramphenicol, linezolid, tetracycline (MICs ≤ 2 mg/L), and resistant to trimethoprim (MICs >128 mg/L) and ciprofloxacin (MICs ≥8 mg/L). Resistance to clindamycin (27% n=16), erythromycin (3.3%, n=2), moxifloxacin, nitrofurantoin and rifampicin (1.7%), was present. All but one *C. difficile* isolate demonstrated intermediate resistance to ampicillin and penicillin (MICs >1mg/L). One UK isolate of the UK 2007-8 lineage was classified as multidrug-resistant (MDR). The μ_{max} of non-UK 2012-14 strains was significantly higher (0.92 h^{-1}, $p<0.001$) than that of strains from the UK (2007-2014). Cytotoxin titres did not differ significantly between lineages (median titres 2-3 RU). The sporulation formation capacities for recent CD002 (UK and Non- UK) were significantly

higher ($p<0.001$) than those of the older isolates. Spore adherence capability did not differ significantly between CD002 lineages. Recent CD002 (UK 2011-13 & Non-UK 2012-14) strains formed significantly more profuse biofilms *in vitro* than the older strains ($p<0.001$). The recent CD002 (UK 2011-13 & Non-UK 2012-14) appeared to have more expanded nutrient utilisation profiles than older CD002 isolates, and one recent UK strain outcompeted a recent Non-UK strain for glucose. Analysis of whole-cell proteomes revealed similarities and differences between strains that suggest a minimal adaptation of the proteome in CD002 has occurred over time.

To conclude, the study uncovered some differences between the different lineages of CD002. The increased sporulation, higher μ_{max}, greater biofilm formation, abundance of spores in mature biofilms, and the utilisation of several nutrient substrates, demonstrated by recent *C. difficile* PCR ribotype 002, suggests that they may have a competitive advantage over other ribotypes, therefore increasing their prevalence in recent years. However, whether these factors, have a greater *in vivo* implication for this ribotype, remains to be determined.

This work is dedicated to my beloved parents Lt. Col A.M and Mrs G.O Ameh, who passed away in 2010 and 2012, I miss them dearly.

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All glory to the almighty God, for giving me unexplained peace and resilience through several difficult seasons. Indeed, *it is not him that willeth, nor him that runneth, but of the Almighty God that showeth mercy* (Romans 9:16).

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  Ameh, IL; Freeman, J; Fawley, WN; Wilcox, MH, Baines, SD

  The 26th European Congress of Clinical Microbiology and Infectious Disease (ECCMID, 2016)

- *In vitro* Assessment of Biofilm Formation in Clinical *Clostridium difficile* PCR ribotype 002 from different time lineages

  Ameh, IL; Freeman, J; Goyal, M; Wilcox, MH, Baines, SD

  The Microbiology Society Annual Conference 2017

ABBREVIATIONS

Abbreviation	Full text
AAD	Antibiotic-associated diarrhoea
agr	Accessory gene regulator
ANOVA	Analysis of variance
BaiCD	Bile acid - inducible *C. difficile*
BHI	Brain Heart Infusion
BHIS	Brain Heart Infusion supplemented with 5% Yeast extract and 1% L-cysteine Hydrochloride
BOC	British Oxygen Company
BSAC	British Society for Antimicrobial Chemotherapy
CA-CDI	Community associated *Clostridium difficile* infection
CBA	Columbia blood agar
CCEY	Cefoxitin/Cycloserine Egg Yolk
CCNA	Cell culture cytotoxicity neutralisation assay
CcpA	Carbon catabolite control/repressor protein
CD002	*Clostridium difficile* PCR ribotype 002
CDAD	*Clostridium difficile* associated disease
CDC	Centre of Disease Control and Prevention
CDI	*Clostridium difficile* infection
CDRN	*Clostridium difficile* ribotyping network for England and Northern Ireland
CDT	*Clostridium difficile* binary toxin
Cfr	Chloramphenicol florfenicol resistance
CFU	Colony-forming unit
ClosER	*Clostridium difficile* European Resistance
CLSI	Clinical & Laboratory Standards Institute
CPE	Cytopathic effect
Cwp	Cell wall protein
DMEM	Dulbecco Modified Eagle's medium
DMSO	dimethyl sulfoxide

DNA	Deoxyribonucleic acid
ECDC	European center of disease prevention and control
EIA	Enzyme Immunoassays
EMEM	Eagle's Minimum Essential Medium
Erm	Erythromycin ribosomal methylase
ESCMID	European Society for Clinical Microbiology and Infectious Diseases
ESGCD	European Study Group on *Clostridium difficile*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EUCLID	EUropean, multicentre, prospective biannual point prevalence study of *CLostridium difficile* Infection in hospitalized patients with Diarrhoea
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
FMT	Faecal microbiota transplantation
G+C	guanine-cytosine
GDH	glutamate dehydrogenase
GM MIC	Geometric mean minimum inhibitory concentration
Gyr	Gyrase
HAI	Health Care-Associated Infections
ICDS	International *C. difficile* Symposium
ITS	Intergenic spacer region
LC-MS/MS	Liquid Chromatography-Tandem Mass spectrometry
LCT	Large clostridial toxins
LCFT	Long term care facilities
MATH	Microbial adhesion to hydrocarbon
MDR	Multidrug resistance
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
MLSb	Macrolide, lincosamide and streptogramin B
MLST	Multi-locus Sequence Typing
MLVA	Multi- Locus variable number tandem repeat analysis
MRSA	Methicillin resistant *Staphylococcus aureus*

NAAT	Nucleic acid amplification test
NAP1	North American pulsed-field type 1
NTCD	Non- toxigenic *C. difficile*
OD	Optical Density
PaLoc	Pathogenicity locus
PBP	Penicillin-binding protein
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
p-cresol	Para-cresol
PFGE	Pulse- field gel electrophoresis
PHE	Public Health England
PM	Phenotype Microarray
PMC	Pseudomembranous colitis
PSMF	phenylmethanesulphonylfluoride
PYG	Peptone yeast glucose
QRDR	Quinolone resistance determining region
REA	Restriction Endonuclease Analysis
RCDI	Recurrent *Clostridium difficile* infection
RNA	Ribonucleic acid
Rpo	RNA polymerase
RT	Ribotype
SAB	Schaedler's Anaerobe Broth
SDS	Sodium dodecyl sulfate
SDW	Sterile Distilled Water
SHEA	Society for Healthcare and Epidemiology of America
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
TBE	Tris-Borate EDTA buffer
TC	Toxigenic Culture
TcdA	Toxin A
TcdB	Toxin B

TFA	trifluoroacetic acid
UN	United Nations
VNTR	Variable number tandem repeat
WBC	White blood cell count
WCA	Wilkins Chalgrens Agar
WGS	Whole-genome Sequencing
WHO	World Health Organisation

1 INTRODUCTION

1.1 *CLOSTRIDIUM DIFFICILE*

1.1.1 Historical Background

The bacterium *Clostridium difficile* was first reported in 1935, when Hall and O' Toole isolated a new obligate anaerobe, from the meconium and stools of new-born infants(Hall & O'toole, 1935). They named this organism *Bacillus difficilis* (In Latin 'difficile stands for 'difficult') to reflect the difficulty involved in its isolation and characterisation(Bartlett, 2008). Hall and O'Toole further evaluated the organism's ability to cause disease in an experiment using rabbits and guinea pigs. *B. difficilis* was found to be highly pathogenic to animals studied, as cultures or filtrates caused marked oedema and eventually death following 48h subcutaneous injection(Hall & O'toole, 1935). They suggested that an exotoxin released by the organism was responsible for its pathogenicity. Consequently, Hall and O'Toole postulated that toxins produced by the organism played a role in conditions such as the formation of occult blood and febrile convulsions of the new-born(Snyder, 1937). Two years later, Snyder performed a follow-up study and found *B. difficilis* in 10% of infant faecal samples. Snyder's study further confirmed the bacterium characteristics described in Hall and Toole's study. *B. difficilis* was later renamed *Clostridium difficile* and placed in the genus of *Clostridium spp* (Kuipers & Surawicz, 2008). Since then, it has been proposed to be re-classified as *Peptoclostridium difficile* (Yutin & Galperin, 2013). More recently, a further reclassification to a novel genus, *Clostridioides*, was proposed, to reflect the organism's phenotypic, chemotaxonomic and phylogenetic characteristics which differ from other members of the genus *Clostridium*. As a result, it has been officially reclassified from *Peptoclostridium difficile* to *Clostrioides difficile*

(Lawson *et al* .,2016). However, for the purpose of this thesis the more familiar and popular name, *Clostridium difficile*, is retained.

1.1.2 *Clostridium difficile* as a pathogen

Following initial reports, Smith & King reviewed eight cases of *C. difficile* of human infections. *C. difficile was* found to exist as a commensal rather than a pathogen in all the cases reviewed. As a result, the authors concluded that *C. difficile was* not pathogenic to man(Smith & King, 1962). Subsequently, *C. difficile was* not regarded as a pathogenic organism until an experiment using germ-free rats by Hammarström and colleagues (1969) demonstrated the organism's pathogenic ability. The authors reported the development of transient diarrhoea in rats mono-contaminated with *C. difficile,* which occasionally led to death (Hammarström *et al*., 1969). Little was known about the organism until the late 1970s when three coincidental independent reports provided evidence to show that *C. difficile* was the etiological agent for the development of antimicrobial-associated pseudomembranous colitis (PMC) (Bartlett *et al*.,1978; George *et al*., 1978; Tedesco *et al*., 1974).

In 1974, Tedesco *et al* found a significant link between clindamycin administration and the development of PMC in patients(Tedesco *et al*., 1974). In the same year, Green reported the presence of cytotoxin in the stools of guinea pigs that developed diarrhoea after receiving penicillin treatment(Green, 1974). Three years later, Bartlett and colleagues described clindamycin–induced colitis in hamsters and reported an unidentified toxin-producing *Clostridium* species present in the faeces of symptomatic animals(Bartlett *et al*.,1977). This organism was subsequently identified as *C. difficile.* Larson and colleagues later reported the presence of a cytotoxin in the faeces of four out of five patients with histologically proven PMC(Larson *et al*., 1978). Other studies soon followed and this provided confirmation that *C.*

difficile was a cause of antibiotic-associated diarrhoea and PMC in man(Bartlett *et al*., 1978, 1977; Larson *et al*., 1978; Tedesco *et al*., 1974).

1.1.3 **Morphology**

C. difficile is a Gram-positive, rod-shaped, spore-forming, and obligatory anaerobic bacterium, which is a member of the family phylum Firmicutes(Lawson *et al*., 2016). As a member of Firmicutes, *C. difficile* possesses low G+C content chromosomal DNA, and a cell wall peptidoglycan that contains *meso*-DAP (a diagnostic di-amino acid) (Lawson *et al*., 2016).*C. difficile* can exist in two forms; as vegetative cell and an endospore (Fig 1-1). Vegetative cells of *C. difficile* are 3- 16.9 µm in length, 0.5 - 1.9µm in width and produce oval-shaped sub-terminal spores that are highly resistant to a variety of physical and chemical conditions, thus prolonging *C. difficile* survival in the environment (Hafiz & Oakley, 1976). *C. difficile* is a heterotrophic organism with an optimal growth temperature of 37˚C, most strains are motile and possess peritrichous flagella and multiple fimbriae(Lawson *et al*., 2016; Sebaihia *et al*., 2006). Colonies of *C. difficile* on blood agar plates following 48 hours incubation in anaerobic conditions at 37℃ appear typically large (2-5 mm), flat, rhizoidal edge and slightly grey in colour with a matt to a glossy surface(Lawson *et al*., 2016). One characteristic feature that differentiates *C. difficile* from other species of clostridia is its ability to decarboxylate parahydroxyphenylacetic acid to produce *para*-cresol (*p*-cresol), which gives *C. difficile* its characteristic pig-like smell (Hafiz & Oakley, 1976). Recent evidence suggests that *p*-cresol production contributes to the virulence of *C. difficile in vivo* (Passmore *et al*., 2018).

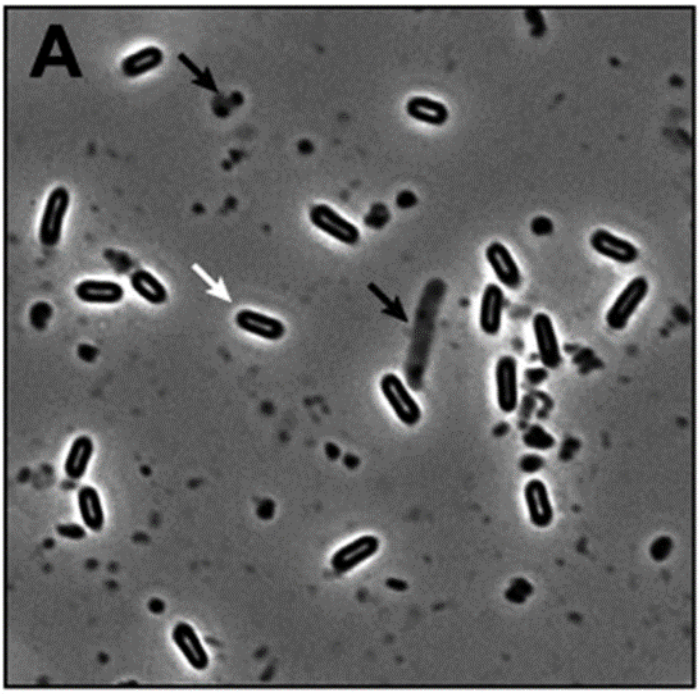


Figure 1-1 Phase-contrast micrograph of a *C. difficile* spore preparation. Showing phase-bright endospores (white arrow) and vegetative cells (black arrow) (Dembek *et al*., 2013)

1.1.4 *C. difficile* Infection (CDI)

Since the identification of *C. difficile* as a pathogen, it is recognised as one of the leading causes of healthcare-associated infections worldwide (Planche & Karunaharan, 2017). In 2013, *C. difficile* became classified as an urgent public health threat by the US Centre for Disease Control and Prevention (CDC, 2013). The annual incidence of nosocomial *C. difficile* infection (CDI) in the USA is almost 500,000 cases, resulting in 29,000 deaths, with estimated costs of over $4.8 billion in acute care facilities (Lessa *et al*., 2015). Several studies showed an increased incidence, severity and recurrence rate in CDI, surpassing methicillin-resistant *Staphylococcus aureus* (MRSA) as the most common hospital-acquired infection in the USA(Gupta & Khanna, 2014; McGlone *et al*., 2012; Miller *et al*., 2011). In the UK, the annual number of cases which had been increasing steeply since 2001, declined significantly from

2007- 2014 (Figure 1-2) and has now plateaued (Planche & Karunaharan, 2017). This is attributed to the introduction of the *Clostridium difficile* Ribotyping Network (CDRN) in 2007, as well as the mandatory surveillance and reporting of CDI cases (Both Trust and Non- Trust Apportioned) in England(Public Health England, 2018).

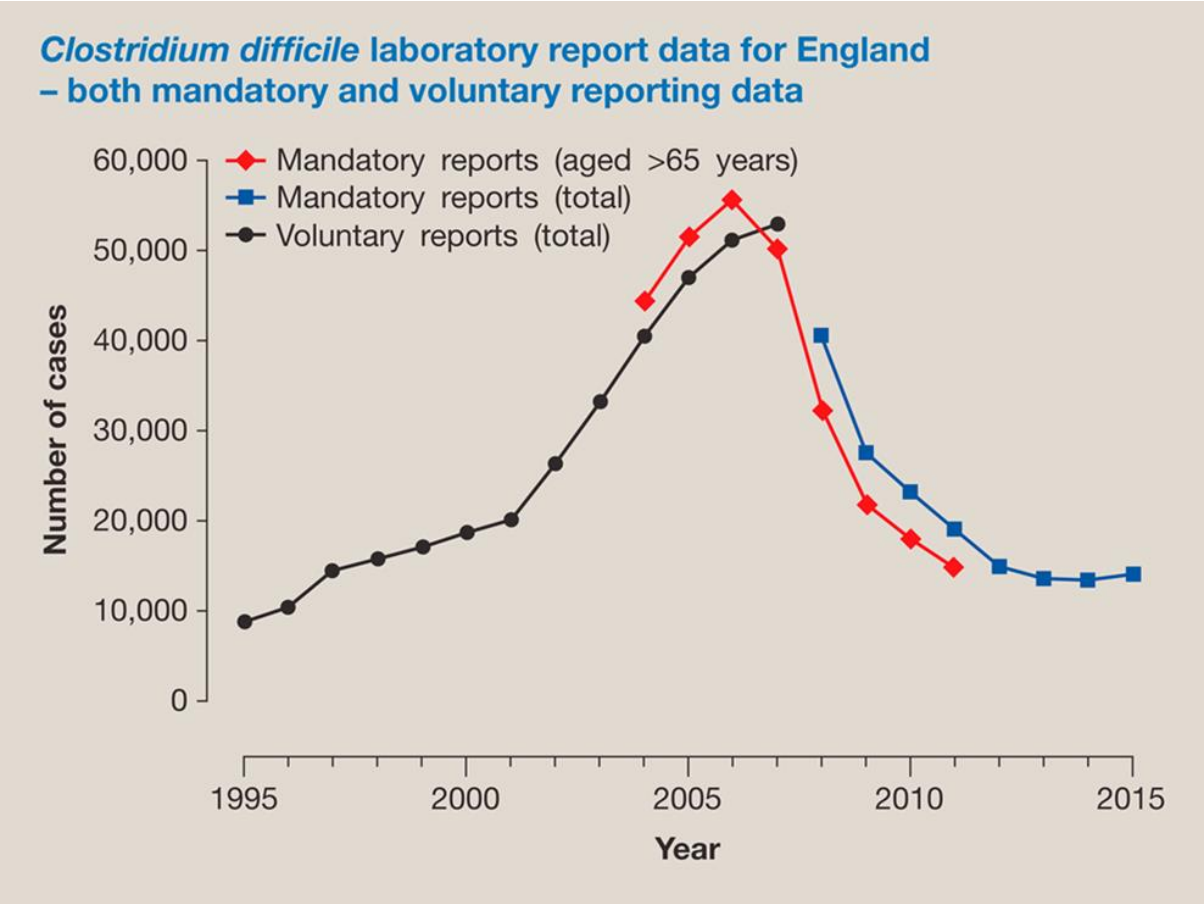


Figure 1-2 *Clostridium difficile* laboratory report data for England, both mandatory and voluntary reporting data (Planche & Karunaharan, 2017).

1.2 VIRULENCE FACTORS AND PATHOGENESIS OF *C. DIFFICILE*

1.2.1 **Toxin A and B**

C. difficile produces two main toxin virulence factors, toxin A (TcdA*)*, an enterotoxin, and toxin B (TcdB), a cytotoxin. These exotoxins TcdA and TcdB have a molecular mass of 308kDa and 270kDa respectively. Both toxins belong to the large clostridial toxin (LCT) family together with *C. sordellii* lethal and haemorrhagic toxin and *C. novyii* alpha-toxin(Braun *et al*., 1996). *C. difficile* exists naturally as either toxigenic (toxin-producing) or non-toxigenic strains, and both are capable of colonising their host.

TcdA and TcdB are encoded on a 19.6-kb pathogenicity locus (PaLoc), together with three other regulatory genes *tcdC, tcdE,* and *tcdR* (Figure 1-3). The two toxin genes are closely aligned, separated by an intervening sequence (*tcdE*). The toxins are greatly expressed during the late-log and stationary phases of growth due to environmental stimuli(Di Bella *et al*., 2016). Baines *et al* reported that some hypervirulent strains produce toxins earlier in their growth cycle, but peak toxin titres were not greater than strains of other ribotypes (e.g. 001)(Baines *et al*., 2008).

tcdE is an accessory gene, with homology to phage holin proteins and is positioned in between *tcdA* and *tcdB* on the PaLoc(Wee *et al*., 2001). The role of TcdE in CDI pathogenesis is poorly understood. Prior studies provided conflicting evidence on the role of TcdE, in facilitating the release of toxins into the extracellular environment(Govind & Dupuy, 2012; Olling *et al*., 2012; Wee *et al*., 2001). Subsequently, Govind and colleagues validated the role of TcdE as they demonstrated its capacity to release of TcdA and TcdB in *C. difficile* R20291(Govind *et al*., 2015). The authors suggested that the disparity in evidence from earlier studies(Govind &

Dupuy, 2012; Olling *et al*., 2012) may be attributed to the use of different strains by both studies.

tcdR encodes an alternative RNA polymerase sigma factor, and the major positive regulator of toxin expression that responds to the environmental stimuli(Di Bella *et al*., 2016; Girinathan *et al*., 2017; Voth & Ballard, 2005). It is located next to *tcdB* on the PaLoc and increases during stationary phase(Voth & Ballard, 2005). A recent study suggested that the role of *tcdR* may be strain-specific, as a mutation in this gene affected both sporulation and toxin production in *C. difficile* R20291 strain, but not in *C. difficile* 630Δerm strain (Girinathan *et al*., 2017).

tcdC is located next to *tcdA* on the PaLoc and transcribed differently from toxin genes*,* it is highly expressed in the early exponential phase, and its expression declines as growth moves to stationary phase(Bakker *et al*., 2012; Hundsberger *et al*., 1997). This decline is shown to be consistent with the increase in toxin production, suggesting that *tcdC* negatively regulates toxin production for both toxin genes(Merrigan *et al*., 2010; Vohra & Poxton, 2011).

The relative significance of the two toxins in CDI pathogenesis has long been a debate. Initial reports indicated that TcdA was the only toxin associated with the development of disease and therefore the major virulence factor(Lyerly *et al*., 1985). Subsequently, Lyras *et al* refuted this claim when their study revealed that TcdB, was 100-1000 fold more toxic to cultured cells than TcdA, suggesting that TcdB and not TcdA was essential for virulence(Lyras *et al*., 2009). Contrastingly, Kuehne *et al* indicated that both toxins play an important role in the development of CDI(Kuehne *et al*., 2010).In that study, $TcdA^{+}$ $TcdB^{-}$ were as likely as wild type strains expressing both toxins to cause disease in animal models, suggesting that both toxins contribute equally to the pathogenesis (though toxin B makes a larger contribution to virulence)(Kuehne *et al*., 2010). It is noteworthy that some ribotypes have deletions in their

PaLoc that abrogate the production of Toxin A or Toxin B (e.g. RT 017, RT033 & RT047)but not their capacity to cause disease (al-Barrak *et al*., 1999; Alfa *et al*., 2000; Kim *et al*., 2008; Pituch *et al*., 2001; Toyokawa *et al*., 2003; Wang *et al*.,2018).

1.2.1.1 Regulation and Expression of *C. difficile* toxins A and B

The regulation and expression of *C. difficile* toxins A and B are influenced by a variety of environmental stimuli that are activated by the synthesis of the *tcdR* gene. Short chain fatty acids (such as butyric acid) and subinhibitory concentrations of antibiotics are known to induce toxin production(Aldape *et al*., 2013; Chilton *et al*., 2012; Karlsson *et al*., 2000). Conversely, several amino acids (such as cysteine) within the bacterial environment repress toxin production through the action of a GTP-sensing transcriptional pleiotropic repressor CodY (Dineen *et al*.,2010; Karlsson *et al*., 2000; Nawrocki *et al*., 2016). Additionally, rapidly metabolisable carbohydrates such as glucose have been shown to inhibit the production of toxins through the action of the global transcriptional regulator, carbon catabolite control/repressor protein (CcpA) (Antunes *et al*., 2012; Fletcher *et al*., 2018). The sigma factor *sigD*, that is associated with the expression of motility genes, promotes toxin expression through binding to a *SigD* dependent promoter sequence(Mackin *et al*., 2013). The signalling molecule cyclicdi-guanosyl-5'monophosphate(c-di-GMP), associated with adhesion, aggregation and biofilm formation has been shown to repress the transcription of toxin encoding genes and *tcdR*(Mackin *et al*., 2013). The master sporulation regulator, Spo0A, has recently been shown to regulate toxin expression, but only in some strains of *C. difficile*(Mackin *et al*., 2013; Pettit *et al*., 2014). Also, quorum sensing studies show that toxin synthesis is regulated by an accessory gene regulator (*agr*) quorum-signalling system, which is mediated through the action of a novel thiolactone quorum signalling peptide (Figure 1-3)(Darkoh *et al*., 2015; Mackin *et al*., 2013). Several studies indicated that many of these

factors are strain-dependent, which suggests that toxin regulatory mechanisms have evolved independently to modulate the pathogenesis of CDI(Baban *et al*., 2013; Girinathan *et al*., 2017; Lyon *et al*., 2016; Mackin *et al*., 2013).

1.2.1.1 Mechanism of action *C. difficile* toxin A and B

TcdA and TcdB display a high degree of similarity at the amino acid level, suggesting that they may have arisen from a gene duplication event (von Eichel-Streiber *et al*.,1992). Both toxins harbour four functional domains: a catalytic glycosyltransferase domain, cysteine protease domain (responsible for autocatalytic processing), a translocation domain that mediates the entry of the toxin into the cell and a C-terminal receptor binding domain, consisting of a series of repeating oligopeptides (also known as CROP: combined repetitive oligopeptides) that is truncated in toxin B (Albesa-Jové *et al*., 2010; Pruitt *et al*., 2009; Pruitt *et al*., 2010; Pruitt & Lacy, 2012).

C. difficile toxins are taken up into the cells by receptor recognition and binding to the C-terminal domain, which triggers endocytosis of the toxins into the cellular endosome. The endosome is then acidified, resulting in a structural conformational change of the toxin, consequently, leading to the formation of a pore, and translocation into the cell cytosol (Figure1-4)(Pruitt *et al*., 2010). Once inside the cell, a host cytoplasmic inositol hexakisphosphate ($InsP_6$) induces autocatalytic cleavage, mediated by a *C. difficile* aspartate protease(Pruitt *et al*., 2009). This cleaving event results in the release of glycosyltransferase domain into the host cell cytosol where glycosylation of the Rho family of GTPases (Rho, Rac, and Cdc42) occurs. Subsequently, there is a disruption of the actin cytoskeleton, inhibition of cell division, membrane trafficking and eventual alteration of the intestinal epithelial. This results in increased intestinal permeability and fluid accumulation which subsequently manifests as diarrhoea, a hallmark symptom of CDI (Smits *et al*.,2016)

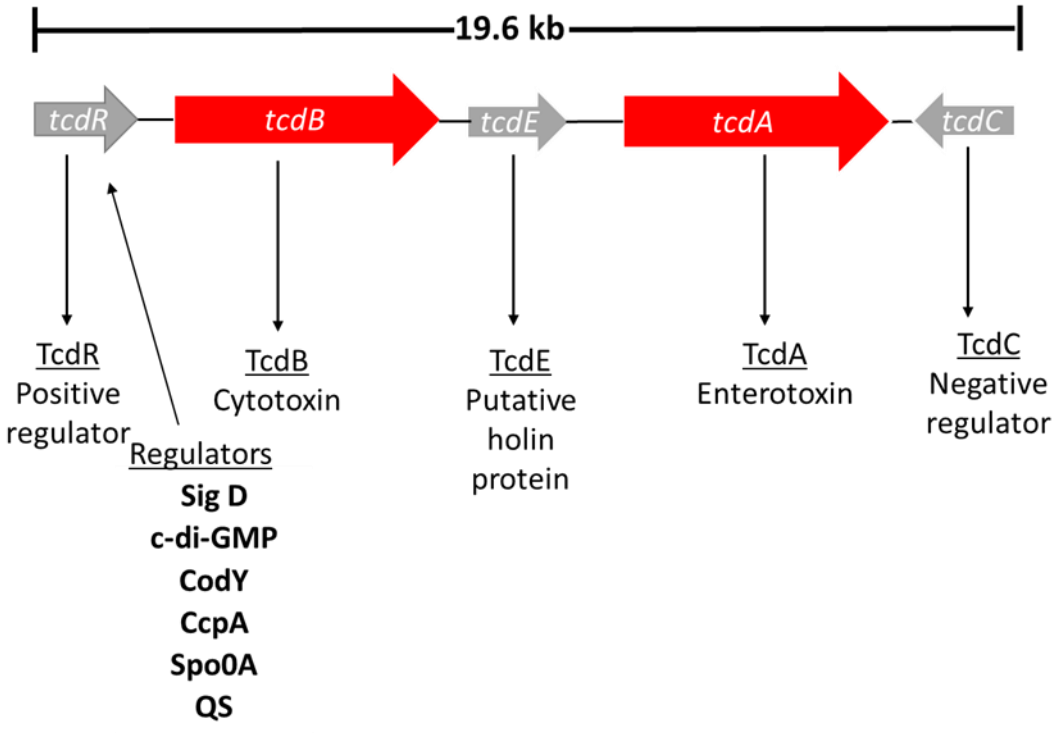


Figure 1-3 The pathogenicity locus of *Clostridium difficile* The schematic of the 19.kb *C. difficile* PaLoc, showing the two major toxins; TcdA and TcdB (encoded by *tcdA* and *tcdB* genes), the negative and positive regulatory genes *tcdC* and *tcdR* and the putative holin-like protein *tcdE* gene. Other regulators (sigma D (SigD), cyclicdi-guanosyl-5'monophosphate(c-di-GMP), the nutritional repressor CodY (known as GTP-sensing transcriptional pleiotropic repressor CodY), catabolite control protein A (CcpA), Stage 0 sporulation protein A (Spo0A) and quorum sensing (QS)(Thiolactone) that affect toxin gene transcription mostly act via expression of the *tcdR* gene(Adapted from, Voth & Ballard, 2005).

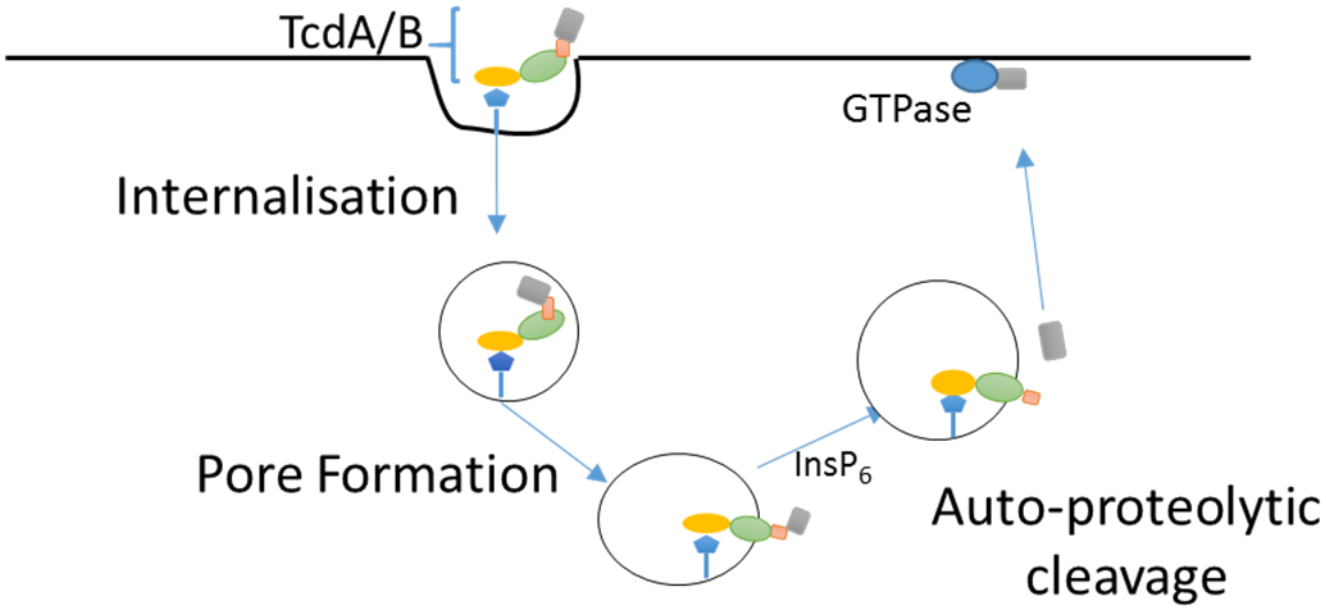


Figure 1-4 Mechanism of TcdA and TcdB entry into the cells. TcdA and TcdB consist of four domains; catalytic glycosyltransferase domain (GTD)(grey), cysteine protease domain(orange), translocation domain(green)and the C-terminal receptor binding domain(yellow). Toxins bind to the cell membrane via the C- terminal and are internalised by receptor-mediated endocytosis. Once Internalised, the acidic environment results in a conformation change to the toxins that allows insertion into the endosomal membrane and pore formation. Subsequently, autoproteolytic processing of the toxins occurs, and GTD is released into the cytosol by inositol hexakisphosphate $InsP_6$. GTD glycosylated the Rho family of GTPase that results in damage to the actin cytoskeleton and cell integrity(Adapted from Pruitt & Lacy, 2012).

1.2.1.2 The emergence of Hypervirulent *C. difficile*

In the early 2000s, the epidemic of CDI significantly increased due to the emergence and predominance of a hypervirulent strain, causing several outbreaks and increasing disease severity (Loo *et al*., 2005; Pepin *et al*., 2005; Pépin *et al*., 2004). This strain was characterised as restriction endonuclease analysis group BI, pulse-field gel electrophoresis type NAP1, and polymerase chain reaction ribotype 027, designated BI/NAP1/027(Killgore *et al*., 2008; McDonald *et al*., 2005). This strain has been recovered from patients in many countries across Europe, North America, Asia, South America and Australia(Collins *et al*., 2013; Hernández-Rocha *et al*., 2012; Kuijper *et al*., 2006; Quesada-Gómez *et al*., 2010; Riley *et al*., 2009; Tae *et al*., 2009). In addition, BI/NAP1/027 has been associated with increased purported toxin production, high sporulation rates and fluoroquinolone resistance (Akerlund *et al*., 2008; He *et al*., 2013; McDonald *et al*., 2005; Warny *et al*., 2005). Initial studies suggested that mutations (Δ117 frameshift mutation and 18bp deletion) observed in the *tcdC* gene is responsible for the elevated toxin production demonstrated by BI/NAP1/027 strains (Curry *et al*., 2007; MacCannell *et al*., 2006; Warny *et al*., 2005). However conflicting reports did not support the role of *tcdC* as a significant repressor of toxin production. Contrary to initial studies, Merrigan and colleagues found no significant difference in the amounts of toxin produced by both hypervirulent and non-hypervirulent strains in the exponential phase, suggesting that TcdC played a modulatory rather than a repressive one (Merrigan *et al*., 2010). Subsequently, studies by Vohra and Poxton supported these findings as increasing levels of *tcdC* transcription also coincided with increased transcription of toxin genes(Vohra & Poxton, 2011). Furthermore, Cartmen *et al* reported no association with *tcdC* and toxin production in

both hypervirulent strain (R20291) and wild type (*C. difficile* 630). Suggesting that *tcdC* is not the major repressor of toxin production(Cartman *et al*., 2012).

The emergence of other hypervirulent strains and has since been reported. These include strains of PCR ribotypes 017, 018 and 078, which have been linked to outbreaks of CDI (Barbanti & Spigaglia, 2016; Berger *et al*., 2019; Cairns *et al*., 2017, 2015; Goorhuis *et al*., 2008; Hung *et al*., 2016). PCR ribotype 078 (Toxinotype V), produces toxin A and B, as well as the binary toxin, while PCR ribotype 018 only produces toxin A and B. Although strains of PCR ribotype 017 (Toxinotype VIII) harbour almost the entire *tcdA* gene, prior studies revealed a 1.8 kb deletion in the repeating region (3' end)(Figure 1-5(Imwattana *et al*., 2019)), and a point mutation in the 5' end that abrogates the production of the toxin A(Drudy *et al*., 2007;Von Eichel-Streiber *et al*., 1999). In addition, strains of this ribotype do not produce the binary toxin and possess no mutations in the *tcdC* gene. Isolates belonging to ribotype 017 and 018 and are reportedly multi-drug resistant (MDR), as they are resistant to more than two or antimicrobial classes (Barbanti & Spigaglia, 2016; Freeman *et al*., 2018; Isidro *et al*., 2018; Spigaglia *et al*., 2010).

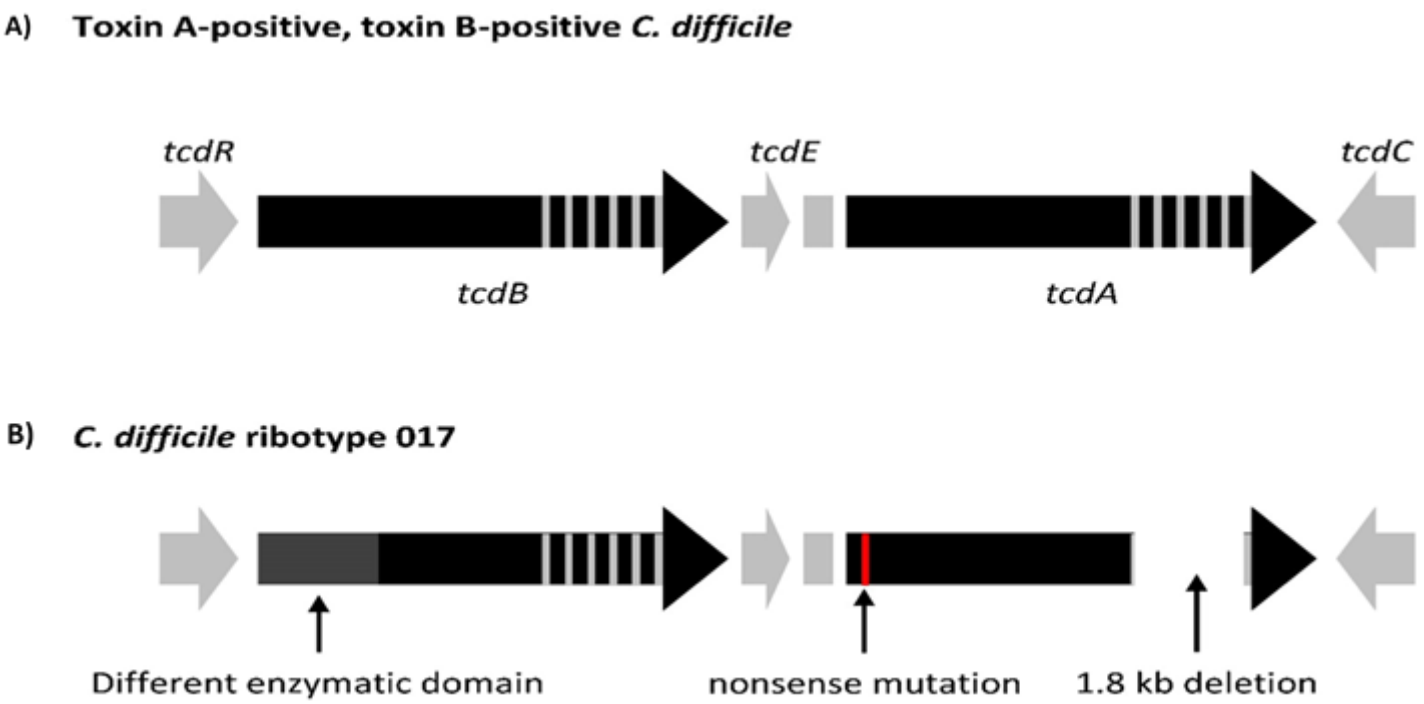


Figure 1-5 A diagram showing the variations in the Paloc of *C.difficile* strains. A)The PaLoc of toxin A positive and toxin B positive *C.difficile* strains. **B)**The Paloc of *C.difficile* ribotype 017 (A- B+) strains showing the nonsense mutation near the 5' terminal of the *tcdA* and the 1.8 kb deletion near the 3' end that abrogates the production of TcdA. The arrows indicate open reading frames and the direction of transcription. (Adapted from Imwattana *et al*., 2019).

1.2.2 *C. difficile* binary toxin (CDT)

Some *C. difficile* strains, particularly ribotype 027 and 078, produce a third toxin, known as a *C. difficile* binary transferase (CDT)(Lyon *et al*., 2016).CDT was first described in 1988 as a member of binary ADP-ribosylating toxin family, specifically iota-like subfamily of binary toxins which includes *C. perfringens* iota and *C. spiroforme* toxin (Barth *et al*., 2004; Popoff *et al*., 1988).

CDT is encoded by two genes *cdtA* (50kDa), and *cdtB*, (100kDa), that are located on a 4.3 kb genomic region referred to as Cdt locus (CDTLoc)(Carter *et al*., 2007). This region contains both toxin genes and a regulatory protein *cdtR* (Figure 1-6), recently *cdtR* has been shown to regulate the production of TcdA and TcdB in epidemic 027 strains (Lyon *et al*., 2016). Strains lacking this toxin have a conserved 68-bp sequence in place of the CDT Locus (Carter *et al*., 2007). As with all binary toxins, CDT comprises an enzymatic (CdtA) and a binding (CtdB) component. CdtB binds to a cellular receptor, leading to the internalization of CdtA into the cytosol. This, in turn, catalyses the ADP- ribosylation of actin, leading to disruption of the actin cytoskeleton, excessive fluid loss, cell rounding and eventual cell death(Carter *et al*., 2007).

The exact role of this toxin in disease pathogenesis is controversial, with animal studies providing conflicting evidence. Geric and colleagues suggested that the binary toxin is not essential for disease, but maybe important for colonisation, as supernatants from four A-/B-/CDT^{+} strains resulted in colonisation and not disease or death in hamsters(Geric *et al*., 2006). Contrastingly, Kuehne *et al* indicated that knock out strains of epidemic strain R20291 with only CDT gene were virulent in a hamster model (Kuehne *et al*., 2014). In addition, Schwan *et al* reported that CDT aids adherence to intestinal epithelial cells by the formation of microtubule protrusions (Schwan *et al*., 2009).CDT has been shown to be more prevalent in

strains isolated from animals, with the CDT structural genes being found in approximately 23% to 100% of these isolates(Carter *et al*., 2007). In addition, several cases of CDI were reported in France that was linked to $TcdA^-$ $TcdB^-$ CDT^+, suggesting that this toxin may play an important role in disease pathogenesis(Eckert *et al*., 2015). A retrospective study by Bacci *et al* indicated that human infections with CDT-producing strains of *C. difficile* yielded greater mortality when compared to infections caused by strains that did not produce CDT (regardless of ribotype) suggesting that the binary toxin contributes to the severity of CDI(Bacci *et al*.,2011).

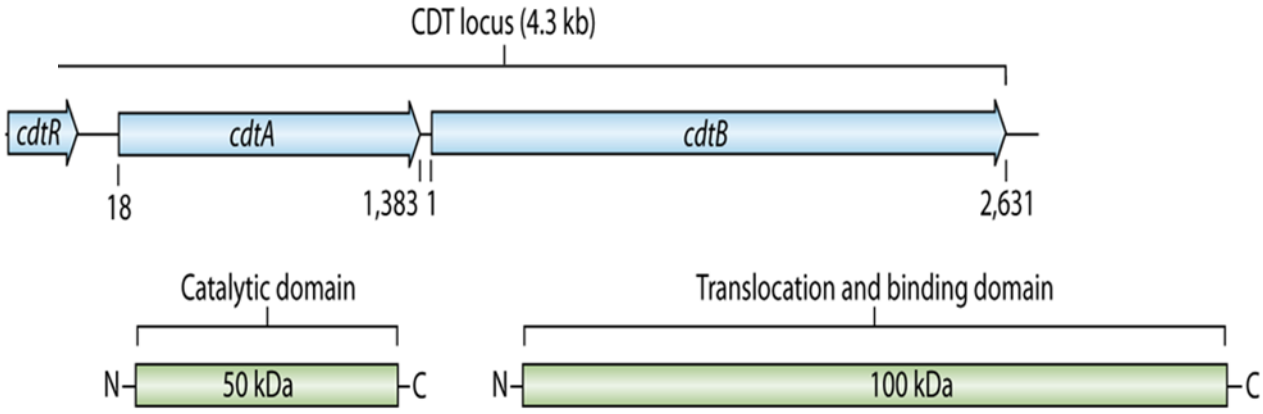


Figure 1-6 The CDT locus of *C. difficile*, showing the toxin and regulatory genes (cdtA, cdtB and cdtR)(Carroll & Bartlett, 2011)**.**

1.2.3 **Adhesion factors**

The colonisation of the colon is a key factor in the full manifestation of CDI. *C. difficile* colonisation of the gut is facilitated by adhesion to the mucosal epithelium, making this step crucial to disease pathogenesis. The ability of *C. difficile* to adhere to the human gut was first reported in by Borriello, who detected the presence of *C. difficile* cells under the microscope, in a washed biopsy sample from patients with PMC(Borriello, 1979). Subsequently, several

non-toxin related virulence factors that play a key role in adherence and colonisation have been identified and partially characterised.

1.2.3.1 Surface layer proteins (SLP)

The S-layer proteins (SLP) are proteinaceous layers produced by many bacteria including *C. difficile.* They are mainly composed of two protein subunits commonly referred to as high molecular weight and low molecular weight proteins which display inter-strain variability in *C. difficile* (Calabi *et al*., 2002). These proteins are encoded by *slpA* and formed from the post-translational cleavage by the protease Cwp84 (Dang *et al*., 2010). There is evidence to show that these proteins play a role in colonisation, acting as adhesins assisting the interaction between *C. difficile* cells and the gut cells (Calabi *et al*., 2002). In a recent study, antibodies against SlpA were able to inhibit the adherence of *C. difficile* to cultured cells (Merrigan *et al*., 2013).In addition to adherence, several studies show that SLPs play an important role in immune recognition and inflammatory response(Bianco *et al*., 2011; Fagan *et al*., 2011; Ryan *et al*., 2011). Thus far, the best-characterised S-layer proteins are Cwp84 (84kDa) and Cwp66. As initially stated, Cwp84 is involved in the processing of the S-layer while Cwp66 (66kDa) is implicated in adherence (de la Riva *et al*., 2011). Cwp66 contains two domains, each carrying three imperfect repeats and one showing a correlation with the autolysin CwIB in *Bacillus subtilis*(Waligora *et al*., 2001). Studies suggest that the adhesive capability of Cwp66 in *C. difficile* may be heat shock-mediated. As reduced adherence of heat-shocked *C. difficile* to cultured cells was observed in the presence of antibodies against Cwp66 (Waligora *et al*., 2001).

1.2.3.2 Other Adhesion Factors

C. difficile strains are known to possess flagella for motility, although this is a variable trait among clonal isolates (Pituch *et al*., 2002). The two best characterised *C. difficile* flagellar

proteins include FliC (structural monomer) and FliD (cap protein), both of these have been shown to participate in the attachment to the intestinal mucus(Tasteyre *et al*., 2002). Currently, the role of flagella in disease pathogenesis is debatable and appears to be strain-dependent (Baban *et al*., 2013; Dingle *et al*., 2011). Some studies suggest that flagella play an important role in adherence to intestinal epithelial cells including hypervirulent strain (ribotype 027). Conversely, other studies have indicated that the absence of the flagella in non-epidemic strains (*630Δerm*) had little or no effect on adherence, rather mediated increased adherence to cells (Baban *et al*., 2013; Dingle *et al*., 2011). Flagella have also been shown to play a role in the development of *C. difficile* biofilms (Đapa *et al*., 2013).

Some strains of *C. difficile* have fimbriae which are polar, 4-9nm in width and up to 6µm long (Borriello *et al*., 1988). Earlier studies investigated the importance of fimbriae in disease pathogenesis, however, no correlation was established between the presence of fimbriae in a strain and its ability to cause disease(Borriello *et al*., 1988; Taha *et al*., 2007). A recent analysis of Type IV pilus genes in the epidemic strain ribotype 027 (R20291) revealed the presence of nine pilin and putative pilin-like genes. Immunization studies using proteins produced by 6 of these genes yielded immunological responses in animals (Maldarelli *et al*., 2014).

A 58kDa heat shock protein, GroEL, is expressed by *C. difficile* in response to various environmental stress. GroEL was found in the membrane as well as the extracellular space following heat-shock (Hennequin *et al*., 2001). Additionally, the importance of GroEL was demonstrated, when GroEL specific antibodies and purified GroEL protein inhibited *C. difficile* adherence to eukaryotic cells *in vitro* (Hennequin *et al*., 2001). Vaccination with recombinant

GroEL, reduced gut colonization in infected mice, thus supporting the importance of this protein in intestinal adhesion (Péchiné *et al*., 2013).

1.2.4 **Sporulation**

C. difficile forms highly resistant metabolically dormant endospores as a result of exposure to unfavourable conditions such as temperature, nutrient limitation, and extremes of pH (Rupnik *et al*., 2009).The spore is formed within the mother cell, which ensures its preservation until spores encounter favourable conditions for germination and outgrowth. *C. difficile* spores are ubiquitous, especially in the nosocomial environment, where they persist on hospital surfaces for long periods (Vedantam *et al*., 2012). The ability of spores to persist in the environment is believed to be a key factor in the acquisition and transmission of CDI.

It is hypothesised that spores ingested by a susceptible host, are able to survive the low pH environment in the stomach and reach the small intestine where germination occurs upon exposure to bile salts(Paredes-Sabja *et al*., 2014). There are several bile salts that induce the germination of *C. difficile,* however, sodium taurocholate is the most effective, glycine and thioglycolate serve as co-germinants (Crobach *et al*., 2018; Sorg & Sonenshein, 2008). An earlier study indicated that patients with CDI can excrete high levels of spores to the environment for 1 to 4 weeks after CDI treatment (Sethi *et al*., 2010). Additionally, an *in vitro* study by Paredes-Sabja & Sarker (2012) demonstrated the adherence of *C. difficile* spores to the surface of the intestine. Spores, which are not affected by antimicrobial therapies commonly used in the treatment of CDI, can then germinate and recolonise the host gastrointestinal tract before the normal microflora recovers after antibiotic treatment, causing a relapse of infection (Hopkins & Wilson, 2018; Paredes-Sabja & Sarker, 2012).

There is some debate regarding the association between strains of the hypervirulent ribotype 027, and the greater production of spores. Several studies noted that some clinical *C. difficile* isolates, especially those of the hypervirulent ribotype 027, germinated more efficiently and formed more spores *in vitro* than non-epidemic ribotypes(Akerlund *et al*., 2008; Merrigan *et al*., 2010; Moore *et al*., 2013; Vohra & Poxton, 2011), however, subsequent studies reported no association(Burns *et al*., 2011; Heeg *et al*., 2012).

1.2.4.1 Sporulation pathway in *C. difficile*

Sporulation is an ancient process of bacterial cell differentiation largely conserved among *Clostridiales* and *Bacillales* (Dembek *et al*., 2015; Higgins & Dworkin, 2012). Sporulation in *C. difficile,* like other spore-forming bacterial organisms, is a complex process that results in the formation of a resistant spore from a vegetative cell. This process is largely regulated by external signals which trigger a signalling cascade of sigma factors to initiate sporulation (Fimlaid *et al*., 2013). Little is known about the signals that trigger *C. difficile* sporulation, however, studies suggest that it could be related to environmental stimuli such as nutrient starvation, quorum sensing, and other unidentified stress factors (Higgins & Dworkin, 2012). Although the sporulation pathway in *C. difficile* has been extensively studied, the molecular mechanisms that govern their composition and formation remain poorly understood. Sporulation studies in *Bacillus subtilis* (*B. subtilis*) provides insight into the sporulation pathway in *C. difficile* (Fimlaid *et al*., 2013).

The entry into sporulation of *B. subtilis* is controlled by Spo0A, a master regulatory protein which becomes activated by a phosphorelay cascade (Higgins & Dworkin, 2012). Accordingly, Spo0A activates downstream regulators which initiate the sporulation process and repress vegetative cell functions. Activated Spo0A triggers an asymmetric division, yielding two distinct cells, a pre-spore (also known as forespore), and the mother cell (Figure1-7) (Pereira

et al., 2013). Soon after division, sporulation-specific RNA sigma factors required for gene expression in the forespore and mother cell are sequentially activated. The four-cell type-specific sigma factors ($\sigma^{F,}$ σ^{E}, σ^{G}, and σ^{K}) are alternated between the two cells. Sigma F and E control the early stages of development in the two cells and are replaced by sigma G and K in the later stages of development (Fimlaid *et al*., 2013; Pereira *et al*., 2013). Sigma F is activated in the forespore, while sigma E is activated in the mother cell. Sigma E activates production of spore coat proteins and communicates with the forespore for the activation of sigma G. Sigma G initiates the signalling cascade that results in the activation of sigma K, this leads to the assembly of the spore outer coat (Pereira *et al*., 2013).

During the asymmetric division, a septum is formed which divides the prespore from the mother cell. Whilst the septum is being formed, the chromosome replicates to form an axial filament (Dembek *et al*., 2015; Fimlaid *et al*., 2013). The remaining section of the chromosome is actively transported into the forespore and segregated from the mother cell with the completion of the septum formation. This results into two distinct cells, both with a complete chromosome. The mother cell surrounds the forespore and compresses the membrane off in the way to completely engulf the forespore. Following this, the chromosome in the forespore is reconstructed into a circular structure, a thick cell wall and the protective protein coat is formed, this completes the full synthesis of the spore. Subsequently, the mother cell is lysed (programmed cell death), liberating the mature spore (Pereira *et al*., 2013).

Although the sporulation activities in *B. subtilis* are quite similar to those in other *Clostridium* species, recent studies have highlighted some differences (Pereira *et al*., 2013). The main periods of activity for the four cell-type sigma factors are highly conserved in *C. difficile*

relative to *B. subtilis* (Pereira *et al*., 2013). The main observable difference with *B. subtilis* was that sigma E activity was partially independent of sigma F and sigma G or K did not require sigma E or sigma G. Pereira *et al* suggested that the connection between the forespore and mother cell lines of gene expression in *C. difficile* were weaker compared to those observed in *B. subtilis* (Pereira *et al*., 2013).

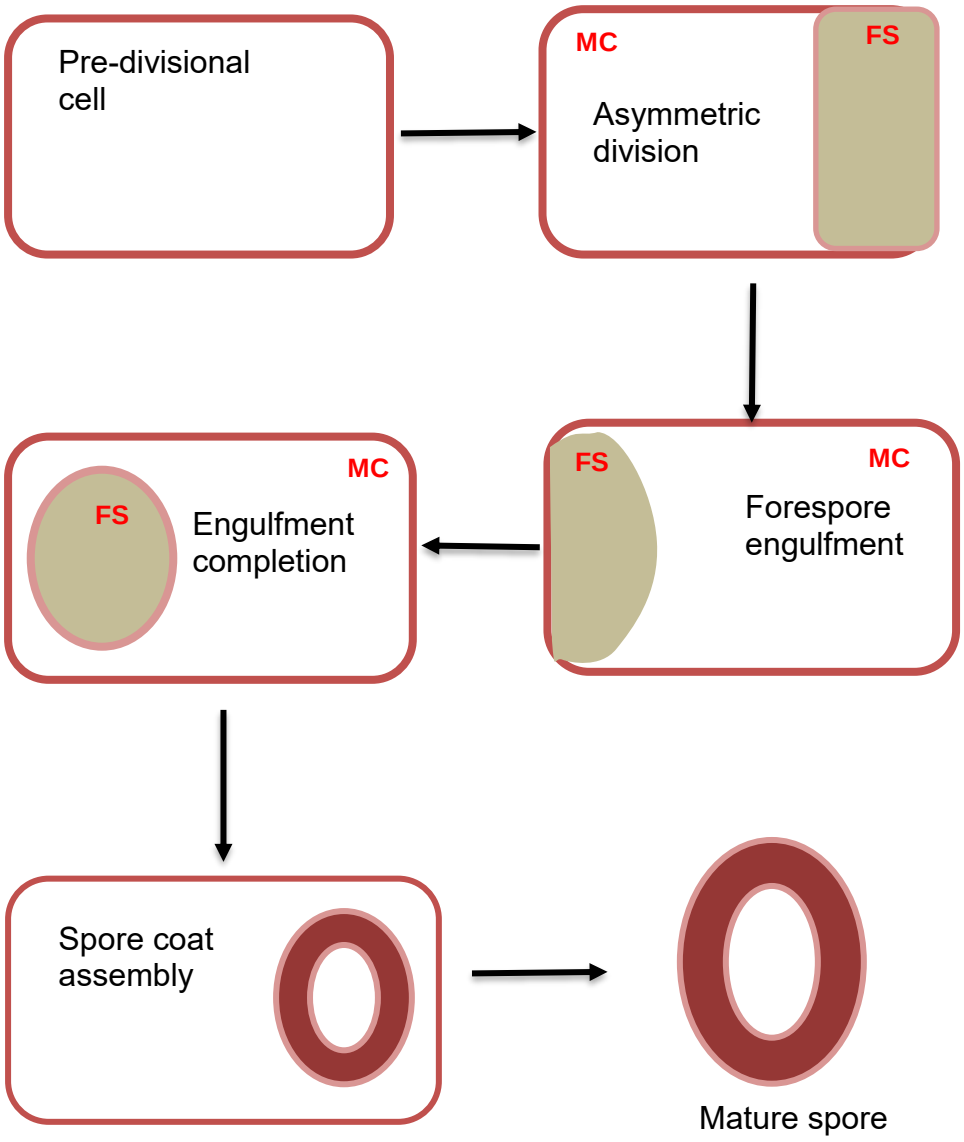


Figure 1-7 Schematic of the main morphogenetic stages in the process of sporulation. Mother cell (MC) and forespore (FS) (Adapted from Pereira *et al*., 2013)

1.2.5 **Biofilms**

Biofilms are complex communities of microorganisms enclosed in a self–produced extracellular matrix composed primarily of exopolysaccharides(Costerton *et al*., 1999). The transition of a bacterial cell from planktonic to biofilm growth occurs as a survival strategy in response to environmental stresses such as nutrient starvation (Semenyuk *et al*., 2014). Numerous studies have established the link between biofilms and many human infections(Costerton *et al*., 1999; Delcaru *et al*., 2016; Høiby *et al*., 2010; Sonesson *et al*., 2017). However, biofilm formation by individual gut species, particularly anaerobic species, is poorly understood (Dapa & Unnikrishnan, 2013). The formation of *C. difficile* biofilms has been shown to be modulated by central regulators such as Spo0A and LuxS and by adhesion factors such as flagella and the cysteine protease Cwp84 (Dapa & Unnikrishnan, 2013; Dawson *et al*., 2012; Pantaléon *et al*., 2015).

The role of biofilms in CDI has not been fully established. Nevertheless, there is evidence to show that *C. difficile* forms microbial communities in the host gastrointestinal tract (Lawley *et al*., 2012). Using murine studies, the adherence of *C. difficile* to mucus and intestinal cell lines with the formation of cellular aggregates was revealed(Lawley *et al*., 2012). This suggested that *C. difficile* can form part of intestinal biofilms, thus facilitating recurrent infections through the germination of *C. difficile.* Initially, the presence of spores in *C. difficile* biofilms was debatable but recent studies revealed the abundance of spores in biofilms of the organism (Crowther *et al*., 2014; Dawson *et al*., 2012; James *et al*., 2018; Semenyuk *et al*., 2014).

However, the gastrointestinal tract biofilms are composed of complex mixtures of gut microorganisms, therefore pure culture studies of *C. difficile* biofilms may elicit data that is of limited relevance to the complex situation likely to be observed *in vivo.* Consequently, complex gut model biofilms have been developed in order to study the interplay between the gut microflora, *C. difficile* (spores and vegetative forms), and antimicrobial agents during simulated CDI(Crowther *et al*., 2014b). An *in vitro* gut model study by Crowther and colleagues observed dormant *C. difficile* spores in the sessile gut communities, in comparison to their planktonic counterpart(Crowther *et al*., 2014a). The authors simulated recurrent CDI, following the cessation of antimicrobial therapy in the gut model and suggested that intestinal biofilms may serve as a potential reservoir for *C. difficile* spores. Additionally, consistent with other biofilms, *C. difficile* in biofilms have been shown to have reduced susceptibility to vancomycin, which is one of the first-line therapies for CDI (Đapa *et al*., 2013).

1.2.5.1 Biofilm formation in *C. difficile*

Like many other bacteria, the formation and development of biofilms in *C. difficile* are characterised by three important processes; initiation, maturation and disassembly (Kostakioti *et al*., 2013; Vlamakis *et al*., 2013). The process begins with the formation of a heterogeneous community through the attachment of planktonic cells to a surface, leading to the formation of microcolonies (Semenyuk *et al*., 2014). After initial attachment, the bacterial population increases as a result of cell division and density-dependent cell-cell communication known as quorum sensing is initiated. The resident bacteria become enclosed in an extracellular polymeric substance (EPS) that can be made up of polysaccharides, amino acids, and nucleic acids. The EPS serves as a protective barrier for the organisms.

Subsequently, there is development and maturation of the biofilm architecture, this stage is also aided by cell-cell communication between microbial colonies. The next phase is the development and maturation of the biofilm architecture, and this is facilitated by cell-cell communication. As cells mature, biofilms release D-amino acids, consequently leading to the dissolution of mature biofilms, and the released bacteria can colonise new surfaces (Vlamakis *et al*., 2013).

1.3 RISK FACTORS FOR CDI

1.3.1 **Prior hospitalisation**

C. difficile is usually nosocomially acquired, as a result, most cases arise due to hospitalisation or use of long-term care facilities. The prevalence of *C. difficile* spores in the nosocomial environment is considered a great risk factor as patients are more likely to become colonised or develop CDI in the hospital setting. Studies have described a high rate of *C. difficile* colonization (10-35%) among hospitalised patients and 4-20% of residents in long- term care facilities(Barbut & Petit, 2001; Kuijper *et al*., 2006; Simor *et al*., 2002).In addition, isolation of *C. difficile* from stool samples of the hospital inpatients is reported to be proportional to the length of hospital stay (Kuijper *et al*., 2006).

1.3.2 **Antibiotic exposure**

The major risk factor in the development of CDI is the prior use of one or more courses of antibiotics for the treatment of an infection. Reportedly, most antibiotics have potential to induce CDI, some antibiotics such as clindamycin, cephalosporins, aminopenicillins and fluoroquinolones are associated with a relatively higher risk of *C. difficile* infection (Rupnik *et*

al., 2009). Several studies have demonstrated that by restricting the use of these antibiotics, the rates of CDI significantly reduced (Carling *et al*., 2003; Kallen *et al*., 2009; Gaynes *et al*., 2004; Valiquette *et al*., 2007). The use of antimicrobial agents is known to disrupt the protective host microbiota, leading to the loss of colonization resistance by the host, as a result, patients become susceptible to infections caused by opportunistic bacterial pathogens (Johanesen *et al*., 2015). Studies have shown that antibiotics in the gut facilitate the germination and outgrowth of *C. difficile* spores, increased toxin production and the expression of colonization factors that all aid in full manifestation of CDI (Adams *et al.*, 2007; Aldape *et al*., 2013; Chilton *et al*., 2012; Heinlen & Ballard, 2010; Saxton *et al.*, 2009).

1.3.3 **Age**

The risk and severity of CDI increase with age due to the reduction in health status. In one study, it was revealed that the risk of CDI during an outbreak was 10 times great in the elderly (<65years of age) than the younger population (Depestel & Aronoff, 2013). Although CDI is mostly reported in the elderly population, there is an increasing incidence of CDI in the younger population (Baker *et al*., 2010; Benson *et al*., 2007; Dumyati *et al*., 2012; Khanna *et al*., 2012). A population-based study by Khanna *et al* showed a striking increase of CDI among the younger population over the last 20 years, with most cases arising from the community setting(Khanna *et al*., 2012). However, the drivers of increased CDI prevalence among the younger population in the community are yet to be identified.

1.3.4 **Proton pump inhibitors (PPIs)**

The use of proton pump inhibitors has been linked to the development of CDI, however this remains a controversial risk factor as many studies appear to conflicting (Aseeri *et al*., 2008; Cunningham *et al*., 2003; Dial *et al*., 2004; Janarthanan *et al*., 2012; Kim *et al*., 2010;

Kwok *et al*., 2012; Linsky *et al*., 2010; McDonald *et al*., 2015; Ro *et al*., 2016). A potential mechanism for this phenomenon is the reduction of gastric acid secretion by PPIs in the stomach. Which results increased stomach pH that facilitates the passage of ingested *C. difficile* that can colonise the gut and cause disease (Gupta & Khanna, 2014). It is not exactly clear how gastric acid suppression may increase the risk of CDI, however, it has been proposed that spores may be stimulated to germinate by bile salts while increased pH may allow the survival of vegetative cells (Dial *et al.*, 2006). The link between PPI exposure and CDI was demonstrated by Hung and colleagues in an animal study. Hung and colleagues observed an increase in the severity of *C. difficile* associated colitis in mice after PPI exposure, suggesting that PPIs play an important role in CDI (Hung *et al*., 2013). It is reported, that incessant use of PPIs in the community (being one of the most prescribed medications for acid – related disorders) is particularly significant to a subset of community-acquired CDI without prior antibiotic exposure (Chitnis *et al*., 2013). Suggesting that, the disruption of the microbiota, following PPI exposure, may be sufficient to cause disease(Clooney *et al*., 2016; Imhann *et al*., 2016; Seto *et al*., 2014).

1.4 CLINICAL MANIFESTATIONS OF CDI

C. difficile can cause a spectrum of clinical manifestations, ranging from asymptomatic carriage to life-threatening infections that can be fatal (Smits *et al*., 2016).

1.4.1 **Asymptomatic Colonisation**

Asymptomatic *C. difficile* colonisation is a condition whereby *C. difficile* is detected in individuals without clinical symptoms of CDI (Furuya-Kanamori *et al*., 2015). Evidence from earlier studies shows that asymptomatic *C. difficile* colonisation provides a protective effect

against CDI through an immune-mediated response(Kyne *et al*., 2000, 2001). Recently, it was proposed that asymptomatic carriage may lead to *C. difficile* co-colonisation with other members of the gut microbiota, and therefore serve as a protective factor for CDI (Vincent *et al*., 2016). The prevalence of asymptomatic *C. difficile* colonisation varies depending on age and geographical location. According to reports from several studies, approximately 17.5% of healthy adults are colonised by *C. difficile*, with 1-5 % being colonised by toxigenic strains(Eyre *et al*., 2013; Galdys *et al*., 2014; Ozaki *et al*., 2004; Terveer *et al*., 2017). Prevalence of up to 50% is found in elderly patients in long term care facilities, health-care workers and patients with underlying diseases (e.g. cystic fibrosis) (Arvand *et al*., 2012; Bauer *et al*., 2014; Hell *et al*., 2012; Riggs *et al*., 2007). In addition, a high prevalence of colonisation has been reported in neonates, and healthy infants of up to 2 years of age, as they have been considered insensitive to free *C. difficile* toxins due to their lack of the mature toxin- binding necessary for disease causation(Jangi & Lamont, 2010; Rousseau *et al*., 2012; Schäffler & Breitrück, 2018). It is suggested that asymptomatic carriers serve as reservoirs for disease transmission in the environment(Eyre *et al*., 2013; Riggs *et al*., 2007). Riggs *et al* suggested that asymptomatic carriage of epidemic and non-epidemic *C. difficile* strains have the potential to contribute disease transmission in long term care facilities(Riggs *et al*., 2007).

1.4.2 **Mild or moderate CDI**

C. difficile is the cause of approximately 25-30% of all cases of antibiotic-associated diarrhoea (AAD)(Bartlett & Gerding, 2008). While a large proportion of the remainder is due to an osmotic imbalance within the large intestine(Walk & Young, 2008). In this scenario, the bacteria responsible for breaking down fermentable starch are eliminated by antimicrobial therapy, as a result, dietary sugar is not being metabolised (Walk & Young, 2008).

Consequently, there is an increased intestinal permeability and fluid accumulation that results in diarrhoea.

For mild to moderate CDI patients experience watery diarrhoea occurring between 1-2 hours, within 2 weeks after antimicrobial therapy (Goudarzi *et al*., 2013). Other clinical manifestations include abdominal cramps, sub-febrile temperature, nausea and leucocytosis (Postma *et al*.,2015). Overall, fever, leucocytosis and abdominal cramps occur in about 15%, 50%, and 22% of cases, respectively(Bartlett & Gerding, 2008). Elevated creatinine, elevated lactate and hypoalbuminemia are the common laboratory findings in mild-moderate CDI cases. Physical examination demonstrates lower abdominal tenderness (Potsma *et al*., 2015). Evidence of CDI colitis includes abdominal pain, nausea, malaise, anorexia, watery diarrhoea, and in some cases, a trace of blood in the stool(Postma *et al*., 2015). In addition, low-grade fever, dehydration, pyrexia and leukocytosis may be present. The sigmoidoscopic examination may reveal a nonspecific diffuse or patchy erythematous colitis without pseudomembranes (Bartlett & Gerding 2008).

1.4.3 **Severe CDI**

Pseudomembranous colitis (PMC) is one of the severe forms of CDI, and *C. difficile* causes 95% of PMC(Farooq *et al*., 2015). Unlike mild or moderate CDI, PMC is characterised by the formation of pseudomembranes in the colorectal mucosa. Clinical symptoms include abdominal cramp, dehydration, hypoalbuminemia, watery diarrhoea, rising inflammatory cells, serum proteins and mucus (Vaishnavi, 2010). Sigmoidoscopic examination reveals 2-10mm yellow plaques in the colorectal mucosa (Figure 1-8) and in rare cases the terminal ileum.

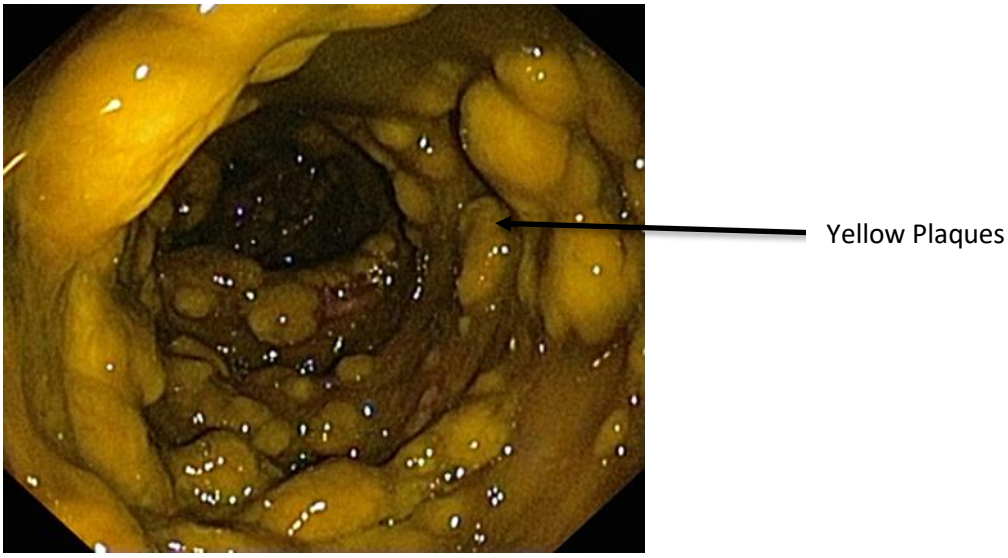


Figure 1-8 The appearance of the colon from a patient with pseudomembranous colitis, with yellow plaques in the colorectal mucosa (Adapted from Natural health news, 2017**).**

Fulminant colitis is considered the most serious form of CDI occurring in approximately 3-8% of cases.The increase in fulminant colitis has been attributed to the emergence of hypervirulent *C. difficile* strain BI/NAP-1/027(Dallal *et al*., 2002; Nakamura *et al*., 2014; Oguri *et al*., 2019; Tagashira *et al*., 2013).Fulminant colitis results in the development of symptoms, multiple organ failure and increased mortality (Goudarzi *et al*., 2013).Patients with fulminant colitis experience severe lower abdominal pain, watery diarrhoea, abdominal distention, hypovolemia and lactic acidosis. Some symptoms are accompanied by fever and marked leukocytosis (up to 40,000 WBC/µL). Occasionally, little or no diarrhoea may be experienced due to paralytic ileus resulting from colon dilatation (Potsma *et al*., 2015). Advanced complications of fulminant colitis may result in bowel perforation and toxic megacolon. Patients with signs and symptoms of bowel perforation may experience abdominal rigidity, tenderness and reduced bowel sound. Abdominal radiography may also reveal free abdominal air (Postma *et al*., 2015). Patients with toxic megacolon experience clinical signs of severe systemic toxicity (e.g. fever, tachycardia, leukocytosis, anaemia) and colonic dilation

(i.e. diameter >6cm) (Vaishnavi, 2010). Surgical interventions are often required to prevent further complications and death.

1.4.4 **Recurrent CDI (RCDI)**

One of the characteristic features of CDI is its propensity to recur. Recurrences occur in approximately 20-30% of primary CDI cases, and 40-60% of recurrent cases (McFarland *et al*., 2002; Paredes-Sabja & Sarker, 2012). Recurrence may be either due to relapse or reinfection and this has been demonstrated using several enhance typing methodologies, including whole-genome sequencing (WGS)(Eyre *et al*., 2014; Marsh *et al*., 2012). Approximately 10-40% of patients experience recurrent symptoms of CDAD within 8 weeks after successful initial therapy (Vancomycin or metronidazole)(McFarland *et al*., 1999). Recurrence is often associated with treatment failure where *C. difficile* has not been successfully eradicated from the gut following the cessation of antibiotic therapy, and patients become infected with the same strain or another strain(Marsh *et al*., 2012). Many studies show that fewer recurrences occur after treatment with oral vancomycin, however, recent studies indicated that oral fidaxomicin is more effective in the treatment of recurrent CDI(Cornely *et al*., 2012; Eyre *et al*., 2014; Chilton *et al.,* 2016; Louie *et al*., 2012).*In vitro* CDI gut model studies suggest that fidaxomicin becomes sequestered into biofilms, persists on spores by adhering to the exosporium and therefore inhibits outgrowth and proliferation by remaining active in the gut for longer than vancomycin(Chilton *et al*., 2016, 2014). Recurrent CDI is a significant socioeconomic burden as it increases the length of hospital stay and overall cost of hospitalization. The pathogenesis of recurrent CDI is not clear, however, studies suggest that recurrence may be due to an impaired immune response to *C. difficile* toxins or and alteration of the colonic microbiota (Cornely *et al*., 2012; Louie *et al*., 2012).

1.5 DIAGNOSIS

1.5.1 Clinical diagnosis

Due to the incidence and severity of CDI, rapid and accurate diagnosis has been sought after for timely patient management and prevention of nosocomial transmission. Accurate diagnosis of CDI relies on the combination of both clinical and laboratory diagnosis(Napolitano & Edmiston, 2017). The Society for Healthcare and Epidemiology of America (SHEA) guidelines on CDI diagnosis recommend that laboratory testing should be performed when one or either of the following clinical presentation are observed; the presence of diarrhoea (≥3 unformed stools in a 24 hour period) or a radiographic evidence of ileus or toxic megacolon or histopathologic findings indicating the presence of pseudomembranous colitis(Bartlett & Gerding, 2008; Cohen *et al*., 2010; Debast *et al*., 2014; Rao & Higgins, 2016).

1.5.2 Laboratory diagnosis

There is currently no single diagnostic test that is used for CDI, instead several methods exist, and are divided into three groups: test for *C. difficile* products (glutamate dehydrogenase (GDH), TcdA and or TcdB); culture methods for the detection of toxin-producing *C. difficile* (Cell culture cytotoxicity neutralisation assay (CCNA) and toxigenic culture (TC)) and Nucleic acid amplification test (NAAT) for *C. difficile* genes (detecting 16S rRNA, toxin genes or genes encoding GDH)(Crobach *et al*., 2016).

1.5.2.1 Cytotoxin detection

Cytotoxin detection techniques (CCNA and TC), are considered the gold standard technique for CDI diagnosis, and both methods detect the presence of *C. difficile* toxins in stool samples (Timothy *et al*., 2011).CCNA is performed by inoculating the monolayer of an appropriate cell

line (i.e. Vero cells) with stool filtrates, which is then observed at 24 and 48h intervals, for the presence of *C. difficile* toxin. This is demonstrated by a cytopathic effect (CPE)(Delme´e, 2001) along with neutralisation of the CPE with a *C. sordellii* antitoxin. Toxigenic culture (TC) is similar to CCNA, however, faecal samples are cultured on a selective differential medium (usually cefoxitin, cycloserine egg yolk agar) first, which in theory will inhibit the growth of other faecal organisms other than *C. difficile* (Wren, 2010). The recovered isolate is then tested for the ability to produce toxin. In addition, toxigenic culture only gives information on whether the *C. difficile* strain can produce toxin. While both methods are reportedly sensitive (94%-100), they appeared to be laborious and time-consuming, which limited their practicality for diagnostic testing (Burnham & Carroll, 2013).

1.5.2.2 Enzyme Immunoassays (EIA)

Enzyme immunoassays (EIAs) have been adopted by most laboratories because of their simplicity, and faster turnaround times (Burnham & Caroll, 2013). Toxin A/B EIAs directly detect free toxins in stools, while GDH EIAs, detects glutamate dehydrogenase (GDH), a metabolic enzyme that is produced by both toxigenic and non-toxigenic strains (Crobach *et al*., 2016). Although both methods are economical, their sensitivity and specificity are very low. Compared to CCNA and TC, the pooled sensitivity of EIAs ranged between 57- 83%, however, their specificity was reported to be 99% (Crobach *et al*., 2016). The pooled sensitivity of GDH EIAs compared to CCNA and TC is 94-96% respectively (Crobach *et al.,* 2016). However, they cannot make a distinction between the presences of toxigenic or non-toxigenic strains and are thus less specific to detect disease.

1.5.2.3 Nucleic Acid amplification tests (NAAT)

The use of NAATs for the detection of *C. difficile* from stool specimens was reported in 1993 (Gumerlock *et al*., 1993). Since then, a variety of NAATs targeting genes encoding TcdB, TcdA,

and/or the binary toxin genes have been developed (Burnham &Carroll, 2013). Available NAATs, include PCR assays, helicase dependent amplification assays and loop-mediated isothermal amplification assay. NAATs are reportedly as sensitive as CCNA and TC (95-96% respectively) and more specific (compared to CCNA and TC 94% and 98 % respectively) than GDH EIA as the only detect toxigenic strains of *C. difficile.* Although NAATs are superior to other diagnostic methods for CDI, this testing method only detects the presence of toxin genes. As a result, it will also detect asymptomatic carriers of toxigenic *C. difficile* (Crobach *et al*., 2016; Peng *et al*., 2018).

1.5.2.4 Two-step algorithms

Owing to the large variation in sensitivity and specificity of the various diagnostic test for CDI, the ability to accurately distinguish between *C. difficile* colonization and disease is difficult (Smits *et al*., 2016). Consequently, the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) recommends a two-step algorithm (Figure 1-9), with either NAAT or GDH-EIA tests (First Step) to maximise diagnostic accuracy(Polage *et al*., 2015). Samples with a negative result from either NAATs or GDH-EIA tests can be reported as negative for CDI, but samples with a positive result are further tested by a toxin-EIA as a second step (Crobach *et al*.2016). Samples confirmed positive by the second toxin- EIA test and then reported as CDI positive (Peng *et al*., 2018).

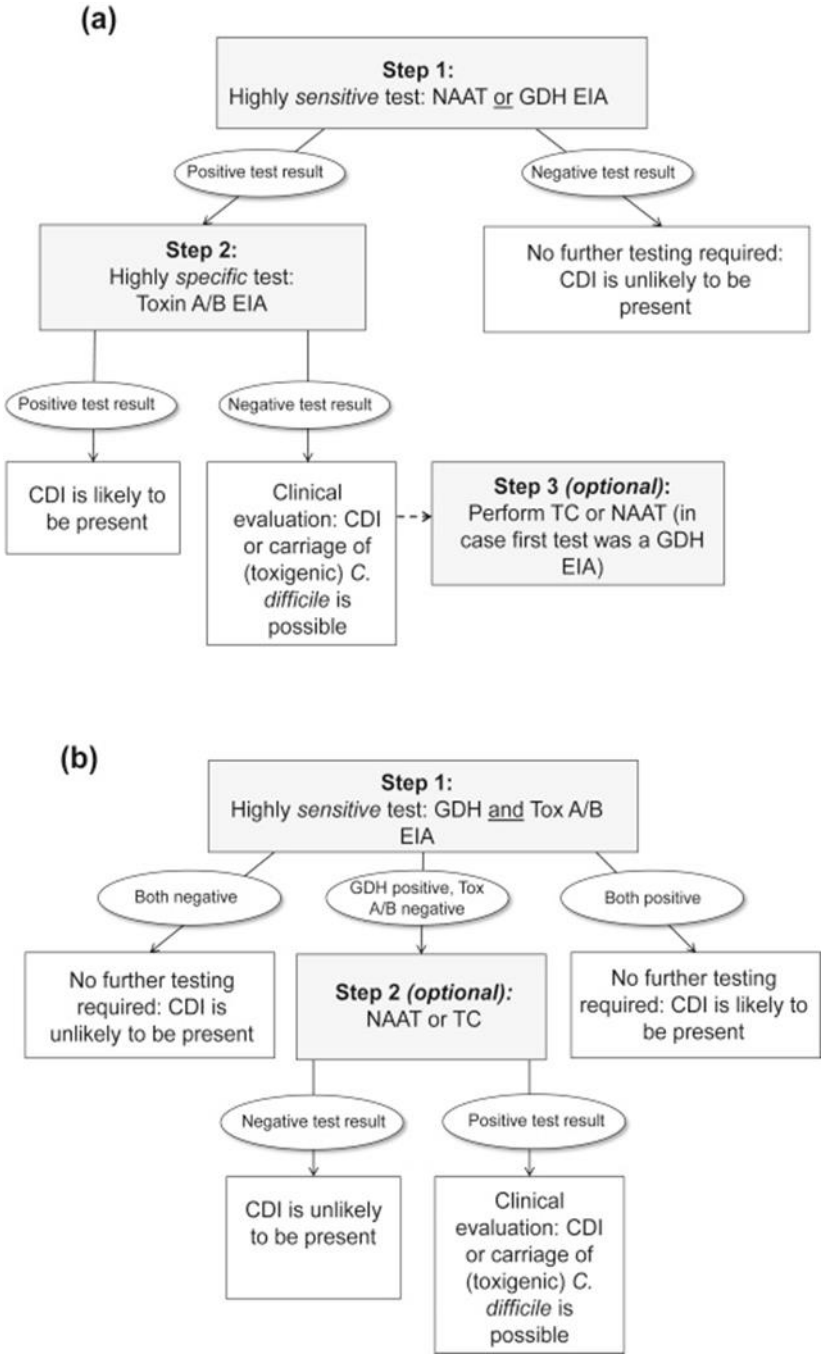


Figure 1-9 Algorithms for CDI testing as recommended by ESCMID guidelines. a) GDH or NAAT- Tox A/B algorithm, b) GDH and Tox A/B- NAAT/TC algorithm (Crobach *et al*., 2016)**.**

1.6 TREATMENT OF CDI

1.6.1 **Metronidazole**

Metronidazole, a nitroimidazole compound has been recommended as a treatment for CDI since the late 1990s (Gerding *et al*., 2008) It is the first choice therapy for treatment for mild to moderate CDI due to its good antimicrobial activity against *C. difficile* strains (Debast *et al*., 2014; Owens *et al*., 2008). Metronidazole, a prodrug, executes bactericidal activity by reduction of the 5-nitro group of the imidazole ring to become cytotoxic to bacterial cells(Edwards, 1993). In anaerobic organisms, metronidazole is reduced by microbial nitroreductases to a cytotoxic nitro radical, which binds non-specifically to bacterial DNA, resulting in damage characterised by helix disruption and strand breakage(Löfmark *et al*., 2010).

Although resistance to metronidazole is currently rare, prior studies have identified treatment failure and relapse post-metronidazole therapy(Musher *et al*., 2005). Reduced susceptibility (MIC >2mg/l) and resistance to metronidazole in *C. difficile* strains (with MIC values of ≥8 to 32mg/l) have been reported in different regions of the world (Baines *et al*., 2008; Freeman *et al*., 2015).A recent pan- European surveillance (*Clos*ER) study, reported metronidazole resistance in 0.11% (MIC <8µg/ml) of strains investigated (Freeman *et al*., 2015). A recent investigation in Eastern China showed that 15.6% of strains recovered from hospitalised patients (between 2012-2015) were metronidazole resistant, including one non-toxigenic isolate (MIC>256µg/ml)(Jin *et al*., 2017). Meanwhile, in the Middle East, 5-5.3% of clinical isolates tested between 2010 and 2011 in Iran are reportedly resistant to metronidazole and 18.3% of strains in Israel were metronidazole resistant (Adler *et al*., 2015; Goudarzi *et al*., 2013; Shayganmehr *et al*., 2015).

Some studies indicated that *C. difficile* exhibits hetero-resistance to metronidazole (Peláez *et al*., 2008). *In vitro* studies suggest that sub-inhibitory concentrations of metronidazole had a role in selecting and maintaining colonies with increased MICs(Moura *et al*., 2013; Peláez *et al*., 2008), making this phenomenon a matter of concern in the treatment of CDI. *C. difficile* mechanism of reduced susceptibility and resistance to metronidazole is yet to be elucidated Metronidazole resistance studies, in *Helicobacter pylori* and *Bacteroides fragilis*, identified the presence of *nim* genes (*nimA*-*G*) associated with resistance (Albert *et al*., 2005; Baines *et al*., 2008; Gal & Brazier, 2004; Kaakoush *et al*., 2009). Recent studies on RT027 and RT010 strains suggest that the mechanism of resistance may be multifactorial, involving alterations in different metabolic pathways, such as the activity of nitroreductases, iron uptake and DNA repair (Chong *et al*., 2014; Moura *et al*., 2014). Data obtained from recent *in vitro* studies demonstrated that subinhibitory concentrations of metronidazole can enhance biofilm formation of RT010, suggesting that biofilms may play a role in *C. difficile* resistance to metronidazole(Vuotto *et al*., 2016).

1.6.1 **Vancomycin**

Vancomycin, a glycopeptide is a first-line therapy for moderate to severe CDI (Debast *et al*., 2014). Vancomycin exerts bactericidal activity by the inhibition of peptidoglycan synthesis (Arthur *et al*., 1996). Vancomycin is a large hydrophilic molecule which is orally administered due to its poor absorption in the gastrointestinal tract and achieves a high concentration in the stool(Gonzales *et al*., 2010; Spigaglia, 2016). Resistance to vancomycin has rarely been reported, however, the emergence of decreased susceptibility (MIC ≥ 4mg/L) has been reported in several studies(Freeman *et al*., 2015; Peláez *et al*., 2002). The resistance mechanism of *C. difficile* to vancomycin has not yet been reported, however, published

reports on vancomycin resistance in *Enterococcus* identified the acquisition of *vanABDEG* genes resulting in the production of peptidoglycan precursors with reduced affinity to glycopeptide antibiotics (Courvalin, 2006). These genes have not been identified in *C. difficile,* however *C. difficile* does possess *vanRSGXYT* putative proteins (VanG-like gene cluster), which is functional but somehow prevented from conferring resistance (Ammam *et al*., 2012; Ammam *et al*., 2013). Leeds *et al* identified mutations in the *rpoC* gene, CD2725, CD3659 and *sdaB* of *C. difficile* isolates with reduced susceptibility to vancomycin, and suggested that this may contribute to reduced susceptibility(Leeds *et al*., 2014).

1.6.2 **Fidaxomicin**

Fidaxomicin is a macrocyclic narrow spectrum, a bactericidal antimicrobial agent, used for the management of CDI with the risk of recurrences (Debast *et al*., 2014). Its mechanism of action results in the inhibition of protein synthesis. It has a minimal impact on the composition of the indigenous gut microbiota, particularly *Bacteroides* species, due to its narrow spectrum of activity (Artsimovitch *et al*., 2012; Louie *et al*., 2012). Fidaxomicin is very active against *C. difficile* isolates *in vitro*, exhibiting lower MICs (0.02-0.025mg/L) (Baines &Wilcox, 2015; Freeman *et al*., 2015). When administered orally, fidaxomicin is poorly absorbed in the gut, resulting in high faecal concentration (>1000µg/g) that exceed the MIC of *C. difficile* (Sears *et al*., 2012). Evidence of reduced susceptibility (MIC 2-4 mg/L) and resistance (MIC 16mg/L) of *C. difficile* to fidaxomicin has been reported (Finegold *et al*., 2004; Goldstein *et al*., 2011; Leeds *et al*., 2014). Whole-genome sequencing analysis of reduced susceptible isolates of *C. difficile* to fidaxomicin revealed mutations in the RNA polymerase β subunit (*rpoB*)gene and CD22120 (*marR* homologue), indicating that these mutations contribute to reduced susceptibility (Leeds *et al*., 2014).

1.6.3 Potential Newer Antibiotics for CDI

1.6.3.1 Rifaximin

Rifaximin is a semisynthetic, nonabsorbable antibiotic that inhibits protein synthesis(Neff *et al., 2013*). It is primarily used for the treatment of traveller's diarrhoea and hepatic encephalopathy (Neff *et al*., 2013). It has demonstrated excellent *in vitro* activity against *C. difficile* isolates(Marchese *et al*., 2000)and has also been shown to be as efficient as vancomycin in animal studies (Kokkotou *et al*., 2008). It has been shown to be effective in the treatment of first episodes and recurrent CDI (Basu *et al*., 2010; Garey *et al*., 2009; Johnson *et al*., 2009; Rubin *et al*., 2011). Although promising, resistant strains of *C. difficile* have already been recovered from patients(Liao *et al*., 2012; Marchese *et al*., 2000).

1.6.3.2 Cadazolid

Cadazolid is a novel fluoroquinolone- oxazolidinone antibiotic which works by the inhibition of protein synthesis and weak inhibition of bacterial DNA synthesis as a second effect (Locher *et al*., 2014). This antibiotic has been shown to exhibit potent *in vitro* activity against clinical *C. difficile* isolates and vancomycin-resistant enterococci, a major risk factor for vancomycin-treated patients(Chilton *et al*., 2014; Seiler *et al*., 2016). In addition, cadazolid reduced spore and toxin production as well as the recurrence of a simulated CDI, in an *in vitro* gut model study (Chilton *et al*., 2014).In phase II clinical trial, cadazolid was effective in the treatment of first episodes or recurrences of CDI when compared with vancomycin(Louie *et al*., 2015). The efficacy of cadazolid was further investigated in two phase III clinical trials but failed to reach its primary endpoint of non-inferiority to vancomycin, in one of the two phases 3 clinical trials. As a result, the further development of cadazolid as a treatment for CDI is highly unlikely(Gerding *et al*., 2019).

1.6.3.3 Ridinilazole

Ridinilazole (formerly SMT-19969) is a narrow-spectrum antibiotic that inhibits DNA synthesis with poor oral bioavailability(Fehér *et al*., 2017). Ridinilazole is reportedly highly active against multiple isolates of *C. difficile* and has demonstrated efficacy in both *in vitro* gut and *in vivo* hamster models(Baines *et al*., 2015; Bassères *et al*., 2016; Freeman *et al*., 2016; Sattar *et al*., 2015). The precise mechanism of action is not fully known, however, it has been shown to significantly reduce toxin production levels, and demonstrated anti-inflammatory activity *in vitro* on human intestinal cells(Baines *et al*., 2015; Bassères *et al*., 2016). Ridinilazole was superior to vancomycin in two phase II trials as it sustained clinical response in the treatment of CDI(Snydman *et al*., 2018; Vickers *et al*., 2017). A Phase III trial is currently ongoing.

1.6.3.4 Tigecycline

Tigecycline is a broad-spectrum glycylcycline antibiotic that inhibits protein synthesis (Feher *et al*., 2017). There is evidence to show that tigecycline is effective in the treatment of CDI (Fantin *et al*., 2015; Kim *et al*., 2014; Kundrapu *et al*., 2015; Larson *et al*., 2011). Tigecycline has proven to be efficient in the inhibition of toxin production and sporulation of *C. difficile in vitro* (Aldape *et al*., 2015; Garneau *et al*., 2014). However, due to its wide spectrum activity, tigecycline may alter the intestinal microbiota significantly, thus there is a potential of facilitating primary and recurrent CDI(Bassis *et al*.,2014).

1.6.4 Microbiome therapy

1.6.4.1 Faecal microbiota transplantation

Faecal microbiota transplantation (FMT) has emerged as a promising therapy for multiple recurrent CDI (Debast *et al*., 2014). Prior to FMT, donors are rigorously screened in order to prevent the transmission of communicable diseases(Bakken *et al*., 2011). Subsequently, the

faeces of a healthy donor (Figure 1-10) is introduced into the gut of patient with recurrent CDI through a nasogastric tube, colonoscopy or rectal enema (Lee *et al*., 2016; Youngster *et al*., 2014). The aim of FMT is to restore intestinal microbiota by re-colonising the gut with the population of commensal organisms that have been disrupted by antibiotics and rebuild colonisation resistance(Braun *et al*., 1996; Vincent *et al*., 2016).FMT has shown efficacy in the treatment of multiple recurrent CDI and fulminant CDI(Emanuelsson *et al*., 2014; Gravito-Soares *et al*., 2017; Lagier *et al*., 2015; Lee *et al*., 2016; Tabbaa *et al*.,2018; Youngster *et al*., 2014). Although FMT has been successful over the years, the long -term safety and efficacy of this therapy still remain unclear(Sbahi & Di Palma, 2016; Tabbaa *et al*., 2018).

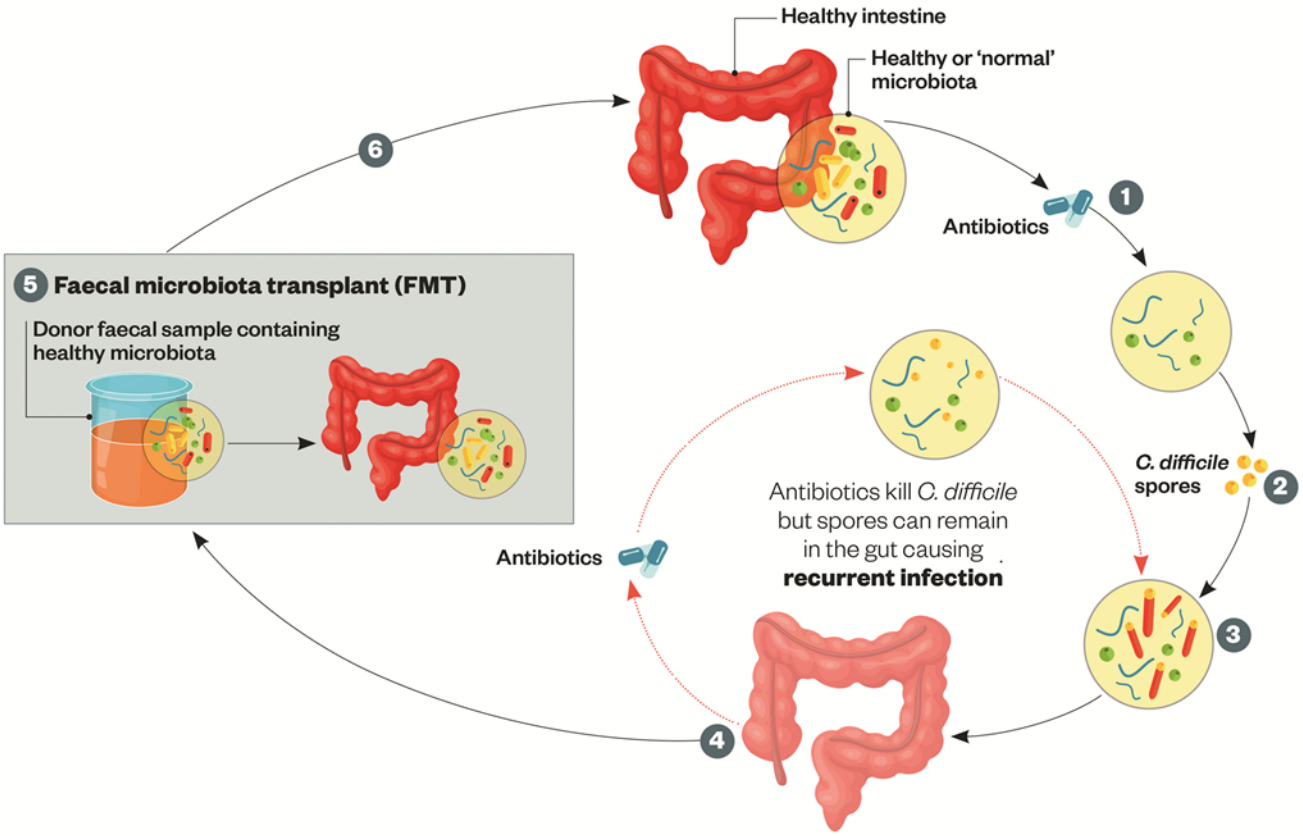


Figure 1-10 *C. difficile* infection and the role of faecal microbiota transplantation. Ingestion of antibiotics results in disruption of the gut flora, **2** Ingestion of spores, **3** Spores germinate resulting in dysbiosis of the gut flora, **4** Development of CDI, **5** FMT and **6** Restored microbiota(Geoghegan *et al*.,2017).

1.6.4.2 Stool- substitute therapies

Several studies have investigated the efficacy of stool- substitute therapies in the treatment of CDI. Khanna *et al* investigated the efficacy of a spore mixture (SER-109) to prevent recurrent CDI in a phase Ib clinical trial(Khanna *et al*., 2016). The spore mixture was derived from stool samples of healthy donors. After elimination of the rest of the microbiota with ethanol, the spore mixture comprised of *Firmicutes* spores. The aim of this therapy is to restore the microbiota diversity through the competition of strains with *C. difficile* in the gut. Promising results were achieved in the phase Ib trial as the gut microbiota diversity was increased, and 29 (96.7%) out of 30 patients with multiple recurrent CDI, had no further recurrences during the study period(Khanna *et al*., 2016). Despite initial results in phase Ib, SER-109 failed to meet the primary efficacy endpoint of reduced CDI occurrences after 8 weeks in a phase II trial. Sere Therapeutics initiated another phase II clinical trial, and recent reports show that SER-109 was able to reduce the abundance of antibiotic resistance genes in recurrent CDI cases while increasing the microbiome diversity in subjects(Ford *et al*., 2018). In addition, higher doses of SER-109 resulted in an increase of secondary bile acids and the prevention of recurrent CDI in the gut model (Henn *et al*., 2018). A phase 3 clinical trial on the efficacy of SER-109, has been initiated by Seres therapeutics.

In a small proof- of principle trial, representative species of the gut (33 purified bacterial strains, MER-1) derived from a single healthy donor stool sample was used in the treatment of two patients with recurrent CDI. The treatment was successful as patients remained free of recurrent CDI episodes for six months (Petrof *et al*., 2013). A clinical pilot study for MER-1 was initiated in 2016 to assess the efficacy of this drug in comparison to vancomycin for the treatment of recurrent CDI.

An earlier study, reported the reduced risk of developing CDI in patients asymptomatically colonised with non- toxigenic *C. difficile* strain (NTCD)(Shim *et al*., 1998). Administration of non-toxigenic *C. difficile* strains has been reported to provide gastrointestinal colonization and prevent CDI in hamsters (Nagaro *et al*., 2013). NTCD strains are thought to compete with toxigenic CDI strains in the gut and thus prevent CDI. Advanced research is being done with the nontoxigenic *C. difficile* strains M3, with encouraging results in a recent phase II trial(Gerding *et al*., 2015). However, the possibility that NTCD strains may acquire toxin genes by toxigenic strains *in vivo* may be a matter of concern as this phenomenon has been demonstrated *in vitro* by Brouwer *et al*. (2013). The transconjugation of the pathogenicity locus, which contains toxin A and B genes from toxigenic strains to NTCD was accomplished *in vitro* however, it remains to be determined if this transfer would be possible *in vivo* or in laboratory conditions which closely reflect the conditions within the human colon(Brouwer *et al*., 2013).

1.6.4.3 Probiotics

As defined by the Food and Agriculture Organisation (FAO) of the United Nations (UN) and the World Health Organisation (WHO), probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit to the host” (FAO/WHO, 2002). Probiotics have been suggested as both a prophylactic and treatment in CDI, however, studies have given variable results with regard their effectivity in the treatment of CDI (Lawrence *et al*., 2005; McFarland *et al*., 1994). Various species of probiotics have been studied, but the most common probiotics used are *Saccharomyces boulardii* and *Lactobacillus rhamnosus* (Lau & Chamberlain, 2016; Lawrence *et al*., 2005; McFarland *et al*., 1994). It has been reported that *Saccharomyces boulardii*, secretes a protease which digests toxin A and B molecules and its brush border membrane receptor(Castagliuolo *et al*., 1996; Castagliuolo *et al*., 1999). In

addition, this yeast has been shown to inhibit *C. difficile* adherence to Vero Cells *in vitro* (Tasteyre *et al*., 2002). The aim of probiotics as therapy is to re-populate the gut and prevent CDI through the action of colonisation resistance (Lau & Chamberlain 2016). Many studies that have investigated the effectiveness of probiotics in either the prevention or treatment of CDI, however, these results appear to be conflicting(Allen *et al*., 2013; Johnston *et al*., 2012; Susanne *et al*., 2012). Recent randomised control trials, demonstrated the effectiveness of this therapy, as probiotic supplementation led to a significant reduction in the risk of developing CDI in patients receiving antibiotics (Lau & Chamberlain 2016). Although argued by some that there is no harm in taking probiotics as a preventative measure; in a severely immunocompromised patient and infant's, probiotics can still cause infection (Munoz *et al*., 2005)

1.6.5 Therapies based on toxins

1.6.5.1 Tolevamer

Tolevamer is a novel high molecular mass (>400kDa) non-antimicrobial anionic polymer proposed for the treatment of CDI. Tolevamer targets *C. difficile* toxins A and B and binds non-covalently to neutralise their activity. Unlike traditional antibiotics, a major advantage of this therapy is that tolevamer has no antimicrobial activity, as a result, there is no disruption of the normal gut microflora (Barker *et al*., 2006). The original tolevamer compound (GT160-246) attenuated CDI severity and recurrences in preclinical studies(Kurtz *et al*., 2001). Despite, its demonstrated efficacy in the treatment of CDI Phase III clinical trials were ended prematurely as results showed that tolevamer was not as effective as vancomycin in treating CDI (Louie *et al*., 2006; Peppe *et al*., 2008).These results were further replicated in an *in vitro* gut model study(Baines *et al*., 2009).

1.6.6 **Immunotherapy**

1.6.6.1 ***Clostridium difficile*** **toxoid vaccine (ACAM-CDIFF)**

ACAM-CDIFF is a *C. difficile* toxoid vaccine against *C. difficile* toxin A and B, developed by Sanofi Pasteur. This vaccine stimulates an immune response through the production of serum IgG anti-toxin A and serum IgG anti-toxin B antibodies(Anosova *et al*., 2013; Anosova *et al*., 2015). After a positive immunological response was obtained in both phase I and II trials(de Bruyn *et al*., 2016; Foglia *et al*., 2012; Greenberg *et al*., 2012), a phase III randomised control trial was launched and is currently ongoing.

1.6.6.2 **Monoclonal antibodies (MAbs)**

Monoclonal antibodies (MAbs) against *C. difficile* cytotoxins have been proposed for the treatment of CDI. Initially, the efficacy of a single administration of MAbs for either toxin A or B was debatable (Corthier *et al*., 1991; Steele *et al*., 2013). Until enhanced efficacy and lower recurrences were observed when the two antibodies (toxin A; actoxumab and toxin B; bezlotoxumab) were administered together in a hamster model(Babcock *et al*., 2006).This combination was further compared to standard CDI treatment in a phase II trial, significantly lower recurrence rates were reported(Lowy *et al*., 2010). Despite good results with this combination, the efficacy of bexlotoxumab in preventing CDI recurrence was confirmed in phase III trials, however, its combination with actoxumab did not show any additional benefit (Wilcox *et al*., 2017). Based on these results the US Food and Drug Administration (FDA) recently approved bexlotoxumab, it was made available in the first quarter of 2017 (FDA, 2016), it is now administered as standard therapy for CDI.

1.6.6.3 Passive immunization

Previous studies have indicated the potential of colostrum hyper-immune bovine colostrum (HBC) as a therapy for CDI (Kelly *et al*., 1996; Steele *et al*., 2013). HBC was reportedly as effective as metronidazole in the treatment recurrent CDI in a discontinued randomised phase II study (Mattila *et al*., 2008). It was recently demonstrated to alleviate the symptoms of CDI in gnotobiotic piglets without disrupting the normal gut microbiota (Sponseller *et al*., 2015). Meanwhile, a murine study demonstrated the ability of HBC to prevent and treat primary CDI and prevent recurrences(Hutton *et al*., 2017).

1.6.7 **Phage Therapy**

Phages are viruses that infect and replicate within bacteria and their use as therapeutic agents began shortly after their discovery in the 20th century(Twort, 1915). Previous studies have described isolated phages that can specifically lyse clinically relevant *C. difficile* ribotypes but their use in CDI treatment is still limited(Fortier & Moineau, 2007; Goh *et al.*, 2005; Govind *et al.*, 2006; Sekulovic *et al.*, 2014).This mainly due to the lack of strict virulent phages that target *C. difficile*. A recent study examined the therapeutic potential of seven isolated *C. difficile* phages (CDHM 1-6) on multiple *C. difficile* ribotypes(Nale *et al*., 2016). CDHM3 was identified as the phage with broadest therapeutic potential as it was able to infect 31 out of 80 strains from 12 different ribotypes. Additionally, Nale and colleagues observed complete lysis of *C. difficile in vitro* following treatment with a four-phage cocktail (CDHM1, 2, 5, &6)(Nale *et al*., 2016). Similarly, the same phage cocktail reduced *C. difficile* colonisation in a hamster model and in *Galleria mellonella* larva models(Nale *et al.*, 2016a; Nale *et al*., 2016b; Shan *et al*., 2018). More recent studies using *in vitro* batch fermentation human colon models have provided useful insights into the specificity of *C. difficile* phages and shown that phages do

not have a deleterious impact on members of the gut microbiota(Meader *et al*., 2010; Meader *et al*., 2013; Nale *et al*., 2018).

1.7 ANTIMICROBIAL SUSCEPTIBILITY AND RESISTANCE IN *C. DIFFICILE*

1.7.1 Antibiotic resistance patterns

The antimicrobial resistance patterns in *C. difficile* varies considerably according to different countries and different geographical location. The recent pan- European surveillance study that included 22 European countries, report resistance to rifampicin, moxifloxacin and clindamycin in multiple ribotypes(Freeman *et al*., 2018). In addition, antimicrobial susceptibility studies of *C. difficile* published between 2012 and 2015 from different countries show that most *C. difficile* isolates are resistant to clindamycin, cephalosporins, erythromycin and fluoroquinolones (Goudarzi *et al*., 2013; Obuch-Woszczatyński *et al*., 2014; Pirš *et al*., 2013; Tenover *et al*., 2012).

1.7.2 Antibiotics linked to induction of CDI

Fluoroquinolones are a broad spectrum of antimicrobial agents strongly associated with the development of CDI (Spigaglia *et al*., 2010). The *in vitro* activity of the older fluoroquinolones, such as ciprofloxacin, has been reported to be moderate or poor against anaerobes, including *C. difficile.* Conversely, the third- and fourth-generation fluoroquinolones (e.g. moxifloxacin, levofloxacin) are characterised by improved activity against anaerobic bacteria(Redgrave *et al.*, 2014). Fluoroquinolones are direct inhibitors of DNA synthesis; by binding to the enzyme-DNA complex, they stabilize DNA strand breaks created by DNA gyrase and topoisomerase IV. Alterations in the quinolone – resistance determining region (QRDR) of either *gyrA* and/or *gyrB* genes, which encode the A and B subunits of enzymes responsible for bacterial DNA supercoiling and separation of the two DNA fragments after replication, are reportedly the

major mechanism of resistance identified in *C. difficile* (Baines & Wilcox, 2015). Several amino acid substitutions have been identified in both *gyrA* and *gyrB* genes, but the most common substitution identified in resistant strains is a point mutation Thr82Ile in GyrA(Ackermann *et al*., 2001; Dridi *et al*., 2002; Kuwata *et al*., 2015; Spigaglia *et al*., 2011). In the recent 3- year pan-European antimicrobial susceptibility surveillance study (*Clos*ER), moxifloxacin resistance was particularly common among prevalent *C. difficile* ribotypes(RT027, RT001, RT017, RT018, RT356 and RT198), and characterised by MIC ≥32mg/L(Freeman *et al*., 2018; Freeman *et al*., 2015).

Penicillin's including cephalosporins and aminopenicillins are broad-spectrum β-lactam antimicrobial agents that are well recognised for their propensity to induce CDI (Baines & Wilcox, 2015). Resistance to β-lactams (particularly cephalosporins) has been reported in *C. diffficile*, however, the mechanisms of resistance is yet to be elucidated(Baines *et al*., 2013; Isidro *et al*., 2018; Snydman *et al*., 2011). Genomic analysis of *C. difficile* 630 genomes revealed coding sequences for β-lactamase like proteins and penicillin-binding proteins (PBPs) that may be potentially involved in resistance to cephalosporins(Sebaihia *et al*., 2006). However, further studies are required to clarify their role. Studies on imipenem resistance in ribotype 017 isolates revealed two amino acid substitutions in the transpeptidase domain of the penicillin-binding protein (*pbp*$_3$-Cys721Ser and *pbp*$_1$-Ala555Thr)(Isidro *et al*., 2018a; Isidro *et al*., 2018b).They authors suggested this may contribute to imipenem resistance by reducing the affinity of imipenem binding to PBPs.

Majority of *C. difficile* strains are resistant to members of the antimicrobial group macrolide-lincosamide-streptogramin B (MLS_B), particularly erythromycin and clindamycin (Solomon *et al*., 2011). Resistance to erythromycin (MIC ≥ 8mg/L) and clindamycin (MIC ≥16-256 mg/L) has been identified in multiple *C. difficile* ribotype, especially ribotype 001, 017, 018 and 027.

MLS_Bs are protein synthesis inhibitors which act by binding to the ribosome 30S subunit. Multiple resistance mechanisms have been identified, however, ribosomal methylation, mediated by erythromycin ribosomal methylase (*erm*) genes is the widespread mechanism of resistance (Patterson *et al.*, 2007; Solomon *et al*., 2011; Spigaglia *et al*., 2011; Spigaglia *et al*., 2005).MLS_b resistance in *C. difficile* is encoded by the *ermB* gene, which is located on a 9.6 kb mobilisable nonconjugative element called Tn*5398* (Farrow *et al*., 2001; Mullany *et al*., 1995). ErmB mediates resistance by methylation of bacterial 23S rRNA, leading to inhibition of antimicrobial activity. In addition, other transposable elements such as Tn*5398*-like derivatives, Tn*6194* and Tn*6215* and the *cfr* (chloramphenicol florfenicol resistance) gene have been implicated in *C. difficile* resistance to MLS_B (Dingle *et al*., 2014; Wasels *et al*., 2015).

1.7.3 **Other antibiotics**

Tetracyclines are broad-spectrum antibiotics that consist of four fused cyclic rings and are effective against aerobic and anaerobic Gram-positive and Gram-negative bacteria (Chopra & Roberts, 2001). This family of antimicrobials are considered a low risk for CDI induction. Like MLS_B antibiotics, tetracyclines are protein synthesis inhibitors that act at the ribosomal level, preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (Chopra & Roberts 2001). Published reports indicated that *C. difficile* resistance to tetracycline is primarily due to either energy-dependent efflux or ribosomal protection via *tet* genes(Dong *et al*., 2014; Mullany *et al*., 2012). The *tet* class of genes are usually carried on several transposable elements such as Tn*5397*, Tn*916* or Tn*916*-like family and Tn*6164*. Many studies have shown that resistant *C. difficile* strains carry the *tetM* gene on the conjugative transposon Tn*5397*(Dong *et al*., 2014; Jasni *et al*., 2010; Mullany *et al*., 2012)and in some cases the *tetW* and *tetX* genes (Fry *et al*., 2012; Spigaglia *et al*., 2008). Both TetM and TetW

are cytoplasmic proteins with homology to elongate factors (EF-Tu and EF-G) that protect ribosomes from the action of tetracyclines by reducing their susceptibility (Baines & Wilcox 2015). Spigaglia and colleagues demonstrated the concurrent presence of *tetM* and *tetW* in three isolates of ribotype 048 and 012 (Spigaglia *et al*., 2011).

Recently, linezolid a synthetic antibiotic belonging to a new class of antimicrobial agents, known as oxazolidinones has been reported to reduce toxin levels in *C. difficile*(Baines *et al*., 2011). Linezolid is principally active against Gram-positive bacteria and is not currently used to treat CDI(Marín *et al*., 2015). The mechanism of action of linezolid is by inhibiting protein synthesis at the ribosomal level through interaction with 23s rRNA(Baines *et al*., 2011). Resistance to linezolid in Gram-positive bacteria is uncommon but has been reported mainly in clinical isolates of coagulase-negative staphylococci, *Staphylococcus aureus*, *Enterococcus faecaelis*, and *Enterococcus faecium* (Shen *et al*., 2013). One or more of 3 resistance mechanisms to linezolid in these organisms have been reported: 1) point mutations within the domain V region of 23S rRNA gene, 2) mutations/deletions in ribosomal proteins L3 (*rplC* gene) and L4 (*rplD* gene), and 3) methylation of position A2503 of 23SrRNA mediated by an rRNA methyltransferase(Long *et al*., 2010; Marín *et al*., 2015; Tewhey *et al*., 2014).*C. difficile* resistance to linezolid has been described in clinical isolates, although possible mechanisms of resistance in this species have not been elucidated (Ackermann *et al*., 2003; Freeman *et al*., 2016; Marín *et al*., 2015; Peláez *et al*., 2002). Sequencing results by Marín *et al* (2015) revealed the presence of the *cfr* gene in 7 out 9 *C. difficile* linezolid-resistant strains, suggesting that the presence of this gene may be the mechanism of resistance. However, the exact contribution of these genes in *C. difficile* resistance to linezolid is still unknown. Consistent with these reports, Freeman *et al* (2016), found a high level of linezolid resistance

(≥16mg/L) in RT001, RT017, RT027 & RT356, however, the mechanism of resistance in these isolates was not investigated.

1.7.4 Multi-Drug Resistance in *C. difficile*

Multidrug resistance (MDR), is defined as resistance to more than two antimicrobial classes. The emergence of multidrug resistance (MDR) among *C. difficile* isolates, particularly hypervirulent BI/NAP1/027, RT078, RT001/072 has been reported in many studies (Goudarzi *et al*., 2013; Senoh *et al*., 2015; Tenover *et al*., 2012). Analysis performed by Spigaglia and colleagues in a European based study on 316 clinical isolates of *C. difficile* indicated that 55% of clinical isolates were MDR (Spigaglia *et al.,* 2011). Data extrapolated from 13 published studies indicated that MDR patterns among *C. difficile* isolates are characterised by resistance to clindamycin, fluoroquinolones, erythromycin and cephalosporins (Spigaglia *et al*., 2016). Recent reports indicated that MDR of *C. difficile* isolates in Europe is associated with emergent ribotypes, notably 017, 018, 198 and 356 (Freeman *et al*., 2015, 2018). These strains are resistant to clindamycin, erythromycin, moxifloxacin and rifampicin, however, in Korea and Japan, MDR strains of RT018 are resistant to clindamycin, erythromycin and moxifloxacin(Kim *et al*., 2012; Senoh *et al*., 2015). In Poland and the Czech Republic, MDR is associated with strains of RT176, which is resistance to erythromycin, moxifloxacin, ciprofloxacin and rifampicin(Krutova *et al*., 2015; Obuch-Woszczatyński *et al*., 2014).

1.8 EPIDEMIOLOGY AND TYPING

1.8.1 *C. difficile* Epidemiology in Europe

In 2005, the European Study Group on *Clostridium difficile* (ESGCD) performed a 2-month survey of 38 hospitals in 14 different European countries. This survey was carried out to obtain an overview of the incidence of CDI in Europe. The mean incidence of CDI was 2.45

cases/10,000 patient days, but this figure varied widely among the participating hospitals (minimum to maximum range; 0.1-7.1)(Barbut *et al*., 2007; Freeman *et al*., 2010). PCR ribotype 001 was the most common toxigenic isolate (1.), followed by ribotype 014 (9%); PCR ribotypes 002, 012, 017, 020 and 027 contributed to 6% of all toxigenic isolates, whereas ribotype 078 was found in 3% of toxigenic isolates. The distribution of PCR ribotypes varied among the countries studied; however, PCR ribotype 002 was predominant in Switzerland (28.6%) France (12.1), and Italy (10.5 %). Higher incidence of CDI was observed in countries that had experienced recent outbreaks attributed to ribotype 027 strains (i.e. Netherlands, Belgium and France) than countries where 027 had not been reported (Italy, Turkey and Greece) (Barbut *et al*., 2007). Additionally, patients infected with ribotype 027 were more likely to have more severe diseases than patients who were infected by another PCR ribotype.

In November of 2008, the European Society of Clinical Microbiology and Infectious Disease (ECDIS) Study Group performed the first pan-European Surveillance study. This study was sponsored by the European Centre of Disease Prevention and Control (ECDC) and was carried out in 69 hospitals in 28 countries in Europe (Bauer *et al*., 2011). The incidence of *C. difficile* varied across hospitals (weighted mean 4.1 per 1000 patient's days per hospital, range 0.0-36.3). PCR ribotype 014/020 (16%), 001 (9%) and 078 (8%) were the most prevalent ribotypes. Additionally, ribotypes 001, 002, 012, 014, 015 018, 027 and 078 were the most common ribotypes seen across Europe (Public Health England, 2016).

In November 2014, reports from a European multicentre, prospective, bi-annual point-prevalence study of *C. difficile* infection in hospitalised patients with diarrhoea (EUCLID study) were released. This study included 20 European countries, with 482 participating hospitals which were queried for their methods and testing policy for CDI. The reported cases of CDI

varied between countries with a mean of 7.0 cases per 10,000 patient bed days (Davies *et al*., 2014). The diversity of *C. difficile* ribotypes across Europe was much greater in this study compared with prior European studies (Bauer *et al*., 2011; Freeman *et al*., 2010). The most common PCR ribotypes encountered in this study included: 027, 001/072, 014, 002,140, 010, 020, 018, 015, and 005.The prevalence of 027 had risen more than threefold (from 5% to 18 %) although striking intercountry variation was noted: high endemicity of ribotype 027 was shifted from the UK and Ireland in 2008 to Germany and eastern Europe in 2012-13 (Davies *et al*., 2014). In a recent study, ClosER *(Clostridium difficile* European Resistance), the most common RTs encountered were RT: 027(12%), 001/072 (9%), 078 and 014 (both 8 %). Ribotype prevalence differed markedly according to country, with some exhibiting predominant RTs, while others showed a diversity of types. Recent surveillance studies of *C. difficile* in children show that PCR ribotype 265 is predominantly found in children, in the Netherlands(van Dorp *et al*., 2017).

Since the creation of the *Clostridium difficile* Ribotyping Network (CDRN) in the UK, the incidence of CDI has declined dramatically. According to CDRN reports (2008/2009), the prevalent *C. difficile* ribotypes in the UK were PCR ribotypes 027 (36%), 106 (13%) and 001 (7%). Recently; the CDRN reported (2013-2015) a decrease in the once prevalent ribotypes (027, 106 and 001) while other ribotypes have emerged (078, 005, 014/020 and 015), including ribotype 002(Figure 1-11), which is now the most prevalent ribotype in the UK (Fawley *et al*., 2016; Health Protection Scotland, 2018; Public Health England, 2016; Public Health Wales, 2019). The drivers for its increased prevalence (CD002) remain unclear. The potential drivers may be associated with the organism, antimicrobial prescribing policies in the hospital or greater community-associated CDI importing into the nosocomial environment.

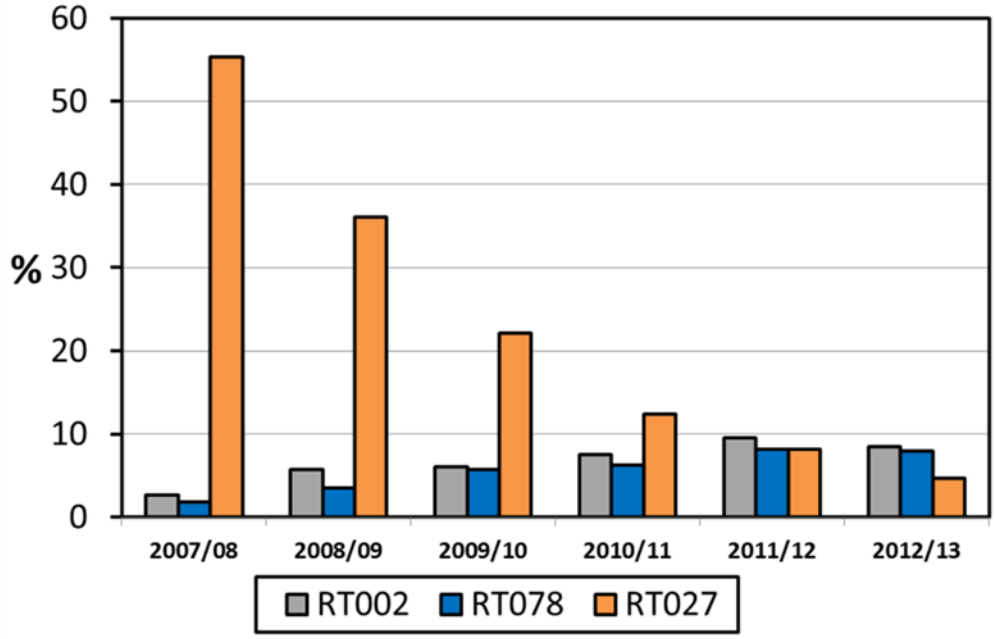


Figure 1-11 UK *C. difficile* RT prevalence 2007-2013 (Data supplied courtesy of CDRN).

1.8.2 *C. difficile* Epidemiology in North America

Since the early 2000s, there has been a significant increase in the incidence and severity of CDI in North America (Freeman *et al*., 2010; Lessa *et al*., 2012). Reports from 28 community hospitals in southern USA suggested that *C. difficile* had become the most common cause of healthcare-associated infection (replacing MRSA) (Miller *et al*., 2010). Additional reports indicated that hospital discharge of CDI cases in the United States had increased from 3.82 to 8.75 per 1000 discharge from 2000 to 2008 respectively (Lessa *et al*., 2012).In Quebec, Canada, a retrospective study of all CDI cases revealed a fourfold increase in incidence from 35.6 to 156.3 cases per 100,000 population from 1991 to 2003 (Pepin *et al*., 2004).

The increased incidence of CDI in the United States and Canada has been largely attributed to the spread of emergent hypervirulent strain BI/NAP1/027 (ribotype 027) strain. This epidemic strain 027 has now been found in at least 40 states in the United States and all Canadian provinces (O'Connor *et al*., 2009).

1.8.3 *C. difficile* Epidemiology in Asia and Australia

The distribution of *C. difficile* ribotypes in Asia and Australia is significantly different from Europe and North America. Although outbreaks in North America and Europe have been caused by *C. difficile* PCR ribotypes 027 and 078, they appear to have only caused sporadic cases of CDI in Asia (Singapore, Hong Kong, Korea and Japan)(Collins *et al*., 2013). Based on published data from different Asian countries, the most predominant ribotypes in this geographic region are 001, 002, 014, 017 and 018(Borren *et al*., 2017; Chow *et al*., 2017; Collins *et al*., 2013; Hung *et al*., 2016).

In 2009, a surveillance study was carried out in Hong Kong, China. This study included 345 *C. difficile* toxigenic isolates from 307 patients in Hong Kong. *C. difficile* PCR ribotype 002 was the most predominant ribotype (9.4%), with a higher sporulation frequency (20.2%) and resistance to fluoroquinolones. Also, there was a significant increase the incidence rate (from 0.53 to 0.95 per 1000 admissions) and positive detection rate of toxigenic *C. difficile* (from 4.17% to 6.28% between 2004-2008 and 2009). This increase was temporally related to the predominance of CD002 (Cheng *et al*., 2011). Meanwhile, in Japan ribotype smz/018 (referred to as ribotype 018 in Europe) has persisted for over a decade as the most common prevalent *C. difficile* ribotype (Senoh *et al*., 2015).

In 2012, a prevalence study involving 175 hospitals in Queensland, Australia was performed. The three most common ribotypes isolates were *C. difficile* PCR ribotype 002 (22.9%), 014 (13.3%) and the binary toxin-positive PCR ribotype 244 (8.4%)(Huber *et al*., 2014).

1.8.4 *C. difficile* in Animals

C. difficile has been found as a commensal organism and pathogen to farm, wild and domesticated animals. Although no direct evidence of zoonotic transmission of *C. difficile* to

humans has been established, several studies identified, farm, domesticated and wild animals as potential reservoirs for *C. difficile*(Andrés-Lasheras *et al*., 2018; Bakri, 2018; Hensgens *et al*., 2012; Knetsch *et al*., 2018; Rodriguez-Palacios *et al*., 2007; Zhang *et al*., 2019). Several studies identified a considerable ribotype overlap between bovine, porcine, canine and human isolates of *C. difficile*, with certain strains being largely species-specific, while others were found across multiple species, including humans (Lessa *et al*., 2012; Pirš *et al*., 2013; Pirs *et al*., 2008; Rabold *et al*., 2018).In particular, PCR ribotype 078 has been increasingly identified as predominant strain in cattle and pigs in the United States and Europe and also as important pathogens in humans (Goorhuis *et al*., 2008). In the Netherlands, 4%- 11% of humans with CDI carried ribotype 078, between February 2005 and February 2008 the incidence of infection with this strain increased, making it the second most common ribotype isolated from humans in the Netherlands. Studies in the United States and Europe have indicated that certain *C. difficile* genotypes (ribotypes) may be found in farm animals (Hensgens *et al*., 2012; Janezic *et al*., 2014; Schneeberg *et al*., 2013) and retail meats and the same ribotypes are known to cause community-associated human infections. PCR ribotype 078 is often reported as the major animal associated *C. difficile* typically found in pigs. However, PCR ribotypes (014/020 and 002) are also among the most prevalent animal-associated *C. difficile* strains worldwide (Janezic *et al*., 2014).

1.8.5 **Community-acquired CDI (CA-CDI)**

Although CDI is historically regarded as a nosocomial infection, it has increasingly been recognised in the community as the cause of diarrhoea (Fawley *et al*., 2016; Furuya-Kanamori *et al*., 2017; Hensgens *et al*., 2012; Kotila *et al*., 2016; Lessa *et al*., 2015). Community-acquired CDI (CA-CDI) is defined as CDI occurring in the community or within 48 hours of admission to

hospital, with no onset symptoms and no prior hospitalisation in the past 12 weeks(Khanna *et al*., 2012). Several studies reported that strains isolated from patients in the community (Ribotypes 002, 014/20, 018), are similar to strains isolated from patients in the nosocomial environment, suggesting a common source of infection or transmission between both settings (Eyre *et al*., 2013; Fawley *et al*., 2016; Furuya-Kanamori *et al*., 2017; Hensgens *et al*., 2012). Nevertheless, certain ribotypes have been frequently implicated in CA-CDI cases across different geographical locations. In the United States, the estimated incidence of CA-CDI at 30 to 120 cases per 100,000 persons per year (Lessa *et al*., 2015). Meanwhile, the incidence in the Netherlands is estimated at 390 to 730 per 100, 000 person per year (Bouwknegt, van Dorp, & Kuijper, 2015). The most commonly recovered ribotypes were 002 and 078, each identified in 11% of samples. These same ribotypes accounted for 7.6% and 3.4%, respectively, of CA-CDI in the US (Bouwknegt *et al*., 2015; Lessa *et al*., 2015), suggesting a zoonotic mode of transmission in the community, as these ribotypes have been recovered from animals (Bouwknegt *et al*., 2015; Janezic *et al*., 2014). In a recent study ribotype, 002 was the most predominant ribotype, frequently associated with CA-CDI in Australia (Furuya-kanamori *et al*., 2017). Recent Eurosurveillance data on CA-CDI cases in England (2011- 2013) identified RT 002(13.5%) as the most frequently occurring ribotype in CA- CDI cases (Fawley *et al*., 2016). While RT078 and RT027 were fourth and eighth most frequently identified ribotype in CA-CDI cases. More recently Dauby and colleagues (Dauby *et al*., 2017) reported a fatal incident of *C. difficile* bacteraemia in the community, the *C. difficile* strain recovered from the patient's stools was identified as PCR ribotype 002 through multi-locus variable number tandem repeat analysis. Most patients who develop CA-CDI do not have typical risk factors for CDI, such as antibiotic treatment or recent hospitalisation (Hensgens *et al*., 2012; Wilcox *et al*., 2008).

1.8.6 **Typing of *C. difficile***

1.8.6.1 Pulse- field gel electrophoresis (PFGE)

PFGE is one of the first molecular typing methods used for *C. difficile.* PFGE involves the resolution of large fragments of DNA generated from the digestion of genomic DNA using rare-cutting enzymes such as *SmaI* and *SacII* (Janezic & Rupnik, 2010; Killgore *et al*., 2008). The resultant banding patterns are referred to as pulsotypes. Initially, the attempt to apply PFGE to typing *C. difficile* was challenging as many isolates were un-typeable due to the sensitivity of their DNA degradation. However, this issue was resolved when a newly improved protocol (incorporating 200µM of thiourea) was introduced, eventually, un-typeable isolates were designated(Fawley & Wilcox, 2002; Gal *et al*., 2005). It is considered the standard typing *C. difficile* in North America (Canada &USA)(Huber *et al*., 2013).

1.8.6.2 PCR ribotyping

PCR ribotyping is a strain typing method that is mostly used in Europe and Australia for epidemiological investigations to track transmission and identify emerging variants of *C. difficile*(Janezic & Rupnik, 2010). This method distinguishes strains based on the amplification of the 16S-23S rRNA intergenic spacer (ITS) region using primers that target the 3' end of the 16S RNA and 5' end 23S RNA. The potential of this technique for typing *C. difficile* was initially described in 1993, however, the protocol was long and laborious(Gurtler, 1993). It was later modified by O'Neil and colleagues, who designed new primers closer to the ITS region in order to obtain smaller fragments for improved analysis on agarose gels(O'Neill *et al*., 1996). Using a modified PCR ribotyping technique, Stubbs *et al* identified 116 distinct ribotypes of *C. difficile*(Stubbs *et al*., 1999). This approach was later adopted for routine use by the UK Anaerobe Reference Laboratory, Cardiff. Subsequently, Bidet and colleagues further

optimised the PCR ribotyping technique using new, and more specific primers and obtained bands that are more stable and readable(Bidet *et al*., 1999). Although this technique exhibits good discriminatory power, it is not sufficient for the discrimination of strains of closely related ribotypes such as 027, 106 and 017 (Manzoor *et al*., 2011). There are now more than 900 distinct PCR ribotypes that have been identified and the use of capillary electrophoresis has allowed further discrimination of closely related ribotypes (Dr Jane Freeman– personal communication).

1.8.6.3 Toxinotyping

Toxinotyping is used to differentiate *C. difficile* isolates based on variation in the *PaLoc* (Rupnik *et al*., 1998). The principle of toxinotyping is based on amplifying regions within PaLoc and restricting these regions with enzymes such as; *EcoRI, AccI* and *HincII*. The resultant pattern is then compared to the *C. difficile* reference strain VPI 10463 to determine the toxinotype (Rupnik *et al*., 1998).To date, 34 toxinotypes have been identified(Rupnik & Janezic, 2016). While a good correlation between this method and other typing methods such as; PFGE, ribotyping and serogrouping was found, it is not as discriminatory as some other techniques (Martin *et al*., 2008).

1.8.6.4 Multi-Locus Sequence Typing (MLST)

MLST is a technique that involves examining the DNA sequence variations within multiple housekeeping genes of microbial species after PCR amplification. Sequence variants of each housekeeping gene is assigned a distinct allele number and a combination of these numbers provides a sequence type (ST)(Knetsch *et al*., 2013). MLST was first described for *C. difficile* typing by Lemee and colleagues, who developed typing scheme that involved the analysis of seven housekeeping genes (*aroE, ddl, dutA, tpi, recA, gmk and sodA*) to study the genetic relationship and population structure of a group of *C. difficile* isolates(Lemee *et al*.,2004).

Allelic profiling allowed the definition of 34 different sequence types (STs). In a later scheme by Griffiths *et al*, a different set of seven housekeeping genes (*adk, atpA, dxr, glyA, recA, sodA, and tpi*) were used for epidemiological analysis of a diverse panel of *C. difficile* isolates(Griffiths *et al*., 2010). This was a more robust MLST scheme as it yielded 40 STs and allowed genotyping of *C. difficile* directly from stool specimens. The discriminatory power of MLST appears to be similar to PCR ribotyping, but not sufficient to delineate outbreak cases (Griffiths *et al*., 2010; Killgore *et al* 2008). MLST is recommended as an appropriate tool for phylogenetic studies of *C. difficile* (Knetsch *et al*., 2013).

1.8.6.5 Multi-Locus variable number tandem repeat analysis (MLVA)

MLVA has emerged as the most superior typing method for *C. difficile* due to its higher discriminatory power in monitoring transmission events involved in outbreaks (Eckert*et al*., 2011; Manzoor *et al*., 2011; Marsh *et al*., 2006; van den Berg *et al*., 2007). This method uses PCR to amplify multiple variable number tandem repeat (VNTR) loci and determine the number of repeat sequences per loci by fragment analysis. The level of diversity within the genome is used to resolve phylogenetic relationships of isolates. Since the identification of this technique for typing of *C. difficile,* different methods have been published with the number of VNTR loci used ranging from 2-12 (Eckert *et al*., 2011; Manzoor *et al*., 2011; Wei *et al*., 2011). Wei *et al* suggested that a 10 loci MLVA scheme should be used to detect epidemic clones and a 4 loci scheme to detect outbreaks(Wei *et al*., 2011). MLVA was shown to be a highly discriminatory tool in the investigation of *C. difficile* outbreaks in France, Argentina and the UK (Eckert *et al*., 2011; Fawley & Wilcox, 2011; Goorhuis *et al*., 2009).In England, CDRN currently offers MLVA analysis of *C. difficile* using seven VNTR loci or an expanded panel of 12, as an enhanced service for investigating outbreaks.

1.8.6.6 Whole Genome Sequencing (WGS)

Whole-genome sequencing (WGS) is a high-throughput typing method that differentiates strains based on single nucleotide polymorphisms (SNP), within the bacterial genome(Knetsch *et al*., 2013).WGS has proven to be advantageous in surveillance studies, outbreak identification, and the study of strain transmission events in several bacterial pathogens including *C. difficile*(Eyre *et al*., 2013; He *et al*., 2013, 2010; Köser *et al*., 2012).WGS was able to discriminate ribotype 027 isolates from the US and Europe into 25 distinct genotypes, as well as reveal distinct evolution lineages, and unique antibiotic resistance genes (He *et al*., 2010). Eyre and colleagues used WGS to study the transmission events involved in many cases of *C. difficile* in Oxfordshire. The authors, reported that many cases of *C. difficile* infection thought to be genetically related were in fact genetically distinct, suggesting diverse sources of infection within the nosocomial environment (Eyre *et al*., 2013; Knetsch *et al*., 2013). Although this approach has proven successful, it is predominantly used retrospectively due to its cost, as a result, MLVA is usually performed to determine the genetic relatedness of strains in an outbreak.

1.9 AIMS AND OBJECTIVES

C. difficile PCR ribotype 002 (CD002) is now the most commonly isolated *C. difficile* strain in the United Kingdom. According to statistics generated by the *C. difficile* Ribotyping Network for England and Northern Ireland (CDRN), its prevalence has increased incrementally over the past 7 years, yet the drivers for this increased prevalence remain unclear. The aim of this study is to characterise UK *C. difficile* PCR ribotype 002 (CD002) isolates from different time lineages and assess any phenotypic traits that may help explain the emergence of this ribotype. The objectives are the following:

1. Determination of antimicrobial susceptibilities and resistance mechanisms of CD002 isolates from different time lineages *in vitro* using standard techniques (Agar incorporation MICs and PCR/sequencing of resistance genes)
2. Quantification of biofilm formation of CD002 isolates from different time lineages in different culture media using a crystal violet microtitre plate assay
3. Evaluation of the cytotoxin production profiles, total sporulation capacities and spore adherence abilities of CD002 isolates from different time lineages using standard techniques (Vero cell cytotoxicity assay, batch culture growth experiments, spore adherence assay)
4. Nutrient utilisation analysis of CD002 isolates using a nutrient microarray.
5. Assessment of the comparative *in vitro* fitness using continuous flow fermentation techniques.
6. Proteomics analysis CD002 isolates using gel electrophoresis and LC-MS/MS mass spectrometry analysis.

2 GENERAL MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND CULTURE

2.1.1 *C. difficile* Strains

A total of 60 *C. difficile* ribotype 002 strains (provided by Prof. Mark Wilcox via the CDRN) isolated between 2007- 2014 from the UK and across Europe were used in this study. Thirty-five of these strains were isolated from the UK while twenty-five from different geographical locations across Europe (Figure 2-1). Strains were grouped according to their time lineage; fourteen of the UK strains formed part of the older lineage of CD002 strains (UK 2007-2008), while twenty-two were part of the recent group of strains (UK 2011- 2013). For the non-UK isolates, only one strain isolated from Dublin in 2008 formed part of the old group, while twenty-three strains formed of the recent group of strains (Table 1& 2). *C. difficile* ATCC® 700057 was used as a control strain in all experiments performed in this study.

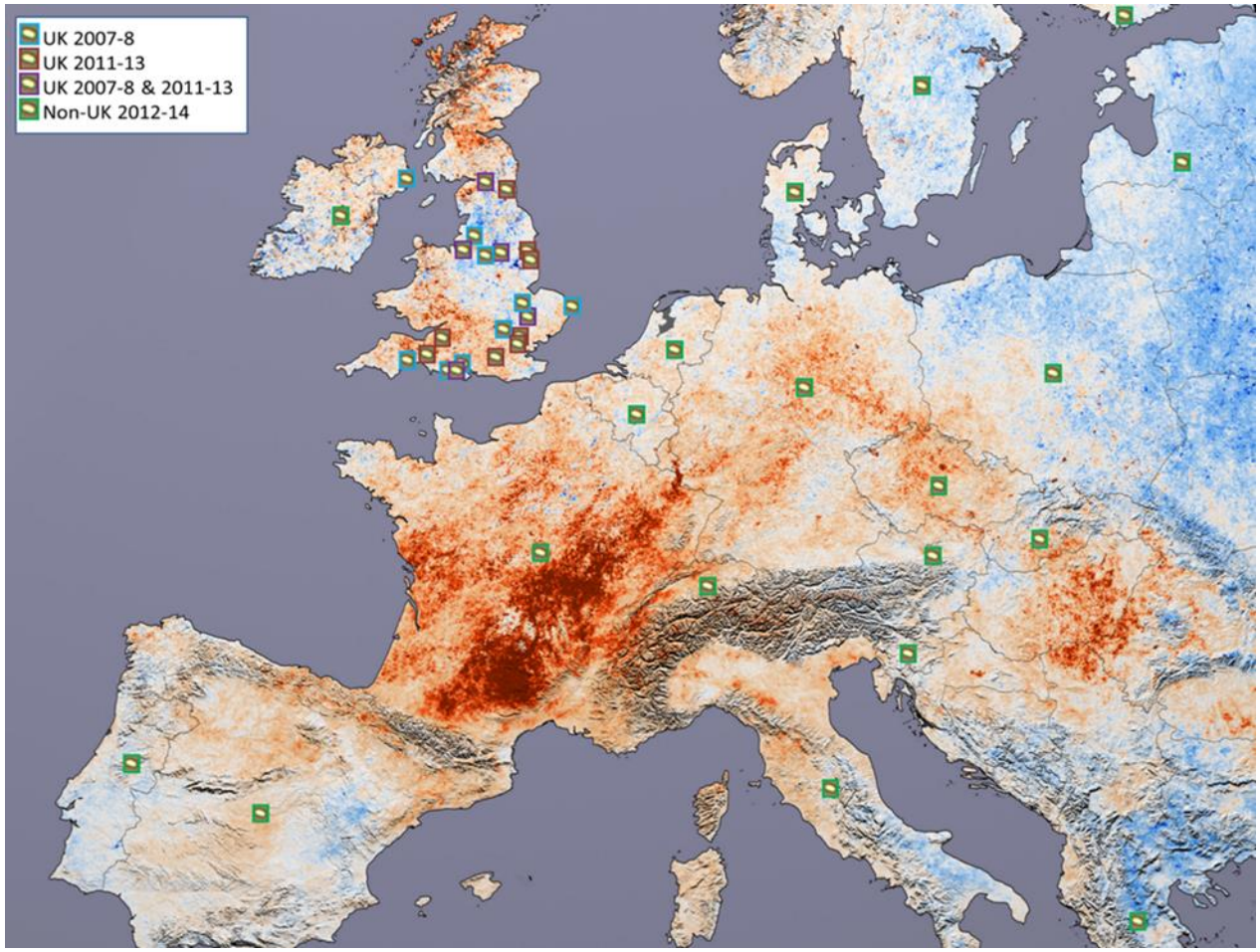


Figure 2-1 Distribution of CD002 isolates evaluated in this study. Non-UK CD002 makers are correct to country but not exact geographical location (Contour colouration is not relevant to this study map acquired from www.nasa.gov.uk

Table 2-1 UK CD002 isolates used in this study

Strain Group	Strain code	Location	Year of Isolation
UK 2007-08	174	Leeds	2007
	173	Great Yarmouth	2007
	172	Luton	2007
	171	Cambridge	2007
	170	Preston	2007
	169	Exeter	2007
	168	Bournemouth	2007
	167	Liverpool	2007
	166	Carlisle	2008
	165	Manchester	2008
	163	Poole	2008
	162	Belfast	2008
	161	Peterborough	2008
	160	Salisbury	2008
UK 2011-13	159	Exeter	2011
	158	Bournemouth	2012
	157	Taunton	2012
	156	Carlisle	2012
	154	Liverpool	2012
	39	Cumberland	2012
	40	Cumberland	2012
	53	Leeds	2012
	54	Leeds	2012
	66	Harrogate	2012
	68	Harrogate	2012
	71	Maidstone	2012
	153	Cambridge	2013
	151	London	2013
	150	Grimsby	2013
	149	Surrey	2013
	148	HPA South East	2013
	147	Hull	2013
	146	Bristol	2013
	145	Leeds	2013
	144	Durham	2013

Table 2-2 Strain selection of Non- UK CD002 isolates used in this study

Strain Group	Strain code	Location	Year of Isolation
Non- UK 2008	164	Dublin	2008
Non- UK 2012-14	1	Ireland	2012
	4	Ireland	2012
	5	Ireland	2012
	6	Ireland	2012
	155	Ireland	2012
	139	Switzerland	2013
	141	Portugal	2013
	140	Poland	2013
	138	Latvia	2013
	142	Germany	2013
	143	Belgium	2013
	134	Austria	2013
	128	Sweden	2014
	137	Spain	2014
	126	Slovenia	2014
	131	Netherlands	2014
	133	Italy	2014
	136	Hungary	2014
	135	Greece	2014
	130	France	2014
	132	Finland	2014
	129	Denmark	2014
	127	Czech Rep	2014
	152	Dublin	2013

2.1.2 **Control Strains**

C. difficile ATCC® 700057 was used as a control strain in all experiments performed in this study. However, for some individual experiments, additional controls were included. In the antimicrobial susceptibility testing, four additional strains were used as controls. In the biofilm formation assay, a *C. difficile* strain of ribotype 078 (Strain 24) was used as an additional control due to its elevated biofilm production. In the cytotoxicity assay, a *C. difficile*

PCR ribotype 001 (P24) strain was cultured for 72 hours to produce a cytotoxin-positive control sample, which was neutralised by *C. sordellii* antitoxin (PL 6508, Prolab Diagnostics, Bromborough, UK).

2.1.3 Media, buffers and solutions

All reagents, liquid media, solid media and solutions used in this study are described in **Appendix 1**.

2.1.4 *C. difficile* culture growth conditions

All *C. difficile* cultures were incubated in an anaerobic work station (DG500, Don Whitley, United Kingdom) with a gas mixture of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen (British Oxygen Company (BOC Gases), UK) at 37°C.

2.1.5 Culture of *C. difficile* on solid media

All strains were cultured onto Brazier's Cefoxitin/Cycloserine Egg Yolk (CCEY) agar (LAB160, Lab M, Lancashire, UK) supplemented with 2% lysed defibrinated horse blood (SR0050C, Thermo-scientific) and incubated anaerobically at 37°C for 48 hours before any test was performed. All solid media were pre-reduced in an anaerobic workstation (DG500, Don Whitley, United Kingdom) for 24h at 37°C.

2.1.6 Culture of *C. difficile* on liquid media

Liquid media used for the culture of *C. difficile* isolates were prepared according to the manufacturers' instructions. This included: Schaedler's anaerobe broth (CM0497, Oxoid), and Brain heart infusion (BHI) Broth (CM1135, Oxoid). All liquid media were pre-reduced for 24 hours at 37°C in the anaerobic work station prior to inoculation.

2.1.7 **Preparation and harvesting of *C. difficile* spores**

C. difficile was inoculated onto Braziers CCEYL agar and incubated anaerobically at 37°C for 48 hours. Single pure colonies of *C. difficile* were cultured on to pre-reduced Columbia blood agar (CBA) plate and incubated anaerobically at 37°C for 7 days. Subsequently, CBA plates were recovered from the anaerobic cabinet, and all growth was removed from CBA using a sterile swab and immersed into a 5ml solution of 50% ethanol. The cell suspension was left at room temperature for 1 hour and stored at 4°C until required. A schematic representation of *C. difficile* spore preparation is provided in Figure 2.1.

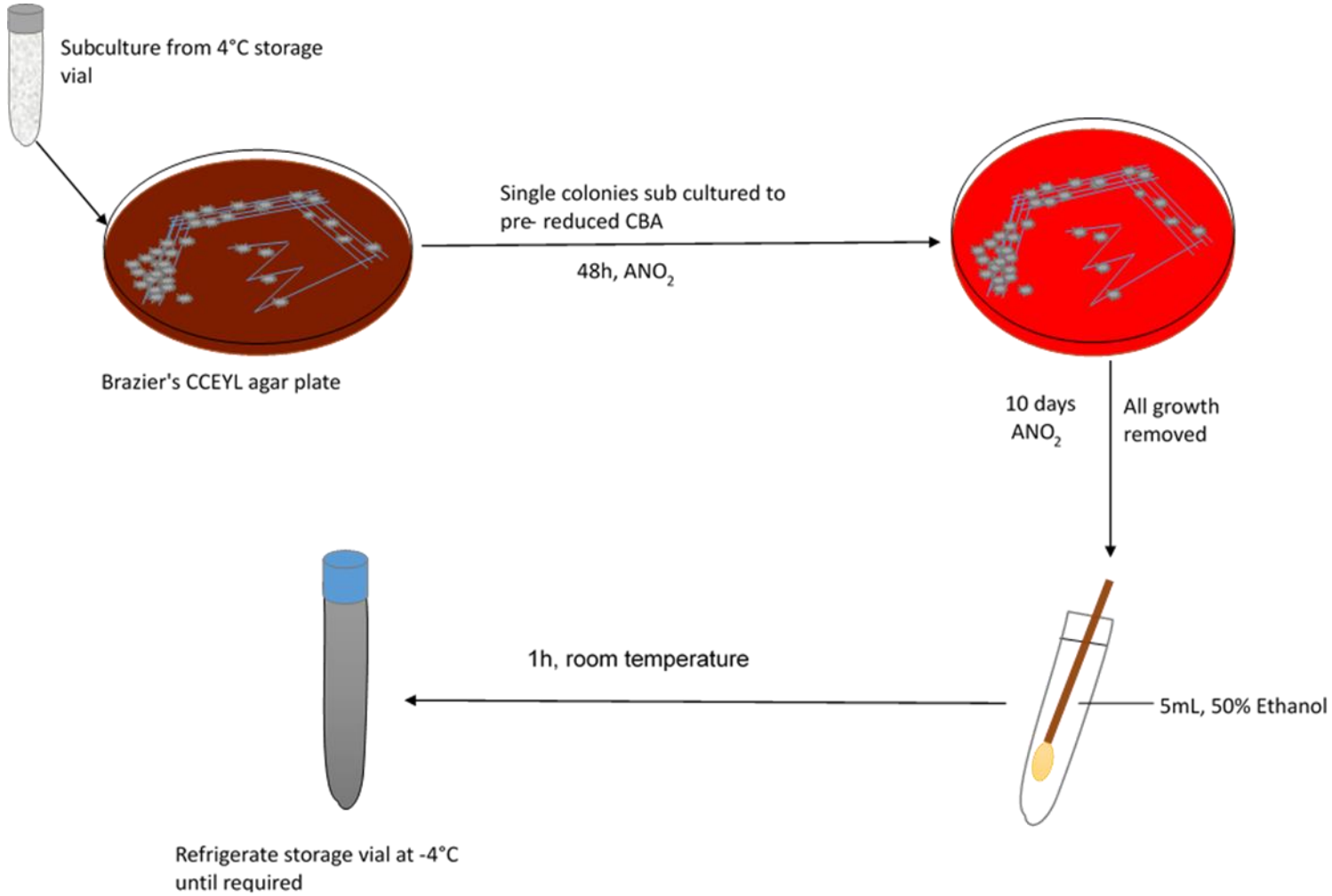


Figure 2-2 Preparation and harvest of *C. difficile* spores

2.2 ANTIMICROBIAL SUSCEPTIBILITY TESTING AND MINIMUM INHIBITORY CONCENTRATION (MIC) DETERMINATION

2.2.1 Antimicrobial agents

Susceptibility of CD002 to a diverse panel of antimicrobial agents was performed using the agar dilution method in accordance with the Clinical and Laboratory Standards Institute guidelines for Antimicrobial Susceptibility Testing of Anaerobic Bacteria (Wilcox, Fawley, Freeman, & Brayson, 2000). The following antimicrobial agents were used in this study are listed in Table 2-3.

Table 2-3 List of Antimicrobial agents used in this study

Antimicrobial agent	Product code	Manufacturer
Metronidazole	M3761-25G	Sigma, UK
Vancomycin	V2002-1G	Sigma, UK
Erythromycin	E6376-25G	Sigma, UK
Ampicillin	A9393-5G	Sigma, UK
Nitrofurantoin	N7878-10G	Sigma, UK
Tetracycline	T3383-25G	Sigma, UK
Penicillin G	13752-5G	Sigma, UK
Chloramphenicol	C0378-25G	Sigma, UK
Clarithromycin	C9742-250MG	Sigma, UK
Trimethoprim	T7883-5G	Sigma, UK
Moxifloxacin	06669204	Bayer plc, Newbury, UK
Clindamycin	PNU21251F	Pfizer, Tadworth, UK
Ciprofloxacin	11939800	Bayer plc, Newbury, UK
Linezolid	PF00184033	Pfizer, Tadworth, UK
Meropenem	S1381	Selleckchem
Fidaxomicin	S4227	Selleckchem
Rifampicin	BP2679-5	Fluka

2.2.2 **Preparation of Antimicrobial Agar Incorporation Plates**

Antimicrobial stock solutions were prepared in their respective solvents as shown in Table 2-4. Antimicrobial stock solutions other than those prepared in DMSO (D8418-50ml, Sigma), DMF (D4551-100ml, Sigma) and ethanol (E/0600DF/21, Fisher Scientific) were sterilised by filtration through 0.22µm syringe filters (SLGP033RS, Millipore, Carrigtwohill, Ireland). Accordingly, serial two-fold dilutions of the antimicrobial stock solution were prepared in respective diluents to give a final concentration range of 0.03-128mg/L when 2mL was added to 18mL of molten Wilkins-Chalgrens Agar (WCA) (CM0619 Oxoid). Each antibiotic incorporated agar was mixed thoroughly and distributed into a sterile petri dish. Following solidification, antibiotic incorporated agars were left at room temperature for 24h or dried in sterile micro safety class II cabinet to removes excess moisture prior to inoculation.

2.2.3 **Preparation of Bacterial Inocula**

C. difficile strains were cultured anaerobically in pre-reduced 5ml Schaedler's anaerobe broth (CM0497, Oxoid) using a sterile swab and incubated overnight at 37°C. Subsequently, 400µl of overnight *C. difficile* cultures (~$1x10^7$ CFU/ml) were transferred to the appropriate well of a sterile inoculating block. Using a multipoint inoculator (UriDot, Mast Group, UK), 1µl (10^4 CFU) of the bacterial culture from an inoculating block was applied onto the surface of pre-dried WCA plates containing doubling dilution of antibiotics and control plates containing no antibiotic and the respective solvent control plate. Pins of the multipoint inoculator were sterilised by flaming in 100 % absolute ethanol (1.02428, EMD Millipore) between each set of antibiotic incorporated agar plates. Control plates containing no antibiotic were inoculated at the start and end of the procedure. All plates and growth controls were incubated anaerobically for 48h at 37°C. Aerobic controls were incubated for 24h at 37°C. The plates

were recovered from the anaerobic cabinet after 48h incubation and MIC values were recorded. The MIC was determined as the lowest antimicrobial concentration where an absence or marked reduction of growth (multiple tiny colonies, haze or fine film of growth or one or two colonies) compared with the growth control was observed.

Table 2-4 Antimicrobial agents, solvents and diluents used in this study (adapted from Andrews (2001)

Antimicrobials	Solvent	Diluent
Metronidazole	Water	Water
Vancomycin	Water	Water
Ampicillin	Saturated $NaHCO_3$ (2056338, Fluka Biochemika, Location)	Water
Chloramphenicol	Ethanol	Water
Ciprofloxacin	Water	Water
Clarithromycin	DMSO	Water
Clindamycin	Water	Water
Erythromycin	Ethanol	Water
Linezolid	Water	Water
Moxifloxacin	Water	Water
Nitrofurantoin	DMF	DMF
Penicillin G	Water	Water
Tetracycline	Water	Water
Trimethoprim	Water (1ml + 10µl glacial acetic acid (10365020, Fisher Scientific)	Water
Meropenem	Water	Water
Fidaxomicin	DMSO	10% DMSO
Rifampicin	DMSO	Water

2.3 MAMMALIAN CELL CULTURE EXPERIMENTS

2.3.1 Vero Cell Cytotoxicity assay

2.3.1.1 Preparation of *C. difficile* cytotoxin extracts

C. difficile cytotoxin extracts were produced by culturing strains in 5ml of Brain Heart Infusion broth supplemented with 5% yeast extract (LP0021, Oxoid) and 1% L-cysteine hydrochloride

(C7477, Sigma) (BHIS) broth and incubating anaerobically for 72 hours at 37°C. After incubation was complete, liquid cultures were retrieved from the anaerobic cabinet, 1ml of each sample was aliquoted into sterile microcentrifuge tubes and centrifuged at 13,000 rpm for 5mins using a Progen Genfuge 24D Digital Microcentrifuge (Progen Mexborough, UK). The supernatant was gently removed and sterilised by filtration through 0.22µm syringe filters (SLGP033RS, Millipore, Carrigtwohill, Ireland) into fresh sterile tubes, while the pellet was discarded. *C. difficile* culture supernatants were stored at 4°C until the cytotoxicity assay was performed.

2.3.1.2 Vero cell preparation

Vero cells (African green monkey kidney cells, PHE Culture collection, ECACC 84113001) were retrieved from liquid nitrogen storage vials and thawed in a 5% CO_2 incubator at 37°C. Thawed Vero cells were cultured in 25cm^3 flasks containing 7ml Dulbecco Modified Eagle's medium (DMEM) (D6546, Sigma). DMEM (500ml) was supplemented with 50mL new-born calf serum (N4637, Sigma), 5mL antibiotic/antimycotic solution (A5955, penicillin 100U/mL, 100mg/L streptomycin, 0.25mg/L amphotericin, Sigma) and 5mL L-glutamine (G7513, Sigma). Vero cells were incubated in a 5% CO_2 incubator at 37° C until a confluent monolayer was achieved upon examination under an inverted microscope (Olympus CKX41). Vero cells were passaged before cytotoxicity assays were performed.

2.3.1.3 Passaging of Vero cells

Passaging of Vero cells was performed by discarding DMEM covering confluent Vero cell monolayers and washing of the monolayer with 0.5ml of Hanks Balanced Salt Solution, HBSS (H9394, Sigma), supplemented with 0.25g/L trypsin EDTA (T4174, Sigma) (HBSS trypsin EDTA) to remove dead cells. Following gentle agitation, excess HBSS trypsin EDTA was discarded and 3ml of HBSS trypsin EDTA solution was added. Vero cell monolayers were incubated in 5%

CO_2 for 10 minutes until the monolayer was dissociated (Trypsinised). Trypsinised Vero cells were diluted either 1:10 into fresh DMEM or 1:20 dilution and aliquoted into flat-bottomed sterile 96-well microtitre plates (DK-4000, ThermoFisher) depending on when the cells were required for cytotoxin assays. Microtitre plates containing diluted Vero cells were incubated in 5% CO_2 at 37°C until a confluent monolayer was reached.

2.3.1.4 Cytotoxicity assays

Twenty microliters of *C. difficile* culture supernatants were serially 10-fold diluted in 180µl of sterile PBS (to 10^{-5}). Subsequently, 20 µl of diluted *C. difficile* supernatant was added in duplicate to Vero cell monolayers (as shown in the flow chart in Figure 2-3). Additionally, 20µl of undiluted *C. difficile culture* supernatant was added into all wells in rows A and B, while 20 µl *C. sordelli* antitoxin (PL6508, Pro-lab, Diagnostics, South Wirral Cheshire, UK) was added into all wells in row B only. *C. sordelli* antitoxin was added to neutralise *C. difficile* cytotoxin to confirm the specificity of the cytotoxic effect. Microtitre trays were incubated in 5% CO_2 at 37° C and examined under the inverted microscope at 24 and 48 hours. A positive reaction was considered when ≥80% of Vero cell rounding was present in the wells of the microtiter tray (as shown in figure 2-). The total cytotoxin titre was expressed as $\log_{10}$ relative units (RU), where 1 RU represented ~80% cell rounding by the undiluted sample, 2 RU represents ~80% cell rounding by the 10^{-1} dilution of the sample (Underwood *et al*. 2009).

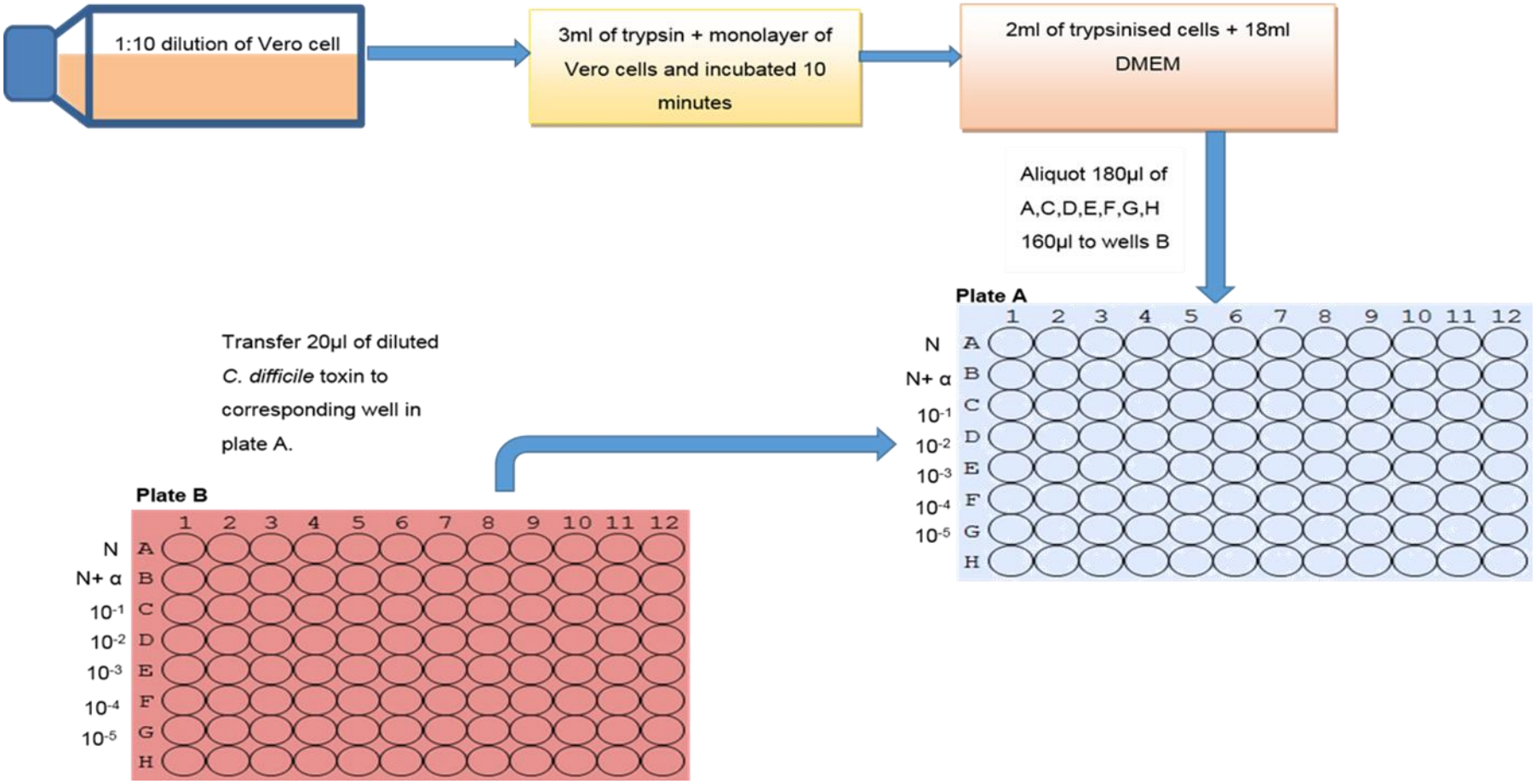
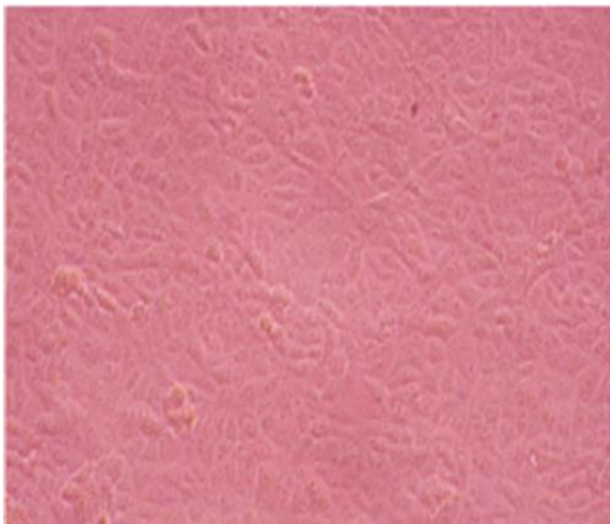


Figure 2-3 Flow chart of the Vero cell cytotoxicity assay

a)



b)

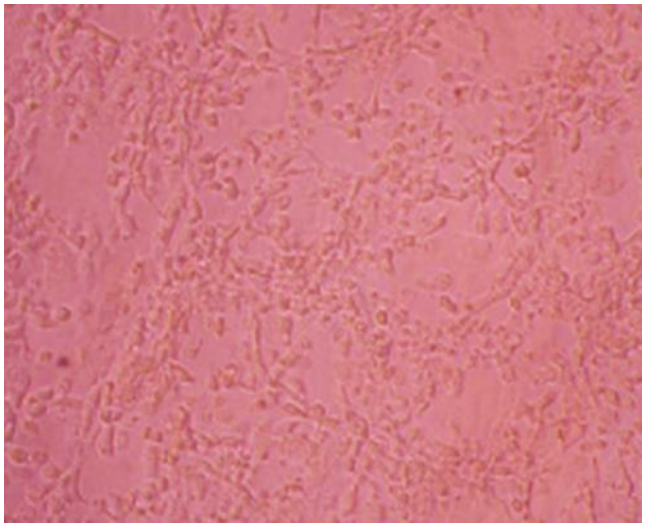


Figure 2-4 Vero cells a) Negative control: Vero cell monolayer (200x magnification). b) Cytopathic effect of *C. difficile* cytotoxin on Vero cell (200x magnification).

2.3.2 **Caco-2 cell culture preparations**

Caco-2 cells (Human adenocarcinoma, cells, PHE Culture collection, ECACC 86010202) were retrieved from liquid nitrogen storage vials and thawed in a 5% CO_2 incubator at 37°C. Thawed Caco-2 cells were transferred into 15ml falcon tubes and centrifuged at 15000*g* for 10 minutes. Subsequently, the supernatant was carefully removed and the cell pellet was re-suspended in 7ml of Eagle's Minimum Essential Medium (P04-08050, Pan Biotech) supplemented with 100mL fetal bovine serum (P30-1302, Pan Biotech), 5mL antibiotic/antimycotic solution (A5955, penicillin 100U/mL, 100mg/L streptomycin, 0.25mg/L amphotericin, Sigma), 5mL L-glutamine (G7513, Sigma), 5mL Non-essential amino acids (P08-32100, Pan Biotech) and 5mL sodium pyruvate (11360070, ThermoFisher). The suspension was transferred to a 25cm^3 Nunc flask (163371, Fisher Scientific) and incubated in a 5% CO_2 incubator at 37 ^oC until 70-80% of cell confluence was achieved (Figure 2-5). Cell culture media were changed media was changed every 2-3 days.

2.3.2.1 Passaging of Caco-2 cells

Passaging of Caco-2 cells was performed after 70-80% confluence was achieved (Figure 2-5). To achieve this, the culture medium covering confluent monolayers was discarded, and monolayers were washed with 5ml of Dulbecco phosphate-buffered saline, in order to maintain pH and osmotic balance of the cells. Subsequently, 2ml of 1% trypsin was added to cell monolayers incubated in 5% CO_2 for 4-6 minutes until the monolayer was dissociated (Trypsinised). Trypsinised cells were viewed under an inverted microscope (CKX41, Olympus), and 5ml of cell culture medium was added to the flask, to neutralise the effects of trypsin. Subsequently, the suspension was transferred into a 15ml falcon tube and centrifuged at 15,000*g* for 10 minutes (IEC CL30R, Thermo-Scientific). Subsequently, the supernatant was

discarded, and cells were resuspended in 3ml of culture medium. Trypsinised Caco-2 were diluted 1:5 and transferred into T-75 flask. For seeding of cells in 24 well plates, cell counting was performed (as described in 2.3.2.2) before cells were seeded at a density of 2 x10^5 per well. 24 well plates and flask were incubated in 5% CO_2 at 37°C until a confluent monolayer. Cell culture media was changed every 24-72 hours until 70-80% confluence was attained.

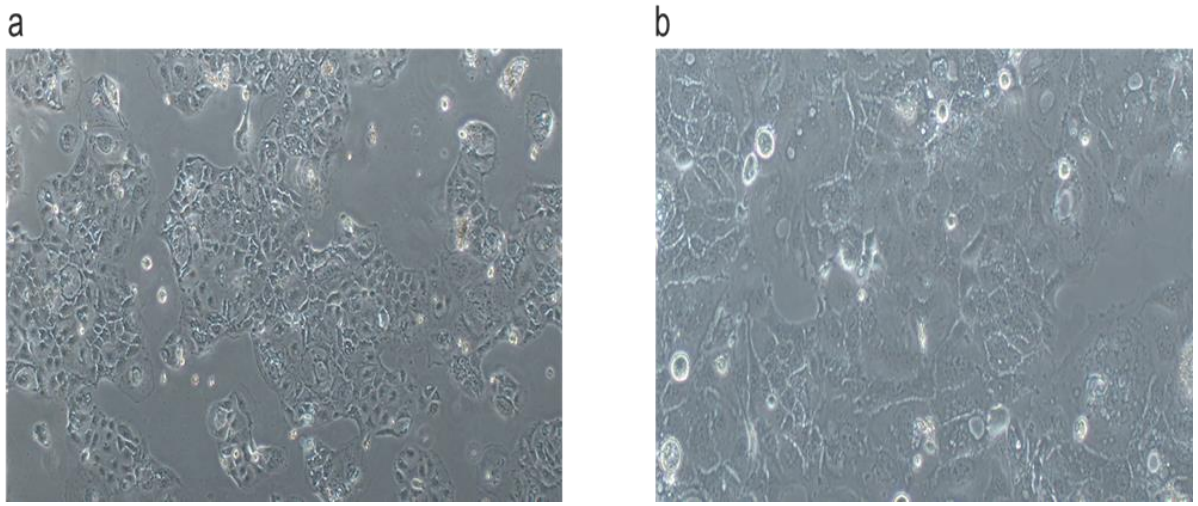


Figure 2-5 Caco-2 cells. a) Caco-2 cells at 50-60% confluency at 40x magnification. b) Caco-2 cells at 50-60% confluency 100x magnification.

2.3.2.2 Counting of Caco- 2 cells with Trypan blue

To confirm the presence of viable cells in a cell suspension (post trypsinisation and centrifugation) cells were stained with Trypan blue to enable visualisation of the cell morphology. Fifty microliters of 4% (v/v) Trypan blue solution (T8154, Sigma) was added to 50µl of an evenly mixed cell suspension and allowed to incubate at room temperature for 1-2 minutes. Subsequently, 10µl of the Trypan blue mixture was carefully transferred to one chamber of a haemocytometer and viewed under a phase-contrast microscope (Olympus CKX41) 100X magnification. Viable cells were counted (unstained cells) in four 1x1 mm

squares of one chamber and the average number of cells was determined. To calculate the cell concentration per ml, the following equation was used:

Cell concentration (cells/ml) = Average number of cells x dilution factor x 10^4

2.4 GROWTH RATE DETERMINATION

2.4.1 Growth rate measurement

To determine the growth rates of CD002, all strains were grown overnight in pre-reduced BHIS broth and incubated anaerobically 37^{o}C. In order to ensure a standardised starting OD_{600} for all strains, a starter culture was prepared by diluting overnight *C. difficile* cultures to OD_{600} of 0.1 in sterile pre-reduced BHIS using the equation below:

([Volume of New broth (ml) x desired OD_{600}]/ (Overnight OD_{600})) = Volume of overnight broth to add to new broth (ml)

The desired volume of the overnight broth obtained from the calculations (for each individual strain) was added to fresh pre-reduced BHIS broth and incubated anaerobically at 37^{o}C for growth. To ensure that absorbance readings fell within the linear range of the spectrophotometer, samples with absorbance values above 0.700 were diluted in sterile BHIS and absorbance readings were retaken. This value was then multiplied by the dilution factor to determine the actual OD_{600} value of the sample.

2.4.2 **Determination of the Maximum Specific Growth rate (µmax)**

The maximum specific growth rate for all strains (h^{-1}) was calculated from the exponential phase (4-6h) of *C. difficile* growth by determining the gradient of the ln (biomass) versus time plot.

2.5 VIABLE COUNTING OF *C. DIFFICILE* SPORES AND VEGETATIVE CELLS

To measure the total viable counts present in *C. difficile* cultures, 100µl of each sample was aliquoted into a 96 well microtitre tray. Subsequently, 20µl of each sample were diluted tenfold in 180µl sterile PBS (P4417, Sigma) in a dilution series to 10^{-6} (as shown in Figure 2-6). Twenty microliters of four appropriate dilutions were inoculated to quarter plates of Brazier's CCEY agar (Cefoxitin/Cycloserine Egg Yolk, Lab M, Lancashire, UK) supplemented with 2% lysed defibrinated horse blood (SR0050C, Thermo-scientific) and incubated anaerobically at 37^{o}C. Between 20-200 colony-forming units were counted and converted into CFU/mL in order to determine the total viable counts.

To measure the colony-forming units (CFU) formed from spores, 100µl of 50% ethanol, was added to 100µl of *C. difficile* cultures that had been aliquoted into a 96 well microtitre tray and allowed to incubate at room temperature for 1h. Subsequently, viable counting was performed as described above.

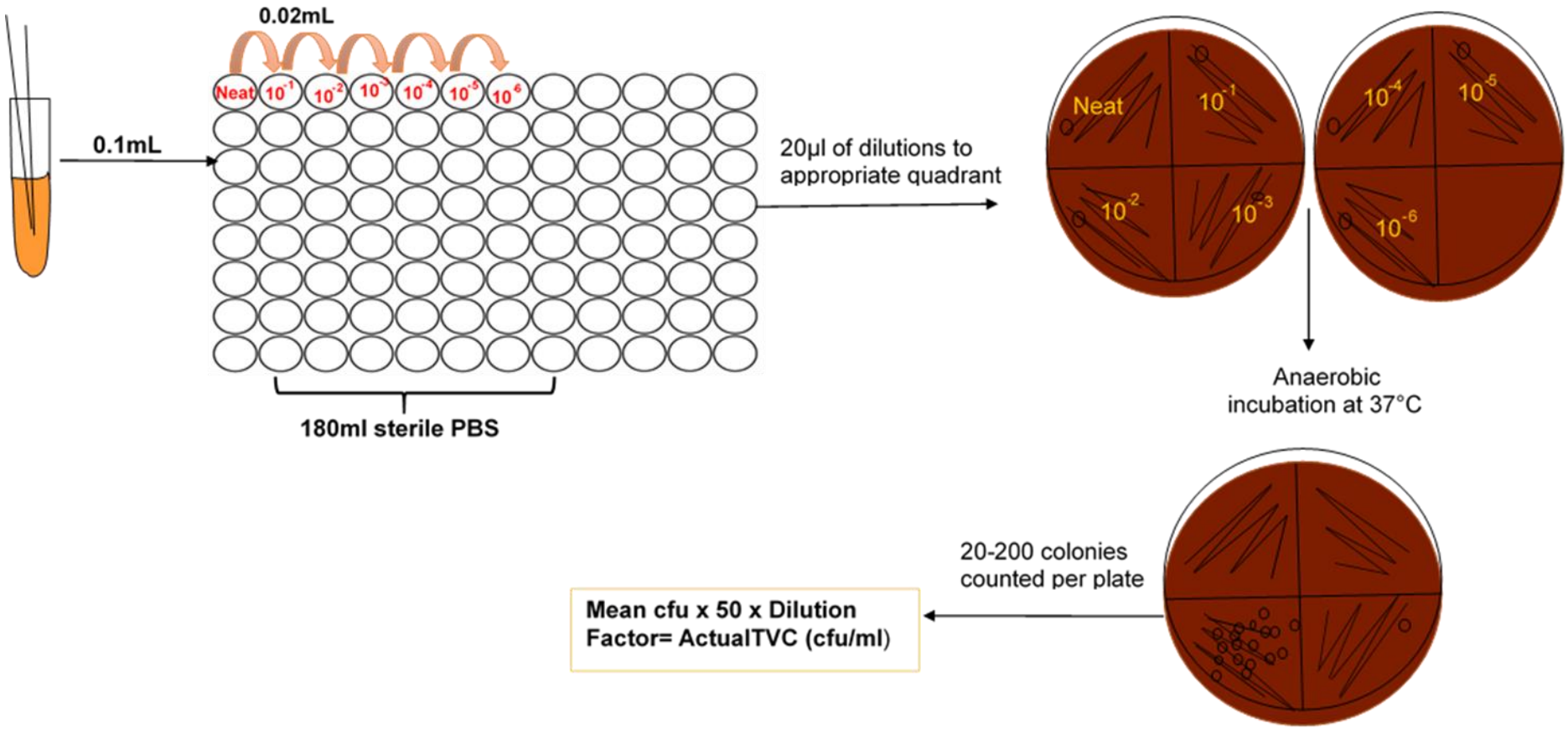


Figure 2-6 Schematic representation of total viable counting of *C. difficile*

2.6 PHENOTYPE MICROARRAY EXPERIMENT

2.6.1 Deoxygenation of PM panels prior to inoculation

Prior to phenotypic microarray experiments, all PM panels were converted to an anaerobic state by deoxygenation. To achieve this, PM panel packaging bags were cut open at one end and placed in a W- Zip plastic pouch (2 PM panels per plastic pouch bag), along with two anaerobic oxygen sachets (AN0010, Oxoid), an anaerobic strip indicator, and original desiccant sachets. The bags were sealed and kept at room temperature for 1 day and subsequently stored at 4°C for an additional 2 days or longer before use. Deoxygenated PM panels were warmed up to room temperature on the laboratory bench.

2.6.2 Inoculum preparation for the phenotype microarray experiment

Using a sterile deoxygenated swab, the inoculum for the phenotype microarray (PM) experiment was prepared by removing colonies of *C. difficile* (48h hours old) from a Columbia blood agar plate (LAB001, Lab M, Lancashire, UK). The retrieved *C. difficile* cells were resuspended in a pre-reduced suspension liquid (AN IF-0a) (72268, Biolog, Hayward, USA) supplemented with sodium bicarbonate (S6014, Sigma), sodium thioglycolate (T0632, Sigma) and methylene green (05208636, MP Biomedicals) at final concentrations of 10mM, 0.4mM, and 1µM respectively. This bacterial suspension was adjusted in AN IF-0a to achieve a 40% transmittance cell suspension (which measured spectrophotometrically as 0.139± 0.002 at OD_{750} using a CE1011 1000 series spectrophotometer. This suspension was further diluted at a ratio of 1:16 in an anaerobic Biolog Mix B solution. The mix B solution consisted of AN IF-0a supplemented with 0.2% (w/v) yeast extract (LP0021, Oxoid) for PM1 and 2, and 0.05% (w/v) of yeast extract for PM3-8. To provide a carbon source in PM3-8, glucose (41095-5000, Fisher Scientific) at a final concentration of 5mM was added to the inoculating fluid. The bacterial

suspension was used for inoculation of all PM panels. As a preliminary check to determine the cell density of the inoculating fluid (T40 1:16) prior to inoculation of the PM panels, the inoculum was plated on Columbia blood agar. This inoculum yielded an average colony count of 4.54 x 10^7 CFU/mL.

2.7 FERMENTATION EXPERIMENTS

2.7.1 Sterilisation and Calibration of pH probes fermentation vessels

Fermentation experiments were performed in a one-litre New Brunswick Bioflo 115 bioreactor with a working volume of 700ml (Figure 2-7). Prior to setting up the fermenter, the bioreactor vessel and its working accessories were sealed and sterilised in an autoclave. The bioreactor pH probe was calibrated using a two-point calibration system, which determines the electrode parameters zero (pH 7) drift and slopes with two buffer solutions, pH 4 (BS04, ThermoFisher) and pH 7 (BS07, ThermoFisher). The calibrated probe was then sterilised before attachment to the culture vessel.

2.7.2 Fermentation media

Peptone yeast glucose (PYG) media was used for fermentation experiments in accordance to the published recipe by Hardy diagnostics except that the concentration of glucose was set at 1g per litre and 0.005g per litre for resazurin (R7017, Sigma) was added as an anaerobic indicator. Ingredients used for the preparation of the media are listed in Table 2-5. The media was prepared at a deficit volume of ingredients that were added post autoclaving, to ensure that the final concentrations were maintained. Following autoclaving and cooling down to room temperature, 20g of glucose, 100ml of 0.1 % hemin solution and 0.1g of resazurin was added by filter sterilization using 0.22µm sterile syringe filters (SLGP033RS, Millipore, Carrigtwohill, Ireland) to a 20L fermentation medium.

Additionally, 700ml of the fermentation medium was prepared and sterilised (autoclaved) *in situ* in the bioreactor vessel. Post sterilisation, media additives (glucose, hemin and resazurin), were aseptically added to the bioreactor vessel through the sample port, using a sterile needle (Z192414, Sigma) and sterile syringe. To avoid contamination, the sample port was first sprayed with 70 % ethanol and then allowed to dry before media additives were aseptically added.

Table 2-5 Ingredients used to make up 1L of peptone yeast glucose (PYG) broth

Ingredients	Volume	Product code and manufacturer
Pancreatic digest of casein	20 g	70169, Sigma
Yeast extract	10 g	LP0021, Oxoid
L-Cysteine	0.5 g	C7477, Sigma
Glucose *	1g	410955000, Fisher Scientific
Sodium Bicarbonate ($NaHCO_3$)	0.4 g	S56297, Fisher Scientific
Sodium Chloride (NaCl)	0.08 g	S3171, Fisher Scientific
Monopotassium Phosphate (KH_2PO_4)	0.04 g	P5379, Fisher Scientific
Dipotassium Phosphate	0.04 g	P3786, Fisher Scientific
Calcium Chloride ($CaCl_2$)	0.008 g	C3881, Fisher Scientific
Magnesium Sulphate ($MgSO_4$)	0.008 g	P1880, Fisher Scientific
0.1% haemin Solution	5ml	H9039, Sigma
1% (v/v) Vitamin K_1	0.1ml	V3501, Sigma
Resazurin	0.005g	R7017, Sigma

2.7.3 **Fermenter set up and sterility test**

Post sterilisation of the culture vessels and the working accessories, the fermenter was set up by aseptically connecting the necessary components to the culture vessel (as shown in figure

2-7). The pure nitrogen supply for the bioreactor was fed into the bioreactor vessel via a sterile membrane filter and sparged through the culture medium. Temperature and agitation were maintained at 37^{o}C and 200 rpm respectively and the dissolved oxygen was set at zero. For pH control, a mixture of sterile 1M NaOH and 1M HCL was used. The fermenter was run under these conditions for 24h prior to inoculation in order to validate the sterility of the bioreactor. As an additional step, 1ml of the culture medium was aseptically retrieved from the culture vessel, and Gram staining was performed prior to inoculation to screen for any bacterial contamination.

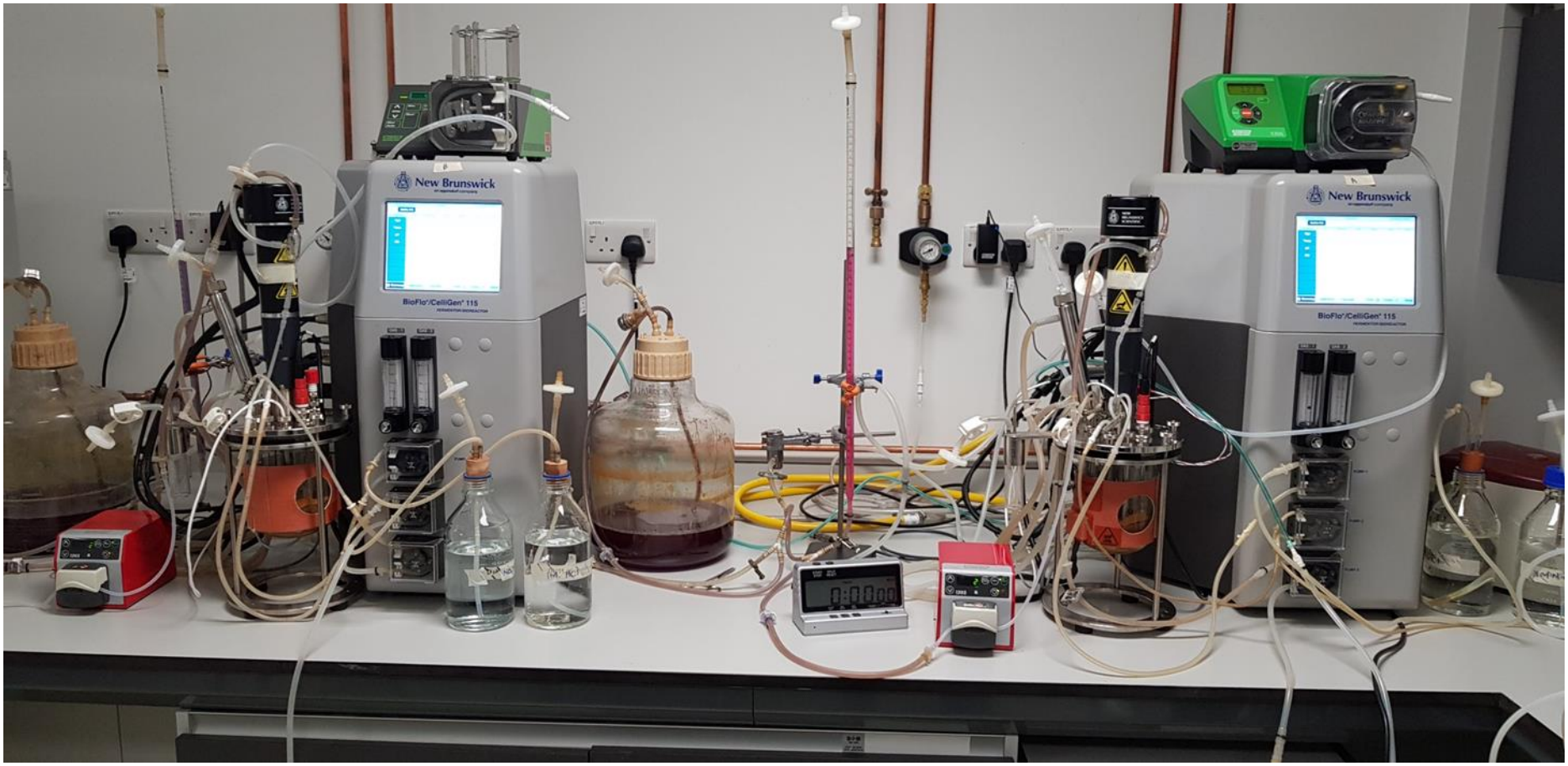


Figure 2-7 Photograph of the single-stage fermenter set up for continuous culture competition studies

2.8 MOLECULAR METHODS

2.8.1 **Bacterial DNA Extraction**

For all PCR amplifications, bacterial DNA was extracted by the boiling method. Three to five colonies of *C. difficile* were suspended into 100µl of sterile nuclease-free PCR grade water (SH30538.02, GE Healthcare). This suspension was boiled at 100°C for 10 mins on a Dry Block Thermostat (11456367, BTD, Grant, UK), and centrifuged at a low speed (3,000*g*) for 1min to remove cell debris. The DNA- containing supernatant was used for PCR amplification.

2.8.2 **Primers**

Table 2-6 is a list of primers used for amplification of antimicrobial resistance genes and investigation of the presence of the binary toxin genes in all isolates.

Table 2-6 Primers used in this study

Gene	Primer	Sequence 5'-3'	DNA sequence length in (bp)	References
ermB	*ermB*-F	GAA AAG GTA CTC AAC CAA ATA	639	Solomon *et al*., 2011
	ermB-R	AGT AAC GGT ACT TAA ATT GTT TAC		
gyrA	*gyrA*- F	AAT GAG TGT TAT AGC TGG ACG	390	Spigaglia *et al*., 2009
	gyrA-R	TCT TTT AAC GAC TCA TCA AAG TT		
gyrB	*gyrB*-F	AGT TGA TGA ACT GGG GTC TT	390	Spigaglia *et al*., 2009
	gyrB-R	TCA AAA TCT TCT CCA ATA CCA		
ermA	*erm (A)-F*	TCT AAA AAG CAT GTA AAA GAA	645	Patterson *et al*., 2007
	erm (A)-R	CTT CGA TAG TTT ATT AAT ATT AGT		
ermC	*erm(C)-F*	TCA AAA CAT AAT ATA GAT AAA	642	Patterson *et al*., 2007
	erm(C)-R	GCT AAT ATT GTT TAA ATC GTC AAT		
ermF	*erm(F)-F*	CGG GTC AGC ACT TTA CTA TTG	466	Patterson *et al*., 2007
	erm(F)-R	GGA CCT ACC TCA TAG ACA AG		
ermQ	*erm(Q)-F*	AAG TTA TTG GGT TAC AGC TA	771	Patterson *et al*., 2007
	erm(Q)-R	CAC CTC CTA ATT TAA ATC TAC TA		
rpoB	*CDrpoB2-F*	ATG GAA GCT ATA ACG CCT CAA	200	Curry *et al*., 2009
	CDrpoB2-R	ACA GCA CCA TTT ACA GTT CTA		
cdtA	*cdtApos*	TGA ACC TGG AAA AGG TGA TG	375	Stubbs *et al*., 2000
	cdtArev	AGG ATT ATT TAC TGG ACC ATT TG		
cdtB	*cdtBpos*	CTT AAT GCA AGT AAA TAC TGA G	510	Stubbs *et al*., 2000
	cdtBrev	AAC GGA TCT CTT GCT TCA GTC		

2.8.3 **PCR Amplifications**

PCR amplifications were performed in a reaction volume of 50µl, consisting of 3µl template DNA from CD002 strains, 1µl of each primer (0.5 µM in final concentration), 25µl of amplitaq gold master mix (439881, Thermo-Fisher) and 20µl of sterile PCR grade water (SH30538.02, GE Healthcare). The detailed volumes and concentrations for the PCR reactions are shown in Table 2-7.

Table 2-7 PCR reaction

Reagent	Final concentration	50 µl
25 µM forward primer	0.5 µM	1µl
25 µM reverse primer	0.5 µM	1µl
Amplitaq mix	-	25 µl
Template DNA	-	3 µl
Sterile PCR grade Water	-	20 µl

2.8.4 **Agarose gel electrophoresis**

Agarose gel electrophoresis was used to detect PCR amplification. Agarose gel (1.4%) was prepared by adding 1.4g of agarose powder (A9539, Sigma) into 70ml of TBE (Tris-Borate EDTA) buffer (T4415, Sigma). This mixture was heated in a microwave until dissolved and cooled. Subsequently, 7µl of SYBR safe DNA gel stain (S33102, Thermo Fisher) was added to the cooled solution and mixed gently. This mixture was poured into a casting tray, with an appropriate comb for loading samples. After 10-15 minutes, the tray was placed in an electrophoresis tank, TBE Buffer was added, and the comb was removed. Consequently, a 10µl aliquot of each PCR product mixed with 2µl of loading buffer (G2526, Sigma) was loaded into the gel along with DNA Ladder. The gels were run at 100 V for 60-90 min. Gels were visualised under UV light using the InGenius Gel documentation system (Syngene, UK) and images were captured using GeneSnap software (Syngene, UK).

2.8.5 **Purification and analysis of DNA quantity and purity**

The resulting PCR products were purified using Wizard SV gel and PCR clean-up system (A9281, Promega, USA), according to the manufacturer's protocol. Nucleic acid quantification and purity analyses were carried out using a Qubit 4 fluorometer (Q33226, Invitrogen) and a NanoDrop 1000 spectrophotometer (ND-1000, Thermofisher) respectively. Both processes were carried out according to the manufacturer’s protocol. The 260/280nm absorbance ratio

of 1.8-2.0 was used to assess the purity of DNA, and samples outside this range were not regarded as pure as they indicate the presence of protein, phenol or other contaminants.

2.8.6 **Sequencing**

PCR products were sequenced using the same amplification primers for target genes (as shown in Table 2-6). Sequencing of PCR products was done by Eurofins Genomics Germany (Eurofins GATC, Ebersberg, Germany), and Source BioScience (Cambridge, UK). Sequence data were visualized on SnapGene viewer and Pairwise alignments of DNA sequences were carried out using the BLAST server of the National Centre for Biotechnology Information (NCBI): http://blast.ncbi.nlm.nih.gov/Blast.cgi and Clustal Omega sequence alignment software: http://www.ebi.ac.uk/Tools/msa/clustalo/. Sequences that differed from the CD630 reference genome were translated with EMBOSS Transeq to identify amino acid substitutions within the gene: http://www.ncbi.nlm.nih.gov/gorf/gorf.html

2.8.7 **Proteomic Analysis**

2.8.7.1 Growth conditions and whole-cell protein extraction

Protein extraction was carried out according to methods previously described by Chilton *et al*. (2014). All strains were grown anaerobically on Columbia blood agar (CBA) at 37^{o}C for 64h to obtain optimum reproducible protein profiles. Subsequently, CBA plates were recovered from the anaerobic cabinet, and all growth was harvested using a sterile swab. Cells were re-suspended in 1ml of lysis buffer (50mM phosphate buffer, containing 0.5mM phenylmethanesulphonylfluoride (PSMF (10837091001, Sigma), protein inhibitor) and kept on ice in order to reduce proteolysis. FastPrep 0.1 mm silica spheres lysing beads (116911100, MP Biomedicals) were added to the cell suspension, and homogenisation was performed on the FastPrep-24 system (116004500D, MP Biomedicals). Samples were shaken for 2 runs of

60 seconds at 4m/s and incubated on ice, before centrifugation for 30 minutes at 21,000g (4°C), to remove cell debris. Subsequently, the supernatant was removed and transferred to the sterile 0.5ml microcentrifuge tube. Protein concentrations of the lysates were determined using the Qubit protein assay kit (Q33211, Invitrogen) according to the manufacturer's protocol. Protein extracts were stored at -20°C until use.

2.8.7.2 1D gel electrophoresis

Single dimension gel electrophoresis was carried out using the NuPAGE gel system (A25977, ThermoFisher). Five microliters of reduced 2x SDS sample buffer was added to 10µg of protein sample and made up to 16µl with distilled water. Each sample was loaded onto a sample well of a NuPAGE 10% Bis-Tris protein gels (NP0301, ThermoFisher) alongside 10µl of SeeBlue Plus2 pre-stained Protein standard (LC5925, Invitrogen). Gels were run in NuPAGE MES SDS running buffer for 35 minutes at 200V (120mA, 25 W). Gels were stained in Coomassie dye overnight and destained using a destaining solution. Gels were visualised under UV light using the InGenius Gel documentation system (Syngene, UK) and images were captured using GeneSnap software (Syngene, UK).

2.8.7.3 In-gel tryptic digestion

Trypsin digestion was carried out using the In-gel tryptic digestion kit (89871, Thermofisher, UK) according to the manufacturer's protocol. Briefly, using a sterile scalpel, bands from 1D gels were excised and transferred into sterile 0.5ml micro-centrifuge tubes containing 200µL of destaining solution (25mM ammonium bicarbonate, in 50%(v/v) acetonitrile) added. Samples were incubated in a shaking incubator (SHKE8000, ThermoFisher) for 30 mins at 37°C. The destaining step was then repeated, to ensure full decolourisation in the gel slices. The reduction step was performed in 30µl of reducing buffer (50mM TCEP (Tris [2-carboxyethyl] phosphine), for 10 mins at 60°C, and alkylation in 30µL alkylation buffer (50mM

iodoacetamide (IAA)) for 1 hour in the dark. The gel slices were washed in 200 µL of destaining buffer for 15 mins at 37°C (in a shaking incubator), followed by in 50 µl of 100% acetonitrile for 15mins at room temperature, and then air-dried for 10 mins. Trypsin digestion was carried out in 10 µL of activated trypsin solution (10ng/µl of pierce Trypsin protease in 9 µL of 25mM ammonium bicarbonate solution) for 15 mins at room temperature, followed by overnight incubation at 37°C in 25 µL digestion (25mM ammonium bicarbonate solution). The peptide extracts were transferred into a clean tube, further peptide extraction was performed by adding 10 µL of 1% (v/v) trifluoroacetic acid (TFA) (1082620025, EMD Millipore) to gel pieces and incubated for 5mins. The extraction mixture was added to the peptide extracts. Peptides extracts were dried down in a speed vacuum and stored in -20°C until use.

3 ANTIMICROBIAL SUSCEPTIBILITIES AND RESISTANCE MECHANISMS OF CD002 ISOLATES

3.1 BACKGROUND

Clostridium difficile (C. difficile) is a Gram-positive pathogenic bacterium which is a leading cause of antibiotic-associated diarrhoea (AAD)(Planche & Karunaharan, 2017). Since the identification of this pathogen, it emerged globally, causing several epidemics with significant morbidity and mortality(McGlone *et al*., 2012). Currently, *C. difficile* infection (CDI), accounts for approximately 453,000 cases in the United States, 172,000 in Europe and 18,005 in England(Borren *et al*., 2017; Lessa *et al*., 2015). Recent estimates in Asia, reveal similar rates of CDI reported in Europe and North America (Borren *et al*., 2017).

Prior or ongoing antimicrobial therapy is known to be an important risk factor for the development of CDI. Antibiotics disrupt the normal intestinal microbiota leading to the loss of colonisation resistance, thus creating an environment for *C. difficile* to grow, proliferate and cause a toxin-mediated disease(Peng *et al*., 2017; Pérez-Cobas *et al*., 2015). The first established link between the use of antibiotics and the development of CDI was reported in the 1970s, when cases of pseudomembranous colitis (PMC) were linked to the use of clindamycin(Bartlett *et al*., 1978; George *et al*., 1978; Tedesco *et al*., 1974). Since then several antibiotics, commonly used for the treatment of bacterial infections in the clinical settings have been implicated in the development of CDI (Baines & Wilcox, 2015). Clindamycin, a third-generation cephalosporin, fluoroquinolones and aminopenicillins are reported of greater propensity to induce CDI(Loo *et al*., 2005; Owens *et al*., 2008; Pepin *et al*., 2005).

Majority of *C. difficile* strains are susceptible to metronidazole and vancomycin, which are the first-line therapies for CDI, however, resistance to other antimicrobial agents in recent years

is a matter of great concern. Antimicrobial resistance enables *C. difficile* to thrive in the presence of increased levels of an antibiotic in the gut environment(Baines & Wilcox, 2015). Consequently, strains of *C. difficile,* that are resistant to multiple agents may have a selective advantage in the colon, possibly enhancing the risk of primary or recurrent CDI. Antimicrobial resistance does not only drive epidemiological change but also the emergence of new strain types. This was noted during the global emergence of hypervirulent *C. difficile* strain 027/B1/NAP1, due to the use of fluoroquinolones(He *et al*., 2013). These strains exhibited mutations in DNA gyrase genes which were absent in reportedly historic strains of the same type. Currently, there is no universal resistance pattern that has been reported in *C. difficile.* However reports from different antimicrobial susceptibility studies (between 2012-2015) indicated that clinical isolates of *C. difficile* were commonly resistant to clindamycin, cephalosporins, erythromycin and fluoroquinolones (55%,51% and 47% respectively) according to the Clinical Laboratory Standards Institute (CLSI) and EUCAST interpretative breakpoints(Freeman *et al*., 2018; Freeman *et al*., 2015; Goudarzi *et al*., 2013; Oka *et al*., 2012; Pirš *et al*., 2013; Senoh *et al*., 2015; Spigaglia, 2016; Tenover *et al*., 2012).

Many investigations have been made on the determinants of *C. difficile* antimicrobial resistance. Several resistance mechanisms have been identified, including the acquisition of genetic elements, alterations of antibiotic targets and the contribution of biofilm formation on persistence and tolerance(Chilton *et al*., 2016; Đapa *et al*., 2013; James *et al*., 2018; Vuotto *et al*., 2016). Approximately 11 % of the *C. difficile* genome, harbours an abundance of mobile genetic elements (MGEs), and some of these have been implicated in *C. difficile* antimicrobial resistance (Mullany *et al*., 2015). An example of this is seen in *C. difficile* resistance to Macrolide-Lincosamide-Streptogramin B (MLS_B) antibiotics, which is mediated by at least four kinds of transposons, including Tn*5398*, Tn*5398*-like derivatives, Tn*6194* and Tn*6215*

(Spigaglia, 2016). Tn*5398* have been shown to facilitate the transfer of the *ermB* gene which encodes a 23S rRNA methylase and induces the resistance to the MLS_B family antibiotics (Wasels *et al*., 2015). Transfer of Tn*6215* between *C. difficile* strains via phage transduction has been demonstrated (Goh *et al*., 2013), and recent data suggest that both Tn*6215* and Tn*5398* can be transferred to a recipient *C. difficile* strain through a transformation mechanism (Wasels *et al*., 2015). The transfer of Tn*6194* from strains of *C. difficile* to *Enterococcus faecalis* was also demonstrated *in vitro* (Wasels *et al*., 2015).

Alterations in the antibiotic target is another mechanism for mediating antimicrobial resistance in *C. difficile.* Many amino acid substitutions have been identified in target genes of different antibiotics in *C. difficile.* An example of this is seen in *C. difficile* resistance to fluoroquinolones, which is as a result of amino acid substitutions in the DNA gyrase genes (*gyrA* and/or *gyrB*). Mutations in the beta subunit of the *rpoB* gene, which encodes a bacterial RNA polymerase is known to mediate reduced susceptibility and resistance to rifamycins (Curry *et al*., 2009; O'Connor *et al*., 2008). Multiple factors may induce alterations in the antibiotic targets, however selective pressure from exposure to antibiotics in the environment is the most common mechanism. Recent reports by Vuotto and colleagues showed enhanced biofilm formation by *C. difficile* strains, due to selective pressure from exposure to antibiotics (Vuotto *et al*., 2016). It is suggested that biofilm formation may contribute to the acquisition of antimicrobial resistance in *C. difficile,* however, the mechanism is still poorly understood.

Since the continued use of antibiotics drives epidemiological prevalence and the emergence of new strain types, it is imperative to test the susceptibility of emerging ribotypes of *C. difficile* to different antibiotics. As a result, the antibiotic susceptibility and resistant patterns *of C. difficile* ribotype 002, which emerged recently as the most prevalent ribotype in the UK

will be investigated in the present study. Numerous methods exist for antimicrobial susceptibility testing (AST); however, the agar dilution method is the recommended gold standard by the CLSI. Agar dilution methods employ the diffusion of the antibiotic into the agar, and susceptibility to *C. difficile* is determined by comparing the minimum inhibitory concentration (MIC) values to the MIC interpretative breakpoint values.

3.1.1 **Aims and objectives**

To characterise clinical isolates of *C. difficile* ribotype 002 from different time lineages and geographical locations, according to their sensitivity and resistance to different antibiotics. The aims will be achieved by the following objectives

1. Determination of the minimum inhibitory concentrations to a diverse panel of antibiotics using the agar dilution method
2. Determination of mechanisms of resistance using PCR and sequencing of resistance genes, clindamycin inducible resistance, and beta-lactamase assay

3.2 MATERIALS AND METHODS

3.2.1 **Bacterial Strains**

The *C. difficile* isolates used for this study have been described in chapter 2.1.1. Control strains used included; E4 (PCR ribotype 010), which was previously characterised as being reduced susceptible to metronidazole(Brazier *et al*., 2001), p62 (PCR ribotype 001) as a clindamycin-resistant control (Fawley *et al*., 2003), *Bacteroides fragilis (B. fragilis*) ATCC 25285 as a metronidazole-susceptible control, and *Staphylococcus aureus* (*S. aureus*) ATCC 29213 was included as an indicator for aerobic contamination. *Escherichia coli (E. coli)* NCTC 9001 was used as a nitrofurantoin-susceptible control.

3.2.2 Antimicrobials Susceptibility testing and Minimum Inhibitory Concentration (MIC) Determination

Antimicrobial susceptibility testing was carried out according to methods described in 2.2. The MIC was determined as the lowest antimicrobial concentration where an absence or marked reduction of growth (multiple tiny colonies, haze or fine film of growth or one or two colonies) compared with the growth control was observed. The MIC values for individual strains were categorised as susceptible, intermediate and resistant based on the CLSI/EUCAST/BSAC MIC interpretative breakpoint values (Table 3-1). However, there were no approved breakpoints for the following antimicrobials: nitrofurantoin, clarithromycin, and trimethoprim against *C. difficile*; therefore, MIC breakpoint values for these antibiotics antimicrobials against *S. aureus* were used.

3.2.3 Clindamycin inducible resistance

Resistance to clindamycin was investigated by the clindamycin Inducible resistance ('D test') test. This test has been previously used to identify strains of *Staphylococcus* species that are inducible resistant to clindamycin(Prabhu *et al*., 2011). The aim of the test is to induce the expression of *erm*-mediated resistance through the exposure of isolates to both clindamycin and erythromycin, adjacent to one another on an agar plate. This test was performed on *C. difficile* isolates with MICs ≥8 mg/L, which according to CLSI guidelines were resistant to clindamycin. One hundred microliters of *C. difficile* overnight culture in Schaedler's anaerobe broth was inoculated onto pre-dried Wilkins Chalgren agar (WCA). Using a sterile swab, the bacterial suspension was evenly spread out on the WCA agar plates. Subsequently, a 30µg disc of erythromycin (CT0021B, Oxoid) was placed at 15mm from a 10µg clindamycin (CT0015B, Oxoid) using an Oxoid disc dispenser and incubated anaerobically for 48h at 37°C.

Following incubation, plates were retrieved from the anaerobic cabinet and examined for flattening of inhibition zone (D-shaped) around the clindamycin disc in the area between two discs (Figure 3.1). This phenomenon is an indication of inducible clindamycin resistance.

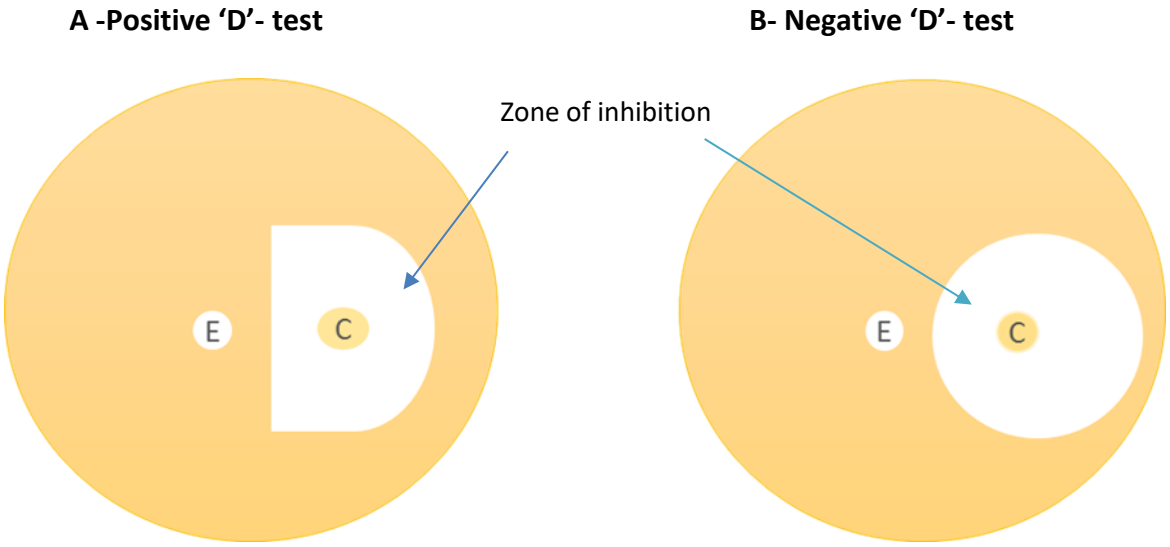


Figure 3-1 Schematic diagram showing clindamycin inducible resistance: A) is a representation of a positive D- test (D+ Phenotype). B) Is representation of a negative D- test phenotype E- Erythromycin, C-Clindamycin.

3.2.4 Beta-Lactamase Activity Assay

Beta-lactamase activity was assayed based on the hydrolysis of nitrocefin, a chromogenic cephalosporin that results in the generation of a coloured product. All isolates were investigated for β-Lactamase activity, using the broth method. Four drops of rehydrated nitrocefin (SR0112, ThermoFisher) solution was added to 1mL of *C. difficile* culture grown in Wilkins Chalgren broth. β- Lactamase activity was monitored by a visible colour change from yellow to red within 30 minutes of incubation.

3.2.5 **Molecular analysis of mechanisms of resistance**

Molecular analysis of the mechanisms of resistance to erythromycin, clindamycin, rifampicin, ciprofloxacin and moxifloxacin were investigated using a PCR assay. PCR products were purified using Wizard SV gel and PCR clean-up system (A9281, Promega, USA), and sequencing was done by Eurofins Genomics Germany (Eurofins GATC, Ebersberg, Germany), and Source BioScience (Cambridge, UK). Pairwise alignments of DNA sequences were carried out using the BLAST server and Clustal W Omega as described in 2.6.6.

Ciprofloxacin and moxifloxacin resistant isolates (MIC ≥8mg/L) were investigated for the mutations in the DNA gyrase genes (*gyrA* & *gyrB*). The quinolone resistance determining region (QRDR) of both *gyrA* and *gyrB* genes was amplified using two different pairs of primers, listed in Table 2-6. PCR amplification consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C (*gyrB*)/ 58°C (*gyrA*) for 30 s, and extension at 72°C for 30s(Spigaglia *et al*., 2011). Using agarose gel electrophoresis, described in 2.8.4, the resulting PCR products were assessed for the presence of a 390bp internal fragment of the *gyrB* and *gyrA* (Figure 3-5) by using standard molecular weight markers; Hyper ladder1-kb (BIO-33053, Bioline, London UK).

Erythromycin and clindamycin-resistant isolates (MIC ≥ 8mg/L) were investigated for the presence of the *erm (B)* gene by using the primers pair *ermBf* and *ermBr* listed in Table 2-6, (Sutcliffe *et al*., 1996). Strain p62 (PCR ribotype 001), previously described as resistant to clindamycin (Fawley *et al*., 2003), was included as a positive control for *erm (B)* gene. According to published methods by Solomon *et al,* PCR amplification consisted of 35 cycles of denaturation at 94°C for 30s, annealing at 52°C for 60s, and elongation at 72°C for 60s. The

last elongation step was performed at 72°C for 5 minutes(Solomon *et al*., 2011). Using agarose gel electrophoresis as described in 2.8.4, the resulting PCR product were assessed for the presence of a 639bp internal fragment of the *erm*(B) (Figure 3-5) by using standard molecular weight markers; 1kb DNA ladder (D3937, Sigma).

To detect the presence of other classes of *erm* (A, C, F and Q) in *ermB* negative isolates, primers described by Patterson *et al* (2007) as listed in Table 2-5 were used. PCR amplifications consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C (*ermA*), 43°C (*ermC*), and 52°C (*ermQ*) for 1 min, and elongation at 72°C for 2 mins. For *ermF*, PCR amplification consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 2 min. Using agarose gel electrophoresis as described in 2.8.4, the resulting PCR products were assessed for the presence of 645bp, 642bp, 466bp, and 771bp internal fragments of *erm* (A, C, F and Q respectively) (Figure 3-5) by using standard molecular weight markers; 1kb DNA ladder (D3937, Sigma).

Rifampicin resistant isolates with an MIC ≥16mg/L were investigated for mutations in *rpo (B)* gene using previously published primers CDrpoB2F and CDrpoB2R (Curry *et al*., 2009) as listed in Table 2-6. PCR amplifications consisted of 35 cycles of denaturation at 95°C for 1 min, annealing at 49.9°C for 1 min, and elongation at 72°C for 1min. Using agarose gel electrophoresis as described in 2.8.4, the resulting PCR product were assessed for the presence of 200bp, internal fragments of *rpo (B)* (Figure 3-5) by using standard molecular weight markers; 100bp DNA ladder (D3687, Sigma).

3.2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). In order to determine the appropriate statistical test to use, data were assessed for normality of distribution using both D'Agostino-Pearson and Shapiro-Wilk normality test. Statistically significant differences were tested using Kruskal-Wallis tests and differences were considered significant at a P-value of <0.05. An additional post hoc testing using Dunn's multiple comparison tests was used to determine the significant differences that existed between groups.

3.3 RESULTS

3.3.1 Antimicrobial susceptibility in all isolates and differences between groups

The susceptibility of all isolates was determined according to the CLSI/BSAC/EUCAST MIC recommended breakpoint values (as shown in Table 3-1). All isolates were fully susceptible to metronidazole, vancomycin, fidaxomicin, chloramphenicol, meropenem, linezolid and tetracycline (Table 3-1). Nitrofurantoin susceptibility was observed in 98.3% (n=59) of isolates; 96.7% (n=58) were susceptible to both erythromycin and moxifloxacin; 93.3% (n=56) were susceptible to clarithromycin; 91.6 % (n=55) were susceptible to rifampicin, and 6.7% (n=4) were susceptible to penicillin while 5% (n=3) and 3.3 % (n=1) were susceptible to ampicillin and clindamycin respectively. The MIC_{50} was the same value as MIC_{90} for all antimicrobial agents in all lineages, except for metronidazole, vancomycin, clindamycin, and nitrofurantoin.

As shown in figure 3-3, the older strains (UK 2007-8) had higher geometric mean MIC values for metronidazole (GM MIC= 0.4 mg/L, $p<0.05$), erythromycin (GM MIC= 2.6 mg/L, $p>0.05$),

clarithromycin (GM MIC=1.5 mg/L, p<0.05), moxifloxacin (GM MIC= 2.3 mg/L, p>0.05), and ciprofloxacin (GM MIC = 18.4 mg/L, p>0.05). The geometric mean MICs for meropenem (2.7 mg/L) and nitrofurantoin (6 mg/L) were higher in UK 2011-13 isolates, however, this was not statistically significant (p>0.05). Non-UK 2012-14 isolates had higher geometric mean MIC values for vancomycin (GM MIC =1.1 mg/L, p<0.05), and clindamycin (GM MIC= 9.6 mg/L, p>0.05).Conversely, the recent isolates (UK 2011-13 & Non- UK 2012-14) had higher geometric mean MICs for chloramphenicol (GM MIC= 3.8 mg/L, p>0.05).No statistical significant differences (p>0.05) existed between lineages for geometric mean MICs to penicillin, ampicillin, rifampicin, linezolid, fidaxomicin and tetracycline.

3.3.2 **Antimicrobial resistance of CD002**

Fluoroquinolone resistance was common in this study, as shown in Figure 3-2, all isolates were resistant to ciprofloxacin at an MIC of ≥8mg/L, only one UK isolate belonging to UK 2007-08 lineage was resistant to moxifloxacin (MIC >128mg/L). Amplicons produced following amplification of the quinolone resistance determining region (QRDR) of *gyrA and gyrB* (Figure 3-4a) were sequenced to detect sequence polymorphisms. A point mutation ACT- ATT in *gyrA*, resulting in the amino acid substitution Thr-82-Ile, was identified in two strains, one strain was of the recent non-UK lineage (Non- UK 2012-2014) while the second belonged to the older UK lineage (UK 2007-8). A novel nucleotide substitution Arg-98-Thr, Asp-103-His was identified in a non-UK isolate (Non- UK 2012-2014). No other nucleotide substitutions were identified. No nucleotide substitutions were identified in the *gyrB* gene.

Resistance to rifampicin (MIC of ≥32mg/L) was noted in one non-UK strain. Five isolates (1 each in both UK lineages and 3 in the Non-UK lineage) were reduced susceptible to rifampicin

at an MIC ≥0.004-16 mg/L. Resistant and reduced susceptible isolates were investigated for mutations in the *rpoB* gene (Figure 3-5c). Sequence analysis revealed a point mutation AGA-AAA in *rpoB*, resulting in the amino acid substitution Arg-505-Lys. This substitution was identified in one strain (MIC ≥ 16mg/L) of a non-UK lineage (2012-14). Additionally, a novel amino acid substitution Leu-509-Phe (CTT- TTT) was identified in a reduced susceptible strain (MIC ≥ 0.016mg/L), this strain belonged to the UK 2007-8 lineage.

Clindamycin resistance was present in 27% (n=16) of isolates, with many resistant isolates being of the non-UK lineage (n=8). Only two isolates (3.3%) belonging to two different UK lineages (UK 2007-8 & UK 2011-13) were resistant to erythromycin. The mechanisms of resistance to MLS_B antibiotics were further investigated by clindamycin inducible resistance test and PCR amplification to assess the presence of the *erm* (B) gene. The clindamycin inducible resistance test was negative for all isolates tested as shown in Figure 3-4. The *erm* (B) gene was not detected in any of the clindamycin resistant or erythromycin-resistant strains (Figure 3-5b). These strains were further investigated for the presence of other erm genes (A, C, F and Q), but none of these genes were present in any of the resistant isolates (Data not shown).

Intermediate resistance to ampicillin, penicillin, and clindamycin was observed in 93.3% (n=56), 93.3% (n=56), and 73.3%(n=44) of isolates respectively. To investigate the presence of beta-lactamase activity conferring resistance to penicillin, a beta-lactamase assay was performed. No isolates were positive for the Nitrocefin assay. All isolates were resistant to trimethoprim at an MIC breakpoint ≥128. Two isolates (3.3%), belonging to two different UK

lineages (UK 2007-8 & UK 2011-13) demonstrated resistance to clarithromycin, and while one isolate (1.7%) belonging to the UK 2007-8 lineage was resistant to nitrofurantoin.

Multidrug resistance (MDR), defined as resistance to more than two antimicrobial classes was observed in one UK isolate (UK 2007-8). This isolate was resistant to four antimicrobial classes (Fluoroquinolones, nitrofurans, macrolide-lincosamide-streptogramin B, and beta-lactams).

Table. 3-1 Antimicrobial susceptibilities (Geometric mean MIC, mg/L) of 60 clinical CD002 isolates to antimicrobial agents using agar incorporation MIC testing. PEN, penicillin G, AMP ampicillin, LZD, linezolid, MOX, moxifloxacin CHL, chloramphenicol, CLA, clarithromycin, ERY, erythromycin**;** VAN, vancomycin; MET, metronidazole; NIT, nitrofurantoin; CLI, clindamycin; TET, tetracycline; CIP, ciprofloxacin, MER, Meropenem, FDX, Fidaxomicin, RIF, Rifampicin and TMP, Trimethoprim

Antimicrobial Agent	Lineage	*N*	GM MIC (mg/L) [e]	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC Interpretative Breakpoints			S, n (%)	I, n (%)	R, n (%)
							S	I	R			
MET[a]	UK 2007-8	14	0.4	0.25-2	0.25	0.5	≤ 2	-	>2	14 (100)	0 (0)	0 (0)
	UK 2011-13	22	0.3	0.25-0.5	0.25	0.5				22 (100)	0 (0)	0 (0)
	Non-UK 2012-14	24	0.3	0.25-0.5	0.25	0.25				24 (100)	0 (0)	0 (0)
VAN[b]	UK 2007-8	14	0.8	0.5-2	1	1	≤ 2	-	>2	14 (100)	0 (0)	0 (0)
	UK 2011-13	22	0.9	0.5-2	1	1				22 (100)	0 (0)	0 (0)
	Non-UK 2012-14	24	1.1	0.5-2	1	2				24 (100)	0 (0)	0 (0)
FDX[c]	UK 2007-8	14	0.1	0.02-0.125	0.06	0.125	<1	>1	-	14 (100)	0 (0)	0 (0)
	UK 2011-13	22	0.1	0.02-0.125	0.06	0.125				22 (100)	0 (0)	0 (0)
	Non-UK 2012-14	24	0.1	0.02-0.125	0.06	0.125				24 (100)	0 (0)	0 (0)
RIF[a]	UK 2007-8	14	0.002	0.002-0.016	0.002	0.002	≤0.002	0.004-16	≥16	13(95.45)	1 (4.5)	0(0)
	UK 2011-13	22	0.002	0.002-0.004	0.002	0.002				21(95.45)	1 (4.5)	0(0)
	Non-UK 2012-14	24	0.003	0.002-32	0.002	0.004				21(87.5)	2(8.3)	1(4.1)
ERY[a]	UK 2007-8	14	2.6	2-128	2	2	≤2	4	≥8	13(92.9)	0 (0)	1 (7.1)
	UK 2011-13	22	2.3	2-128	2	2				21(95.5)	0 (0)	1(4.5)
	Non-UK 2012-14	24	1.9	1-2	2	2				24 (100)	0 (0)	0 (0)
CLI[a]	UK 2007-8	14	5.3	4-8	4	8	≤2	4	≥8	0 (0)	11(78.6)	3(21.4)
	UK 2011-13	22	7.1	2-8	4	8				1(4.5)	16(72.7)	5(22.7)
	Non-UK 2012-14	24	9.6	4-8	4	8				0(0)	17(70.8)	8 (29.1)
CIP[a]	UK 2007-8	14	18.4	16-128	16	16	≤4	-	≥8	0 (0)	0 (0)	14(100)
	UK 2011-13	22	16.5	8-32	16	32				0 (0)	0 (0)	22(100)
	Non-UK 2012-14	24	16	8-32	16	16				0 (0)	0 (0)	24(100)
MOX[a]	UK 2007-8	14	2.3	2-32	2	2	≤2	4	≥8	13(92.86)	0(0)	1 (7.14)
	UK 2011-13	22	1.8	1-2	2	2				22(100)	0(0)	0(0)
	Non-UK 2012-14	24	1.9	1-4	2	2				23(95.83)	1 (4.5)	0(0)
PEN[a]	UK 2007-8	14	1	≤1	1	1	≤0.5	1	≥2	0 (0)	14 (100)	0 (0)
	UK 2011-13	22	0.9	0.5-1	1	1				4(18.2)	18 (81.1)	0 (0)
	Non-UK 2012-14	24	1	≤1	1	1				0(0)	24 (100)	0 (0)

Antimicrobial Agent	Lineage	*N*	GM MIC (mg/L) [e]	MIC range (mg/L)	MIC $_{50}$ (mg/L)	MIC $_{90}$ (mg/L)	MIC Interpretative Breakpoints			S, n (%)	I, n (%)	R, n (%)
							S	I	R			
AMP[a]	UK 2007-8	14	1	0.5-2	1	1	≤0.5	1	≥2	1 (7.1)	12 (85.7)	1(7.1)
	UK 2011-13	22	1	0.5-1	1	1				1 (4.5)	21(95.5)	0 (0)
	Non-UK 2012-14	24	1	0.5-1	1	1				1 (4.5)	23 (95.5)	0 (0)
MER[a]	UK 2007-8	14	2.4	2-4	2	4	≤4	8	≥16	14 (100)	0 (0)	0 (0)
	UK 2011-13	22	2.7	2-4	2	4				22 (100)	0 (0)	0 (0)
	Non-UK 2012-14	24	2.5	2-4	2	4				24 (100)	0 (0)	0 (0)
TET[a]	UK 2007-8	14	0.2	0.125-0.25	0.25	0.25	≤4	8	≥16	14 (100)	0 (0)	0 (0)
	UK 2011-13	22	0.2	0.125-0.25	0.25	0.25				22 (100)	0 (0)	0 (0)
	Non-UK 2012-14	24	0.2	0.125-0.5	0.25	0.25				24 (100)	0 (0)	0 (0)
CHL[a]	UK 2007-8	14	3.5	2-4	4	4	≤8	16	≥32	14 (100)	0 (0)	0 (0)
	UK 2011-13	22	3.8	2-4	4	4				22 (100)	0 (0)	0 (0)
	Non-UK 2012-14	24	3.8	2-4	4	4				24 (100)	0 (0)	0 (0)
LZD[b]	UK 2007-8	14	2	≤2	2	2	≤4	-	>4	14 (100)	0 (0)	0 (0)
	UK 2011-13	22	1.9	1-2	2	2				22 (100)	0 (0)	0 (0)
	Non-UK 2012-14	24	2.0	≤2	2	2				24 (100)	0 (0)	0 (0)
CLA[d]	UK 2007-8	14	1.5	1-128	1	2	≤1	2	>2	11(78.6)	2(14.3)	1(7.1)
	UK 2011-13	22	1.2	1-128	1	1				21(94.5)	0(0)	1(4.5)
	Non-UK 2012-14	24	1.1	1-2	1	1				24(100)	0(0)	0(0)
NIT[d]	UK 2007-8	14	5.3	4-32	4	8	≤8	16	≥32	13 (92.9)	0 (0)	1 (7.1)
	UK 2011-13	22	6.0	4-8	8	8				22 (100)	0 (0)	0 (0)
	Non-UK 2012-14	24	4.8	4-8	4	8				24 (100)	0 (0)	0 (0)
TMP[d]	UK 2007-8	14	128	>128	-	-	≤1	-	>1	0 (0)	0 (0)	60 (100)
	UK 2011-13	22	128	>128	-	-				0 (0)	0 (0)	60 (100)
	Non-UK 2012-14	24	128	>128	-	-				0 (0)	0 (0)	60 (100)

[a] MIC breakpoint applied were those recommended by the Clinical and Laboratory Standards Institute (CLSI) [b] Vancomycin and linezolid MIC breakpoint applied was recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) [c] Fidaxomicin MICs were compared to the EUCAST epidemiological cut-off value (1 mg/L) and defined as sensitive or reduced susceptibility. [d]MIC breakpoints applied were those recommended for *S. aureus* by the British society for antimicrobial chemotherapy (BSAC) (http://bsac.org). [e]Geo mean - The central number in a geometric progression (e.g. 9 in 3, 9, 27), also calculable as the nth root of a product of n number.

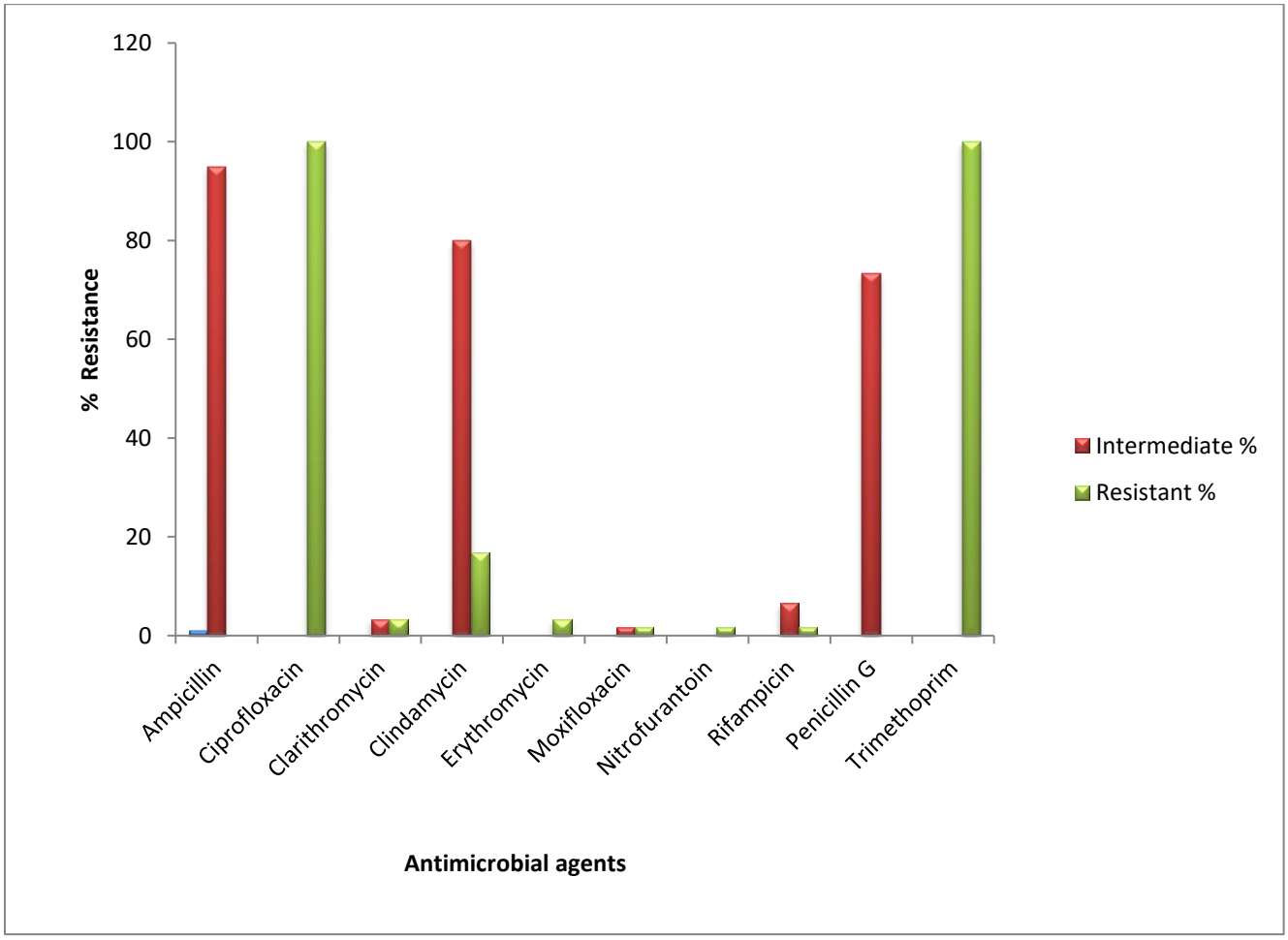


Figure 3-2 Antimicrobial resistance (% of isolates above the susceptible MIC breakpoint) of clinical CD002 (N=60) to a range of antimicrobial agents using agar incorporation MIC testing.

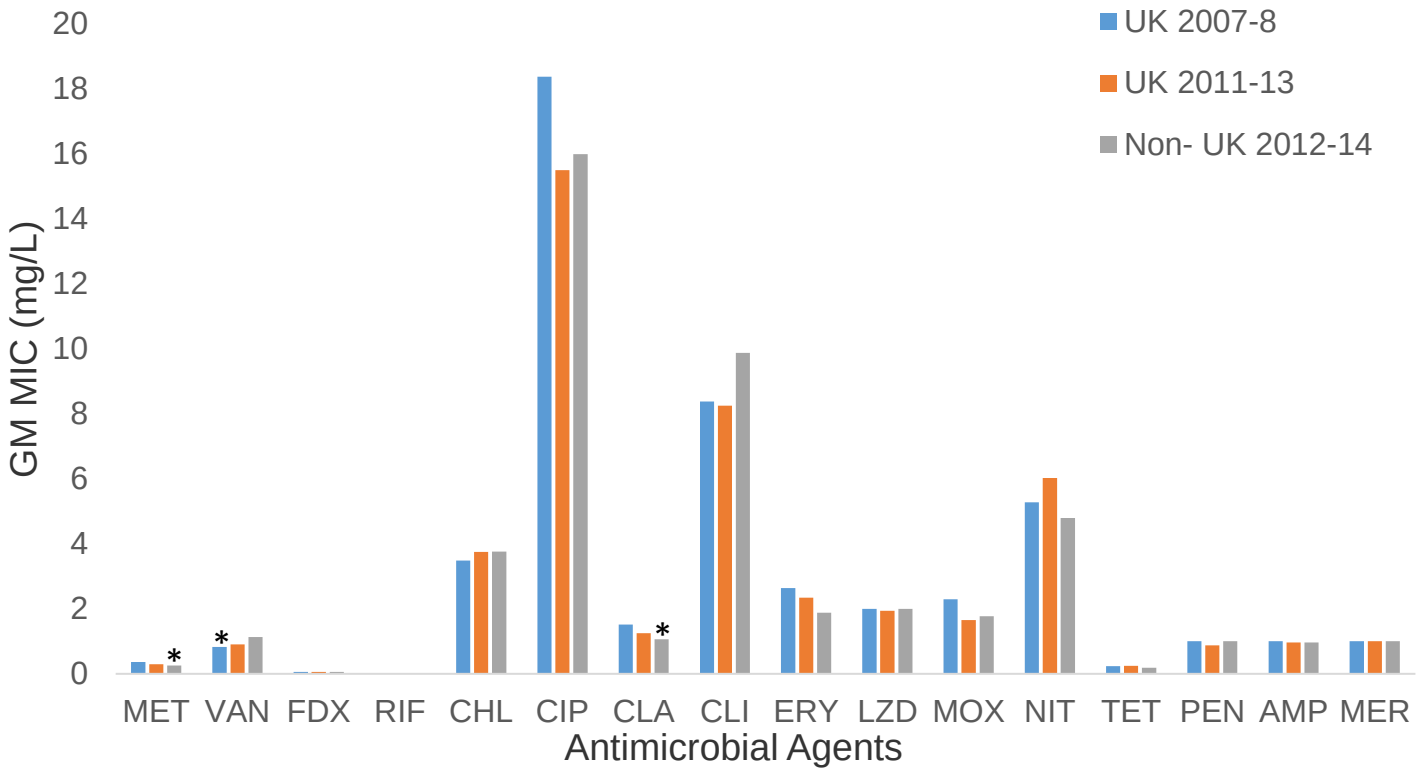


Figure 3-3 Antimicrobial agents geometric mean (GM) MICs (mg/L) for CD002 using agar incorporation MIC testing. PEN, penicillin G; AMP ampicillin; LZD, linezolid; MXF, moxifloxacin; CHL, chloramphenicol; CLA, clarithromycin; ERY, erythromycin; VAN, vancomycin; MET, metronidazole; NIT, nitrofurantoin; CLI, clindamycin; TET, tetracycline; CIP, ciprofloxacin, FDX, fidaxomicin, MER, Meropenem, Rifampicin, RIF. GM MICs (mg/L) for MET, ERY, MOX, CIP, and CLA were higher in UK 2007-8, MER and NIT were higher in UK 2011-13, VAN, and CLI was higher in Non-UK 2012-14 isolates, while CHL was higher in both UK 2011-13 and Non-UK 2012-14 isolates. The asterisk (*) indicates where a statistical difference was observed.

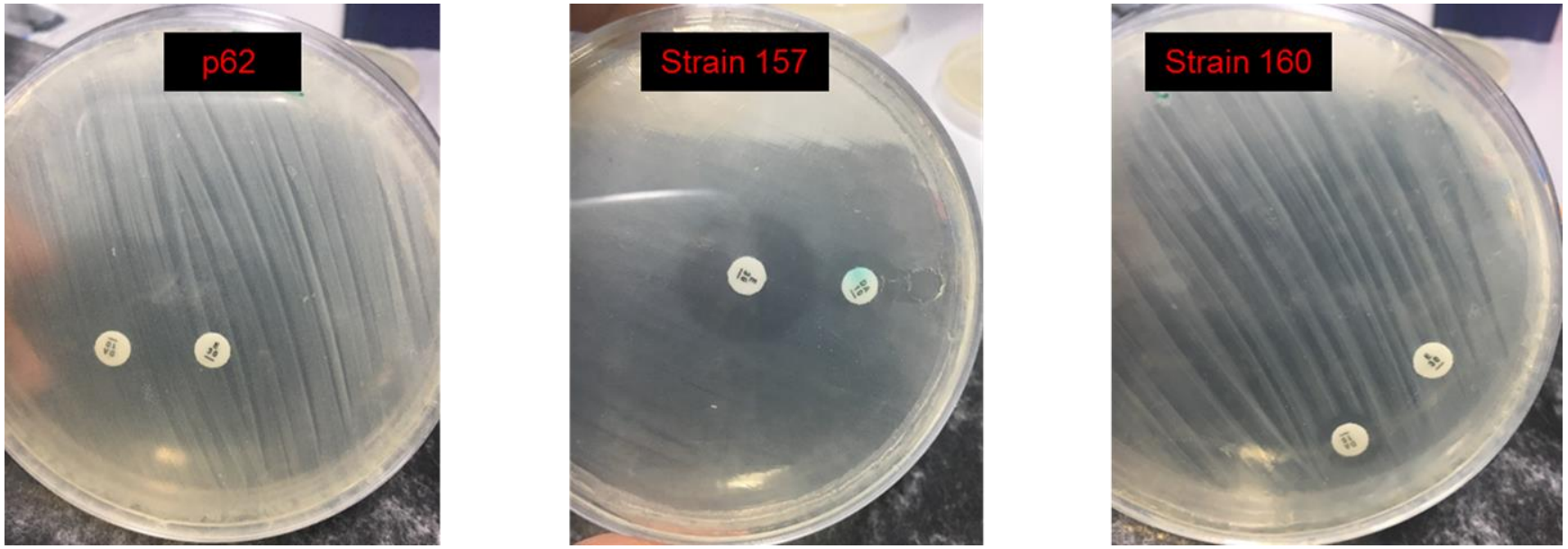


Figure 3-4 Disk diffusion testing for inducible clindamycin resistance of CD002 isolates. Erythromycin disk was placed 15mm apart from Clindamycin disk. **A)** *C. difficile* P62 strain, clindamycin resistant, negative D- test; **B)** CD002 strain 157, Erythromycin susceptible and clindamycin resistant, negative for D- test; **C)** CD002 strain 160, erythromycin and clindamycin resistant, negative D- test.

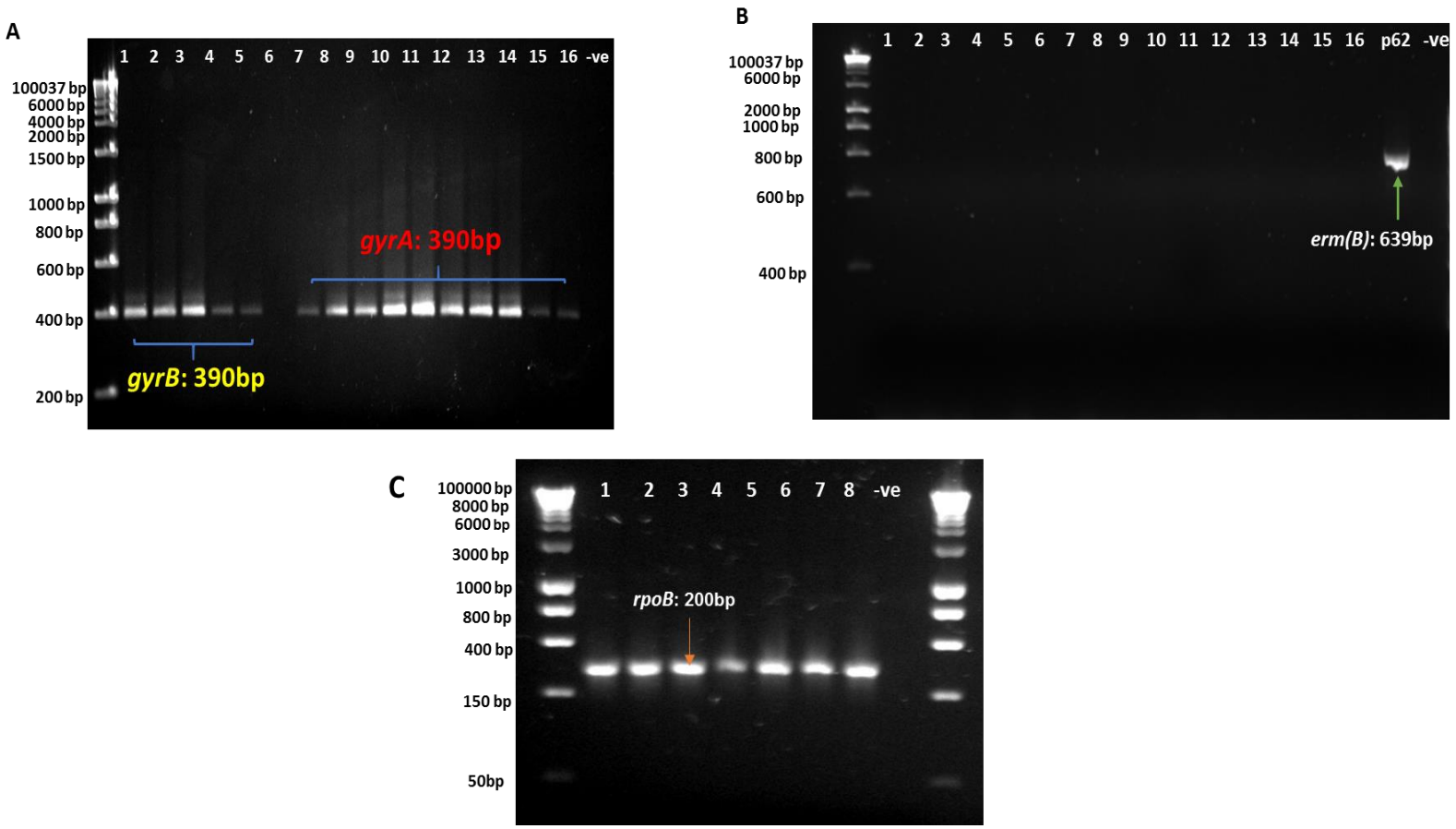


Figure 3-5 PCR analysis of antimicrobial resistance genes in CD002. Genomic DNA was extracted from CD002 isolates, and PCR was conducted to identify different target genes. PCR products were analysed by 1.4 % (w/v) agarose gel electrophoresis and visualized using UV light in a gel doc system. A) A PCR image for investigation of DNA gyrase subunit A and B genes, Lane1 represents 1kb DNA ladder, while lane 2-7 are amplified products for *gyrB*, lane 9-17 are amplified products for *gyrA*, lane 18 is a negative control. B) A PCR image for investigation or *erm*(B) gene; lane 1 is 1kb DNA ladder, while lane 2-16 are amplified products of the *erm*(B) gene, lane 18 is a negative control, while lane 19 is a positive control(P62) for the presence of DNA. C) A PCR image for investigation of *rpoB* gene in reduced susceptible and resistant CD002 isolates; Lane 1 and 10 1kb DNA ladder, lane 2-8 are amplified products of *rpoB*, Lane 9 is a negative control. The investigated gene; *gyrA*, *gyrB*, and *rpoB* were present in all isolates. All strains were negative for the *erm (B)* gene and only positive for the P62 control at 639bp.

3.4 DISCUSSION

Antimicrobial agents are known to play an important role in the induction and development of CDI. Some antimicrobial agents serve as treatments for CDI, while others are non-treatment agents, and both categories have the propensity to induce CDI. For the non-treatment agents, there is the potential that *C. difficile* strains, resistant to these agents might be able to cause CDI at an earlier stage in dosing when gut levels are still above the MIC of the indigenous microflora. On the other hand, *C. difficile* isolates sensitive to either treatment or non-treatment agents, are also able to induce CDI, following cessation of the antimicrobial therapy. CD002 emerged recently as the most prevalent ribotype in the UK, and evidence shows that it is the most frequently isolated ribotype in the community-associated CDI(CA-CDI) cases (Fawley *et al*., 2016). There is a possibility that antimicrobial agents been prescribed in the community for unrelated conditions such as throat infections, might be facilitating the prevalence of this ribotype in the community, consequently, leading to the import of CD002 into the hospital settings. As a result, antimicrobial susceptibility profiles to different agents, particularly agents typically prescribed in the community settings is necessary. In the present study, *C. difficile* PCR ribotype 002 isolates from different time lineages in the UK were tested for their antimicrobial susceptibility to seventeen antimicrobial agents. Using the MIC breakpoint values recommended by CLSI, EUCAST or BSAC as appropriate.

C. difficile resistance to first-line CDI therapies; metronidazole and vancomycin, is rarely reported (Freeman *et al*., 2015, 2018; Peláez *et al*., 2002). However, treatment failures associated with the use of metronidazole and vancomycin have been reported but not linked to resistance to these agents (Johnson *et al*., 2000; Musher *et al*., 2005; Pepin *et al*., 2005;

Vardakas *et al*., 2012). It is hypothesised that the poor efficacy of metronidazole may be associated with the colon concentration (0-9.3mg/L in faeces) which is insufficient to treat cases of CDI caused by strains with higher MICs(Baines *et al*., 2008; Bolton & Culshaw, 1986; Moura *et al*., 2013). Additionally, the development of heteroresistance which is reported in several studies may also be a contributing factor in the poor treatment efficacy of metronidazole (Martínez-Meléndez *et al*., 2018; Peláez *et al*., 2008).

The emergence of reduced susceptibility to metronidazole (i.e. MIC ≥ 4mg/L) was previously reported in *C. difficile* in ribotype 001 and subsequently in other ribotypes such as 027, and 106 (Baines *et al*., 2008; Freeman *et al*., 2015,2018). Concurrently, decreased susceptibility to vancomycin (MIC ≥4mg/L) has also been reported around Europe and the United States (Freeman *et al*., 2015) in a small proportion of isolates. The mechanism(s) of reduced susceptibility to metronidazole and vancomycin are yet to be elucidated. However, a report by Baines and colleagues hypothesised that reduced entry or enhanced efflux of the drug could contribute to the reduced susceptibility of *C. difficile* isolates to metronidazole(Baines *et al*., 2008). Recently, proteomic analysis of a resistant and reduced-susceptible phenotypes of PCR ribotype 027, revealed multiple proteins (such as putative 5-nitroimidazole reductase) involved in DNA repair and iron metabolism (Chong *et al*., 2014). The authors suggested that these proteins may contribute to the resistance and/or reduced susceptibility to metronidazole in *C. difficile.* The clinical impact of reduced susceptibility to vancomycin on treatment response remains undefined, as it is widely known, that high levels of vancomycin is reached in the colon (>2200mg/L) (Martínez-Meléndez *et al*., 2018; Young *et al*., 1985).

In this study, neither resistance nor reduced susceptibility to vancomycin and metronidazole was observed among clinical isolates of CD002. CD002 isolates were inhibited by metronidazole at MIC_{50} 0.25mg/L and MIC_{90} 0.5mg/L, this finding correlates with reports by

Freeman *et al*. (2015). Published data show that metronidazole susceptibility in other *C. difficile* ribotypes varies between 0.125mg/L- 1mg/L respectively, contingent to geographical region(Rahimi, Jalali, & Weese, 2014). Ninety per cent of CD002 isolates were inhibited at a range of 0.5- 2mg/L to vancomycin. This suggests that the emergence of CD002 in the UK and across Europe is not because of the acquisition of antimicrobial resistance elements or acquired resistance due to mutational events following exposure to current CDI treatments.

Fidaxomicin was approved in 2011 as a therapy for CDI (Crawford *et al*., 2012). Since then, it has been reported to have excellent activity against *C. difficile* isolates (Ackermann *et al*., 2004; Finegold *et al*., 2004; Freeman *et al*., 2015, 2018; Goldstein *et al*., 2011; Louie *et al*., 2011).In a recent US-based surveillance study, 925 *C. difficile* isolates were inhibited at a range of (≤0.004-1mg/L) with an MIC_{90} of 0.5mg/L (Snydman *et al*., 2015). A recent analysis of *C. difficile* isolates in Mexico revealed an MIC_{90} of 0.06 mg/L to fidaxomicin(Martínez-Meléndez *et al*., 2018). In a previous study, 1323 isolates of *C. difficile* were inhibited at an MIC range of ≤0.001-1mg/L, with MIC_{90} of 0.5mg/L (Goldstein *et al*., 2012). In a three-year pan-European surveillance study, 2830 isolates were inhibited at a range (≤0.002-0.25mg/L), with an MIC_{90} of 0.125mg/L (Freeman *et al*., 2018). In the present study, all CD002 isolates were inhibited at an MIC range of (≤0.002-0.125mg/L), with an MIC_{90} of 0.125mg/L in all lineages, this is consistent with reports by Freeman *et al*. (2015, 2018).

Reduced susceptibility and resistance of *C. difficile* to fidaxomicin is very rare. One isolate with an MIC of 16 mg/L, recovered from a patient with recurrent CDI has previously been reported (Goldstein *et al*., 2011). Recently, Martinez-Melendez *et al*. (2018) reported four clinical isolates of PCR ribotype 027 with an MIC of 2mg/L, which were recovered from patients with recurrent CDI. Previously, Finegold *et al*. (2004), reported a single isolate with an MIC of 2mg/L. *In vitro* analysis by Leeds *et al*. (2014), demonstrated the presence of mutations in

rpoB or CD22120 which may confer reduced susceptibility or resistance of fidaxomicin to *C. difficile.* The MICs of CD002, presented in this study were much lower than the concentrations of fidaxomicin that are achieved the gut lumen and stool i.e. >1000 µg/g (Sears *et al*., 2012). Consistent with reports by Freeman *et al*. (2015, 2018), reduced fidaxomicin susceptibility was not observed CD002. In addition, the authors reported that fidaxomicin susceptibility is retained among *C. difficile* ribotypes circulating around Europe, following its introduction for use in the clinical setting. Based on reports by Freeman *et al* (2015, 2018) and the present study, we suggest that the emergence of CD002 in the UK is not due to reduced fidaxomicin susceptibility. However, continued surveillance is required, since reduced susceptibility to fidaxomicin in other ribotypes has previously been reported in different geographical locations (Goldstein *et al*., 2011; Martínez-Meléndez *et al*., 2018).

Rifamycins (rifaximin) have been used as a therapy for CDI (Oldfield *et al*., 2014). Recently, rifaximin was proposed as a chaser therapy for the treatment of recurrent CDI (Oldfield *et al*., 2014). *C. difficile* resistance to rifampicin has been reported in many studies, and mutations within the gene encoding the β-subunit of the RNA polymerase (*rpoB*), is the known mechanism for resistance (Huang *et al*., 2013; Martínez-Meléndez *et al*., 2018). Different amino acid substitutions have been identified within *rpoB*, however, Arg505Lys, known to confer a high- level of rifampicin resistance (MIC ≥128mg/L), and a low fitness burden, is the most common (Curry *et al*., 2009; Dang *et al*., 2016; O'Connor *et al*., 2008; Spigaglia *et al*., 2011). In the present study, reduced susceptibility and resistance to rifampicin was uncommon, 91.7%(n=55) of isolates were fully susceptible at concentrations (MIC≤0.002mg/L) below the reported faecal concentrations(8000 µg/g)(Jiang *et al*., 2000). Only one isolate of CD002, from Spain, was resistant, while five other isolates from different lineages and geographical locations were reduced susceptible to rifampicin. In the pan-

European surveillance study, 13.33% of isolates of different ribotypes originating from Spain, were resistant to rifampicin, none of these isolates was ribotype 002 (Freeman *et al*., 2015). Additionally, previous studies have reported higher rifampicin resistance amongst *C. difficile* isolates from Italy and Hungary (Freeman *et al*., 2015, 2018; Miller *et al*., 2011). However, in the present study, CD002 isolates from Italy were fully susceptible to rifampicin.

Molecular analysis of resistant and reduced susceptible CD002 isolates in the present study revealed previously reported amino acid substitution in *rpoB* Arg505Lys, in a Non- UK 2012-14 Strain. Additionally, a novel amino acid substitution (Leu509Phe) was identified in a reduced susceptible isolate belonging to the UK 2007-8 lineage. Mutational changes at this amino acid position 509, is very rare in *C. difficile* other species (including *E.coli, S. aureus*), but has been previously seen in *Mycobacterium tuberculosis* (Tan *et al*., 2012). The amino acid substitutions reported in the present study are located within position 488- 583 of the *rpoB gene* and mutations in this region are known to either disrupt the direct interaction between rifamycins and RpoB, or modify the rifamycin- binding pocket and therefore reduce affinity of the target for the antimicrobial(Baines & Wilcox, 2015; Curry *et al*., 2009). Although resistance to rifamycins was rarely seen in the present study, (which suggests that rifamycins are unlikely facilitating the emergence of CD002 in the UK), we cannot negate the observation from the three year pan-European surveillance study, that reported rifamycin resistance as being common among multiple *C. difficile* ribotypes (Particularly, 027, 001, 018, 356, 017, 176 and 198) (Freeman *et al*., 2018). As a result, continued surveillance in CD002 and other *C. difficile* ribotypes is required to monitor the spread of resistance and its impact on emerging *C. difficile* ribotypes.

Fluoroquinolone resistance in Europe is linked with hospital outbreaks caused by common prevalent ribotypes, notably PCR ribotype 027 (Freeman *et al*., 2015; Spigaglia *et al*., 2010). A

high level of resistance to second-generation (ciprofloxacin) and third-generation (moxifloxacin) fluoroquinolones was reported in studies of clinical isolates(Barbut *et al*., 2007) and was proven to be the main event responsible for the emergence of CD027 globally(He *et al*., 2013). Resistance to fluoroquinolones can be associated with mutations present in the quinolone resistance determining region (QRDR) of *gyr* genes (the gene products of which are involved in the replication of bacterial DNA), with the majority of the resistant *C. difficile* strains showing amino acid substitutions in the *gyrA* gene (Keessen *et al*., 2013; Spigaglia *et al*., 2011).In the present study, all CD002 isolates were resistant to ciprofloxacin (MIC ≥ 8mg/L). This is in concordance with earlier reports by Cheng *et al*, who reported resistance to ciprofloxacin at MICs > 32mg/L in CD002 isolates(Cheng *et al*., 2011). Similarly, all human isolates of *C. difficile* (including CD002) in a previous study, were highly resistant to ciprofloxacin, while no resistance to moxifloxacin resistance was recorded(Pirš *et al*., 2013). Barbut *et al* (2007) and Freeman *et al* (2015) observed moxifloxacin resistance in 37.4 % and 39.9% of *C. difficile* isolates respectively. In the present study, only one isolate of CD002 (UK 2007-8) was resistant to moxifloxacin with an MIC ≥ 32mg/L. Tenover *et al* found three moxifloxacin resistant (MIC >32mg/L) CD002 isolates in North America, however, their methods for antimicrobial susceptibility was different from methods applied in this study(Tenover *et al*., 2012). Previous studies found amino acid substitutions in DNA gyrase genes for strains highly resistant to fluoroquinolones (MIC ≥ 32mg/L)(Solomon *et al*., 2011; Spigaglia *et al*., 2011). In the present study molecular characterisation of specific resistance to fluoroquinolones revealed the common amino acid substitution in *gyrA*, Thr-82-Ile, in two isolates of CD002 belonging to the UK 2007-8 lineage and Non-UK 2012-14 lineage respectively. Additionally, two novel amino acid substitutions, Arg-98-Thr, Asp-103-His in *gyrA*, were identified in a non-UK strain, that was only resistant to ciprofloxacin (MIC= 32

mg/L). Mutations within the *gyrB* subunit or both subunits are known to be associated with strains that attained a higher level of resistance to moxifloxacin (MIC ≤ 32 mg/L), however, this was not the case in the present study. The moxifloxacin resistant (MIC= 32mg/L) isolate only harboured mutations in the *gyrA* subunit, and no other fluoroquinolone-resistant isolates in the present study harboured mutations in *gyrB*. This result suggests that the driving force of fluoroquinolone resistance in the emergence of epidemic strains, particularly ribotype 027, is less likely to be a driving factor in the recent emergence of CD002 in the UK.

C. difficile resistance to MLS_B antimicrobial agents (clindamycin and erythromycin) has been reported in the literature and is commonly associated with the presence of the *erm(*B) gene which encodes an rRNA methylase carried on a conjugative transposon Tn*5398* (Spigaglia, 2016)In the present study, MIC values for the MLS_B group of antibiotics, clindamycin and erythromycin ranged between (≤1-8mg/L and ≥0.5-128mg/L, respectively) for all CD002 isolates. Clindamycin resistance was observed in 26.7% (n=16) of CD002 isolates, with majority of these isolates being of the non-UK lineage. The GM MICs to clindamycin did not differ significantly between lineages (range 3.87- 5.1mg/L), which is slightly lower compared to published results in Europe, where the GM MIC for clindamycin observed in CD002 isolates was 5.4- 6.8mg/L (Freeman *et al*., 2018). Additionally, reduced susceptibility to clindamycin was observed in 71% of isolates (MIC >4mg/L). This finding is similar to published results by Pirs *et al*, in which animal and human isolates of CD002 demonstrated reduced susceptibility to clindamycin at 85% and 9.1% respectively(Pirš *et al*., 2013). Erythromycin resistance was uncommon in this study, only two UK CD002 isolates belonging to different lineages (UK 2007-8, UK 2011-14) were resistant to erythromycin at an MIC of 128mg/L. Cross-resistance to clindamycin and erythromycin was uncommon. Prior studies identified *C. difficile erm*-negative strains, resistant to both erythromycin and clindamycin or only to erythromycin

(Pituch *et al*., 2006; Spigaglia *et al*., 2011). All CD002 strains in the present study were *erm* (B) negative including strains resistant to both erythromycin and clindamycin or only erythromycin. Erm-negative CD002 isolates in the present study were also negative for the predominant alternative *erm* genes found in anaerobes (e*rm A, C, F* and *Q*). This is in agreement with published results by Spigaglia and colleagues, where, fifty-three *C. difficile* strains were *erm* (B) negative and also negative for these other common *erm* genes found in anaerobes(Spigaglia *et al*., 2011). Further investigations by Spigaglia and colleagues were unable to delineate the mechanism of resistance in *erm* (B) negative isolates(Spigaglia *et al*., 2011). Dingle *et al* identified a novel Tn*916-like* transposon, similar to Tn6218, that participates in the transfer of *cfr* and *ermAB* genes, it is suggested that the *cfr* gene could play a role in *C. difficile* resistance to MLS_b antibiotics in the absence of *erm*(Dingle *et al*., 2014). Based on these reports, it would have been plausible to suggest that the *erm (B)* negative isolates identified in the present study, could be positive for the *cfr* gene, however since these isolates were susceptible to linezolid, this might be unlikely.

Historically, beta-lactam antibiotics (aminopenicillins and cephalosporins) have been heavily implicated in the development of CDI (Goudarzi *et al*., 2013). The mechanism by which this occurs is not fully known, however, increased exposures of ampicillin is thought to stimulate the expression of adhesins that are fundamental to *C. difficile* pathogenesis (Dawson, Valiente, & Wren, 2009). Additionally, as broad-spectrum antimicrobials, they perturb the microbiota balance in the gut thereby creating an environment for *C. difficile* to flourish. Resistance to β-lactams is found to be associated with antibiotic- degrading agents, β-lactamases or mutational events resulting in the modification of penicillin-binding proteins (PBPs), which affect the drug affinity(Spigaglia, 2016).In a previous study, patients carrying CD002 were found to have received more β- lactam antibiotics in preceding 3 months,

suggesting that these group of antimicrobial agents may induce CDI mediated by CD002 (Cheng *et al*., 2011). A prominent observation in the present study was the high percentage of intermediate resistance to beta-lactam antimicrobial agents (ampicillin and penicillin G) in CD002. A majority (93.3%, n=56) of isolates demonstrated intermediate resistance to ampicillin and penicillin G. The MIC_{50} and MIC_{90} values of penicillin and ampicillin in all lineages was 1mg/L. This suggests that both antimicrobial agents exhibit the same antimicrobial activity on CD002 isolates. Although investigations into β-lactamase activity in all isolates were negative, reduced susceptibility to beta-lactams (ampicillin & penicillin), maybe a factor influencing the emergence of this ribotype in the UK. As strains exhibiting reduced susceptibility might be able to grow earlier in the pharmacokinetics (PK) cycle, before the recovery of the indigenous microflora, therefore aiding colonisation and subsequent likelihood of developing an infection. However, further studies utilising other model systems and ribotypes is warranted to support this hypothesis.

Trimethoprim, a folate pathway inhibitor primarily used in the treatment of urinary tract infections, had a no activity against all CD002 isolates used in this study. All isolates were resistant to trimethoprim with MIC_{50} and MIC_{90} values that were greater than 128 mg/L. *Clostridium* species and anaerobes are reportedly intrinsically resistant to trimethoprim (Mazuet *et al*., 2016; Then & Angehrn, 1979). Markedly high trimethoprim MICs among *C. difficile* isolates have been recorded in other studies. An earlier study reported resistance to trimethoprim at MIC ≥512mg/L by 30 genotypically distinct *C. difficile* ribotypes(Freeman & Wilcox, 2001). Knight and colleagues, reported MIC_{50} and MIC_{90} of 64 mg/L to *C. difficile* isolates that were recovered from animals and humans in Australia(Knight *et al*., 2016). Despite widespread resistance to trimethoprim in *C. difficile,* this antimicrobial is not linked as having a high propensity to induce CDI, most likely due to its poor anti-anaerobe

activity(Mazuet *et al*., 2016) and therefore inability to disrupt host colonisation resistance. As a result, the use of this agent particularly in the community is unlikely, driving the emergence of CD002.

Many studies highlight the emergence of multi-drug resistance among *C. difficile* strains, particularly hypervirulent RT027 and RT001/072 (Freeman *et al*., 2011; Spigaglia *et al*., 2011). More recently, high levels of multi-drug resistance in Europe is associated with emergent ribotypes, notably 017, 018, 198 and 356 (Freeman *et al*., 2015, 2018). In addition, antimicrobial resistance surveillance studies in Hong Kong reported the emergence of multidrug-resistant *C. difficile* PCR ribotype 017 and 002 (Chow *et al*., 2017). Other studies showed that the percentage of *C. difficile* multidrug-resistant strains vary from one geographic location to another. Tenover *et al*, identified no multi-drug resistant strains in CD002 isolates within North America(Tenover *et al*., 2012). The present study showed only 3.3% of isolates were multi-drug resistant as they demonstrated resistance to at least three different antimicrobial classes. The low percentage rates of multidrug resistance among CD002 isolates in the present study and Tenover *et al*'s study suggests CD002 strains with multi-drug resistance may not be circulating around the UK and other countries across Europe and North America. However, since multi-drug resistant CD002 is rapidly emerging in Asia, continuous surveillance is necessary, in order to prevent potential CDI outbreaks that may be caused by strains of this ribotype.

The data generated in the present study highlighted the antimicrobial susceptibility patterns and resistance associated with CD002 isolates circulating across Europe. In general, the results obtained indicated that CD002 from the different lineages have comparable antimicrobial susceptibility patterns observed within other PCR ribotypes that have been previously studied(Aspevall *et al*., 2006; Freeman *et al*., 2015, 2018; Keessen *et al*., 2013; Pirš

et al., 2013). Therefore, it is highly unlikely that antimicrobial resistance might be a driver for the emergence of CD002 in the UK and in Europe.

4 INVESTIGATION OF VIRULENCE FACTORS AND BIOFILM FORMATION OF CD002 ISOLATES FROM DIFFERENT TIME LINEAGES

4.1 BACKGROUND

The notoriety of *Clostridium difficile* as the leading cause of nosocomial antibiotic-associated diarrhoea worldwide has increased in recent years (Planche & Karunaharan, 2017). The consensus for this dramatic increase is attributed to the emergence of hypervirulent strains. *C. difficile* is an anaerobic, spore-forming Gram-positive bacterium that causes a wide spectrum of diseases, ranging from asymptomatic carriage, mild and self-limiting diarrhoea, to severe life-threatening pseudomembranous colitis, toxic megacolon, sepsis and death(Smits *et al*., 2016).

Clostridium difficile infection (CDI), is acquired through ingestion of *C. difficile* spores that have been shed into the environment by symptomatic or asymptomatic carriers, or potentially from environmental or zoonotic sources (Brown & Wilson, 2018; Eyre *et al*., 2013;Freeman *et al*., 2010; Rabold *et al*., 2018). These highly resistant spores survive the acidic nature of the stomach and proceed to the small intestine, where they germinate into vegetative cells following stimulation by the host-derived germinants, most notably primary bile acids(Britton & Young, 2014). Primary bile acids (cholate and chenodeoxycholate) are derived from the liver, in the colon, they induce *C. difficile* germination through a Ger- type germinant receptor known as CspC (Buffie *et al*., 2015; Francis *et al*., 2013; Paredes-Sabja *et al*., 2014). As a protective mechanism of colonisation resistance in the colon, certain members

of the gut microbiota (particularly *Clostridium scindens*) encode 7α-dehydroxylation enzymes in a bile acid-inducible (bai) CD (known as *baiCD*) gene cluster(Solbach *et al*., 2018; Theriot, Bowman, & Young, 2016). These 7α-dehydroxylating enzymes are responsible for metabolising primary bile acids to secondary bile acids (deoxycholate and lithocholate), that inhibit *C. difficile growth* (Crobach *et al*., 2018). Hence, a disruption of the gut microbiota (principally due to antimicrobial therapy) abolishes 7α-dehydroxylating activity and reduces resistance to *Clostridium difficile* colonisation. This has been demonstrated in several studies (Buffie *et al*., 2015; Solbach *et al*., 2018; Theriot *et al*., 2016).

Following germination of *C. difficile* spores, a proteolytic cascade leads to the degradation of the spore peptidoglycan, release of calcium dipicolinic acid and rehydration of the spore(Paredes-Sabja *et al*., 2014). This ultimately results in the outgrowth of vegetative cells that colonise the host, following proliferation and adherence to host intestinal epithelial cells. Several cell surface proteins (such as Cwp66, Fbp68, and the *C. difficile* flagella) have been shown to aid adherence and colonisation in CDI (Baban *et al*., 2013; Barketi-Klai *et al*., 2011; Mora-Uribe *et al*., 2016). Subsequently, the main virulence factors, toxin A and/or toxin B, are released, for the full manifestation of disease. Toxins become internalised by the host cells and are subsequently transferred to the cytoplasm. Following activation, the toxin inactivates host GTPases, leading to the disruption of the actin cytoskeleton and an impairment of the tight junctions. This results in fluid accumulation and extensive mucosal damage to the large intestine (Smits *et al*., 2016).

In addition to these large clostridial cytotoxins, several other virulence factors are implicated in CDI pathogenesis. An example is the *C. difficile* binary toxin, which is an additional toxin produced by some strains of *C. difficile* such as ribotypes 078 & 027. The exact role of binary toxin in CDI pathogenesis remains unclear, however, there is evidence to show that the binary

toxin facilitates the adherence of *C. difficile* cells to host epithelial surfaces (Gerding *et al*., 2014; Schwan *et al*., 2009). Additionally, Stewart and colleagues identified the binary toxin as a predictor for recurrent CDI(Stewart *et al*., 2013). Furthermore, *in-vitro* studies have demonstrated the ability of *C. difficile* to form biofilms on surfaces, however, their role in disease pathogenesis remains poorly understood (Crowther *et al*., 2014a; Dapa & Unnikrishnan, 2013; Dawson *et al*., 2012; Hammond *et al*., 2014; James *et al*., 2018; Semenyuk *et al*., 2014). It is hypothesised that biofilms within the gut may play a role in recurrent CDI, by serving as a potential reservoir for *C. difficile* (vegetative cells and spores) to re-colonise the host after treatment has been administered(Baines *et al*., 2005; Crowther *et al*., 2014b; Dapa & Unnikrishnan, 2013). Indeed, this was demonstrated in an *in vitro* gut model comparing multi-species intestinal biofilms (Crowther *et al*., 2014). Approximately eighteen days after cessation of antimicrobial therapy, recurrent CDI occurred, following an initial episode of simulated CDI that had been successfully treated with vancomycin(Crowther *et al*., 2014a).

The acquisition and expression of virulence factors by pathogenic bacteria often promote their survival. This phenomenon has been demonstrated in hypervirulent *C. difficile* ribotype 027. Prior studies suggest that the global emergence of hypervirulent *C. difficile* ribotype 027, is attributed to the strain's acquisition of several virulence phenotypes, such as a higher sporulation ability and increased toxin production(Merrigan *et al*., 2010; Vohra & Poxton, 2011). Additionally, some colonisation factors such as biofilm formation, spore formation during biofilm development and a high degree of adherence to intestinal epithelial cells have been identified in hypervirulent *C. difficile* ribotype 027 strains, particularly epidemic strain R20291(Dapa & Unnikrishnan, 2013; Dawson *et al*., 2012; Joshi *et al*., 2012; Mora-Uribe *et al*., 2016; Semenyuk *et al*., 2014). Although controversial, it is suggested that these factors may

have granted hypervirulent *C. difficile* ribotype 027 strains, a competitive advantage over other ribotypes, which enabled their persistence in the human colon and contributed to their epidemiological prevalence alongside the development of antimicrobial resistance to fluoroquinolones. Based on this knowledge, understanding how recent emerging strains may differ from previously circulating strains of the same *C. difficile* ribotype is essential.

Although speculative, it is possible that emerging strains of CD002 in the UK may have acquired strain-specific characteristics that may facilitate their survival and drive increased prevalence in clinical CDI. To investigate the emergence of CD002 it is important to evaluate key virulence determinants of CD002 strains of different epidemiological lineages, in other to ascertain if any specific traits exist that may help explain the recent emergence of this ribotype.

In this study, the classical virulence phenotypes such as toxin production and sporulation were analysed. As well as phenotypes recently conceived to be involved in host persistence of *C. difficile* such as biofilm formation, sporulation during the development of biofilms and spore adherence to epithelial cells.

4.1.1 **Aims and objectives**

To study and quantify virulence characteristics of *C. difficile* ribotype 002 from different time lineages and geographical locations. The aims were achieved by the following objectives;

1. Evaluation of the total sporulation capacities of CD002 isolates from different time and lineages using batch growth in BHIS and enumeration by ethanol shock and viable counting
2. Evaluation of spore adherence properties to human gut epithelial cells at different stages of cell development using a Caco- 2 cells adherence assay

3. Evaluation of *C. difficile* toxins*:* cytotoxin production profiles using Vero cell cytotoxicity assay; and PCR for the *C. difficile* binary toxin gene
4. Quantification of biofilm formation of CD002 isolates using the crystal violet assay and viable counting of spores and vegetative cells present within biofilms

4.2 MATERIALS AND METHODS

4.2.1 **Bacterial strains**

C. difficile PCR ribotype 002 strains and controls used in this study have been described in 2.1. As indicated in Table 4-1, all 60 CD002 strains were investigated for cytotoxin production over time, binary toxins, total sporulation capacities, and biofilm studies. However, in the spore adherence assay, only two strains were selected per lineage (Table 4-2), making a total of 6 CD002 strains being investigated for spore adherence. The criteria for selecting a strain for adherence assay was based on sporulation rates after 24h; i) one had to be below the average sporulation rate of that lineage, ii) and the second had to be above the average sporulation rate of that lineage.

To compare results with hypervirulent strains, an 027 strain and 078 strain were included in cytotoxin studies. All strains were cultured according to methods described in 2.1.3- 2.1.6.

Table 4-1 Number of strains used per assay

Assay	CD002 strains used
Toxin studies	60
Total sporulation capacity	60
Biofilm studies	60
Spore adherence	6

Table 4-2 List of strains selected for spore adherence to epithelial cells

Strain	Spore formation at 24h	Average spore formation per lineage at 24h	Lineage
159	1.25 x10^3	1.04 x10^4	UK 2011-13
146	3.95 x10^4		
165	1.55 x 10^3	2.06 x 10^3	UK 2007-8
174	4.52 x10^3		
140	1.90 x10^3	2.32 x10^4	Non- UK 2012-14
141	6.83 x 10^4		

4.2.2 Growth rate (μ) measurement

The growth rate for all CD002 strains was determined according to methods described in 2.4.

4.2.3 Cytotoxin production profiles and investigations for the presence of binary toxin

The cytotoxin production profiles of all CD002 strains was investigated according to methods described in 2.3.1. Cytotoxin production over time was measured at time points: 0, 8, 12, 24, 48 and 72h in Brain Heart Infusion (BHI) broth. Additionally, to see whether cysteine repressed toxin production, CD002 strains were grown in BHI (supplemented with 0.1% (w/v) L-cysteine HCl (C7477, Sigma) + 0.5% (w/v) yeast extract (LP0021, Oxoid) (BHIS), and cytotoxin production was investigated after 72h. To investigate the presence of binary toxin (*cdtA* and *cdtB*) in all isolates, primers described by Stubbs *et al* (2006) as listed in Table 2-6 were used in a PCR assay. PCR conditions were of 30 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 1 min, and elongation at 72°C for 1 min 20s. Using agarose gel electrophoresis as described in 2.83- 2.8.4, the resulting PCR products were assessed for the presence of a 375bp internal fragment of the *cdtA* (Figure 3-5) and 510bp for *cdtB* (Data not shown) by using standard molecular weight markers; 100bp DNA ladder (D3687, Sigma).

4.2.4 Sporulation Studies

4.2.4.1 Preparation of Sporulation medium and measurement of the sporulation rate

Determination of the total sporulation capacities of all CD002 was achieved based on previously published methods by Burns *et al* (2011). Overnight cultures of *C. difficile* grown in 5mL BHIS broth were diluted 1:10 into a fresh 24-hour pre-reduced 4.5 mL BHIS broth. These cultures were grown to an OD_{600} between 0.2-0.5 (early-log phase) to ensure the lack of spores (that will normally be present at stationary phase) upon inoculation into the sporulation medium. In triplicate, cultures grown to the desired OD_{600} (Between 0.2-0.5) were diluted 1:100 into fresh pre-reduced 4.95 mL BHIS broth (the sporulation medium). All cultures were then incubated anaerobically at 37°C for 120h.

To measure the colony forming units (CFU) formed from spores present in *C. difficile* cultures, 100µl of the sporulation medium was retrieved at time points: 0h, 24h, 48h, and 120h respectively. The total sporulation capacities were determined by enumerating the colony forming units at 0h, 24h, 48h and 120h according to methods described in 2.5.

4.2.5 Spore adherence assay

4.2.5.1 Spore preparation and purification

As listed in table 4-2, six strains were selected for spore adherence assay. The spores of these strains were prepared according to methods described by Joshi *et al* (2012). Strains were grown in 10mL of pre-reduced BHIS and incubated anaerobically for 10 days. To recover sporulating cells, *C. difficile* cultures were centrifuged at 5000*g* (1004, Hettich, EBA 21, Canada) for 15 mins at room temperature, the supernatant was discarded. The retained pellet was resuspended in 10ml of cold sterile distilled water and centrifuged at 5000*g*. This washing cycle was repeated three times to release spores from mother cells. The free spores were

then resuspended in 1mL of 50% ethanol (E/0600DF/21, Fisher Scientific, Loughborough, UK) for 1h to kill all vegetative cells. Crude spore preparations were stored at 4°C until use.

4.2.5.2 Hydrophobicity assay

Microbial adhesion to hydrocarbon (MATH) has previously been described by Rosenberg *et al* (Rosenberg, Gutnick, & Rosenberg, 1980). To determine the hydrophobic characteristics of purified CD002 spores, the MATH test was performed according to methods described by Joshi *et al* (2012). Purified CD002 spores were resuspended in 9 ml of sterile distilled water (SDW) to achieve an OD_{600} between 0.5-0.6. Subsequently, in duplicates 4ml of the spore suspension was transferred into a sterile universal, and 0.4ml of n-hexadecane (H6703, Sigma) was added. This mixture was vortexed at full speed for 1min and incubated at room temperature for 15 mins to allow separation of the different phases of the solution. The loss of turbidity of the aqueous solution was then measured to determine the optical density (OD_{600}) after hexadecane exposure. Changes in hydrophobicity were calculated by the formula below:

=100 - (Final OD_{600}/Initial OD_{600}) x100)

4.2.5.3 Spore adherence to epithelial cells

To investigate the adherence capacity of CD002 spores, purified spores were exposed to human gut epithelial cells (Caco-2 cells- Human adenocarcinoma, cells, PHE Culture collection, ECACC 86010202) at different stages of cell differentiation. Caco-2 cells were prepared according to methods described in 2.3.2. These cells were seeded into 12-well plates (150628, ThermoFisher) to a final density of 2 x 10^5 cells per well and incubated for 7 days and 15 days according to methods described by Joshi *et al* (2012). These time points were selected for testing because Caco-2 cells require 15 days of culture to become fully differentiated, and possess a microvillus brush border that is absent at day 7(Joshi *et al*., 2012). At seven- and

fifteen-days incubation, Caco-2 cells were washed three times with sterile PBS (P4417, Sigma) and monolayers were inoculated with CD002 spores at a ratio of 100:1 (2 x 10^7 spores/mL: 2 x 10^5 of Caco-2 cells) 200 µl of culture medium (EMEM). Spore exposed to Caco-2 cells were incubated for 100 mins at 37°C under aerobic conditions in a 5% CO_2 incubator (15660667, Fisherbrand, Fisher Scientific UK). After incubation, spore-exposed Caco-2 cells were carefully washed three times with sterile PBS to remove non-adherent *C. difficile* spores. Subsequently, spore-exposed Caco-2 cells were lysed with 100µl 0.06 % Triton X-100(T8787, Sigma) for 30 min at 37°C. The spore-cell lysate was serially diluted in sterile PBS, and viable counting was performed as described in 2.5. To determine total *C. difficile* spores counts, a second 12-well-microtitre plate of spore-exposed Caco-2 cells were not washed but directly lysed with 100µl 0.06 % Triton X-100(T8787, Sigma) and viable counting was performed as described in 2.5. All assays were performed with two biological and three technical replicates. The percentage of spore adherence was calculated by using the following formula:

[Final CFU/ml/initial CFU/ml)] X 100

4.2.6 Biofilm formation

4.2.6.1 Generation of *C. difficile* Biofilms

CD002 biofilms were generated based on previously published methods by Dawson *et al*. (2012) and Ethapa *et al.* (2013). For the generation of the biofilms, overnight *C. difficile* cultures in 5mL BHIS broth were diluted 1:10 into fresh pre-reduced 4.5mL BHIS broth. In triplicate, 200 µl of diluted *C. difficile* culture was aliquoted into each well of 96-well microtiter trays (DK-4000, ThermoFisher). To prevent evaporation of liquid, microtiter trays were wrapped in Parafilm® (10018130, Bemis PM992, USA) and incubated anaerobically for three and six days respectively. This process was repeated in BHIS+ 0.1M of glucose (BHISG) broth.

4.2.6.2 Measurement of Biofilm biomass

The estimation of biofilm biomass was performed using a crystal violet staining method (Ethapa *et al.,*2013). After the required incubation time (3 and 6 days respectively), the growth medium (BHIS, BHISG) was carefully removed from each well of the 96- well plates using a multichannel pipette (46300100, ThermoFisher) and discarded. The wells of the 96-well plates were gently washed three times with sterile phosphate-buffered saline (PBS) (P4417, Sigma) using a multichannel pipette, and then allowed to dry for 10 mins. The biofilm was stained with 200µl of 1% (w/v) crystal violet (C6158, Sigma) diluted in 95% industrial methylated spirits (M14450/17, Fisher Scientific), and incubated for 30 minutes at room temperature. Subsequently, excess crystal violet was removed from the wells, and stained biofilms were washed five times gently with sterile PBS. The dye was extracted from the biofilms by adding 200µl of methanol (M/4000/17, Fisher Scientific) per well and allowed to incubate for 15 minutes at room temperature. The absorbance of the 96-well microtitre trays was read at 590nm using a microtitre tray spectrophotometer (Thermo Scientific Multiskan EX, Vantaa, Finland). All experiments were performed with three biological replicates, and each experiment incorporated three technical replicates.

4.2.6.3 Viable counting of vegetative cells and spores in *C. difficile* biofilms

The population of vegetative cells and spores in biofilms was determined from three- and six-day-old biofilms, grown in 96 well microtitre plates (BHIS broth) in triplicate. After three and six days of incubation, BHIS was carefully removed from each well. The wells were washed twice with sterile PBS, and 100µL of sterile PBS was added to the wells of the plate. Three- and six-day-old biofilms were disrupted under anaerobic conditions, by agitating at 1300 rpm for 10 minutes on a microtiter personal plate shaker (1959, Mikura- Orbis, W, Sussex,). Disrupted biofilms were serially-diluted in sterile 24-hour pre-reduced PBS, and the

vegetative cells were enumerated on Brazier's agar (LAB160, Lab M, Lancashire, UK), using the viable counting method described in 2.5. Similarly, spores were enumerated by the alcohol shock method described in 2.5. One hundred microliters of 50% ethanol were added to 100µl of disrupted biofilms and, the mixture was incubated at room temperature for 1h to kill all vegetative cells present. Subsequently, spores present in three day and six-day-old biofilms were enumerated by the viable counting method. All experiments were performed with three biological replicates, and each experiment incorporated three technical replicates.

4.2.7 **Statistical analysis**

Statistical analysis was performed using the GraphPad Prism 6 Software (GraphPad Software, La Jolla, CA). Normality tests were performed on all data to determine the appropriate statistical test to use. Statistically significant differences were tested using ordinary one-way analysis of variance (ANOVA) or two-way analysis of variance at the 95% confidence interval. This was done in conjunction with Tukey's post-hoc test. A p-value of <0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 **Batch culture growth curves and maximum specific growth rate**

To evaluate the virulence characteristics among different isolates, it is important to first understand the various growth patterns that exist between bacterial groups. The maximum specific growth rate (μ_{max}) of *C. difficile* ribotype 002 isolates was determined by growth kinetic analysis. The maximum specific growth rate (h^{-1}) for all strains (μ_{max}) was calculated from within the exponential growth phase (4-6h) by determining the gradient of the ln (biomass) versus time (hours) plot. The values of μ_{max} ranged from 0.23 to 1.50 h^{-1}. Lineage comparisons showed that strains of European origin (Non-UK 2012-14) grew significantly

(mean μ_{max} 0.92, ±SE-0.05, Range 0.23-1.42 h^{-1}, P <0.001) faster than UK 2011-13(mean μ_{max} 0.76, ±SE-0.06, Range 0.23-1.5 h^{-1}) and UK 2007-8 strains (Mean μ_{max} 0.69, ±SE-0.03, Range 0.5-0.95 h^{-1}) (Figure 4-1).Conversely, the values of the maximum specific growth rate ($\mu_{max)}$ of both UK 2007-8 and UK 2011- 13 CD002 strains did not differ significantly (P<0.3), the growth curve distributions between lineages were similar, and no growth differences between strains were observed in the timing of the lag and stationary phases of growth (Figure 4-1a). However, in the exponential phase of growth (between 4-6h), a marked increase in biomass (OD_{600}= 0.6, 1.6, 2.0 at 4, 5, 6h respectively) was observed with the UK 2007-8 strains compared to UK 2011-13 strains (OD_{600}= 0.4, 0.8, 1.4 at 4, 5, 6h respectively) and Non-UK Lineage (2012-14) (OD_{600}= 0.3, 0.6, 1.4 at 4, 5, 6h respectively), but this was not statistically significant (p=0.624). At 8 hours of growth, there was no significant increase in the biomass across all lineages.

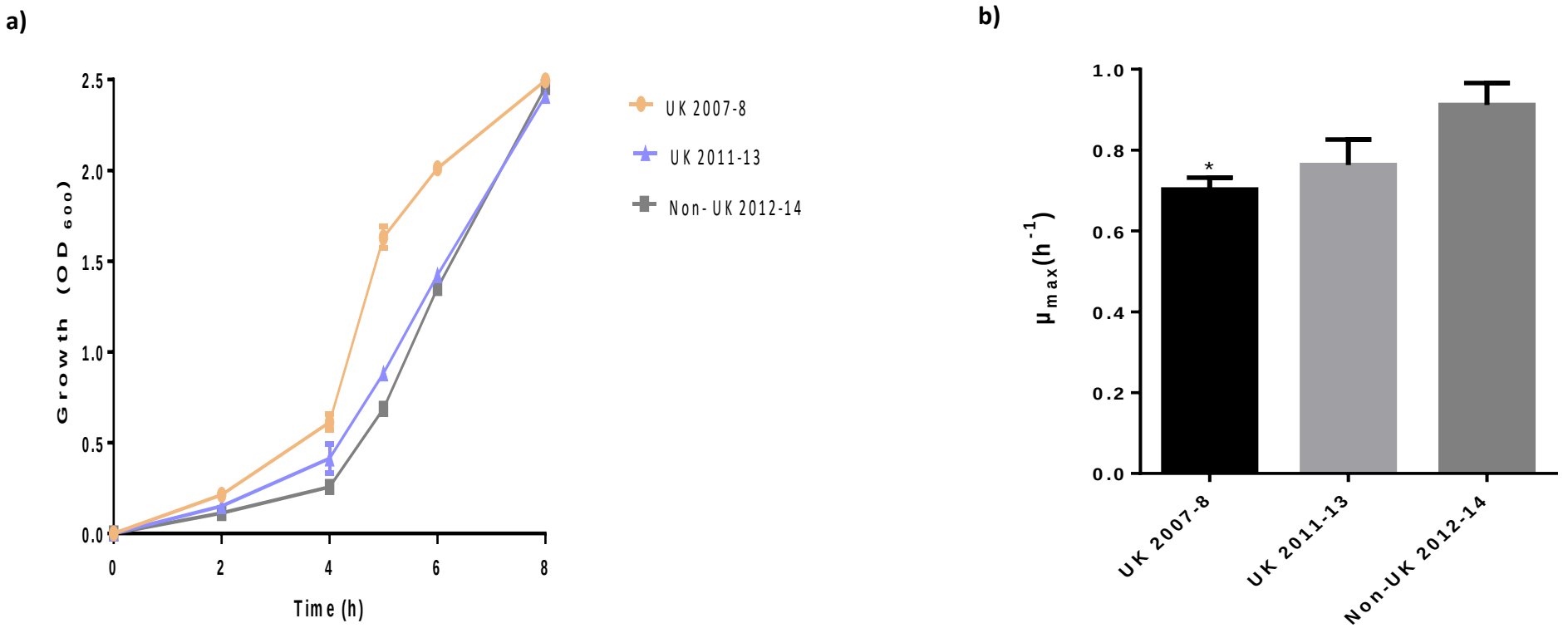


Figure 4-1 Batch growth curve (OD_{600}) and maximum specific growth rate (μ_{max}) of CD002 (h^{-1}, mean ±SE) in brain heart infusion broth. The growth of CD002 from different lineages in BHIS was measured by OD_{600} over an 8-hour period. **(a)** The mean biomass (OD_{600}) at 2, 4, 6, and 8h per CD002 lineage studied. The patterns of growth were similar in all lineages. **(b)** The mean μ_{max} per lineage of all CD002 studied. Strains of the Non-UK lineage had a higher μ_{max}. Data represent the average of three independent experiments and error bars are standard error of the mean. The asterisk (*) indicate where a significant difference was observed.

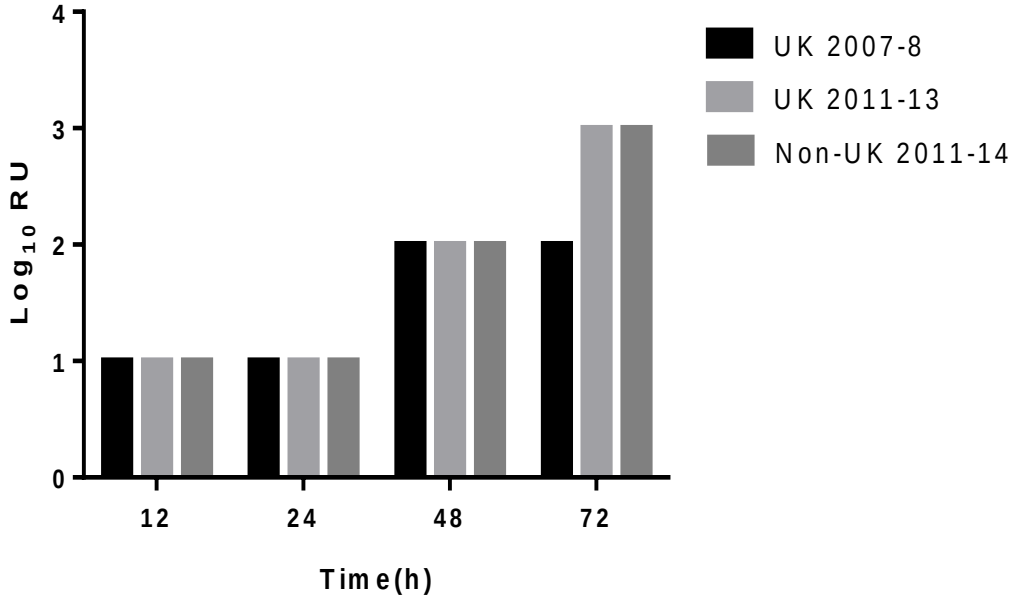
4.3.2 **Cytotoxin production over time and investigation for the presence of CDT locus**

All strains were examined for cytotoxin production using Vero cells cytotoxicity assay at different time intervals. *C. difficile* BHI culture supernatants were retrieved at time points: 0, 8, 12, 24, 48, and 72h of incubation. A sample was regarded as positive for cytotoxin, when a characteristic cytopathic effect against Vero cells (Figure 2-4) was observed, rounding ≥80% of cells within the field of view. No detectable cytotoxin production was observed between 0-8h, for all strains. After 24h of incubation, all strains produced cytotoxin (titre of ≥1RU). As shown in figure 4-2a the median cytotoxin titres in all lineages after 12h and 24h of incubation were 1RU. At 48h of incubation, the median cytotoxin titres in all lineages were 2RU. The recent isolates (UK 2011-13, and Non- UK 2011-14) cytotoxin titres ranged between 1 – 2RU at 48h, while UK 2007-8 isolates ranged between 1 – 3RU. Compared with the UK 2007-8 lineage, the median titre for recent isolates (UK 2011-13, and Non- UK 2012-14) was higher (3RU). The cytotoxin titres at 72h for all isolates grown in BHIS cultures ranged between 2-4RU. These values did not differ significantly from BHI cultures at 72 hours, suggesting that the L-cysteine hydrochloride in BHIS did not repress cytotoxin production. Additionally, all strains were investigated for the presence of binary toxins, through PCR amplification of *cdtA* and *cdtB* genes. As shown in figure 4-2b all strains were negative for *cdtA* gene. CD002 isolates were also negative for *cdtB* gene (data not shown).

Furthermore, in comparison to hypervirulent strains (Data not showed), detectable toxins (1RU) was observed only in 078 after 12h of incubation. After 24h of incubation, the cytotoxin

titre of 078 was 3RU, while 027 was 2RU. Meanwhile, at both 48h and 72h, the cytotoxin titres in 078 were 4RU and 2RU in 027.

a)



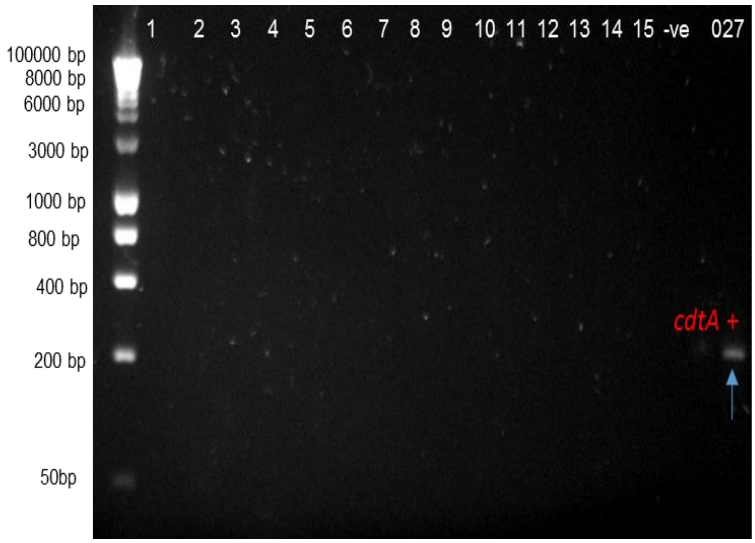


Figure 4-2 Cytotoxin production profile and assessment of binary toxin in CD002. All CD002 strains were investigated for cytotoxin production at time points: 0, 8, 12, 24, 48,72h using a Vero cell cytotoxin assay. The total cytotoxin titres were expressed as log_{10}-relative units (RU), where 1 RU represented ≥80% cell rounding by an undiluted *C. difficile* culture supernatant **(a)** Median cytotoxin titres for all isolates, grouped into different lineages. Median titres at different time points were similar in all lineages (**b)** A PCR image showing the presence of *C. difficile* binary toxin A (*cdtA)* in all CD002 isolates; lane 1 is a 1kb DNA ladder, lane 2-17 are amplified products of *cdtA,* lane 18 is a negative control and lane 19 is a positive control for *cdtA* (derived from a ribotype 027 strain). *cdtA* was absent in all CD002 isolates.

4.3.3 **Sporulation Studies**

4.3.3.1 Sporulation over time

In order to understand the rate at which CD002 strains formed spores *in vitro*, the development of spores that are resistant to alcohol was measured over a period of 5 days in batch culture. Sporulation capacities were determined through the ethanol shock treatment method and CFU were enumerated at time points: 0h, 24h, 48h, and 120h. Colony-forming units (CFU) yielded from ethanol shocked *C. difficile* cultures were assumed to have risen from spores within the culture. The total spore counts were calculated by calculating the mean CFU/mL of three independent experiments at each experimental time-period. All strains produced alcohol-resistant spores, and their numbers in cultures increased over the time-course of the experiment (Figure 4-3).

At the start of the experiment (0h), no spores were recovered in cultures of all lineages, thus the possibility of spores being carried over from previous passages was ruled out. After 24h of growth, recent Non-UK 2012-14, produced significantly ($P<0.0001$) more spores than recent UK 2011-13 strains (Figure 4-3). Additionally, recent strains (UK 2011-13, Non- UK 2012-14) produced significantly ($P<0.0001$) more spores (Average CFU/mL: 1. 04 x 10^4, 2.32 x10^4 respectively) than the UK CD002 from 2007-8 (Average CFU/mL: 2.06 X 10^3). After 48h (Figure 4-4) and 120h (Figure 4-5) of incubation, the spore formation did not differ significantly in any CD002 lineages ($P= 0.093$).

4.3.3.2 Hydrophobicity of spores to hydrocarbons

The ability of a spore to adhere to a surface is influenced by its hydrophobicity. To characterise the relative hydrophobicity of spores of CD002 strains, the MATH test with hexadecane as the organic solvent was used. The relative hydrophobicity (RH) of all CD002 isolates were

investigated (appendix 2). The most hydrophobic isolates belonged to strains of UK 2011-13 lineage, with a mean RH value of 58%. Non- UK (2012-14) isolates, also had high RH values, with an average RH of 51 %. The RH values of isolates belonging to the UK 2007-8 lineage was 33%, which is significantly (P= 0.0382) lower than spores of recent isolates (UK 2011-13, Non-UK 2012-14. As can be seen in figure 4.6a**,** strains selected for the adherence assay had similar hydrophobic characteristics. As shown in figure 4.6a, only one strain (174) belonging to the UK 2007-8 lineage, had a significantly lower (P=0.02) RH value.

4.3.3.3 Adherence of CD002 spores from different times and lineages to intestinal epithelial cells (Caco-2)

Given that the phenotypic difference in microvilli expression between undifferentiated and differentiated Caco-2 cells might affect the specificity of spore adherence, the abilities of CD002 spores to adhere to human gut epithelial cells at different stages of cell development (at 7 and 15 days respectively, figure 4-6b), was determined *in vitro*. Spores of six different CD002 strains of different lineages adhered to 7-day old Caco2 cells at a range of 21-57% (Figure 4-6b), this represents, the percentage of the initial spore count of the strain. All spores exhibited similar adherence capacities, except strain 146 (of the UK CD002 2011-13 lineage), which was significantly (P= 0.024) lower than all other strains tested. Spores adhered more strongly to 15–day old Caco-2 cells, at a range of 50-86% (Figure 4-6b), and the differences between strains were not statistically significant.

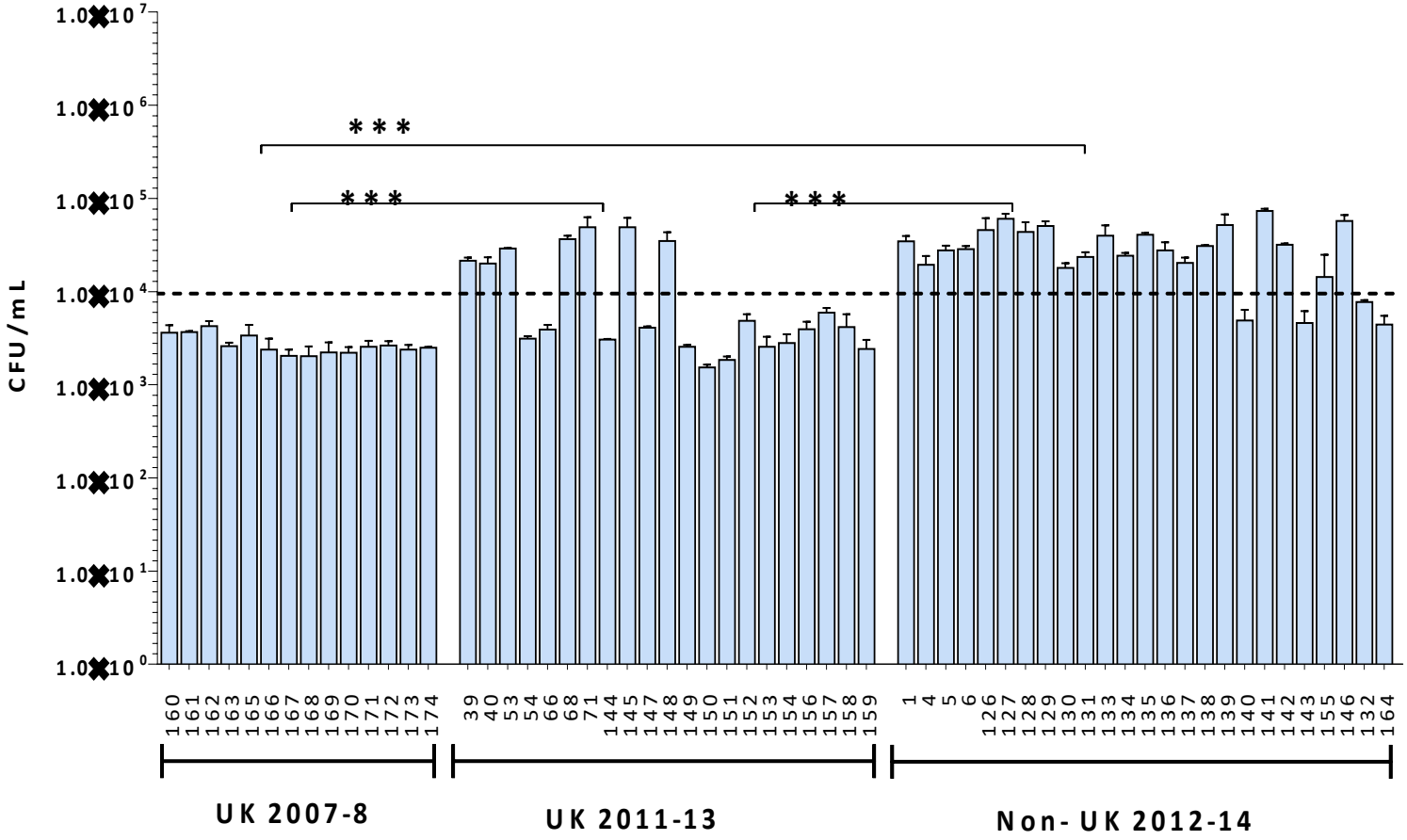


Figure 4-3 Spore formation (CFU/mL) by CD002 in BHIS. The development of CFU after ethanol shock treatment for all CD002, enumerated after 24h of incubation in BHIS. The data represents the average of three independent experiments, and the error bars, indicate the standard error of means. The dotted line represents the average CFU/ml for all isolates. Asterisk (***) indicate where a significant difference was observed.

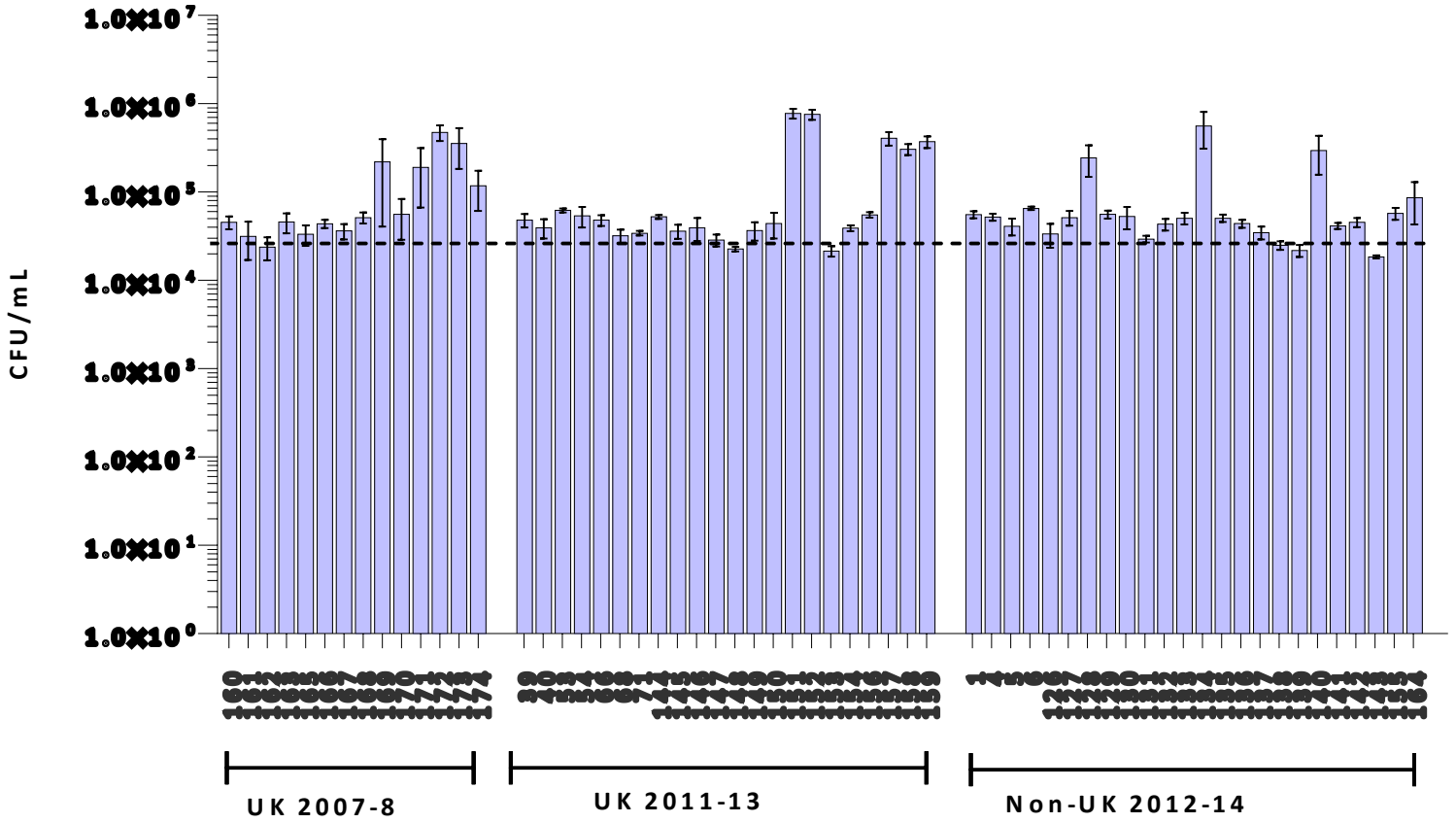


Figure 4-4 Spore formation (CFU/mL) by CD002 in BHIS. The development of CFU after ethanol shock treatment for all CD002, enumerated after 48h of incubation in BHIS. The data represents the average of three independent experiments, and the error bars, indicate the standard error of means. The dotted line represents the average CFU/ml for all isolates.

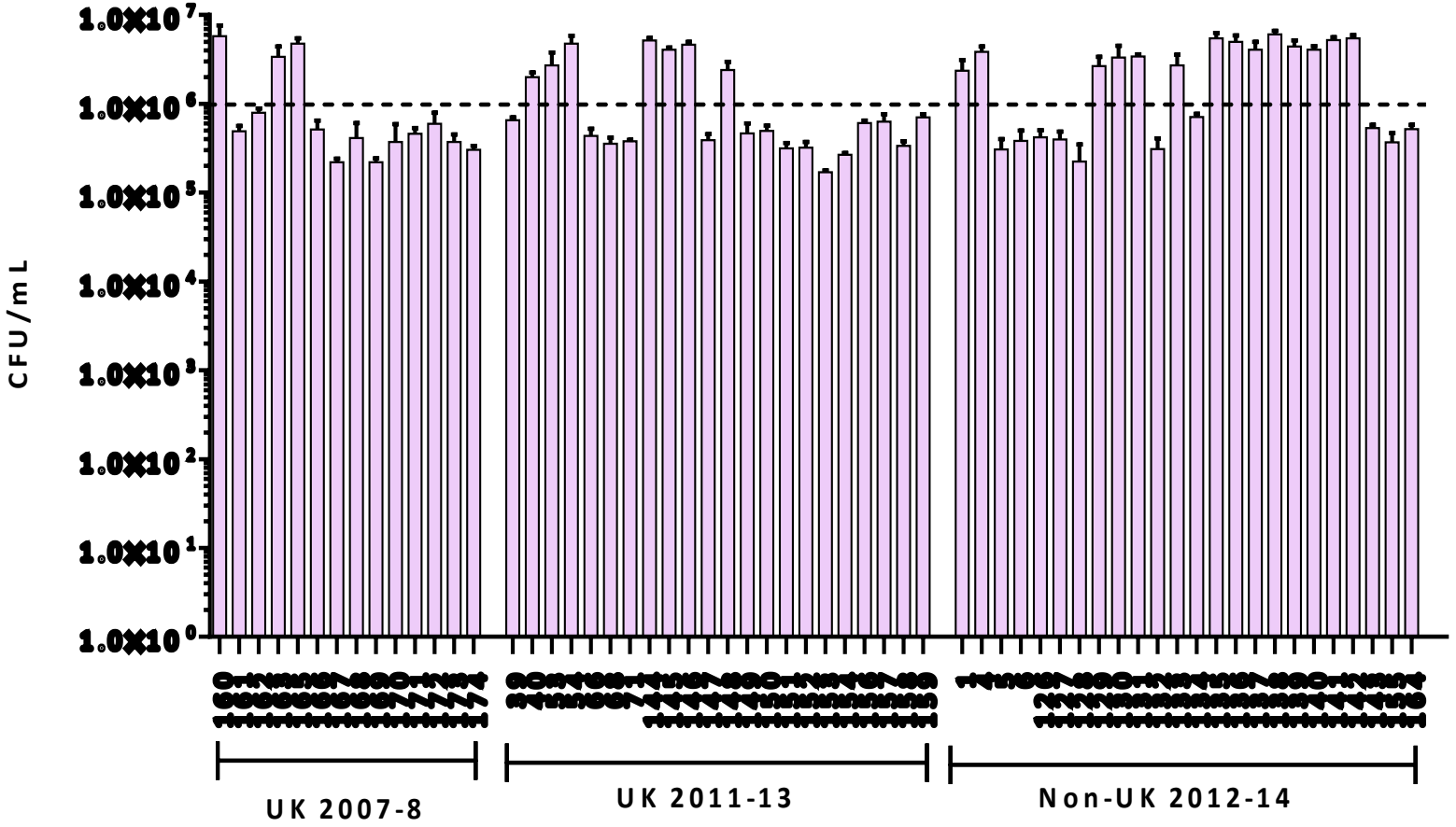


Figure 4-5 Spore formation (CFU/mL) by CD002 in BHIS. The development of CFU after ethanol shock treatment for all CD002, enumerated after 120h of incubation in BHIS. The data represents the average of three independent experiments, and the error bars, indicate the standard error of means. The dotted line represents the average CFU/ml for all isolates.

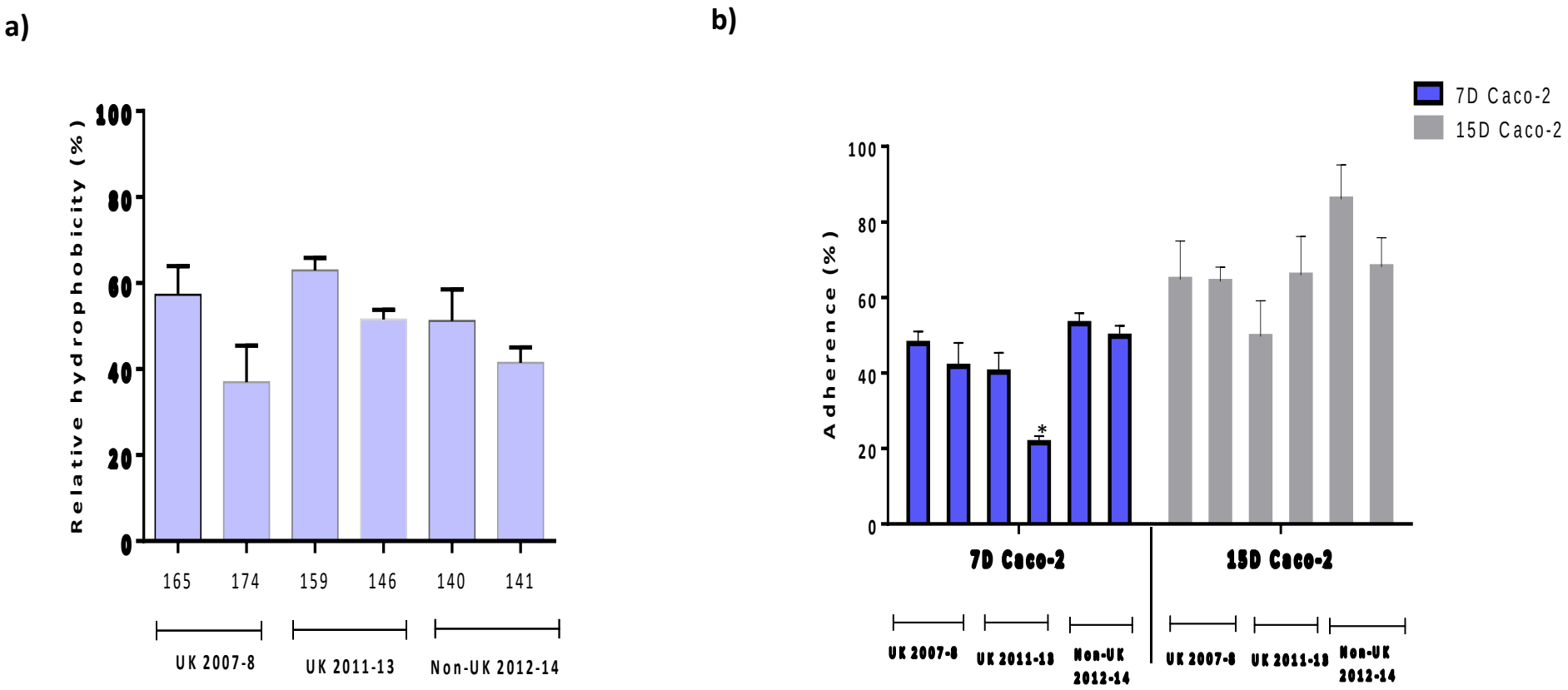


Figure 4-6 Relative hydrophobicity of CD002 spores and adherence to human gut epithelial cell monolayers. **A)** The relative hydrophobicity of six different strains of CD002 (2 strains per lineage) was determined by the MATH test. **B**) The ability of CD002 spores to adhere to monolayers of Caco-2 human colorectal cell line that had been incubated for the 7 and 15 days was enumerated, the adherence capacities at different stages of cell differentiation were similar in all lineages. The data (**a**&**b**) represent the average of two independent assays, and the error bars, indicate the standard error of means. Asterisk (*) indicates where a significant difference was observed.

4.3.4 **Quantification of biofilm formation using a crystal violet assay**

The biofilm biomass in three- and six-day-old biofilms formed by *C. difficile* in BHIS and BHISG media, were quantified using the crystal violet assay following methanol extraction (OD595). To identify the differences in biofilm formation between the lineages, the mean biofilm formation at three and six days was calculated. Lineage-specific and media-specific differences were identified.

As shown in figure 4-7, the recent strains (UK 2011-13 and Non-UK 2012-14) formed significantly higher amounts of biofilm biomass, at three days compared to UK 2007-8 strains ($p<0.0001$) in both BHIS and BHISG. Additionally, Non-UK 2011-14 strains produced significantly ($p=0.0361$) more biofilm biomass in BHISG compared to BHIS. There was no significant difference in the biofilm formation by UK CD002 (UK 2007-8 and UK 2011-13) strains in BHIS and BHISG at three days.

At six days, the biofilm formation in all lineages was significantly reduced (Figure 4-7 $p> 0.001$) in BHIS media. Also, there was no significant difference in the biofilm formation between lineages in BHIS. Conversely, a two-fold increase in BHISG biofilm formation (Figure 4-7, $p>0.001$) was observed in all lineages. However, when compared to three-day-old BHISG biofilms, this did not differ significantly ($p=0.226$). In similarity to three-day-old BHISG biofilms, recent CD002 strains (UK 2011-13 and Non-UK 2012-14) formed significantly greater quantities of biofilm biomass in BHISG compared to UK 2007-8 strains.

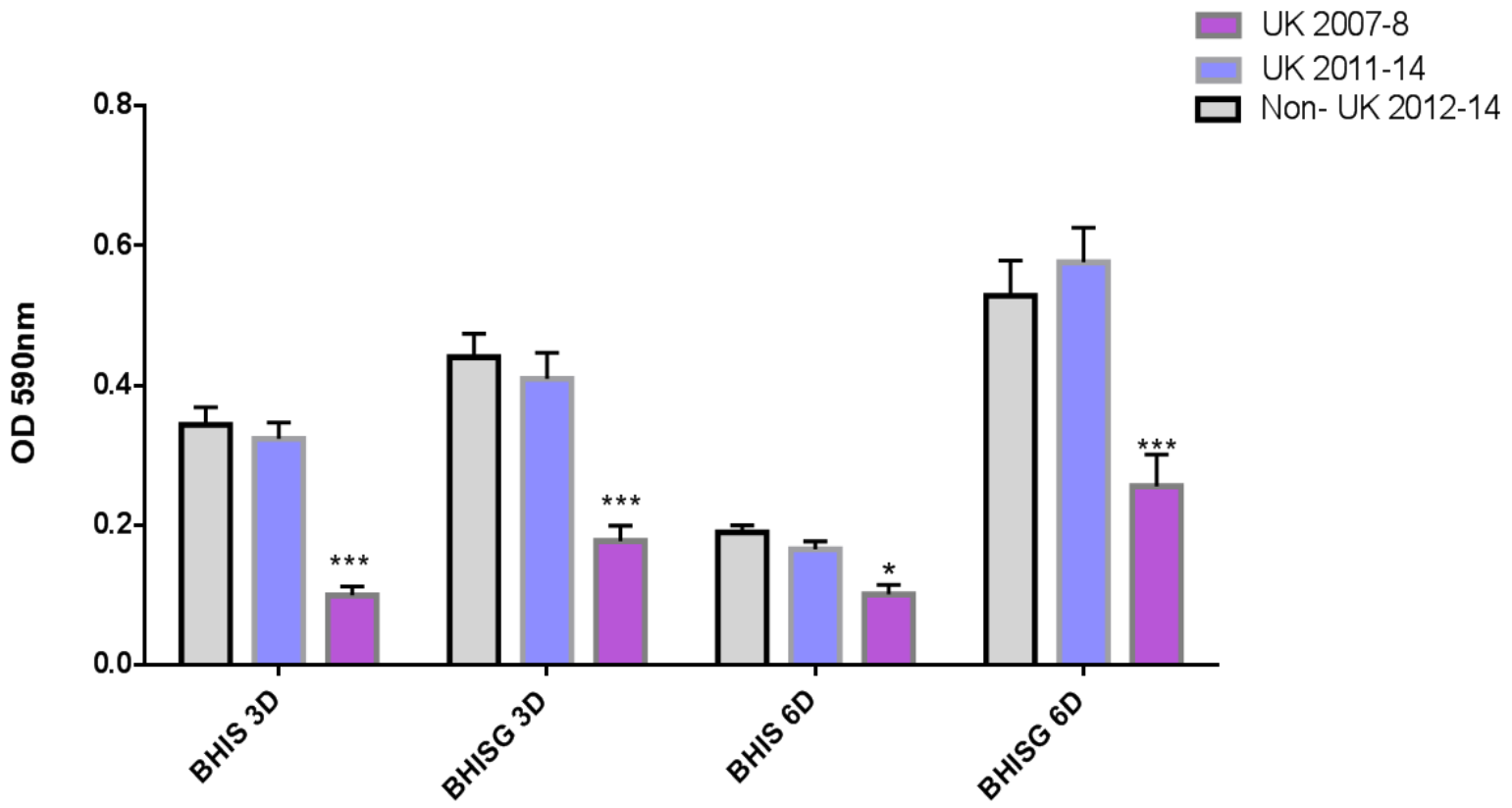


Figure 4-7 Mean biofilm production (OD590, ±SE) in Microtiter plate assay with crystal violet staining Mean OD_{590} derived from methanol elution of crystal violet stain from 3 day old, and 6 day old CD002 biofilms from different times and lineages, grown in BHIS & BHISG in 96-well microtitre plates. Statistical analysis was performed to determine the lineage or media specific differences in biofilm formation, with *** indicating were a significance difference was observed ($p<0.005$).

4.3.5 **Enumeration of the *C. difficile* vegetative cells and spores within the biofilm**

The proportion of vegetative cells and spores within three and six-day-old CD002 biofilms (in BHIS) were quantified in triplicate, and the mean CFU count per lineage was determined. As shown in figure 4.8, CD002 biofilms show an abundance of vegetative cells and spores within three- and six-day-old biofilms. Similar to the observation from the crystal violet biofilm assay, recent UK CD002 (UK 2011-13) and non-UK 2012-14 CD002 formed significantly more pronounced biofilms by 3 days than UK CD002 from 2007-8 ($P<0.009$) as determined using viable counting techniques. Spore counts within biofilms were significantly greater ($P<0.05$) in recent UK CD002 from 2011-13 (1.3×10^6 CFU/ml) than non-UK 2012-14 CD002 (8.2×10^5 CFU/ml) and UK CD002 from 2007-8 (2.9×10^5 CFU/ml). Likewise, vegetative cell counts were significantly greater ($P<0.001$) in recent UK CD002 (UK 2011-13) and non-UK 2012-14 CD002 (5.2×10^6 CFU/ml and 4.2×10^5 CFU/ml respectively).

In comparison to the crystal violet assay, where a decline in BHIS biofilm biomass was observed at six days, the proportion of vegetative cells and spores within six-day-old CD002 biofilms show an increase in biomass. More spores were present in six-day-old biofilms indicating that spore formation within biofilms had increased over time. As shown in figure 4.6, the biofilm total viable counts of non-UK 2012-14 CD002 (1.14×10^7CFU/ml) were significantly greater ($p<0.002$) than UK CD002 from 2011-13 and 2007-8 (8.8×10^6 and 2.7×10^6CFU/ml respectively). Also, recent UK CD002 (UK 2011-13) and non-UK 2012-14 CD002 biofilms contained significantly more spores ($p<0.002$) (2.4×10^6 and 2.7×10^6 CFU/ml) than UK CD002 from 2007-8 (2.3×10^5 CFU/ml).

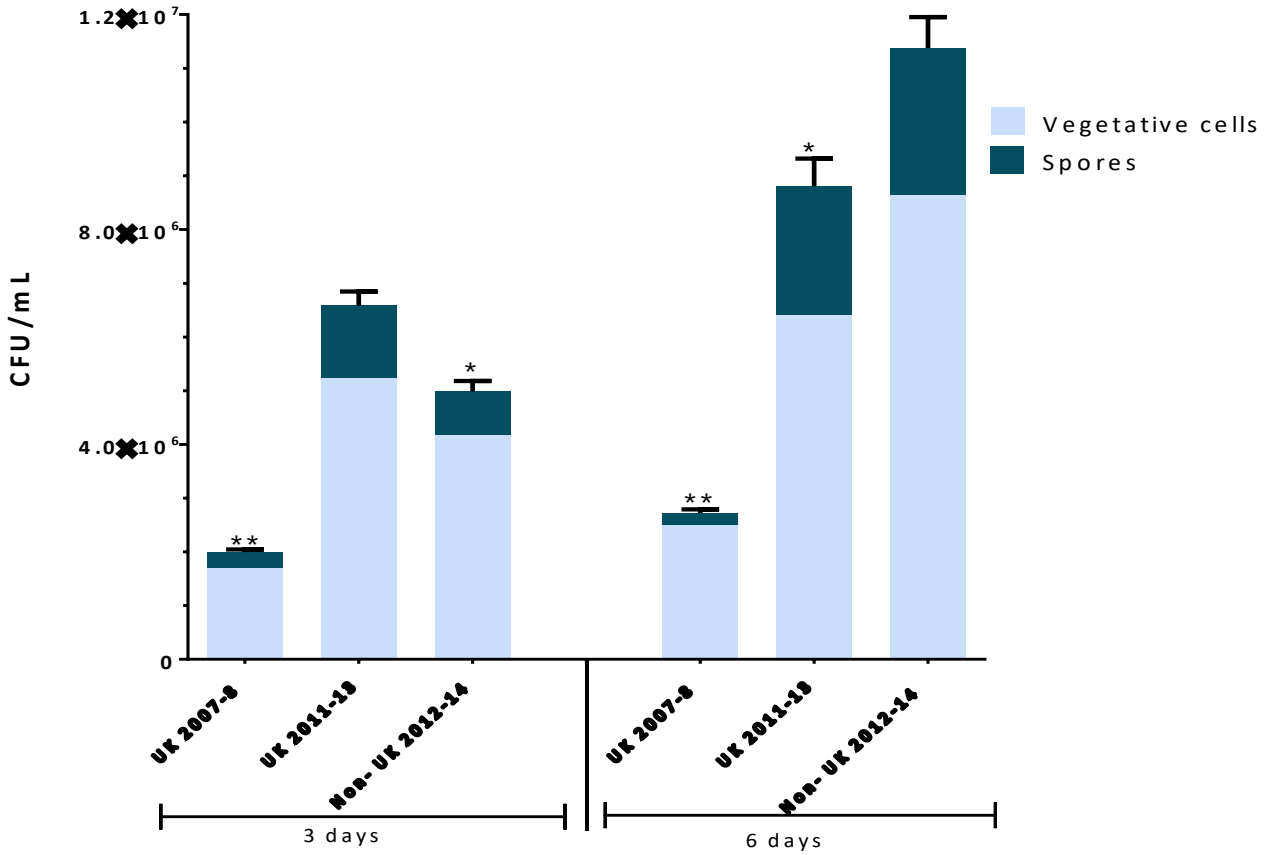


Figure 4-8 Enumeration of vegetative cells and spores within CD002 three- and six-day old Biofilms. Biofilms were grown for three and six days in 96 well microtiter plates. Residual BHIS was removed from the wells, and biofilms were disrupted by vortexing. The biofilm suspensions were serially diluted to determine the proportion of vegetative cells and spores per mL, of three-day and six-day old mature biofilms per CD002 lineage. Asterisk (*) Indicates, where a significant difference was observed (P>0.0001).

4.4 DISCUSSION

Since the identification of *C. difficile* as a pathogenic organism, several investigations have been performed to help explain its pathogenesis. The consensus is that *C. difficile* pathogenesis is a multifactorial process, dependent on virulence factors produced by an infecting strain (Lessa *et al*.,2012; Rupnik *et al*., 2009). Accordingly, it is important to characterise the virulence factors of emerging strains of *C. difficile.* In this chapter, some virulence characteristics and potential host colonisation factors of emerging strains of CD002 were investigated *in vitro*.

Spores are the main morphotype of CDI transmission, and *C. difficile* strains found to have an increased sporulation capacity have been suggested to be more prolific in environmental dissemination and recurrent infections (Akerlund *et al*., 2008; Cheng *et al*., 2011; Merrigan *et al*., 2010). However, Burns and colleagues have since refuted these claims, as they demonstrated considerable variability in the rate at which different isolates of the same ribotype produced spores(Burns *et al*., 2011). They also found sporulation rates of the epidemic and non-epidemic isolates to be broadly similar, suggesting that increased sporulation ability may not largely influence increased severity and disease occurrence. In the present study, the total sporulation capacity of 60 CD002 isolates was assessed, and the data generated indicated that recent strains (UK 2011-13 & Non-UK 2012-14) sporulates more rapidly after 24h (P= 0.005, fig 4-3a) than UK strains isolated in 2007-8. No previous study has investigated the sporulation rates in different lineages of CD002. However, the increased sporulation frequency associated with this ribotype was previously reported by Cheng *et al*. (2011). Accordingly, we can suggest that the greater sporulation capacity demonstrated by recent CD002 isolates, may increase their persistence in the intestinal lumen (or mucosal-

associated biofilm) following cessation of antimicrobial therapy, subsequently allowing re-establishment of the vegetative *C. difficile* population when the conditions in the gut are favourable. Additionally, the persistence of CD002 spores in the intestinal lumen (due to greater sporulation capacity) may indicate a greater propensity for transmission, as spores are normally shed into the environment, approximately 1-4 weeks after CDI treatment(Sethi *et al*., 2010). Spores shed into the environment are difficult to eradicate because of their resistance to heat, chemicals and radiation. Thus, elevated contamination of the environment potentially has contributed to the increased prevalence of this ribotype.

Adherence of gastrointestinal pathogens to the host intestinal cells is a crucial aspect of colonisation (Crobach *et al*., 2018). Initially, *C. difficile* spores were regarded as a means of disease transmission, however, several studies suggested that spores play a major role in host colonisation. There is evidence to show that *C. difficile* spores adhere to intestinal epithelial cells surfaces at relatively high levels (Crowther *et al*., 2014a; Hong *et al*., 2017; Joshi *et al*., 2012; Mora-Uribe *et al*., 2016; Paredes-Sabja & Sarker, 2012; Phetcharaburanin *et al*., 2014). Several spore surface properties have been identified as being involved in mediating adherence of *C. difficile.* An earlier *in vitro* study on spores of *C. difficile* 630, revealed that the exosporium layer was required for adherence and interactions was receptor-mediated(Paredes-Sabja & Sarker, 2012). Phetcharaburanin and colleagues (2014) provided evidence that the BclA1 spore glycoprotein affects the susceptibility to host colonisation and initial stages of infection. Recently an *in vivo* and *ex vivo* study showed that CotE (spore coat protein) facilitated host colonisation by enabling the binding of *C. difficile* spores to mucous (Hong *et al*., 2017).

While spore surface properties involved in mediating adherence were not investigated in the present study, the adherence of CD002 spores to human adenocarcinoma cells (Caco-2) *in*

vitro was evaluated. Joshi *et al* (2012), suggested that spores of a CD002 strain were hydrophilic and thus less efficient in binding to intestinal epithelial cells. Another study showed a correlation between spore hydrophobicity and adherence to undifferentiated Caco-2 cells, suggesting that less hydrophobic spores are less adherent to cell surfaces(Paredes-Sabja & Sarker, 2012). In the present study, spores of CD002 isolates from different lineages were found to be predominantly hydrophilic and exhibited higher levels (21-57%) of adherence to seven-day-old Caco-2 cells than was previously reported by Joshi *et al* (2012). Additionally, Joshi *et al* (2012), revealed the lack of an exosporium layer in less hydrophobic isolates, including spores of CD002 by transmission electron microscopy (TEM). An earlier study revealed that sonication of the exosporium layer in *C. difficile* 630 spores, resulted in reduced hydrophobicity and adherence to Caco-2 cells(Paredes-Sabja & Sarker, 2012), suggesting that the exosporium layer plays a key role in mediating spore adherence to cells. Controversially, Mora-Uribe and colleagues reported higher adherence to Caco- 2 cells (both undifferentiated and differentiated) and mucin, by spores of a *cdeC* mutant R20291 strain, in comparison to wild type spores(Mora-Uribe *et al*., 2016). *cdeC* is a cysteine-rich protein that is required for the morphogenesis of the spore coat and exosporium layer of R20291(Barra-Carrasco *et al*., 2013). Mora-Uribe and colleagues, observed an increased adherence to mucin following the sonication (to remove remnants of the exosporium layer) of *cdeC* mutants R20291 spores, while adherence to the intestinal epithelial cells (Caco-2 & HT-29) did not improve(Mora-Uribe *et al*., 2016). As a result, the authors suggested that the spore coat might hold a higher affinity to mucin, thus spores with a defective exosporium layer will likely colonise a susceptible host, while spores with an intact exosporium are prone to shedding. In an even more recent study, *cdeC* mutants R20291 spores exhibited an increased infection and persistence during recurrent CDI in a mouse model(Calderón-Romero *et al*., 2018).

Additionally, *cdeC* R20291 mutant spores demonstrated an increased fitness over wild type spores in a competitive infection mouse model(Calderón-Romero *et al*., 2018). Based on these reports, it is possible that the hydrophilic nature of the CD002 strains demonstrated in the present study may be due to the lack of an exosporium, as suggested by Joshi *et al* (2012), possibly influencing colonisation ability within the host. Conversely, since recent studies (Hong *et al*., 2017; Mora-Uribe *et al*., 2016)indicated that the spore coat is important for persistence and colonisation, it is tempting to speculate that spores of CD002 are able to persist and colonise a susceptible host through binding to mucin (via means of the spore coat). However, further work investigating the CD002 spore coat, exosporium layer, and their potential *in vivo* implication remains to be determined before any firm conclusions can be made.

It has previously been demonstrated that *C. difficile* spore interacts specifically with proteins and putative receptors on the surface of apical microvilli of Caco-2 cells(Cerquetti *et al*., 2002; Mora-Uribe *et al*., 2016; Phetcharaburanin *et al*., 2014). Fully differentiated Caco-2 cells are known to express apical microvilli and brush border proteins (Joshi *et al*., 2012). Consistent with reports by Joshi *et al* (2012), CD002 spores in the present study, were found to adhere more strongly to fully differentiated Caco-2 cells than undifferentiated Caco-2 cells. This suggests that the spore surface harbours proteins that may have encouraged their adherence to cells, despite being less hydrophobic. However, further investigations into the CD002 spore surface proteins are required to clarify their role in adherence to intestinal epithelial cells.

The severity of CDI symptoms is expected to increase with higher toxin levels(Cohen *et al*., 2018). Recent studies report the emergence of ribotype 002 as a virulent strain associated with severe CDI and high mortality rates (Chow *et al*., 2017; Dauby *et al*., 2017; Wong *et al*., 2016). However, whether severe CDI due to CD002 correlates with higher toxin levels is

unknown. Many studies have described a correlation between sporulation and toxin production as alternate mechanisms for survival by *C. difficile*, though this has long been debated (Akerlund *et al*., 2008; Blanco *et al*., 2018; Carlson *et al*., 2013; Di Bella *et al*., 2016; Koenigsknecht *et al*., 2015; Underwood *et al*., 2009).Recently, Blanco and colleagues (2018), demonstrated a positive correlation between *in vivo* toxin and spore levels in 200 *C. difficile* positive stool samples. Koenigsknecht *et al*. (2015) observed that spores and toxins were produced simultaneously 24h after infection in a murine model, suggesting a positive correlation between faecal toxin and spore levels. Warny and colleagues reported that hypervirulent PCR ribotypes produce quantitatively more toxin, therefore, cause more severe disease(Warny *et al*., 2005). Conversely, *in vitro* CDI gut model study revealed that *C. difficile* PCR ribotype 027 releases toxins earlier in the growth cycle, but peak cytotoxin titres (a maximum of 5RU) did not exceed those of other ribotypes that had been reported in CDI gut model studies(Baines *et al*., 2008). Taken together, it is suggested that strains with increased spore formation might germinate rapidly, and release toxins earlier in the growth cycle, thereby causing infections more readily. In the present study, no link can be established between the sporulation and toxin production, but we can comment on the total toxin production overtime. Detectable toxin titres were present by 12h, which agrees with earlier studies by Vohra and Poxton (2011). Although speculative, the ability of CD002 strains to produce toxins earlier in the growth cycle could mean that they are more efficient in eliciting symptoms, and thus have a greater phenotypic advantage over other ribotypes *in vivo.* Additionally, the increased sporulation observed with recent isolates of CD002 had no significant influence on the total cytotoxin titres between lineages. Perhaps, this was not observed because cytotoxin titres between lineages were not measured at the same intervals as the spore- formation was quantified. The cytotoxin titres ranged between 2- 3RU for all

lineages, this contrasts with the previous study by Keighley *et al*. (2015), which reported 1RU as the cytotoxin titres for all CD002.

The growth rate between lineages was investigated to determine any differences in the growth patterns among all strains. As demonstrated in figure 4-1a, the recent isolates of Non-UK 2012-14 origin had significantly increased μ_{max} values when compared to the UK isolates (UK 2007-8 & UK 2011-13). Previous studies had demonstrated similar growth pattern among strains of *C. difficile* (Burns *et al*., 2011; Vohra & Poxton, 2011). A more recent study demonstrated elevated CD002 μ_{max} (0.84 h^{-1}) compared to hypervirulent ribotypes 027 (0.67 h^{-1}) and 078(0.36 h^{-1}) (Keighley *et al*., 2015). However, fewer strains (n=11) of each ribotype were examined in comparison to the present study. Consistent with reports by Keighley and colleagues (2015), the μ_{max} values of CD002 in the present study ranged between 0.69 - 0.92 h^{-1}. These values are higher than μ_{max} of hypervirulent 027 and 078 (0.67 h^{-1}, and 0.36 h^{-1} respectively) reported by Keighley *et al* (2015). Based on this observation, we can assume that recent isolates of CD002 have a faster μ_{max}, which means that they form vegetative cells faster, produce toxin earlier, sporulates earlier, and therefore better at causing disease and being transmitted or surviving therapy. However, it is noteworthy that the experimental assay used to quantify growth rates in this study may not be fully reflective of what happens *in vivo*. Recurrence is a major challenge encountered in the management of CDI, as 35% of patients reportedly undergo recurrence after the first episode(Marsh *et al*., 2012). Additionally, approximately 25- 85 % of recurrent CDI cases have been associated with the initial infecting strain (Barbut *et al*., 2000; Oka *et al*., 2012), raising questions as to what factors may contribute to this process. Several studies addressed these questions, yet the factors contributing to a relapse of infection remains unknown. There are speculations that biofilms formation by *C. difficile* may be one of the mechanisms, and this has been demonstrated in

many *in vitro* and murine studies(Crowther *et al*., 2014a; Dapa & Unnikrishnan, 2013; Dawson *et al*., 2012; Hammond *et al*., 2014; James *et al*., 2018; Plaza-Garrido *et al*., 2015; Semenyuk *et al*., 2014; Soavelomandroso *et al*., 2017). Similar to earlier *C. difficile in vitro* biofilm studies, the ability of CD002 to form biofilms *in vitro* was demonstrated in the present study (Dapa & Unnikrishnan, 2013; Dawson *et al*., 2012; Plaza-Garrido *et al*., 2015; Semenyuk *et al*., 2014). Consistent with previous studies, an abundance of spores was observed in CD002 biofilms, especially after six days of growth (Dapa & Unnikrishnan, 2013; Dawson *et al*., 2012; Plaza-Garrido *et al*., 2015). A major observation in this study is that more recent isolates of CD002, (UK 2011-13 & Non-UK 2012-14) produced more biofilms *in vitro* compared to older isolates (UK 2007-8). The biofilm biomass also correlated with the spore abundance observed in different lineages. Although colonisation of these strains has not been examined *in vivo*, given the observations *in vitro*, it is plausible that higher biofilm formation by the recent isolates may indicate better colonisation *in vivo*. In this regard, more recent isolates may stimulate recurrent infection after initial therapy is administered, which may have contributed to the recent prevalence of these strains. However, until further evaluation using other model systems is performed, and evidence supporting the association of these strains in recurrent CDI cases, we can only leave room for conjecture as pure culture *C. difficile* biofilms are not reflective of multispecies biofilms that are present in the gut.

Carbohydrates have been reported to modulate the development of biofilms in different pathogens. In *Burkholderia pseudomallei*, the presence of low concentrations of glucose (2mM) enhanced biofilm formation(Ramli *et al*., 2012). Biofilm formation by *Streptococcus gordonii* was influenced by carbohydrates(Gilmore *et al*., 2003). Dapa *et al* demonstrated that addition of glucose, increased the levels of biofilm formation in *C. difficile* strain 630, suggesting that glucose is an important factor in the growth and formation of biofilm in *C.*

difficile(Đapa *et al*., 2013). Contrastingly, the addition of glucose had no effect on the levels of biofilm formed in strain R20291. A recent study provided evidence to show that glucose represses sporulation during the development of *C. difficile* biofilms (Plaza-Garrido *et al*., 2015). The mechanism of glucose-repression of sporulation during biofilm formation is unknown, however, the authors suggested that it may be mediated by CcpA (The glucose and carbon regulator)(Plaza-Garrido *et al*., 2015). In the present study, the presence of spores in BHISG mediated CD002 biofilms were not investigated. Consistent with earlier studies (Dapa & Unnikrishnan, 2013), enhanced biofilm formation was observed when all CD002 isolates were tested in BHISG, supporting the aforementioned statement that carbohydrates facilitate the formation of biofilms. In addition, these pure culture biofilms give an indication of biofilm formation *in vivo*, however, they are likely to be complex multi-species biofilms *in vivo*(Crowther *et al*., 2014a), therefore study of CD002 is warranted in more complex models to see if the biofilm formation results translate.

In conclusion, the virulence and colonisation phenotypes of CD002 strains have been demonstrated in this study. The observed phenotypic differences (Increased sporulation capacity, increased biofilm formation, as well as the abundance of spores during biofilm development) between UK 2007-8 and recent (UK 2011-13 and Non- UK 2011-14) strains may explain the recent emergence of this ribotype. However, other factors such as spore germination frequency reported to vary among different strains of *C. difficile* (Carlson *et al*., 2015; Crobach *et al*., 2018), and the response of CD002 biofilms to current therapies need to be explored.

5 THE NUTRIENT UTILISATION PROFILES OF CD002 STRAINS AND COMPETITION *IN VITRO*

5.1 BACKGROUND

The human gut is inhabited by a diverse population of microorganisms with an estimate of over 100 trillion cells. It is populated mostly by bacteria, but also viruses, fungi and protozoa (Thursby & Juge, 2017). These community of microorganisms, termed the gut microbiota form a mutually beneficial relationship with the host and provide a range of physiological functions such as colonisation resistance, metabolic functions and maintenance of gut integrity (Cameron & Sperandio, 2015). An alteration of the gut microbiota referred to as dysbiosis significantly compromises the host response to pathogenic organisms. A clear illustration of this phenomenon has been demonstrated by Gram-positive spore-forming bacterium, *C. difficile,* which flourishes in the presence of an altered microbiota.

A wide variety of nutrients derived endogenously or exogenously are present in the gut, however, the availability of these nutrients to the diverse population of microbes is often limited (Thursby & Juge, 2017). Therefore, 'survival of the fittest' is the norm for these microbes, leading to fierce competition for finite resources (Pacheco & Sperandio, 2015). Due to these challenging living conditions, many microbes evolved different competitive strategies that are either active or exploitative to ensure their survival the gut. In 1983 Rolf Freter hypothesised, that the ability of a pathogen to thrive during intestinal colonisation depends on its ability to efficiently utilise nutrient sources and a suitable niche for colonisation (Freter *et al*., 1983). Supporting this, Pacheco and colleagues demonstrated how Enterohemorrhagic *Escherichia coli* upregulates its virulence genes following the detection of fucose released

through the enzymatic break down of mucins by *Bacteroides thetaiotaomicron* (Pacheco *et al*., 2012). Maltby and colleagues demonstrated how commensal *Escherichia coli* strains (HS and Nissile 1917) were able to prevent *E. coli* O157 colonisation(Maltby *et al*., 2013). This was achieved through the commensal strains' ability to utilise the five sugars that were used by the pathogenic organism. However, if the mouse was colonised by only one of the commensal strains, the pathogen could exploit the other sugar sources that have not been consumed by the commensal, this is known as exploitative competition.

Similarly, commensal *E. coli* strain Nissile 1917 was able to outcompete *Salmonella enterica serovar* Typhimurium (*S*. Typhimurium) due to its superior iron uptake capacity (Deriu *et al*., 2013). Furthermore, an *in vivo* study on germ-free mice, showed how metabolic competition over monosaccharides by the commensal organism *Bacteroides thetaiotaomicron,* provided colonisation resistance against *Citrobacter rodentium* (*C. rodentium*) (Kamada *et al*., 2012) This was largely possible because *Bacteroides* species can utilise a diverse profile of monosaccharides and polysaccharides, serving as carbon sources, therefore *B. thetaiotaomicron* was able to outcompete *C. rodentium*, an organism with a similar metabolic profile. In addition, the study of the *C. difficile* 630 genomes by Sebiaha and colleagues revealed a vast array of substrates that are utilised by *C. difficile* for its growth and survival within the challenging gut environment (Sebiaha *et al*., 2006). This suggests that *C. difficile* is a uniquely adaptable organism that can exploit several nutrient niches within the host gut.

Antagonism by the production of inhibitory compounds such as bacteriocins or short-chain fatty acids is another mechanism by different bacterial species limit the growth of their competitors (Britton & Young, 2012; Stecher *et al*., 2013) This type of competition is referred to as interference or active competition. A recent study demonstrated the ability of *Clostridium scindens* (*C. scindens*) to inhibit the growth and pathogenesis of *C. difficile* through

its capacity to generate secondary bile acids that are required for the inhibition of *C. difficile* spore germination (Buffie *et al*., 2015). Similarly, a recent study by Passmore and colleagues shows that *para*-cresol production by *C. difficile* affects the biodiversity of the gut microbiota, and thus contributes to the survival and pathogenesis of *C. difficile* (Passmore *et al*., 2018).

Using *in vitro* and *in vivo* studies, Robinson and colleagues, demonstrated, how epidemic *C. difficile* (ribotype 027), was able to outcompete endemic strains (ribotype 001, 002, 014 and 053) in the presence of the complex microbiota(Robinson *et al*., 2014). Scaria and colleagues, reported an expanded nutrient utilisation profile associated with epidemic *C. difficile* strain (ribotype 027), suggesting that this may have also contributed to the virulence of these strains(Scaria *et al*., 2014). More recently, reports suggest that epidemic lineages of *C. difficile* ribotype 027 and 078 acquired independent mechanisms to metabolise trehalose, a food additive, which have helped select for their emergence and contributed to their hypervirulence (Collins *et al*., 2018). Based on this prior knowledge on the epidemic *C. difficile* strain (ribotype 027) nutrient utilisation, the importance of studying the metabolic profiles as well as the competitive fitness of recent emerging strains or ribotype cannot be overemphasized. Understanding the full metabolic profile of a pathogen or strain can give insights into the conditions under which, they are likely to proliferate. Consequently, surveillance measures may be introduced to help curb the eventual spread of that pathogen or strain. Since *Clostridium difficile* ribotype 002 has emerged recently as the most prevalent ribotype in the UK, it is important to investigate the nutrient utilisation profile as well as the competitive fitness of this ribotype, to see if these factors may help explain its prevalence.

5.1.1 **Aims and Objectives**

To identify the nutrient utilisation profiles of selected strains of CD002 from different time and lineages and to investigate their competitive fitness *in vitro.* The aims will be achieved by the following objectives,

1. Nutrient utilization profile using Biolog Phenotype Microarray plates (PM1- 8)
2. Investigation of the competitive fitness of strains in a single-stage continuous flow chemostat

5.2 MATERIALS AND METHODS

5.2.1 **Bacterial strains**

Due to the nature of experiments in this chapter, only five CD002 strains of different time lineages were selected. These strains were selected from the different lineages based on their maximum specific growth rate values (μ_{max}) in BHIS (reported in chapter 4) and antibiotic resistance profiles (reported in chapter 3) in order to allow selective culture. The characteristics of the strains used are given in Table 5-1. *C. difficile strain* (630, ribotype 012), isolated in Zurich Switzerland in 1982*,* was provided by Dr Shan Goh at the University of Hertfordshire and was used as a control in the phenotype microarray experiments.

Table 5-1 Characteristics of CD002 strains used for phenotype microarray comparisons and competition studies *in vitro*

Strain Number	Isolation Year	Isolation location	Lineage	Antimicrobial Resistance *	μ_{max}
71	2012	Maidstone, UK	UK 2011-13	Sensitive	1.04
137	2014	Spain	Non-UK 2012-14	Rifampicin	0.87
157	2012	Taunton, UK	UK-2011-13	Erythromycin	1.13
160	2008	Salisbury, UK	UK 2007-8	Moxifloxacin	0.50
164	2008	Dublin, Ireland	Non-UK 2007-8	Sensitive	0.56

*Strain resistance to an antimicrobial agent was used to prepare selective agars used in the competition study

5.2.2 **Phenotypic microarray experiment**

The metabolic profiles of five CD002 (Listed in Table 5-1) strains were measured using the Biolog Phenotype Microarrays (PM). PM experiments were carried out according to the manufacturer's protocol as described in 2.6.1- 2.6.2. Briefly, strains were grown overnight in 5mL brain heart infusion (BHI) broth (CM1135, Oxoid). Subsequently, 300µL of *C. difficile* culture was plated onto pre-reduced Columbia blood agar plates (LAB001, Lab M, Lancashire, UK), and incubated anaerobically overnight at 37°C. Consequently, *C. difficile* cells were retrieved from the cultured agar plates using a sterile deoxygenated swab and inoculated into a pre-reduced IF-0a (72268, Hayward, USA), to achieve a 40% transmittance cell suspension (which measured spectrophotometrically as 0.139± 0.002 OD at 750nm using a CE1011 1000 series spectrophotometer). This suspension was further diluted (1:16) in anaerobic Biolog Mix B solution. One hundred microliters of the final cell suspension were transferred to each well of the respective deoxygenated PM panels and incubated anaerobically at 37°C for 48 h.

Bacterial growth was then determined by reading the absorbance at 750 nm using a Multiskan FC Microplate photometer (51119000, ThermoFisher). Due to the financial constraints, biological replicates were not included in this experiment, instead, CD630 was used as a control and results were compared to published data by Scaria *et al* (2014), to validate the results of the test strains. However, PM analysis of CD630 was performed in duplicate.

5.2.3 **Competition Studies**

5.2.3.1 Preliminary batch culture growth experiments to investigate glucose limitation

Prior published research indicated that *C. difficile* growth is limited by the absence of glucose in peptone yeast extract (PY) broth(Dupuy & Sonenshein, 1998; Karlsson *et al*., 1999; Karlsson *et al*., 2000). As a result, peptone yeast extract glucose (PYG) broth was used for the glucose competition study. All strains were grown according to conditions described in 2.14- 2.16. *C. difficile* strains were inoculated in pre-reduced peptone yeast extract glucose (PYG) (as described in Table 2-5) broth and allowed to grow overnight. Overnight *C. difficile* cultures were diluted 1:10 in pre-reduced PYG medium and grown for 24h to determine the growth rates (μ and μ_{max}) and inform on the dilution rate (D=F/V) that was to be used in the competition studies. All strains were cultured in duplicate, and samples were retrieved every 2 hours to determine the growth rate of the strains. At the stationary phase of bacterial growth, 1g of sterile glucose (41095-5000, Fisher Scientific) was introduced into the sample medium, the growth rate was then monitored every two hours from this point. After 6 hours, an increase in *C. difficile* growth was observed. This preliminary work was undertaken to mimic growth conditions in the fermenter and to determine the suitable nutrient medium and glucose concentration for the competition of strains.

5.2.3.2 Experimental design for competition experiments

The same medium used in batch culture was used in continuous culture experiments. Continuous cultivation was performed in a one-litre New Brunswick Bioflo 115 bioreactor with a working volume of 700ml. This process was performed in accordance with methods described in 2.7.1- 2.7.4. To perform competition for glucose in continuous culture, individual *C. difficile* strains were grown separately in PYG broth overnight. Overnight cultures of *C. difficile* grown in PYG broth were diluted 1:10 into a fresh 24h pre-reduced PYG broth. These cultures were grown to an OD_{600} between 0.2-0.4, to ensure biomass of 10^3 was achieved by both competing strains, as it was necessary for both strains to have the same starting biomass in the fermenter. The chosen biomass of 10^3 achieved at OD_{600} between 0.2-0.4 was determined prior to the experimental set up through viable counting (Data not shown). Subsequently, 10mL of each competing strain (listed in Table 5-2) was aseptically transferred to a sterile universal for inoculation into the bioreactor.

To inoculate the bioreactor, 20mL of media was aseptically removed from the bioreactor and replaced with 20mL of the mixed *C. difficile* culture (Made up of 10mL of one strain and 10mL of the competing strain). The bioreactor stirrer impeller was set at 400 rpm to allow sufficient time for adaption of both strains within the fermenter. Subsequently, samples were collected at 0h to determine the initial biomass at the start of the experiment. The overall biomass of the mixed culture was monitored every two hours over a period of 12 h, by retrieving samples from the culture vessel and measuring the absorbance at OD_{600}. The Watson Marlow peristaltic pumps were turned on to start the continuous flow of media to the culture vessel at a dilution rate of $0.39h^{-1}$. This dilution rate was chosen because it was below the maximum specific growth rate of the competing strains.

Two millilitres of the sample was retrieved from the culture vessel daily. One hundred microliters of the retrieved sample were serially diluted (according to methods described in 2.5) and plated on to on Brazier's agar with the appropriate selective antibiotic (Table 5-2) and without the antibiotic, to enumerate CFU/ml of the different strains. The antibiotics used were selective for one strain (per pair), therefore enumeration of the second strain (without a selective antibiotic) in the competing pair, was determined by subtracting the number of colonies on antibiotic selective plates from the number of colonies on non-selective plates (Total viable count plate). Prior to fermentation studies, pre-experimental validation was performed on the selective agars to ensure that it yielded selectivity required for the experiment (data not shown).

Table 5-2 The competing pair and selective media used for viable counting of strains during glucose competition studies

Competing pair	Selective agar	CD Strain	MIC to selective antimicrobial(mg /L)	Drug concentration in agar (mg/L)
164+ 137	Rifampicin Braziers Agar	164	0.002	1
		137	32	
160+ 71	Moxifloxacin Braziers Agar	160	32	10
		71	2	
137+157	Erythromycin Braziers Agar	137	1	10
		157	64	

5.3 RESULTS

5.3.1 **Phenotype Microarray experiments**

5.3.1.1 CD002 Carbon source utilisation

The Biolog PM metabolic panels for carbon source metabolism (PMs 1 and 2) consisted of 190 assays. To validate the carbon source utilisation by CD002 strains listed in Table 5-1, the carbon utilisation profile of CD630 (**630B**) in this study was first compared with data (**630A**) obtained from a PM analysis study by Scaria *et al* (2014). Although there was a variation in the number of carbon sources utilised by strain **CD630B** (54 carbon sources) as opposed to CD**630A** (57 carbon sources) from Scaria *et al*'s (2014) study (Figure 5-1 and Figure 5-2), the difference was not statistically significant (P=0.132). In PM 1 (Figure 5-1), strain **630B** utilised some carbon sources that were not utilised by strain 630 in the study of Scaria *et al* (2014). This included: D- threonine, pyruvic acid, D-Galactonic acid-g-Lactone, D- serine, L- Arabinose, l-Lyxose. Conversely, strain **630B** demonstrated a much lower affinity (≥**1.3-1.1**) to some compounds which were of a higher affinity (≥1.4) to **630A** in Scaria *et al*'s (2014) study. This included; α-Hydroxy Glutaric Acid -γ- Lactone, Uridine, α-D-Lactose, α-Hydroxy Butyric Acid, Sucrose. In PM 2, **630B** was negative (**≤ 1**) for L- Arabitol, L- Tartaric acid, butyric acid and L-lysine, with a low affinity (**≥1.3-1.1**) to β-Hydroxy Butyric Acid, Turanose, L-Alaninamide, and L-Phenylalanine**.** A high affinity to these compounds was demonstrated by **CD630A** in the Scaria *et al* (2014) study. Additionally, CD**630B** demonstrated a very strong affinity (**≥ 2**) to five compounds that were negative in Scaria *et al* (2014) study**,** this included: Dihydroxy Acetone, D-Arabinose, 2, 3-Butanedione, Sorbic Acid and 2-Deoxy-D-Ribose.

Out of the 190 carbon sources tested, simple sugars were utilised by most CD002 strains. Complex carbohydrates such as pectin and arbutin were also utilised by all CD002 strains. A

very strong affinity (≥2) for D- ribose, 2-Deoxy-D-Ribose, Dihydroxyacetone, L-Lyxose, D-Xylose, L- arabinose, and D- arabinose was demonstrated by all strains. Among all the CD002 strains studied, UK 2011-13 Strain (71) had the broadest carbon utilisation profile; this strain was positive for 65 carbon sources. Strain 137 (Non- UK 2012-14) was positive for 56 carbon sources, while strain 157 (UK 2011-13) was positive for 55 carbon sources. The older strain, 164 (Non- UK 2008) was positive for 52 carbon sources, while UK 2007-8 strain (160), had the narrowest carbon utilisation profile; this strain was positive for only 43 carbon sources.

In comparison to the hypervirulent outbreak, associated strains R20291 (PCR ribotype 027) and 078 studied by Scaria *et al* (2014), the range of utilised carbon sources by CD002 strains in this study was narrower. Interestingly, UK 2011-13 strain (71) which had the broadest carbon utilisation profile among the CD002 strains studied, had a comparable carbon utilisation profile (in terms of the number of sources) with 078 (Scaria *et al*., 2014). Strain 078 and 027 (strain R20291) utilised 69 and 84 carbon sources respectively (Scaria *et al*., 2014)

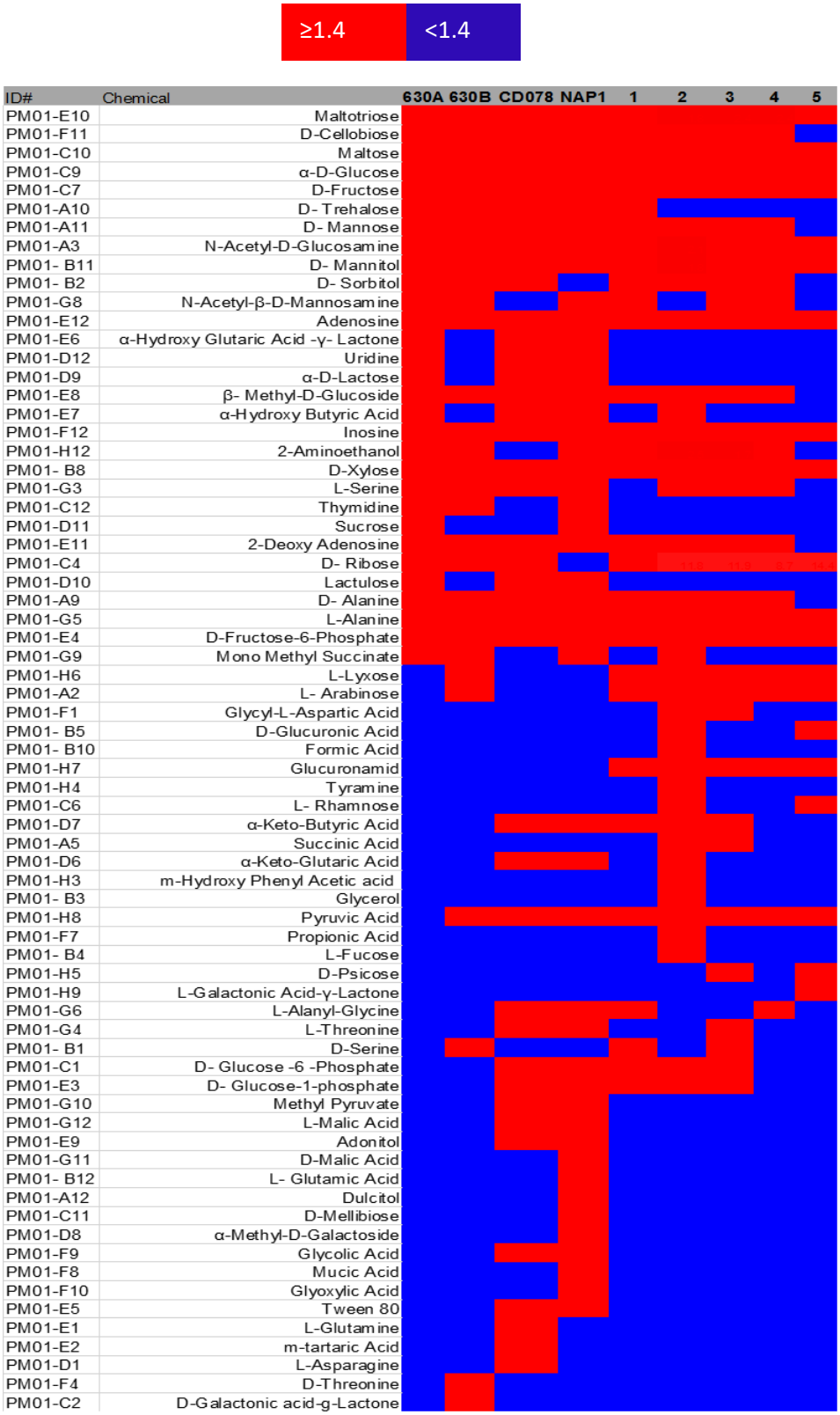


Figure 5-1 Carbon utilisation phenotype microarray (PM1) of CD002 strains from different time lineages. The list includes all positive carbon source phenotypes (red) and negative phenotypes (blue), represented by the scale bar given at the top of the figure. The title given at the top of the column represents the strain as follows: 630A= CD630 data from Scaria *et al* (2014); 630B= CD 630(In the present study); CD078= CD 23m63 (Scaria *et al*., 2014); NAP1= CD R20291 (Scaria *et al*, 2014); *C. difficile* ribotype 002 strains: 1= 157; 2= 71; 3= 137; 4= 164; 5= 160.

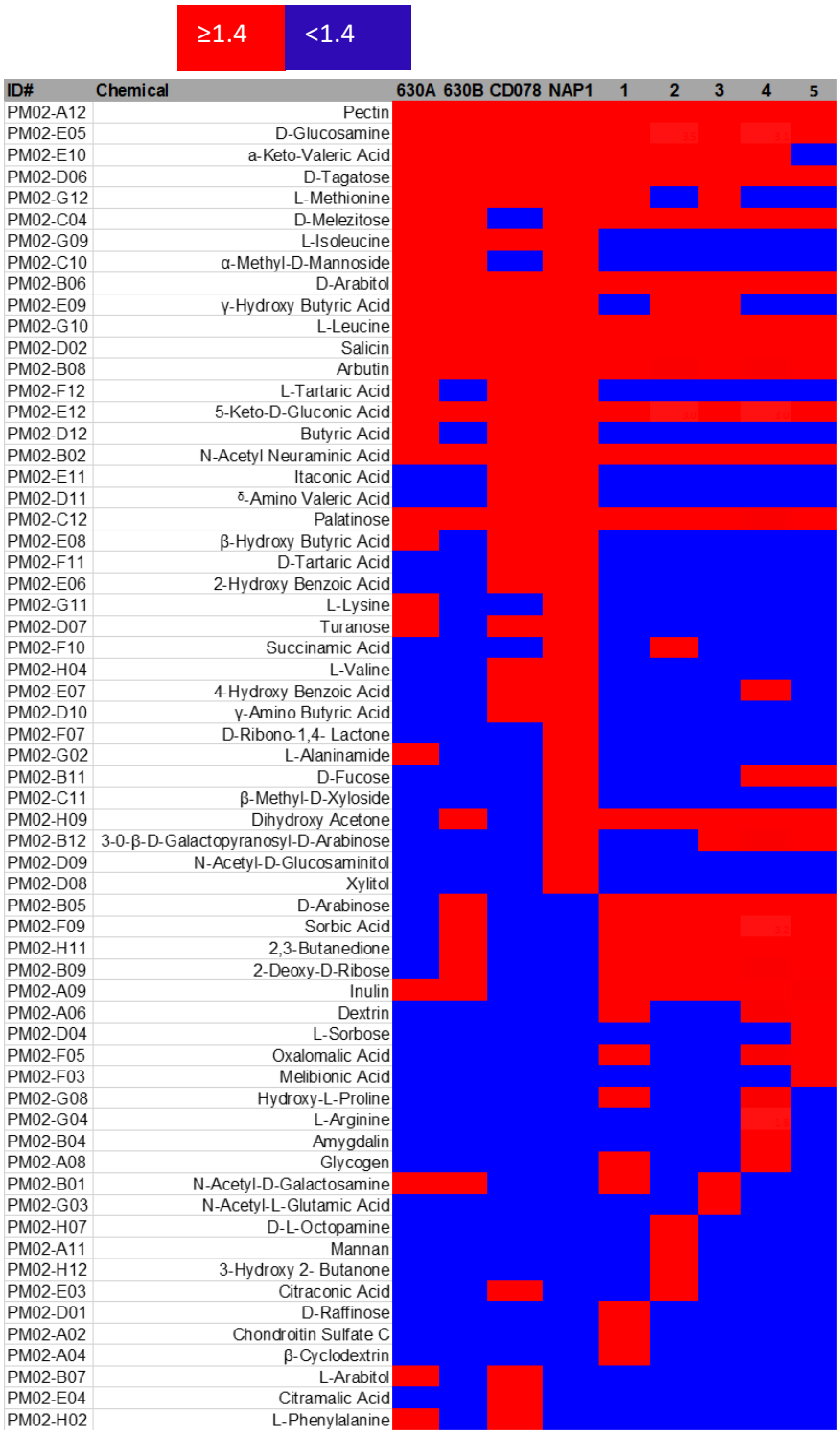


Figure 5-2 **Carbon utilisation phenotype microarray (PM2) of CD002 strains from different time lineages**. The list includes all positive carbon source phenotypes (red) and negative phenotypes (blue), represented by the scale bar given at the top of the figure. The title given at the top of the column represents the strain as follows: 630A= CD630 data from Scaria *et al* (2014); 630B= CD 630(In the present study); CD078= CD 23m63 (Scaria *et al*., 2014); NAP1= CD R20291 (Scaria *et al*, 2014); *C. difficile* ribotype 002 strains: 1= 157; 2= 71; 3= 137; 4= 164; 5= 160.

5.3.1.2 CD002 Nitrogen and Peptide utilisation profile

The Biolog PM metabolic panels for nitrogen source metabolism (PM 3) consisted of 95 assays. As shown in figure 5-3, CD**630B** had a comparable nitrogen metabolic profile as CD630A (Scaria *et al*., 2014). Although CD**630B** was negative for inosine, glucuronamide and Met-Ala, which were positive in CD**630A** (Scaria *et al*., 2014), the difference was not statistically significant (P= 0.0535). Out of the five CD002 strains tested, strain 157 (UK 2011-13) had the broadest nitrogen utilisation profile. This strain was positive for 25 nitrogen sources. Strain 137 (Non- UK 2012-14) was positive for 22 nitrogen sources, while strain 160 (UK 2007-8) was positive for 18 nitrogen sources. Strain 164 (Non- UK 2007-8) and strain 71 (UK 2011-13) were positive for only 13 and 11 nitrogen sources respectively. In comparison to data obtained from Scaria *et al*. (2014), CD002 strains utilised a broader array of nitrogen sources. Strain 078 and 027 (strain R20291) both utilised only 11 nitrogen sources.

The peptide nitrogen sources consisted of 285 assays (PMs 6-8), the utilisation of these peptides as demonstrated by strain CD**630B** in the present study (Figure 5-4- 5-6)**,** was much narrower compared with PM analysis data on CD **630A** in the study of Scaria *et al*. (2014). The differences between CD**630A** and CD**630B** in PM 6 and 7 (Figure 5-4 and figure 5-5) was not significantly different (p= 0.0545), however, there was a significant difference in PM 8 (P= 0.0001) (Figure 5-6). In total, data obtained from Scaria *et al* (2014), showed that CD**630A** utilised 43 peptide sources (Scaria *et al*., 2014), however in the present study, CD630B utilised only 30 peptide sources. Of all CD002 strains tested, strain 137 (Non- UK 2012-14), had the broadest peptide utilisation profile, this strain was positive for 57 peptide sources. Strain 71 (UK 2011-13), strain 164 (Non- UK 2007-8) and strain 157 (UK 2011-13) utilised 35, 30 and 22 peptide sources respectively. Strain 160 (UK 2007-8) had the least peptide utilisation profile, this strain utilised only 6 peptide sources. In comparison to a hypervirulent outbreak-

associated strain R20291 (PCR ribotype 027) and 078 reported by Scaria *et al* (2014), strain 137 (Non- UK 2012-14), had a comparable peptide utilisation profile to ribotype 078, while ribotype 027 was positive for 110 peptide sources.

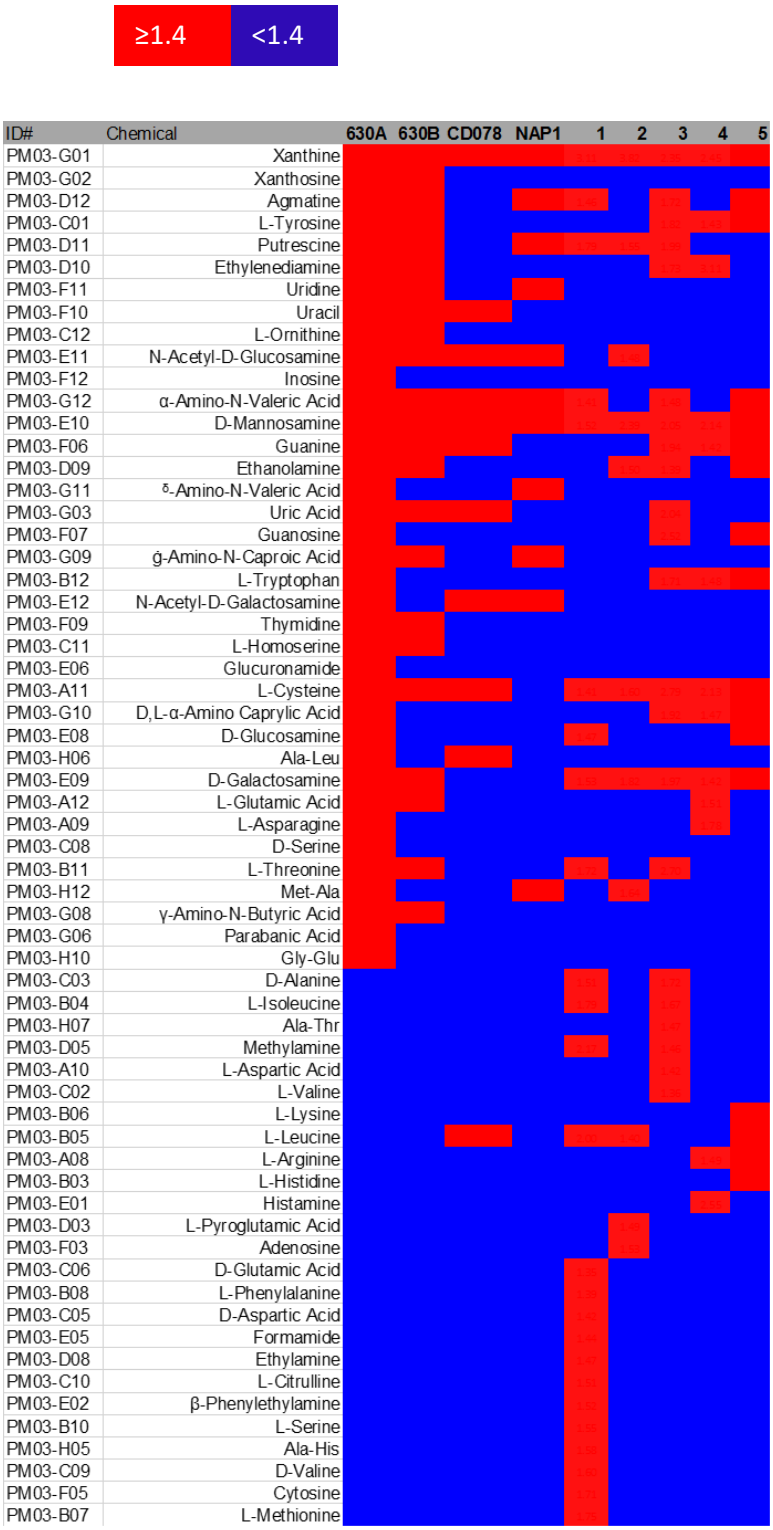


Figure 5-3 **Nutritional Phenotype of CD002 strains from different time lineages determined using Biolog phenotype microarray panel 3**. The affinity of nutrient utilisation is represented by the scale bar given at the top of the figure (red for positive and blue for negative). The title given at the top of the column represents the strain as follows: 630A= CD 630 data from Scaria *et al* (2014); 630B= CD 630(In the present study); CD078= CD 23m63 (Scaria *et al*., 2014); NAP1= CD R20291 (Scaria *et al*, 2014); *C. difficile* ribotype 002 strains: 1= 157; 2= 71; 3= 137; 4= 164; 5= 160. Each row represents a nitrogen source tested.

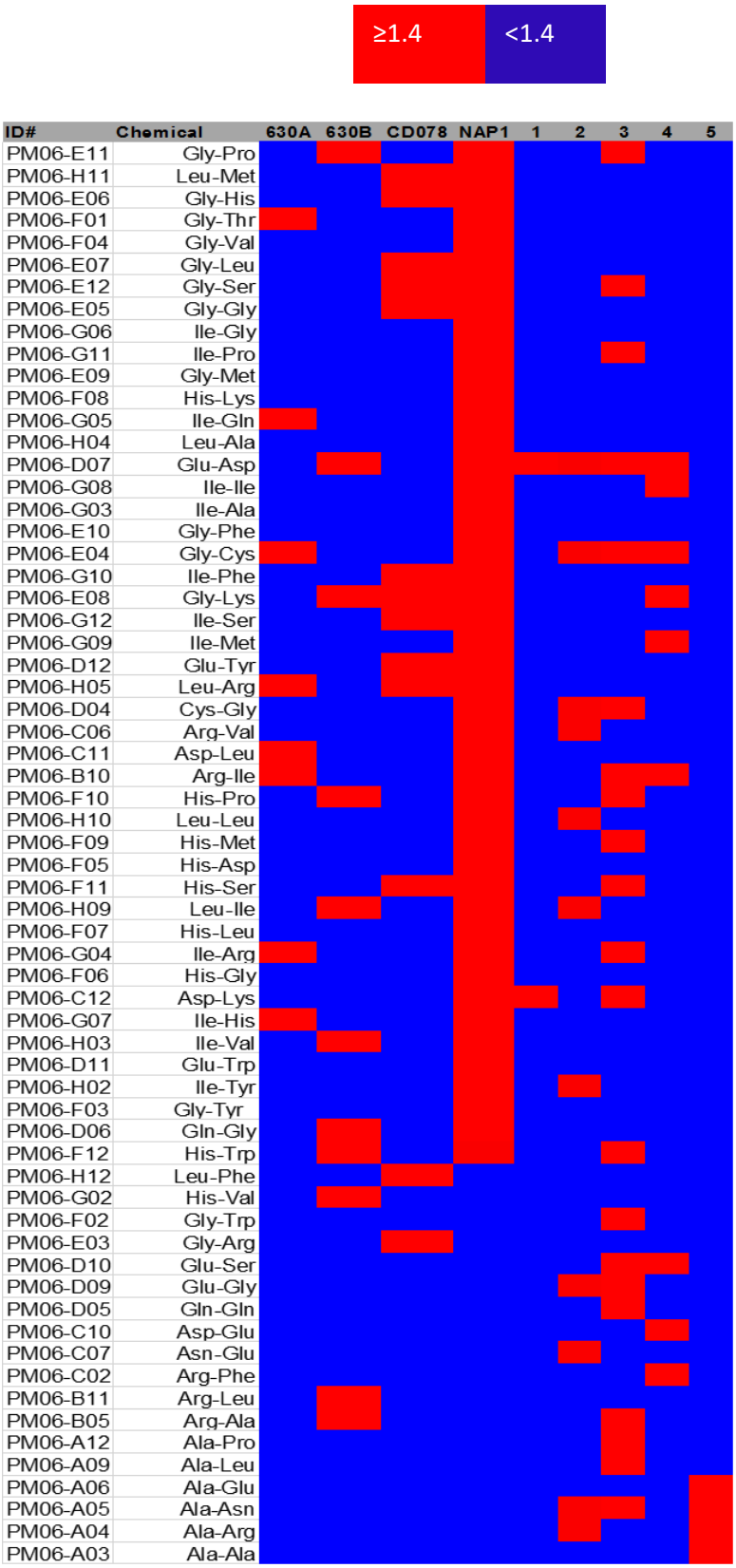


Figure 5-4 **Nutritional Phenotype of CD002 strains from different time lineages determined using Biolog phenotype microarray panel 6**. The affinity of nutrient utilisation is represented by the scale bar given at the top of the figure. The title given at the top of the column represents the strain as follows: 630A= CD 630 data from Scaria *et al* (2014); 630B= CD 630(In the present study); CD078= CD 23m63 (Scaria *et al.*, 2014); NAP1= CD R20291 (Scaria *et al*, 2014); *C. difficile* ribotype 002 strains: 1= 157; 2= 71; 3= 137; 4= 164; 5= 160. Each row represents a peptide source tested.

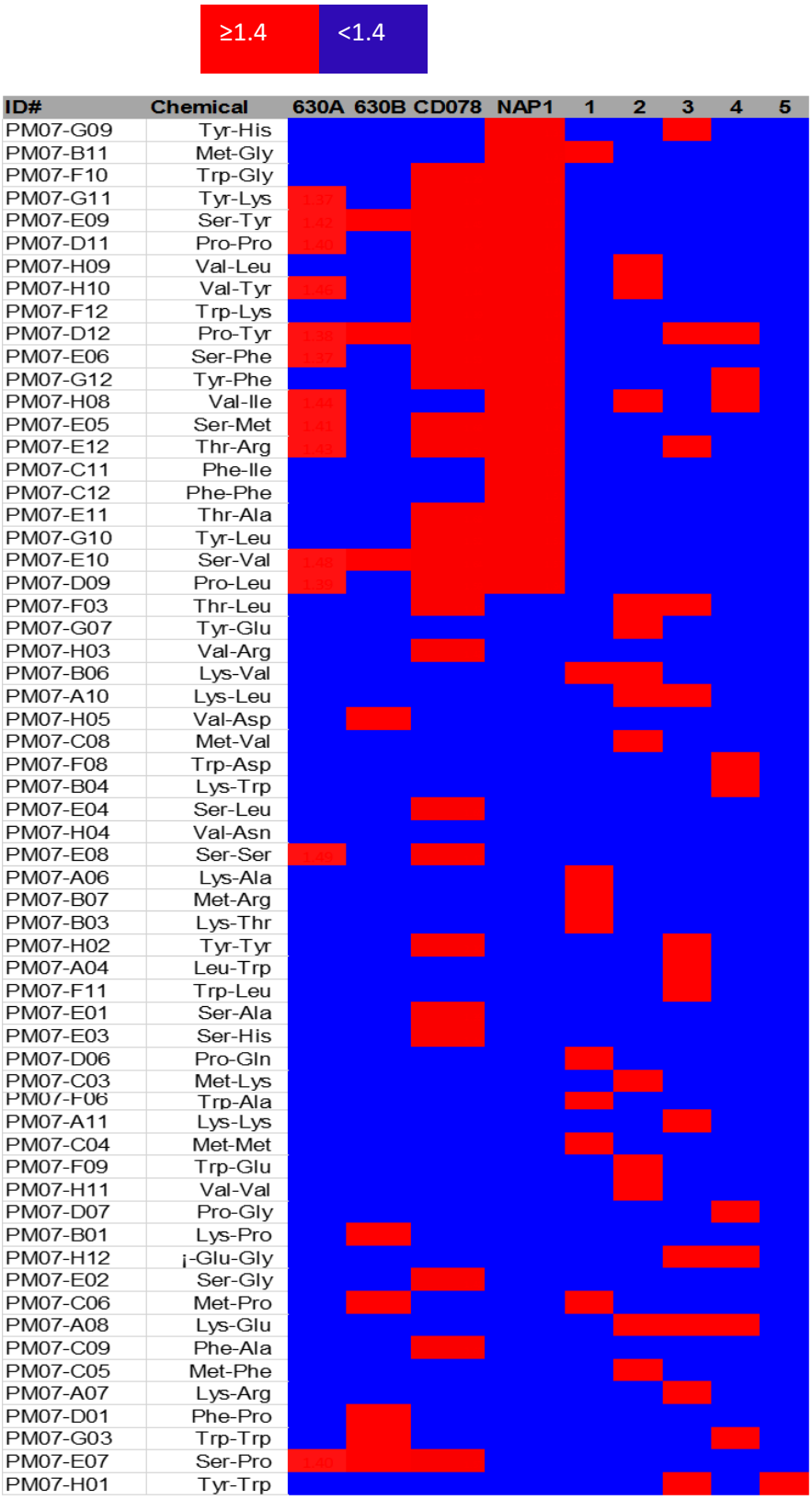


Figure 5-5 **Nutritional Phenotype of CD002 strains from different time lineages determined using Biolog phenotype microarray panel 7**. The affinity of nutrient utilisation is represented by the scale bar given at the top of the figure. The title given at the top of the column represents the strain as follows: 630A= CD 630 data from Scaria *et al* (2014); 630B= CD 630(In the present study); CD078= CD 23m63 (Scaria *et al.*, 2014); NAP1= CD R20291 (Scaria *et al*, 2014); *C. difficile* ribotype 002 strains: 1= 157; 2= 71; 3= 137; 4= 164; 5= 160. Each row represents a peptide source tested.

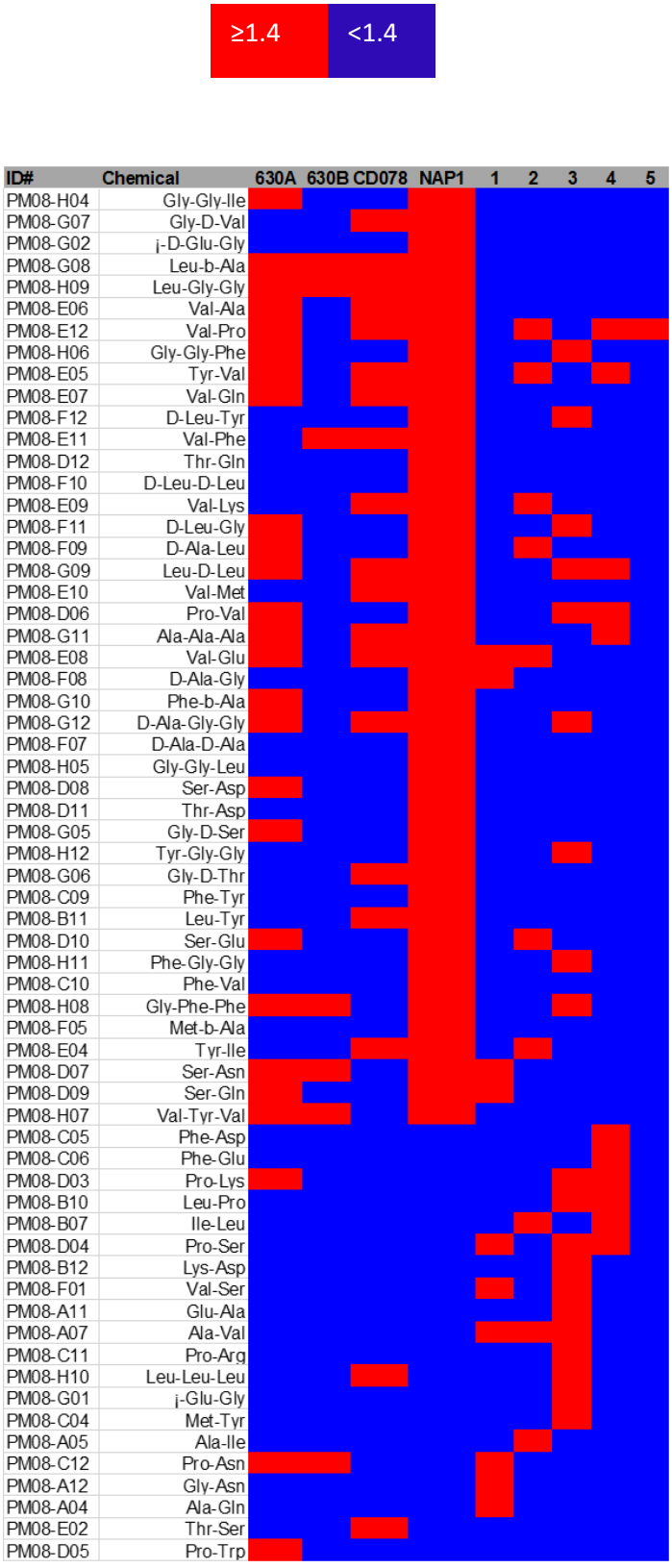


Figure 5-6 **Nutritional Phenotype of CD002 strains from different time lineages determined using Biolog phenotype microarray panel 8**. The affinity of nutrient utilisation is represented by the scale bar given at the top of the figure. The title given at the top of the column represents the strain as follows: 630A= CD 630 data from Scaria *et al* (2014); 630B= CD 630(In the present study); CD078= CD 23m63 (Scaria *et al.*, 2014); NAP1= CD R20291 (Scaria *et al*, 2014); *C. difficile* ribotype 002 strains: 1= CD 157; 2= CD 71; 3= CD 137; 4= CD 164; 5= CD 160. Each row represents a peptide source tested.

5.3.1.3 CD002 utilisation of phosphorus and sulfur

The Biolog PM metabolic panels for phosphorus and sulfur (PM4) metabolism consisted of 95 assays. As shown in figure 5-7, strain **630B** had a similar utilisation pathway as **630A** (Scaria *et al*. 2014), the differences were not statistically significant (P= 0.065). As well as **630A**, **630B** was positive for 16 phosphorus and sulfur sources. The only notable difference is that **630B** utilised pyrophosphate, a source that was not utilised by **630A** in the prior published study (Scaria *et al*., 2014). Out of all the CD002 strains tested, strain 157 (UK 2011-13), had the broadest phosphorus and sulfur utilisation profile. This strain utilised 26 sources, while strain 137 (Non- UK 2012-14) and 71 (UK 2011-13), utilised 18 and 17 sources respectively. The older isolates, strain 160 (UK 2007-8) and strain 164 (Non- UK 2007-8), had the least phosphorus and sulfur utilisation profile. Both strains utilised only two phosphorus and sulfur sources. In comparison to a hypervirulent outbreak-associated strain R20291 (PCR ribotype 027) and 078 reported by Scaria *et al* (2014), recent CD002 (71, 137 and 157) strains demonstrated a comparable phosphorus and sulfur utilisation to strain R2029, while 078 utilised only six sources.

A notable observation is that recent UK (UK 2011- 13) strains, 71 and 157 demonstrated a greater ability to utilise taurocholate and taurine. Similar to the hypervirulent outbreak-associated strain R20291 (PCR ribotype 027) as reported in Scaria *et al* (2014) study, strain 137(Non- UK 2012-14) demonstrated the ability to utilise tetrathionate and taurine.

5.3.1.4 CD002 Utilisation of Nutritional supplements

The Biolog PM metabolic panel for nutritional supplements (PM 5), consisted of 95 assays. As shown in figure 5-8, Strain **CD630B**, utilised similar nutritional supplements as CD630 previously reported (Scaria *et al*., 2014). The differences between the nutrient utilisation by

strain 630 in this study and Scaria *et al* (2014) were not significantly different (p= 0.2512). Out of all the CD002 strains studied, strain 137 (Non- UK 2012-14), had the highest nutrient supplement utilisation profile. This strain was positive for 35 nutritional supplements which are higher than hypervirulent outbreak-associated strain R20291 (PCR ribotype 027) and 078 reported by Scaria *et al* (2014). Strain 164 (Non- UK 2007-8), utilised 19 nutritional supplements, while UK 2011-13 strains (157 and 71), utilised 8 and 6 nutritional supplements respectively. The UK isolate 160 (UK 2007-8) had the narrowest metabolism of the nutritional supplements, this strain only utilised one of the nutritional sources.

5.3.1.5 Overall nutrient utilisation profile

It is a well-known fact that genomic variation exists among *C. difficile* strains, and this was also demonstrated in the phenotypic metabolic profiles of CD002 strains in the present study. Despite strains being of the same ribotype, they exhibited varied metabolic profiles to all the 760 nutrient sources (PM1-8). Among all the CD002 strains studied, the Non- UK (2011-14) CD002 strain (137) of Spanish origin appeared to have the broadest nutrient utilisation capability

. As shown in table 5-3, this strain was found to utilise 183 nutrient sources. Recent UK CD002 (Strains 157 and 71) isolates also had a more expanded nutrient utilisation profile compared to older UK CD002 (160). Both recent UK CD002 (157 and 71) strains utilised 130 nutrient sources, while the older strain (160) utilised only 61. Non- UK strain (164), belonging to the older lineage (Non- UK 2007-8), appeared to have a more expanded nutrient utilisation profile compared with Old UK CD002 (160). This strain was found to utilise 113 nutrient sources.

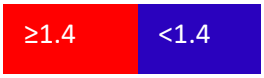
Table 5-3 Nutrient utilisation profile of five CD002 strains and controls

Nutrient	630A*	630B**	CD078	NAP1	157	71	137	164	160
					UK 2011-13		**Non-UK 2012-13**	**Non-UK 2007-8**	**UK 2007-8**
Carbon (PM1 &PM2)	56	53	69	84	55	65	56	52	43
Nitrogen (PM 3)	36	23	11	11	25	11	22	13	17
Phosphorus & Sulphur (PM 4)	16	17	6	32	26	18	20	2	4
Nutrient supplements (PM 5)	16	11	12	12	6	5	34	19	1
Peptide (PM6, PM7 & PM 8)	47	27	58	110	22	35	55	30	6
Total	172	131	156	249	134	131	187	116	65

*630A- Nutrient utilisation profile of *C. difficile* strain 630 reported by Scaria *et al*., 2014.
**630B- Nutrient utilisation profile of *C. difficile* strain 630 reported in the present study.

ID#	Chemical	630A	630B	CD078	NAP1	1	2	3	4	5
PM04-E06	Phosphono Acetic Acid									
PM04-E12	Thymidine 3',5'- cyclic monophosphate									
PM04-E08	Methylene Diphosphonic Acid									
PM04-E07	2-Aminoethyl Phosphonic Acid									
PM04-D12	Uridine- 3',5'- cyclic monophosphate									
PM04-E11	Inositol Hexaphosphate									
PM04-G12	L-Methionine Sulfone									
PM04-D06	O-Phospho-L-Serine									
PM04-E04	Phosphoryl Choline									
PM04-D11	Uridine- 2',3'- cyclic monophosphate									
PM04-D07	O-Phospho-L-Threonine									
PM04-E05	O-Phosphoryl Ethanolamine									
PM04-F11	Cysteamine									
PM04-G10	N-Acetyl-D,L-Methionine									
PM04-E09	Thymidine- 3'- monophosphate									
PM04-E10	Thymidine- 5'- monophosphate									
PM04-G02	S-Methyl-L-Cysteine									
PM04-G03	Cystathionine									
PM04-A02	Phosphate									
PM04-H06	Taurine									
PM04-F04	Tetrathionate									
PM04-H05	Taurocholic Acid									
PM04-F08	D-Cysteine									
PM04-F12	L-Cysteine Sulfinic Acid									
PM04-F09	L-Cysteinyl-Glycine									
PM04-F07	L-Cysteine									
PM04-C11	Cytidine- 2',3'- monophosphate									
PM04-B02	Dithiophosphate									
PM04-G04	Lanthionine									
PM04-F05	Thiophosphate									
PM04-G05	Glutathione									
PM04-G11	L- Methionine Sulfoxide									
PM04-G09	Glycyl-L-Methionine									
PM04-F10	L-Cysteic Acid									
PM04-B08	Guanosine- 2'- monophosphate									
PM04-A05	Tripolyphosphate									
PM04-D05	O-Phospho-D-Serine									
PM04-H12	Tetramethylene Sulfone									
PM04-G06	D,L-Ethionine									
PM04-D04	Phospho-L-Arginine									
PM04-E03	Phosphocreatine									
PM04-B12	Guanosine- 3',5'- cyclic monophosphate									
PM04-D01	D-Mannose-1- Phosphate									
PM04-C12	Cytidine- 3',5'- monophosphate									
PM04-A03	Pyrophosphate									
PM04-A12	Adenosine- 3',5'- cyclic monophosphate									
PM04-D03	Cysteamine-S-Phosphate									
PM04-B11	Guanosine- 2',3'- cyclic monophosphate									

Figure 5-7 **Nutritional Phenotype of CD002 strains from different time lineages determined using Biolog phenotype microarray panel 4**. The affinity of nutrient utilisation is represented by the scale bar given at the top of the figure. The title given at the top of the column represents the strain as follows: 630A= CD 630 data from Scaria *et al* (2014); 630B= CD 630(In the present study); CD078= CD 23m63 (Scaria *et al*., 2014); NAP1= CD R20291 (Scaria *et al*, 2014); *C. difficile* ribotype 002 strains: 1= CD 157; 2= CD 71; 3= CD 137; 4= CD 164; 5= CD 160. Each row represents a phosphorus and Sulphur source tested.



ID#	Chemical	630A	630B	CD078	NAP1	1	2	3	4	5
PM05-E06	Pyridoxamine									
PM05-E12	2'-Deoxy Uridine									
PM05-E11	Uridine									
PM05-D10	Cytosine									
PM05-E08	D-Pantothenic Acid									
PM05-E10	Uracil									
PM05-D06	D-Alanine									
PM05-G12	m-Inositol									
PM05-D12	2'-Deoxy Cytidine									
PM05-E09	Orotic Acid									
PM05-D11	Cytidine									
PM05-F11	Glutathione (reduced form)									
PM05-G10	Pyrrolo-Quinoline Quinone									
PM05-E05	Pyridoxal									
PM05-D09	D,L- α,ε-Diaminopimelic Acid									
PM05-F10	Thymine									
PM05-F12	Thymidine									
PM05-D05	L-Homoserine Lactone									
PM05-H10	Tween 40									
PM05-F05	δ-Amino-Levulinic Acid									
PM05-B06	L-Lysine									
PM05-F06	Hematin									
PM05-B05	L-Leucine									
PM05-C08	trans-4-Hydroxy L-Proline									
PM05-E07	β-Alanine									
PM05-C07	L-isoleucine + L-Valine									
PM05-E04	Pyridoxine									
PM05-B08	L-Phenylalanine									
PM05-D07	D-Aspartic Acid									
PM05-A06	L-Aspartic Acid									
PM05-C12	2'-Deoxy Inosine									
PM05-B10	Guanine									
PM05-B11	Guanosine									
PM05-C04	L-Tryptophan									
PM05-B04	L-Isoleucine									
PM05-B07	L-Methionine									
PM05-B09	Guanosine-3',5'- cyclic monophosphate									
PM05-F07	Deferoxamine Mesylate									
PM05-A09	Adenosine-3',5'- cyclic monophosphate									
PM05-A08	L-Glutamic Acid A9 A									
PM05-A10	Adenine									
PM05-C06	L-Valine									
PM05-C05	L-Tyrosine									
PM05-B12	2'-Deoxy Guanosine									
PM05-A11	Adenosine									
PM05-A12	2'-Deoxy Adenosine									
PM05-C01	L-Proline									
PM05-D01	L-Ornithine									
PM05-F01	Quinolinic Acid									
PM05-G01	Oxaloacetic Acid									
PM05-E03	Spermine									
PM05-H06	D,L-Mevalonic Acid									
PM05-G04	p-Amino Benzoic Acid									
PM05-A04	L-Arginine									
PM05-A05	L-Asparagine									

Figure 5-8 **Nutritional Phenotype of CD002 strains from different time lineages determined using Biolog phenotype microarray panel 5**. The affinity of nutrient utilisation is represented by the scale bar given at the top of the figure. The title given at the top of the column represents the strain as follows: 630A= CD 630 data from Scaria *et al* (2014); 630B= CD 630(In the present study); CD078= CD 23m63 (Scaria *et al.*, 2014); NAP1= CD R20291 (Scaria *et al*, 2014); *C. difficile* ribotype 002 strains: 1= CD 157; 2= CD 71; 3= CD 137; 4= CD 164; 5= CD 160. Each row represents a nutrient supplement tested.

5.3.2 Competition Studies

5.3.2.1 Glucose limitation and Maximum specific growth rate (μ_{max}) in PYG

Having investigated the nutritional phenotypes of CD002 strains (Table 5-1) from different time lineages, it was important to investigate the competitive fitness of these strains to limiting nutrients in a bioreactor. Here, the limiting nutrient chosen was glucose, and prior to competition in the bioreactor, the batch growth rate in peptone yeast glucose media was determined. As shown in figure 5-9, the growth curves obtained for all the strains were similar and the differences were not statistically significant (P= 0.7253). An increase in bacterial biomass was observed six hours after 1g of glucose was added to the growth media in the stationary phase. The maximum specific growth rate (μ_{max}) for all strains was determined (Table 5-4), and the difference between strains was not statistically significant (P= 0.325).

Table 5-4 Maximum specific growth rate of five CD002 strains in peptone yeast glucose broth (PYG)

CD strain	μ_{max} in PYG	Lineage
71	0.75	UK 2011-13
137	0.90	Non-UK 2012-14
157	0.74	UK 2011-13
160	0.87	UK 2007-8
164	0.87	Non-UK 2007-8

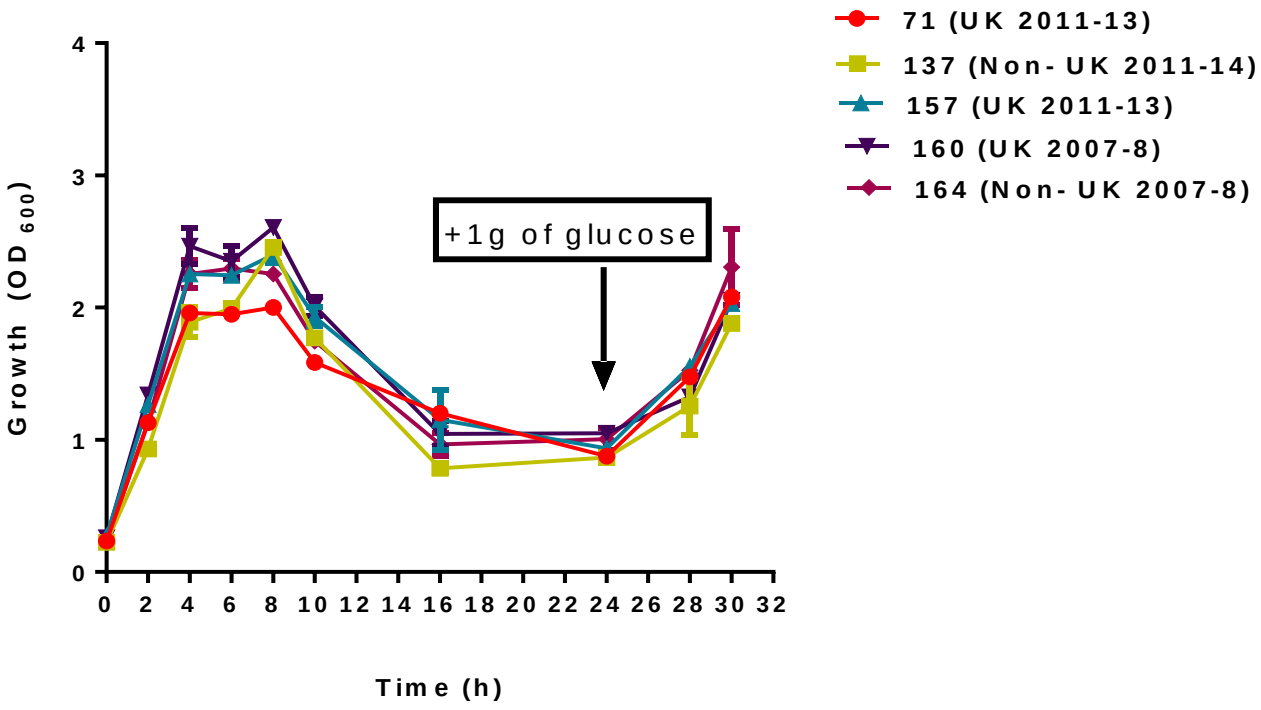


Figure 5-9 The growth curves of five CD002 strains in peptone yeast glucose broth (PYG), measured by OD_{600} over 32h. Data represent the average of three independent experiments and error bars are the standard error of mean.

5.3.2.2 Competition growth studies

After the limiting nutrient and maximum specific growth rate of the strains in PYG was determined, exponentially growing pure cultures of CD002 were inoculated into the bioreactor. Twelve hours after strain mixing and inoculation into the fermenter vessels, the continuous flow was started. As indicated in table 5-2 three CD002 competing pairs were investigated in this study.

The first competing pair consisted of a recent non-UK 2011-14 strain; 137 and an older non-UK 2007-8 strain; 164. As shown in figure 5-10, after 12h of continuous culture growth, strain 164 demonstrated a significantly ($p= 0.0001$) higher biomass compared to strain 137. However, at 48h of growth, a decline in the biomass for strain 164 and 137 was observed

($2.3x10^7$ and 4.65 $x10^7$CFU/ml respectively). Both strains maintained their biomass levels for three consecutive days, although the levels of 137 remained significantly (p= 0.0001) higher than 164. Interestingly, at day six, a dramatic decline was observed in both strain populations, and these levels were maintained until the experiment was complete (at 12 days). However, strain 137 appeared to have a competitive advantage through maintaining a biomass population at one $\log_{10}$ unit higher than strain 164, this was insufficient to wash out 164 completely. When the predicted biomass washout rate was calculated using the mass balance equation, this was similar for both strains. After five days of post continuous culture growth, the predicted biomass washout rate was below a limit of detection (data not shown).

The second competing pair consisted of a recent UK CD002 strain (71) and an Older UK CD002 strain (160). As shown in figure 5-11, 12h post-initiation of continuous culture flow, the biomass concentrations of strains 160 and strain 71 in the bioreactor were 2.8 $x10^8$ and 4.04 x 10^8 CFU/mL respectively. After 48h of growth, there was a slight decline in the biomass of strain 71, while strain 160 had declined significantly (P= 0.0001). Strain 71 appeared to have a competitive advantage over strain 160 to glucose, as this strain thrived at 10^7 CFU/ml until the end experiment, while the populations of strain 160 declined continuously over the course of the experiment. Additionally, after four days post continuous culture flow, the predicted biomass washout rate for both strains was below the limit of detection (data not shown).

A competition for glucose between recent UK CD002 strain (157) and a recent Non- UK CD002 strain (137) was performed. As shown in figure 5-12, 12h post-initiation of continuous culture flow into the bioreactor, the biomass of both strains; 157 and 137 was 7.8 x 10^7 and 5.8 $x10^7$ CFU/mL respectively. Following the 48h of growth, there was a significant (p= 0.0001) decline in the biomass of strain 137 to 3.9 x 10^5 in the bioreactor. Six days into the experiment, the

population of 137 had significantly (p= 0.0001) declined to 167 CFU/mL, while strain 157 thrived. Recent UK CD002 appeared to have a competitive advantage over non-UK CD002.

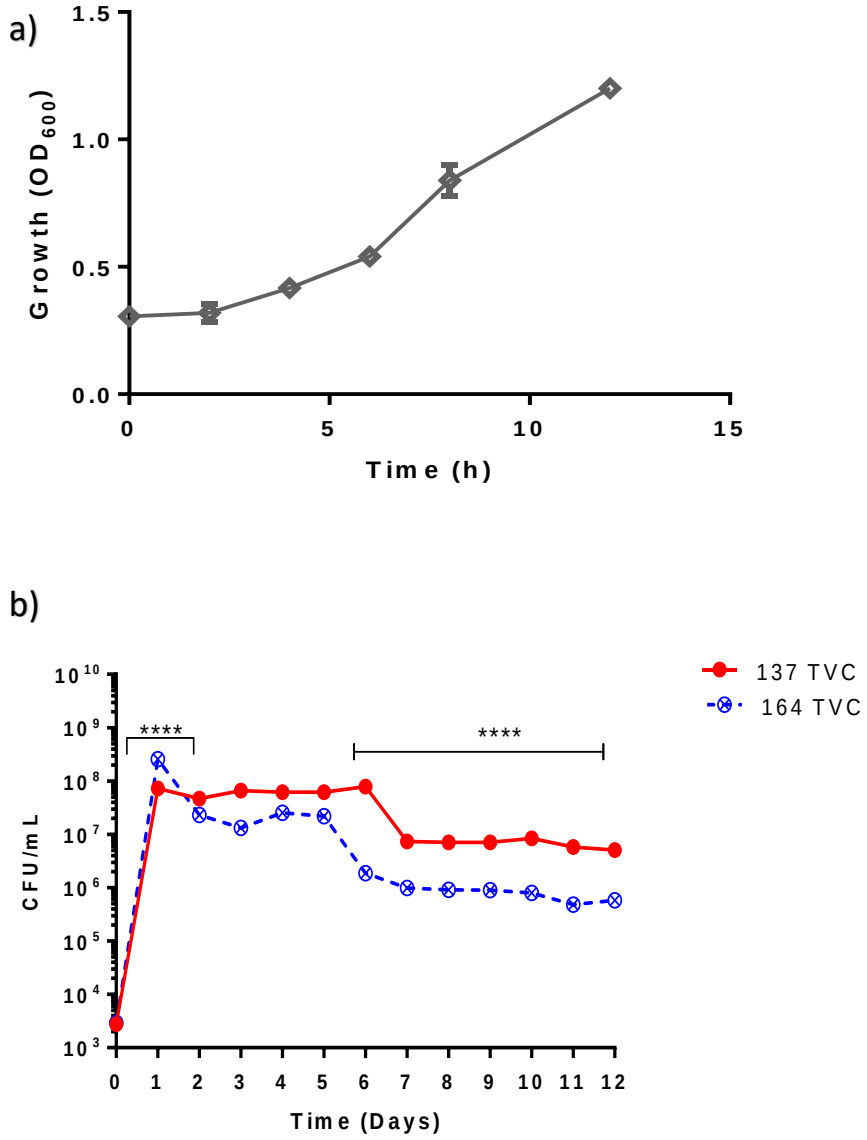


Figure 5-10 **Competition of a recent Non-UK CD002 Strain (137) Vs an Older Non- UK CD002 strain (164) for glucose in a bioreactor**. Growth populations of each strain were measured over a period of 12 days. **A)** The mean batch growth (OD_{600}) of the competing pair(137+164) at 2, 4, 6, 8 and 12h post-inoculation into the bioreactor.**B)** The mean biomass (CFU/ml) of each strain during continuous culture flow, enumerated daily through plating on selective Brazier's agar (with 1mg/L of rifampicin), and Brazier's agar without a selective antibiotic. Enumeration of the colony-forming units was done in triplicate, the asterisk(***) indicates where a significant difference was observed (P=0.0001).

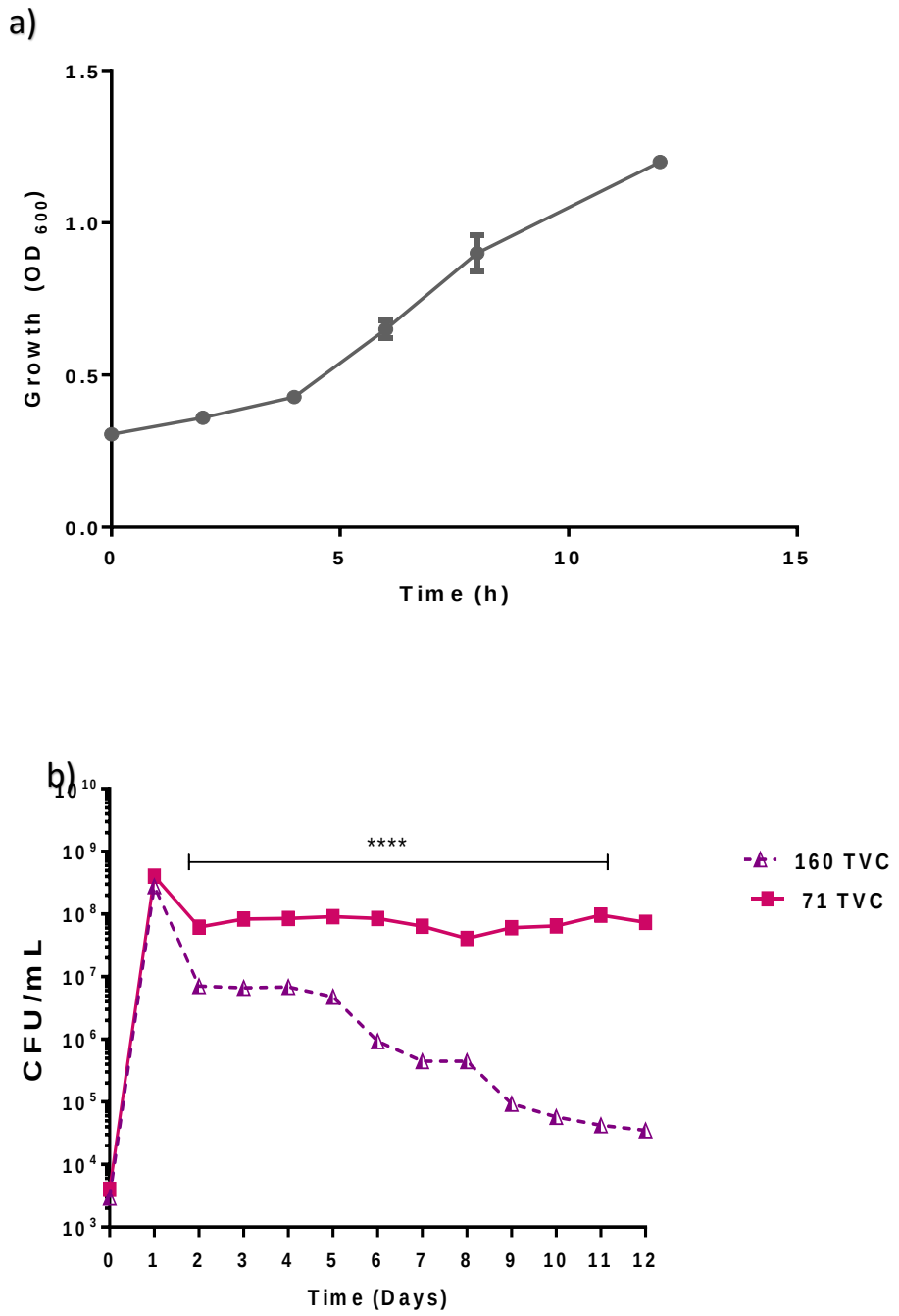


Figure 5-11 **Competition of a recent UK CD002 Strain (71) Vs an Older UK CD002 strain (160) for glucose in a bioreactor**. Growth populations of each strain were measured over a period of 12 days. **A)** The mean batch growth (OD_{600}) of the competing pair(71+160) at 2, 4, 6, 8 and 12h post-inoculation into the bioreactor.**B)** The mean biomass (CFU/ml) of each strain during continuous culture flow, enumerated daily through plating on selective Brazier's agar(with 10 mg/L of moxifloxacin), and Brazier's agar without a selective antibiotic. Enumeration of the colony-forming units was done in triplicate, the asterisk(***) indicates where a significant difference was observed (P=0.0001).

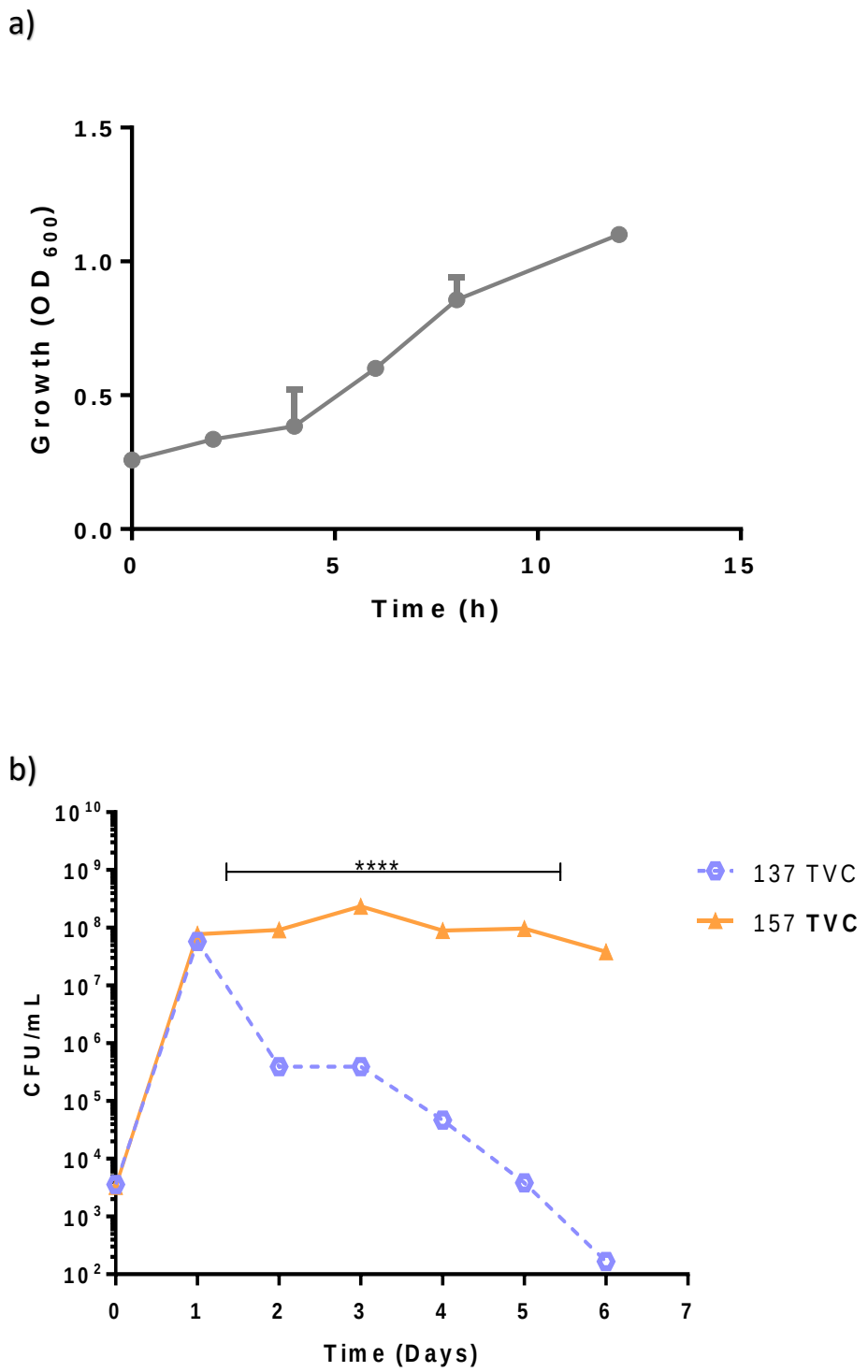


Figure 5-12 **Competition of a recent Non- UK CD002 Strain (137) Vs a recent UK CD002 strain (157) for glucose in a bioreactor**. Growth populations of each strain were measured over a period of 12 days. **A)** The mean batch growth (OD_{600}) of the competing pair (137+157) at 2, 4, 6, 8 and 12h post-inoculation into the bioreactor.**B)** The mean biomass (CFU/ml) of each strain during continuous culture flow enumerated daily through plating on selective brazier's agar(with 1mg/L of rifampicin and 10mg/L of erythromycin). Enumeration of the colony-forming units was done in triplicates, the asterisk(***) indicates where a significant difference was observed (P=0.0001).

5.4 DISCUSSION

Nutrients and space are important for the survival of microorganisms. The nutrients required for microbial growth and metabolic functions include; carbon, nitrogen, potassium, magnesium phosphorus, sulphur, hydrogen, calcium, iron and vitamins (Bren *et al*., 2013; Ghoul & Mitri, 2016). In the human gut, these nutrients are derived from the host diet, mucosal secretions and nutrients released by commensal bacteria. Successful colonisation by pathogens requires scavenging of nutrients, sensing of chemicals, competing with the resident bacteria and regulating the expression of virulence genes (Pacheco *et al*., 2012). Knowledge of a pathogen's full metabolic potential as well as competitive fitness is important in deciphering its virulence potential and factors driving the emergence of epidemic lineages. Due to the increased incidence of *Clostridium difficile* infection (CDI) in the early 2000s, which is attributed to the emergence of hypervirulent strains(He *et al*., 2013; Loo *et al*., 2005; L. C. McDonald *et al*., 2005), several studies have examined the metabolic potential and competitive fitness of these strains, as well as the reference *C. difficile* (strain 630). However, the metabolic potential and competitive fitness of recent emerging ribotypes (002, 005, and 014) of *C. difficile* have not been reported. Here, the nutrient utilisation of different strains of *C. difficile* ribotype 002 was investigated using phenotypic microarray analysis, and the competitive fitness of these strains was investigated in a single-stage fermenter.

Colonisation resistance by the host microbiota is important in preventing CDI and must be overcome in order for *C. difficile* to proliferate in the colon (Robinson *et al*., 2014). Data generated from several mouse model studies have demonstrated *C. difficile* ability to adapt its metabolism to exploit a variety of nutrient sources within the gut environment(Fletcher *et al*., 2018; Janoir *et al*., 2013; Jenior *et al*., 2017; Kansau *et al*., 2016). Despite the ability of *C.*

difficile to conform to a subset of metabolic pathways, carbohydrate and amino acid metabolism are regarded the most important in *C. difficile* colonisation and infection (Jenior *et al*., 2017). Janior and colleagues (2013) reported genes encoding the phosphoenolpyruvate-dependent phosphotransferase system (PTS), which are involved in the up-regulation and down-regulation of a variety of carbon sources during *in vivo C. difficile* colonisation. *In vivo* transcriptomic analysis of *C. difficile* revealed a heterogeneous expression in catabolic pathways for a variety of carbon sources (Jenior *et al*., 2017). Consistent with earlier findings, CD002 strains in the present study, utilised a variety of carbon sources. Interestingly, recent strains (Isolated 2012-2014) demonstrated an expanded carbon utilisation profile, which was comparable to a strain of the common ribotype, 078 (Scaria *et al*., 2014). In agreement with previous studies by Scaria and colleagues (2014), CD002 strains were able to readily utilise simple sugars such as maltose, α-D-glucose, D-fructose and D-mannose.

Genomic diversity exists between strains of *C. difficile*, supporting this, different strains of the same ribotype in PM analysis study by Scaria *et al* (2014) exhibited different nutrient utilisation profiles. An interesting observation in the present study was the ability of CD002 strains to utilise five carbon sources (D-ribose, L-lyxose, L- arabinose, Dihydroxy Acetone, D-Arabinose and 2- deoxy- D- ribose) which were not utilised by hypervirulent *C. difficile* strains (RT 078 and RT 027). These are simple sugars, some of which are derived from the host diet and end products of other commensal gut organisms. The significance of these carbon sources in *C. difficile* physiology and virulence is unknown, however, it is tempting to speculate that it may help select for the emergence and spread of these strains. In view of this hypothesis, a recent study by Collins and colleagues identified independent mechanisms which have been acquired by epidemic lineages of *C. difficile* ribotypes (027 & 078) to

metabolise dietary sugar trehalose(Collins *et al*., 2018). The authors, identified a single point mutation(L172I) in the trehalose repressor gene (*treR*) that was unique to *C. difficile* ribotype 027 strains, and a cluster of four genes involved in trehalose metabolism, as well as a PTS premase which was unique to strains of ribotype 078 (Collins, *et al*., 2018). Subsequent studies on ribotype 017 strains, revealed a single point mutation (C171S) in *treR*, in a location (the predicted effector binding pocket of TreR) adjacent to where the L1721 amino acid substitution is found in 027 strains(Collins *et al.*, 2018). Additionally, the authors suggested a link between the adoption of trehalose as an additive in the human diet in the 21st century, and the emergence of both ribotype 027 and ribotypes 078 outbreaks(Collins, *et al*., 2018a; Collins, *et al*., 2018b). The hypothesized that the introduction of trehalose to human diet may have contributed to driving the epidemiological prevalence of these strains. As a result, the speculation that the aforementioned carbon sources may drive epidemiological prevalence in CD002 may not be farfetched, however, further analysis utilising other model systems is required to support this hypothesis. Additionally, only one CD002 strain (157, UK 2011-13) utilised trehalose in the present study, suggesting that the introduction of trehalose in human diet may not be a factor driving the epidemiological prevalence of CD002.

One of the limited resources in the mammalian lower gastrointestinal tract is nitrogen, and through Stickland reactions, *C. difficile* uses amino acid fermentation to provide energy and nitrogen in the lower gastrointestinal tract (Jenior *et al*., 2017). Stickland reactions involve the fermentation of two amino acids, in which one acts as an electron donor (leucine, isoleucine and alanine) and another acts as an electron acceptor (proline and glycine) (Kansau *et al*., 2016). An earlier *in vivo* study revealed a Stickland pair, leucine and proline as preferential substrates utilised by *C. difficile* to generate ATP (Janoir *et al*., 2013). A recent *in vivo* study by Jenior and colleagues demonstrated how *C. difficile* preferentially metabolised nitrogen-

containing carbon sources derived through fermentation of Stickland substrates (such as alanine, leucine and proline), and host-derived glycans(Jenior *et al*., 2017). Additionally, Moore and colleagues demonstrated how low protein diets were found to be protective against CDI in mice, suggesting that fermentation of proteins is an alternative energy source for *C. difficile*(Moore *et al*., 2015). Consistent with previous studies, recent CD002 strains (strain 157 & 137) in the present study, utilised a wider range of nitrogen sources in comparison to strains of epidemic *C. difficile* ribotypes, 027 and 078 that were reported in a study by Scaria *et al* (2014). Additionally, recent UK CD002 (157 and 71) utilised both L-leucine (PM3) and L-proline (PM5), which are preferential Stickland substrates. Although it might appear trivial, the ability of recent CD002 isolates to readily utilise preferential Stickland substrates could have a profound effect on the strain's dissemination and colonisation.

It has previously been reported that guanosine and methylamine are strong toxin inducers (Lei & Bochner, 2013). In the present study, strain 157 and 137 were positive for two toxin inducers (guanosine and methylamine). After 12h of growth, both strains (In chapter 4) had produced a cytotoxin titre of 1RU, while it took most CD002 strains 24h to achieve a titre of 1RU. The ability of these strains to utilise toxin inducing nutrients may explain why they were able to produce toxins earlier in the growth cycle, also at 72h, the toxin titre of these strains was high (4RU). The phenomenon that high toxin producers may cause severe infections (Di Bella *et al*., 2016) may explain why strains of this ribotype have been implicated in severe CDI cases recently(Chow *et al*., 2017; Dauby *et al*., 2017; Wong *et al*., 2016). However, until clinical information linking these strains to severe forms of CDI in the UK and the rest of the EU is obtained, we can only speculate.

The expanded metabolic potential demonstrated by recent CD002 strain (137 of Spanish origin), in comparison to other CD002 strains investigated in the present study might help explain the emergence of this ribotype. Although speculative, it is reasonable to believe that a strain with a wide utilisation of nutrients coupled with an ability to use spore germinants such as taurocholic acid and alanine, will readily colonise a susceptible host, thereby leading to the increased prevalence of strains of that ribotype. However, it is noteworthy that an expanded metabolic potential demonstrated by one strain of a ribotype does not reflect the behaviour of other strains of that ribotype. Additionally, studies by Jenior and colleagues (2017) revealed that *C. difficile* is able to exploit various nutrient niches within the host, suggesting an ecological generalist rather than a specialist lifestyle(Gripp *et al*., 2011; Jenior *et al*., 2017). This means that in the absence of predisposing factors, a strain with a wide metabolic potential will be unable to outcompete a specialist organism in the gut microbiota. Conversely, we can infer that, in the presence of an altered microbiota, a strain with a wide metabolic potential will have a competitive advantage over strains of other ribotypes with narrower substrate utilisation profiles.

Beyond utilisation of nutrients, competition for nutrients is a key aspect of host colonisation. A study by Janior and colleagues highlighted the importance of glucose as a preferred substrate for *C. difficile* colonisation in the gut(Janoir *et al*., 2013). In the present study, competition for glucose by different strains was investigated *in vitro*. The data generated from the three competing pairs, suggests that recent strains of CD002 may have a competitive advantage in terms of glucose affinity over older strains of CD002. However, due to the predicted biomass washout rate for each strain observed in two (Recent Non-UK CD002 vs Old Non-UK CD002, Recent UK CD002 vs Old UK CD002) out of the three competing pairs, it appears that strains co-existed together and were being washed out a similar rate.

Interestingly, a recent UK CD002 was able to outcompete recent non-UK CD002 in the fermenter. Nevertheless, it is difficult to make assumptions as to whether these strains will behave similarly in the presence of a complex gut microbiota since competition in the present study incorporated the use of pure cultures of CD002. However, previous *C. difficile* competition studies by Robinson and colleagues observed the dominance of *C. difficile* ribotype 027 strains over non- 027 strains *in vitro* as well as *in vivo*(Robinson *et al*., 2014). *C. difficile* ribotype 027 strains appeared to outcompete non-027 strains, leading to a complete loss of non- 027 strains, while 027 strains remained dominant. This suggests that the observations made in the present study could potentially be replicated *in vivo*.

This study had several limitations, the most important, was the inability to include biological replicates in the phenotypic microarray analysis. While steps were taken to ensure validity of results, through comparative analysis of *C. difficile* 630 with data obtained from Scaria *et al* (2014) study, and Gram staining of wells that yield very high biomass after 48h incubation, we cannot rule out the possibility that some of the results could have been false positives. Also, due to the cutoff point for positives, which was 40 % above the negative control (1.4), it is also possible that some other positive nutrient sources which yielded readings of <1.4 may have been excluded in the overall profile. In the competition study, the inability to compete for strains that had each attained a steady state in a fermenter before a competing strain was introduced may be an important limitation. Though steps were taken to ensure that the same biomass of each strain was inoculated into the fermenter, it is reasonable to believe that the experimental design was not conducive for both strains to thrive. As a result, it was difficult to ascertain, if strains which maintained higher biomass, have a more competitive advantage. Another limitation was not determining the substrate utilisation constant of these strains to glucose, as this would have informed about the affinity of the strains to glucose.

The data generated here has provided insights into the nutrient utilisation profiles of CD002 strains which may then translate to their *in vivo* colonisation potential. Whether these factors might contribute to the prevalence and virulence of these strains, is yet to be elucidated using other model systems. Nevertheless, we can conclude that recent CD002 have a more expanded nutrient utilisation profile than older CD002 strains. This suggests, that recent strains of CD002 may have acquired mechanisms to better utilise various nutrient sources and this may have contributed to the recent prevalence of this ribotype.

6 COMPARATIVE PROTEOMIC ANALYSIS OF CD002 ISOLATES FROM DIFFERENT TIME LINEAGES

6.1 BACKGROUND

Clostridium difficile (*C. difficile)* is a Gram-positive spore-forming anaerobic bacterium that has rapidly emerged as the leading cause of nosocomial diarrhoea around the world (Ternan *et al*., 2014). Symptoms range from mild, self-limiting diarrhoea to toxic megacolon, intestinal perforation or death(Chen *et al*., 2013). In recent years, the clinical and molecular epidemiology of this problematic pathogen has received considerable attention due to the emergence of hypervirulent strains types, which are responsible for higher incidence rates and a financial burden on the health care system (Lessa *et al*., 2015; Planche & Karunaharan, 2017).

Advances in next-generation sequencing technologies have proven useful in understanding the evolution, genetic relatedness, and physiology of several problematic pathogens including *C. difficile.* In 2006, Sebaihia and colleagues described the first complete genome sequence of *C. difficile* strain 630 (PCR ribotype 012), a multi-drug resistant strain, originally isolated from a patient with pseudomembranous colitis (PMC) in 1982 in Zurich, Switzerland. Since then, the genome sequence of this strain has been re-annotated by other researchers(Dannheim *et al*., 2017; Monot *et al*., 2011) and the fully sequenced genomes of other *C. difficile* strains have been described(He *et al*., 2010; Janoir *et al*., 2013; Pereira *et al*., 2016; Stabler *et al*., 2009; Suzuki *et al*., 2017) and made publicly available on several databases (such as EMBL-EBI and GenBank). Analysis of the *C. difficile* 630 genomes by Sebaihia and colleagues, revealed its dynamic nature and a vast spectrum of genes involved in resistance to antimicrobial

agents, virulence, host interactions, and various metabolic activities that allow its survival within the challenging gut environment (Sebaihia *et al*., 2006). Subsequent comparative genomic analysis of different strains of *C. difficile* revealed the massive genomic diversity existing between strains, and this genomic variation extends to the core genome of this pathogen(He *et al*., 2010).

Despite the success in delineating *C. difficile* physiology and pathogenesis using whole-genome sequencing technologies, proteomics studies are crucial in better understanding the diversity and adaptive mechanisms of this widespread pathogen. Proteomic studies provide information complementary to the genome information, as the proteome of an organism, translates the genome sequence into cellular functions and structures. During pathogenesis, proteins catalyse crucial metabolic reactions as well as cellular processes (Maaß *et al*., 2018). Access to highly sensitive techniques such as mass spectrometry has made it possible for proteins to be rapidly analysed.

Alterations in *C. difficile* gene expression in response to environmental stimuli have been investigated in several proteomic studies (Boetzkes *et al*., 2012; Chen *et al*., 2013; Chong *et al*., 2014; Dresler *et al*., 2017; Jain *et al*., 2011; Jain *et al*., 2010; Maaß *et al*., 2018; Moura *et al*., 2013; Ternan *et al*., 2014). Mukherjee and colleagues analysed proteins released *in vitro* during high toxin production in *C. difficile* strains 630 and VPI10463. Subsequently, Moura *et al*. (2013) analysed purified *C. difficile* large clostridial toxins (TcdA and TcdB) and identified several surface proteins present in the culture filtrates. Additional studies have characterised the insoluble sub-proteome of *C. difficile* reference strain 630 (Jain *et al*., 2010), the cell surface proteins (Wright *et al*., 2005), and the spore proteins (Lawley *et al*., 2009; Pizarro-Guajardo *et al*., 2018).In addition, proteins involved in antimicrobial resistance, reduced

antimicrobial susceptibility, drug metabolism and stress responses have been reported(Chong *et al*., 2014; Jain *et al*., 2011; Maaß *et al*., 2018)

Comparative proteomic analysis of the secretome of reference strain 630 and two hypervirulent PCR ribotype 027 strains (CD196 and CDR20291), revealed five proteins specifically secreted by hypervirulent strains(Boetzkes *et al*., 2012). Furthermore, differences in the level of expression of proteins, including adhesions, S-layer proteins, cell wall proteins and other potential virulence factors have been identified among different strains, confirming the variation between strains (Boetzkes *et al*., 2012; Chen *et al*., 2013; Chilton, *et al*., 2014). More recently, Dresler and colleagues (2017) analysed the proteins released *in vitro* in eight *C. difficile* PCR ribotypes (001, 005, 010, 012, 014, 027, 078, and 176), and also highlighted the unique expression of specific proteins identified in ribotypes 027 and 176, confirming their genetic relatedness.

The data generated from the aforementioned proteomics studies emphasise the importance of proteomic characterisation of emerging strains and ribotypes. Since *Clostridium difficile* ribotype 002 has emerged as prevalent ribotype in the UK, detailed biological characterisation in conjunction with comparative proteomic analysis is crucial in explaining the emergence of this ribotype in the UK. Analysis of the whole-cell proteome of strains from different geographical locations and lineages could potentially give insights into adaptive changes that may have occurred over time.

6.1.1 **Aims and Objectives**

The aim of these studies was to characterise the whole cell proteome of three CD002 strains (1 per lineage) and identify changes at the proteomic level. This will be achieved through the following objectives:

1. Extraction and analysis of the whole-cell proteome using fast prep system and single dimension gel electrophoresis.
2. Tryptic digestion of peptides and analysis using Liquid Chromatography-Tandem Mass spectrometry (LC-MS/MS).
3. Analysis of spectra using bioinformatics tools such as MASCOT server and Scaffold.

6.2 MATERIALS AND METHODS

6.2.1 **Bacterial strains and proteomic analysis**

To investigate the differences between CD002 isolates at the proteomic level, one strain was selected per lineage (Table 6-1). Whole-cell proteins were extracted according to methods described in 2.8.7.1., and one-dimensional gel electrophoresis was performed according to methods described in 2.8.7.2. The resultant gels (Figure 6-1) were excised into 12 bands for each isolate and in-gel trypsin digestion was carried out according to methods described in 2.8.7.3 for identification by LC-MS/MS.

Table -6-1 List of CD002 Strain per lineage, selected for proteomic analysis

Strain No	Lineage	Origin	Year of Isolation
71	UK 2011-13	Maidstone	2012
137	Non-UK 2012-14	Spain	2014
174	UK 2007-8	Leeds	2007

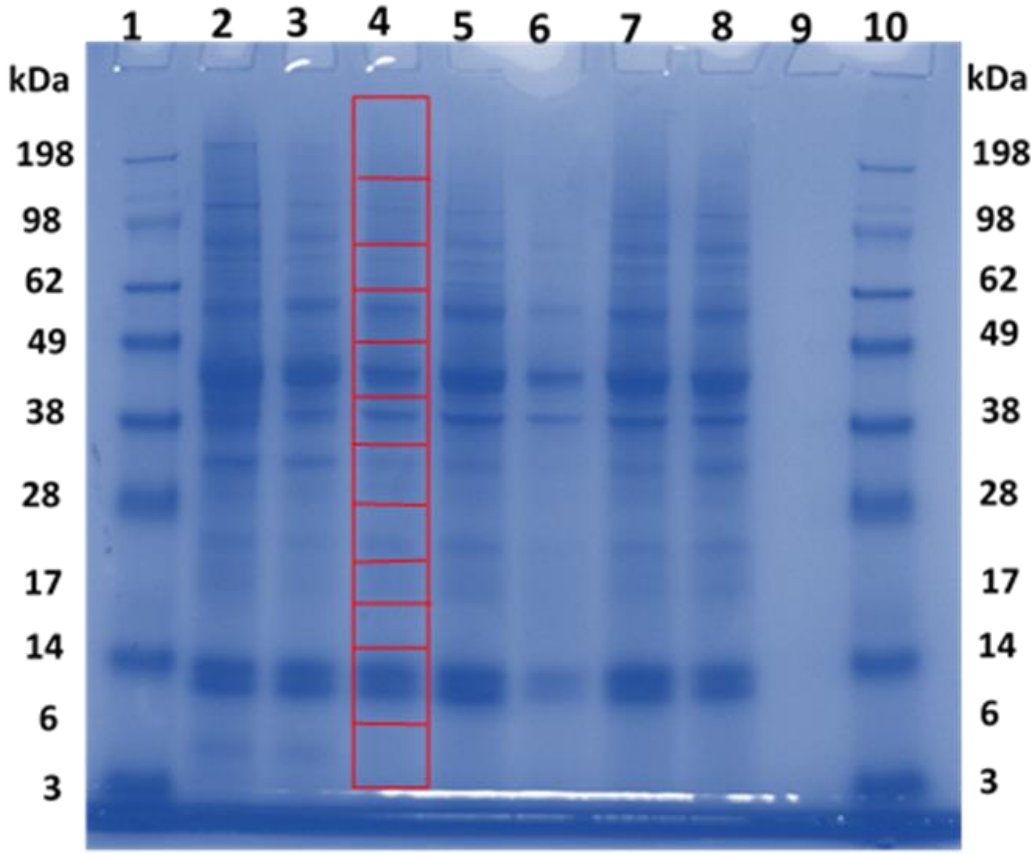


Figure 6-1-Coomassie-stained 1D gel used for LC-MS/MS analysis. Lanes 1 and 10 are the pre-stained see blue molecular markers. Lanes 2 and 3 are biological replicates of the strain 71 extract, lanes 4 and 5 are biological replicates of the strain 137 extract, and lanes 6 and 7 are biological replicates of the strain 174 extracts. Lane 8 is an extract of strain CD630, as a control. Each lane was cut into 12 bands, as shown in lane 4, for digestion and identification by LC-MS/MS

6.2.2 **LC-MS/MS analysis and Database search**

Experts within the mass spectrometry laboratory, UCL School of Pharmacy, London, UK, performed the LC-MS/MS analysis on the proteomes of three CD002 strains (with biological replicates). All LC-MS/MS data were processed using the MASCOT server (Version 2.6, Matrix Science, and London, UK) and searched against NCBI and SwissProt database for all species of bacteria. The peptide tolerance was set at ± 1.2 Da, the MS/MS tolerance was set at ± 0.6 Da

and the search was set to allow two missed cleavages, oxidation of methionine and carbamidomethylation of cysteine and a significance threshold of ≤0.05. Subsequently, MASCOT output files per biological replicate were imported to the Scaffold proteome software (Version 4, Oregon, USA) and re-analysed against the UniProt database with the following parameters: The protein threshold was set at 95%, the peptide threshold was set at 95% and a minimum of at least two peptides per protein was allowed. Although a minimum of two peptides was allowed per protein, identifications based on one peptide was considered valid if the scaffold probability score was ≥95% and if the same peptide had been detected in other samples. The MASCOT and Scaffold search parameters were stringent, so the probability of a wrong assignment was below 0.1%.

6.3 RESULTS

6.3.1 Total number of identified proteins and strain distribution

A single-dimensional (1D) gel electrophoresis followed by LC-MS/MS analysis was used to identify strain-specific differences in the proteome of three CD002 strains from different time lineages and geographical locations (Table 6-1). A total of 250 different proteins were identified in the whole-cell proteome retrieved from strains grown for 64h on Columbia blood agar (LAB001, Lab M, Lancashire, UK) plates. Out of the identified proteins, 165 (66%) of these were shared between all strains (Figure 6.2a, Table 6.2). Some of the proteins identified were only detected in one of the three strains, with 15 (6%) proteins only detected in strain 71 (UK 2011-13), 5(2%) proteins only in strain 137 (Non- UK 2011-14) and 10 (4 %) proteins only in strain 174(UK 2007-8).

The fifteen proteins detected only in strain 71 included: 4-hydroxy-tetrahydrodipicolinate synthase, 50S ribosomal protein L11, anti-sigma F factor, Arginine--tRNA ligase, Biotin

synthase, Energy-coupling factor transporter ATP-binding protein EcfA2, flavodoxin family protein, lysyl-tRNA synthetase, Methionyl-tRNA formyltransferase, Oligoendopeptidase F , Peptide chain release factor 3, putative peptidase, putative purine permease, putative transcriptional regulator and XRE family transcriptional regulator. The five proteins detected only in strain 137 included; Holin-like pore-forming protein, bifunctional protein GlmU, DNA polymerase IV, N-acetylmuramic acid 6-phosphate etherase, and a putative phage protein. The ten proteins detected only in strain 174 include, DNA polymerase III subunit alpha, Fe-S cluster assembly scaffold protein, hypothetical protein BM529_15905, Lrp/AsnC family transcriptional regulator, Peptidylprolyl isomerase, putative septation protein, rubrerythrin (21kDa) small acid-soluble spore protein alpha, small, acid-soluble spore protein beta, Surface layer protein A (37kDa).

Using the scaffold proteome software (Version 4), the identified proteins were annotated using gene ontology (GO terms) to determine their cellular localisation as well as biological processes. Majority of proteins identified were cytoplasmic (74%) proteins and ribosomal proteins (18.3%) (Figure 6.2b) with most proteins being involved in metabolic processes (60-67%).

6.3.2 **Proteins associated with Toxins regulation and synthesis**

Using LC-MS/MS analysis, the major virulence factors for *C. difficile,* toxin A and B were detected in all three CD002 investigated strains. Additionally, the GTP-sensing transcriptional pleiotropic repressor CodY known to repress toxin gene expression was detected in all three isolates. The holin-like pore-forming protein, tcdE was detected in only strain 137 (Non-UK 2011-12) (Figure 6-2c).

6.3.3 **Spore associated proteins**

Spores are the vehicle for CDI transmission, using LC-MS/MS analysis, 10 spore proteins were identified. Four of these proteins were identified in all investigated strain, and they included; Sporulation membrane protein SpoIIIJ, Sporulation sigma-E factor-processing peptidase, Stage IV sporulation protein A, Cell division/stage V sporulation protein. The spore coat protein, peroxiredoxin (CotE), was detected in only strain 71 and 137. Small acid-soluble spore protein alpha, small acid-soluble spore protein beta (sspB), a putative septation protein SpoVG, and a putative cold shock protein (CspA) was detected only in strain 174. While the anti-sigma F factor was detected only in strain 71.

6.3.4 **Proteins involved with the assembly of the *C. difficile* flagellum**

Flagella are essential for motility and are believed to contribute to *C. difficile* virulence. Using LC-MS analysis, eight different flagella proteins were identified and one of these was a putative flagella filament core protein. Flagella proteins, flagellin (fliC), flagellar hook protein (fliD), flagella M-ring protein (fliF) and flagellar export protein (fliJ) were detected in all three CD002 investigated strains. While flagellar assembly factor (fliW), flagellar basal-body rod protein (FlgC) and flagellar biosynthesis protein (FlhA) was detected in only strain 71 and 137. The putative flagella filament core protein was identified in only UK CD002 strains, strain 71 and 174.

6.3.5 **Cell surface-associated proteins**

Cell surface proteins are known to play a role in *C. difficile* adherence. Using LC-MS/MS analysis, a total of 10 cell surface proteins were identified. Eight cell surface proteins were detected in all strains, these included cwp20, cwp22, cwp66, cell wall bind repeat 2 family protein, S-layer protein precursor, S-layer protein and putative cell wall binding protein

Cwp25. The ATPase activity, protein translocase subunit SecA, known to be necessary for the secretion of surface layer proteins (Fagan and Fairweather, 2011) was detected in all investigated strains. Only the cell wall binding protein cwp2 was detected in only two strains; strain 71 (UK 2011-13) and strain 137(Non-UK 2012-14).

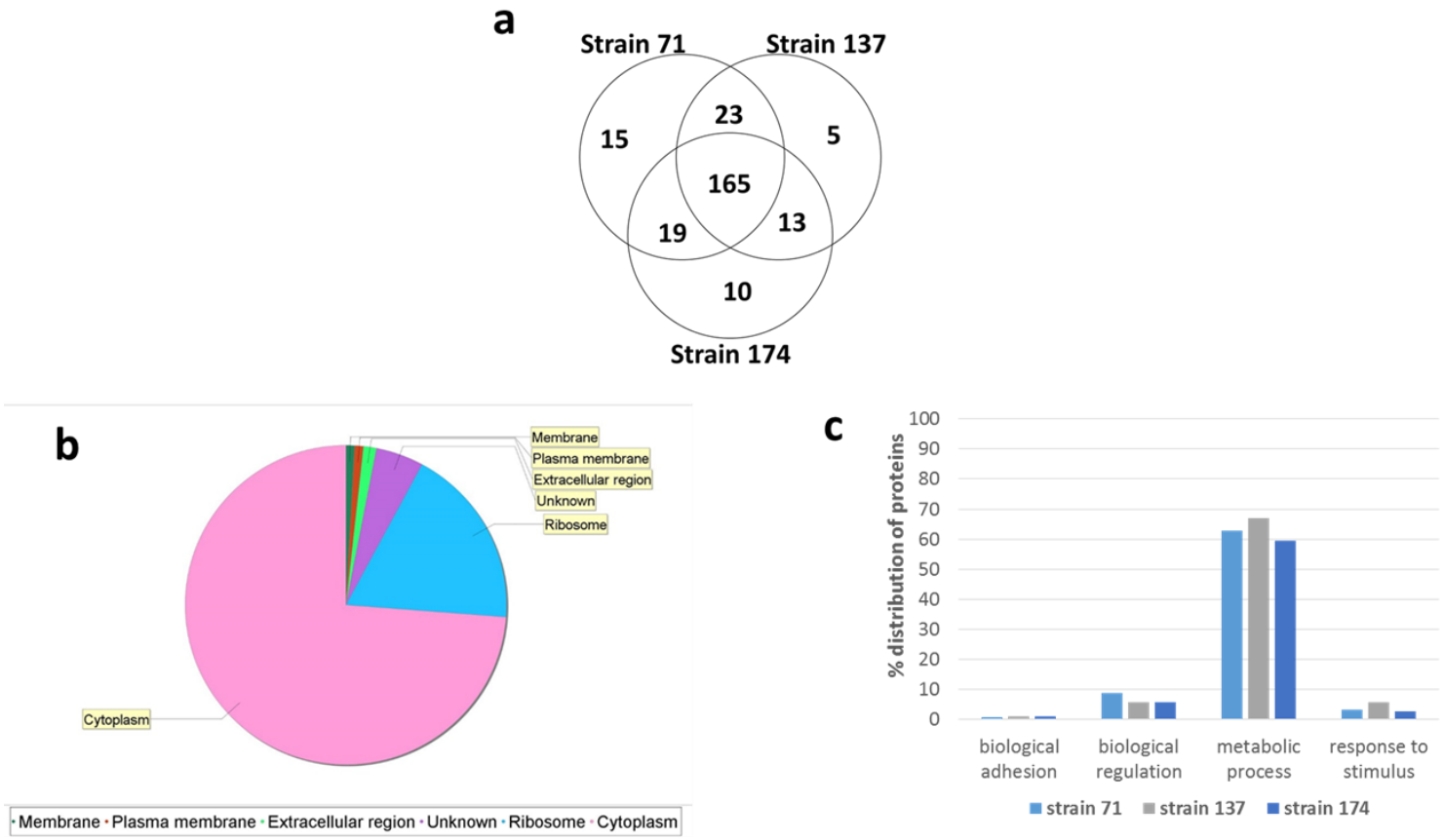


Figure 6-2 Overview of identified CD002 proteins a) A Venn diagram showing the distribution of proteins identified by LC-MS/MS analysis according to the strains in which they were identified. b) A pie chart from the scaffold proteome software, showing the cellular location of identified proteins in the three investigated CD002 strains. c) The percentage distribution of proteins according to the biological processes in which they are involved.

6.3.6 **Heat Shock and stress-related proteins**

Using LC-MS/MS analysis, chaperones and several stress-response related proteins were identified. In total, nine heat shock proteins were identified, and eight of these were detected in all investigated strains. They included; 33 kDa chaperonin, 60 kDa chaperonin (groL), ATP-dependent Clp protease ATP-binding subunit ClpX, ATP-dependent Clp protease proteolytic subunit, Chaperone protein ClpB, Chaperone protein DnaJ, Chaperone protein DnaK (Hsp70), and Hsp90. The 10 kDa chaperonin (gros) protein was identified in only strain 137 and 174. Additionally, one stress-related rubrerythrin was identified in strain 174 only, while rubrerythrin family protein was detected only in strain 71 and 174.

6.3.7 **Proteins associated with leucine pathway metabolism**

Using LC-MS/MS analysis, seven proteins involved in leucine pathway metabolism were identified. Of the seven proteins identified, six of these were detected in all investigated strains. They included; (R)-2-hydroxyisocaproyl-CoA dehydratase alpha subunit (hadB), (R)-2-hydroxyisocaproyl-CoA dehydratase beta subunit (hadC), electron transfer flavoprotein beta-subunit (etfB), electron transfer flavoprotein subunit alpha (etfA), acyl-CoA dehydrogenase , short chain specific (acdA), Isocaprenoyl-CoA:2-hydroxyisocaproate CoA-transferase (hadA). Leucine metabolism associated protein, 2-hydroxyisocaproyl-CoA dehydratase activator (hadI) was detected in only strain 137 and 174.

Table 6-2 List of all proteins identified in the proteome of three CD002 strains investigated by 1D gel electrophoresis followed by LC-MS/MS analysis. Proteins were identified using MASCOT search parameters and further validated as greater than 95% peptide probability, a minimum of 2 peptides and 95% protein probability using Scaffold proteome software.

Protein IDs		Gene	Molecular Weight	Accession Number	No of Unique peptides					
					71		137		174	
No	Ribosomal proteins				A	B	A	B	A	B
1	30S ribosomal protein S10	rpsJ	12 kDa	RS10_PEPD6	2	3	0	0	4	3
2	30S ribosomal protein S12	rpsL	15 kDa	RS12_PEPD6	2	2	2	2	2	3
3	30S ribosomal protein S13	rpsM	14 kDa	RS13_PEPD6	5	4	0	0	3	4
4	30S ribosomal protein S15	rpsO	10 kDa	RS15_PEPD6	0	0	3	4	2	2
5	30S ribosomal protein S19	rpsS	11 kDa	RS19_PEPD6	2	4	2	4	2	3
6	30S ribosomal protein S2	rpsB	26 kDa	RS2_PEPD6	0	0	2	3	2	3
7	30S ribosomal protein S3	rpsC	30 kDa	RS3_PEPD6	2	8	4	2	3	5
8	30S ribosomal protein S4	rpsD	24 kDa	RS4_PEPD6	3	4	3	4	5	2
9	30S ribosomal protein S5	rpsE	18 kDa	RS5_PEPD6	3	2	4	2	2	5
10	30S ribosomal protein S6	rpsF	11 kDa	RS6_PEPD6	3	2	4	2	4	3
11	30S ribosomal protein S7	rpsG	18 kDa	RS7_PEPD6	5	4	2	2	3	3
12	30S ribosomal protein S8	rpsH	15 kDa	RS8_PEPD6	4	6	0	0	5	4
13	30S ribosomal protein S9	rpsI	15 kDa	RS9_PEPD6	3	3	2	2	3	3
14	50S ribosomal protein L1	rplA	25 kDa	RL1_PEPD6	4	2	5	2	4	4
15	50S ribosomal protein L10	rplJ	19 kDa	RL10_PEPD6	3	2	4	3	4	3
16	50S ribosomal protein L11	rplK	15 kDa	RL11_PEPD6	4	3	0	0	0	0
17	50S ribosomal protein L13	rplM	16 kDa	RL13_PEPD6	5	5	8	3	4	2
18	50S ribosomal protein L15	rplO	16 kDa	RL15_PEPD6	4	5	2	2	2	3
19	50S ribosomal protein L18	rplR	14 kDa	RL18_PEPD6	3	2	4	2	2	2
20	50S ribosomal protein L19	rplS	13 kDa	RL19_PEPD6	2	3	0	0	2	5
21	50S ribosomal protein L2	rplB	30 kDa	RL2_PEPD6	2	4	2	3	4	6

22	50S ribosomal protein L21	rplU	11 kDa	RL21_PEPD6	4	3	5	3	0	2
23	50S ribosomal protein L24	rplX	14 kDa	RL24_PEPD6	2	5	0	6	2	7
24	50S ribosomal protein L29	rpmC	8 kDa	RL29_PEPD6	2	3	0	0	4	1
25	50S ribosomal protein L3	rplC	22 kDa	RL3_PEPD6	4	2	5	0	2	3
26	50S ribosomal protein L30	rpmD	7 kDa	RL30_PEPD6	2	0	4	2	2	5
27	50S ribosomal protein L5	rplE	20 kDa	RL5_PEPD6	2	2	0	8	2	2
28	50S ribosomal protein L7/L12	rplL	13 kDa	RL7_PEPD6	2	2	1	2	3	3
29	50S ribosomal protein L9	rplI	17 kDa	RL9_PEPD6	2	2	2	2	2	2
30	Ribosomal protein S12 methylthiotransferase RimO	rimO	51 kDa	Q18BJ2	2	2	2	2	0	0
31	Ribosomal RNA small subunit methyltransferase G	rsmG	24 kDa	C9YSG6	4	2	3	0	2	2
32	Ribosomal RNA small subunit methyltransferase A	rsmA	32 kDa	Q181C1	2	2	2	2	2	3
33	Ribosome maturation factor RimM	rimM	20 kDa	Q18BB6	4	3	4	2	0	0
34	Ribosome-recycling factor	frr	21 kDa	RRF_PEPD6	4	3	3	2	3	0
Cell Surface-associated proteins										
35	Cell surface protein (putative penicillin binding protein)	cwp20	111 kDa	Q18BY9	4	5	3	0	6	2
36	cell wall-binding protein cwp2	cwp2	67 kDa	Q183M5	2	2	2	2	0	0
37	cell wall binding protein cwp22	cwp22	72 kDa	Q183E7	3	3	4	2	4	5
38	cell wall binding protein cwp66	cwp66	68 kDa	Q183M7	2	2	2	2	2	2
39	Cell wall binding repeat 2 family protein	cwp	39 kDa	T4HAJ2_CLODI	2	2	3	5	4	5
40	Surface layer protein A	slpA	37 kDa	BAE48149	0	0	0	0	2	2
41	S-layer protein SlpA	slpA	77 kDa	WP_059026871	18	20	15	11	11	13
42	Protein translocase subunit SecA	secA1	102 kDa	Q18CN0	4	2	0	3	2	4
Proteins involved in sporulation										
43	Small acid-soluble spore protein alpha	sspA	8 kDa	Q183C2	0	0	0	0	2	4
44	Small, acid-soluble spore protein beta	sspB	7 kDa	Q17ZZ3	0	0	0	0	2	3
45	Sporulation membrane protein SpoIIIJ	CD630_36780	27 kDa	Q181T0	2	2	2	2	2	2
46	Sporulation sigma-E factor-processing peptidase	spoIIGA	32 kDa	Q182X8	2	2	2	2	2	2

47	Stage IV sporulation protein A	spoIVA	56 kDa	Q182W3	2	2	2	2	2	2
48	anti-sigma F factor	spoIIAB	16 kDa	Q189W4	4	2	0	0	0	0
49	Cell division/stage V sporulation protein	spoVE	41 kDa	Q182Y5	2	3	0	2	4	3
50	Peroxiredoxin (Spore coat protein)	cotE	81 kDa	Q18BV5	5	2	2	3	0	0
Toxins and transcriptional regulators										
51	Toxin A	toxA	308 kDa	TOXA_CLODI	12	3	5	10	11	5
52	Toxin B	toxB	270 kDa	TOXB_CLODI	2	4	2	5	4	2
53	GTP-sensing transcriptional pleiotropic repressor CodY	Cody	29 kDa	Q18BE1	2	5	2	7	2	3
54	Holin-like pore-forming protein TcdE	tcdE	19 kDa	Q189K6	2	2	2	2	2	2
Proteins involved in Leucine Metabolism										
55	(R)-2-hydroxyisocaproyl-CoA dehydratase alpha subunit	hadB	46 kDa	HADB_CLODI	5	5	6	3	6	2
56	(R)-2-hydroxyisocaproyl-CoA dehydratase beta subunit	hadC	42 kDa	HADC_CLODI	5	2	5	4	5	3
57	2-hydroxyisocaproyl-CoA dehydratase activator	hadI	28 kDa	HADI_CLODI	0	0	2	8	5	2
58	electron transfer flavoprotein beta-subunit/FixA	etfB	29 kDa	Q188I4	2	5	0	3	2	6
59	electron transfer flavoprotein subunit beta/FixA	eftB	28 kDa	WP_009888867.1	6	5	2	3	6	6
60	electron transfer flavoprotein subunit alpha/ FixB	etfA	37 kDa	Q18AQ5	2	3	0	4	4	6
61	electron transfer flavoprotein subunit alpha/FixB	etfA	36 kDa	WP_003428573.1	4	5	3	4	7	8
62	acyl-CoA dehydrogenase , short chain specific	acdA	41 kDa	Q188I5	8	0	7	4	3	9
63	Isocaprenoyl-CoA:2-hydroxyisocaproate CoA-transferase	hadA	44 kDa	Q188I3_PEPD6	5	9	3	11	7	12
Proteins involved in Flagellar assembly of *C. difficile*										
64	Flagellar assembly factor	fliW	15 kDa	FLIW_PEPD6	2	2	2	2	0	0
65	Flagellar basal-body rod protein FlgC	flgC	15 kDa	Q18CY6	9	8	4	6	0	0
66	Flagellar biosynthesis protein FlhA	flhA	76 kDa	Q18CZ8	8	4	3	4	0	0
67	Flagellar Hook Protein (flagellar cap protein)	fliD	56 kDa	Q18CX9	2	2	2	3	3	3
68	Flagellar M-ring protein	fliF	56 kDa	Q18CY4	4	0	2	3	2	2
69	Flagellin	fliC	31 kDa	Q18CX7	5	9	4	11	5	9
70	Flagellar export protein FliJ	flij	17 kDa	A0A386FPC4	4	2	3	2	3	4

Stress related proteins										
71	10 kDa chaperonin	groS	10 kDa	CH10_CLODI	0	0	4	2	1	2
72	33 kDa chaperonin	hslO	33 kDa	Q17ZW5	2	2	2	2	2	2
73	60 kDa chaperonin	groL	58 kDa	CH60_CLODI	13	8	9	6	9	6
74	ATP-dependent Clp protease ATP-binding subunit ClpX	clpX	46 kDa	Q180E8	7	2	5	3	4	2
75	ATP-dependent Clp protease proteolytic subunit	clpP	21 kDa	Q180F0	0	5	2	3	4	5
76	Chaperone protein ClpB	ClpB	98 kDa	Q187Y8	5	3	0	4	2	2
77	Chaperone protein DnaJ	dnaJ	42 kDa	Q182E7	0	5	7	3	3	4
78	Chaperone protein DnaK(Hsp70)(Heat Shock protein 70)	dnaK	66 kDa	DNAK_PEPD6	12	5	10	3	10	13
79	Heat shock protein 90	htpG	75 kDa	Q18D10	2	2	3	4	2	3
80	rubrerythrin	rbr	21 kDa	Q18A24_PEPD6	0	0	0	0	3	2
81	rubrerythrin family protein	rbr	22 kDa	WP_021397258	3	2	0	0	5	2
Elongation factors										
82	Elongation factor G	fusA	76 kDa	EFG_PEPD6	11	13	10	14	17	18
83	Elongation factor P	efp	21 kDa	EFP_PEPD6	4	4	3	2	2	2
84	Elongation factor Ts	Tsf	33 kDa	EFTS_PEPD6	6	2	8	2	2	4
85	Elongation factor Tu	tuf1	44 kDa	EFTU_PEPD6	8	8	10	8	10	7
86	Translation elongation factor G	tuf1	61 kDa	EQK72467.1	7	11	0	0	4	5
87	Transcription elongation factor GreA	greA	18 kDa	GREA_PEPD6	2	2	2	2	2	2
88	Trigger factor	Tig	48 kDa	TIG_PEPD6	2	3	5	4	3	2
Proteins Involved in Histidine pathway metabolism										
89	ATP phosphoribosyltransferase	hisG	23 kDa	Q18C67	4	4	3	2	2	2
90	ATP phosphoribosyltransferase regulatory subunit	hisZ	37 kDa	Q18C68	2	2	3	4	2	3
91	Histidine--tRNA ligase	hisS	48 kDa	Q183H5	7	2	5	3	4	2
Probable and Putative proteins										
92	Probable butyrate kinase	buk2	39 kDa	Q181W4	4	5	0	0	2	3
93	Probable cytosol aminopeptidase	pepA	53 kDa	Q18BF9	2	2	3	4	2	2

94	Probable transaldolase	tal	23 kDa	TAL_PEPD6	2	3	2	2	0	2
95	Probable transcriptional regulatory protein	CD630_07950	26 kDa	Y795_PEPD6	5	2	2	2	0	4
96	putative alanine racemase	alr	43 kDa	CBA64716.1	2	4	5	3	3	5
97	Putative cell wall-binding protein cwp25	cwp25	34 kDa	Q18A40	4	4	3	2	2	2
98	Putative cold shock protein	CspA	7 kDa	Q18A83	2	2	2	3	3	3
99	putative cyclase	CD196_1850	24 kDa	A0A0H3N331	0	2	3	2	0	0
100	putative flagellar filament core protein		34 kDa	EHJ39341	2	2	0	0	5	2
101	Putative flavodoxin	FloX	19 kDa	C9YJJ1	2	3	2	4	0	0
102	putative peptidase	pepT	42 KDa	C9YRZ0	2	5	0	0	0	0
103	Putative phage protein	CD630_09760	17 kDa	Q183Y1	0	0	4	6	0	0
104	Putative pre-16S rRNA nuclease	CD630_12850	15 KDa	Q18BF1	2	2	2	3	3	3
105	Putative purine permease	CD630_31800	47 KDa	Q181U5	3	4	0	0	0	0
106	Putative septation protein SpoVG	spoVG	10 kDa	SP5G_PEPD6	0	0	0	0	2	3
107	putative transcriptional regulator	puuR_2	21 kDa	A0A031WFQ5	2	5	0	0	0	0
108	putative translation elongation factor	tuf	73 kDa	EFH08170.1	0	0	5	4	1	2
Other proteins										
109	1-deoxy-D-xylulose 5-phosphate reductoisomerase	dxr	43 kDa	Q185R8	5	4	3	4	5	2
110	1-deoxy-D-xylulose-5-phosphate synthase	dxs	69 kDa	Q18B68	6	5	2	3	6	6
111	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	dapD	30 kDa	DAPD_PEPD6	0	2	4	3	6	2
112	3-hydroxybutyryl-CoA dehydrogenase	hbd	31 kDa	HBD_CLODI	9	12	6	8	10	8
113	3-methyl-2-oxobutanoate hydroxymethyltransferase	panB	28 kDa	PANB_PEPD6	5	7	4	3	0	0
114	3-methyl-2-oxobutanoate dehydrogenase subunit VorB	vorB	39 kDa	WP_003432442.1	2	3	1	2	3	3
115	3-oxoacyl-[acyl-carrier-protein] synthase 3	fabH	35 kDa	Q18B40	12	6	3	5	1	7
116	3-phosphoshikimate 1-carboxyvinyltransferase	aroA	49 kDa	AROA_PEPD6	4	2	1	2	3	3
117	4-Hydroxybutyrate CoA-transferase	abfT	48 kDa	Q185L2	2	3	1	2	3	3
118	4-hydroxybutyryl-CoA dehydratase	abfD	54 kDa	Q185L4	3	2	0	0	3	4
119	4-hydroxy-tetrahydrodipicolinate synthase	dapA	33 kDa	Q181M9	5	4	0	0	0	0

120	5-methyltetrahydrofolate:corrinoid/iron-sulfur protein co-methyltransferase,	acsE	29 kDa	A0A0N0NZV1	5	4	3	4	5	2
121	ABC-type transport system, oligopeptide-family permease protein	appB	35 kDa	Q183A7	3	6	4	2	7	4
122	Acetate kinase	ackA	43 kDa	ACKA_PEPD6	2	4	1	2	1	2
123	Acetyl-CoA acetyltransferase	thlA	41 kDa	THLA_PEPD6	17	19	17	18	18	19
124	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	accA	35 kDa	Q187P7	2	4	4	3	2	4
125	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	accD	32 kDa	Q187P6	6	3	7	13	12	14
126	Acetylglutamate kinase	argB	26 kDa	ARGB_STRGC	4	3	6	0	5	7
127	Adenylosuccinate synthetase	purA	48 kDa	PURA_PEPD6	7	2	1	5	8	5
128	Alanine-glyoxylate amino-transferase family protein	QQG_0004	45 kDa	T4IP56	2	3	0	0	4	2
129	Alanine racemase	alr	43 kDa	Q180W0	0	0	4	2	2	2
130	Alanine--tRNA ligase	alaS	98 kDa	Q18BE7	1	2	3	4	2	1
131	aldehyde alcohol dehydrogenase family protein	adhE	96 kDa	Q18D77	13	8	9	6	9	6
132	Amino acid-binding ACT domain-containing protein	CDIF1296T	14 kDa	A0A125V679	5	3	2	1	2	4
133	Aminomethyltransferase (Glycine cleavage system T protein)	gcvT	92 kDa	Q186L1	7	1	2	4	2	3
134	aminotransferase	aspB	45 kDa	Q18BK6	2	2	2	3	2	4
135	Arginine--tRNA ligase	argS	65 kDa	SYR_PEPD6	5	4	0	0	0	0
136	Argininosuccinate lyase	argH	50 kDa	ARLY_PEPD6	2	2	2	3	2	4
137	aspartate aminotransferase	OJT83395.1	45 kDa	OJT83395.1	5	1	4	6	3	4
138	Aspartate carbamoyltransferase	pyrB	35 kDa	Q18CS8	0	0	1	2	1	3
139	ATP synthase gamma chain	atpG	32 kDa	ATPG_PEPD6	1	5	6	2	0	0
140	ATP synthase subunit alpha	atpA	55 kDa	ATPA_PEPD6	2	5	2	2	2	2
141	ATP synthase subunit beta	atpD	50 kDa	ATPB_PEPD6	7	2	4	3	1	3
142	ATP synthase subunit delta	atpH	21 kDa	Q180W9	2	2	2	2	2	2
143	ATP-dependent DNA helicase RecG	recG	78 kDa	Q182P8	1	3	4	1	0	0
144	ATP-dependent zinc metalloprotease FtsH	Ftsh	74 kDa	Q181G0_PEPD6	1	3	4	2	3	2
145	Bifunctional protein FolD	folD	31 kDa	FOLD_PEPD6	2	4	0	0	6	3

146	Bifunctional protein GlmU	glmU	50 kDa	Q181B4	0	0	2	2	0	0
147	Biotin synthase	biob	37 kDa	Q18D35	2	5	0	0	0	0
148	butyrate kinase	buk	38 kDa	WP_102822774.1	5	1	3	3	1	4
149	Chorismate synthase	aroC	39 kDa	Q187E5	1	4	2	2	0	0
150	Cyclase family protein	COG1878	24 KDa	WP_003428771	0	0	2	4	3	2
151	cysteine desulfurase	nifS	44 kDa	WP_011861166.1	0	0	10	2	12	2
152	cysteine synthase A	WP_003436514.1	33 kDa	WP_003436514.1	11	7	14	10	7	8
153	Cysteine--tRNA ligase	cysS	54 kDa	Q18CD5	7	3	2	1	2	3
154	D-alanine--D-alanine ligase	VanG	39 kDa	Q186I2	2	2	2	2	0	0
155	Dihydroorotase	pyrC	38 kDa	PYRC_PEPD6	2	2	2	3	2	4
156	D-lactate dehydrogenase	ldhA	37 kDa	Q188H7	4	5	6	7	0	0
157	DNA gyrase subunit A	gyrA	91 kDa	Q18C90	1	2	6	5	4	4
158	DNA gyrase subunit b	gyrB	71kDa	Q18C90	6	3	7	13	12	14
159	DNA ligase	ligA	76 kDa	Q180F3	2	2	5	2	0	0
160	DNA mismatch repair protein	mutS	109 kDa	Q187T6	2	4	2	2	0	0
161	DNA polymerase I	polA	101 kDa	Q18AY7	3	2	0	0	0	0
162	DNA polymerase III PolC-type	polC	162 kDa	Q18BG8	4	1	3	6	2	3
163	DNA polymerase III subunit alpha	dnaE	136 kDa	Q180P3	0	0	0	0	1	3
164	DNA polymerase IV	dinB	42 kDa	Q18A91	0	0	6	3	0	0
165	DNA replication and repair protein RecF	recF	42 kDa	Q18C86	4	2	2	1	5	2
166	DNA-binding protein HU	hbs	10 kDa	A0A031WHB1	4	7	2	6	0	0
167	DNA-directed RNA polymerase subunit alpha	rpoA	35 kDa	RPOA_PEPD6	2	2	2	3	2	1
168	DNA-directed RNA polymerase subunit beta	rpoB	139 kDa	Q18CF1	2	5	4	2	2	1
169	Enamine/imine deaminase	yabj	14 kDa	A0A031WH02	4	2	2	3	1	3
170	Energy-coupling factor transporter ATP-binding protein EcfA2	ecfA2	32 kDa	Q18CI9	1	3	0	0	0	0
171	Enolase	eno	46 kDa	ENO_PEPD6	6	6	4	2	7	4
172	enoyl-CoA hydratase/isomerase family protein		22 kDa	EQG13789.1	3	2	3	1	3	4

173	flavodoxin family protein		18 kDa	WP_009902172.1	2	4	0	0	0	0
174	Fe-S cluster assembly scaffold protein	NifU	16 kDa	WP_003438275.1	0	0	0	0	4	3
175	Formate--tetrahydrofolate ligase	fhs	60 kDa	FTHS_PEPD6	10	12	12	15	19	9
176	Fructose-1,6-bisphosphate aldolase	fba	33 kDa	Q188J1	6	6	12	3	2	1
177	Glucose-6-phosphate isomerase	pgi	51 kDa	G6PI_PEPD6	2	5	4	2	2	1
178	Glutamate--tRNA ligase	gltX	57 kDa	Q18CD6	2	3	4	3	1	2
179	Glyceraldehyde-3-phosphate dehydrogenase	gap	36 kDa	Q181T9	4	2	1	2	3	4
180	Glycine cleavage system H protein	gcvH	14 kDa	GCSH_PEPD6	2	1	3	6	2	4
181	Glycine--tRNA ligase beta subunit	glyS	78 kDa	SYGB_PEPD6	2	3	1	2	4	3
182	Holo-[acyl-carrier-protein] synthase	acpS	14 kDa	Q180W4	2	2	2	3	1	2
183	Imidazole glycerol phosphate synthase subunit HisH	hisH	23 kDa	Q18C72	7	2	1	5	8	5
184	indolepyruvate oxidoreductase subunit beta	iorB	21 kDa	Q181W6	3	2	5	1	4	2
185	Inosine-5'-monophosphate dehydrogenase	guaB	55 kDa	A0A0H3N3T3	0	0	3	2	5	3
186	Isoleucine--tRNA ligase	ileS	120 kDa	Q182V2	5	1	4	6	3	4
187	lactate utilization protein	QCW_2161	24kDa	QCW_2161	2	3	4	3	0	0
188	Leucine--tRNA ligase	leuS	98 kDa	Q182K8	2	2	2	3	2	4
189	LexA repressor	lexA	23 kDa	Q187P1	1	2	3	4	2	1
190	Lrp/AsnC family transcriptional regulator		16 kDa	WP_003421494.1	0	0	0	0	2	2
191	lysyl-tRNA synthetase	LysS	58 kDa	Q181F2	3	1	0	0	0	0
192	Methionine--tRNA ligase	metG	74 kDa	Q181D9	2	3	1	2	4	5
193	Methionyl-tRNA formyltransferase	fmt	34 kDa	Q182S2	4	5	0	0	0	0
194	N-acetylmuramic acid 6-phosphate etherase	murQ	33 kDa	Q184N3	0	0	12	13	0	0
195	NAD-specific glutamate dehydrogenase	gluD	46 kDa	DHE2_CLODI	12	18	16	13	17	17
196	NUDIX hydrolase	CD630_17650	23 kDa	Q186X5	3	2	5	1	3	2
197	Oligoendopeptidase F	pepF	69 kDa	A0A386FR43	4	5	0	0	0	0
198	Orotate phosphoribosyltransferase	pyrE	21 kDa	Q18CS5	4	2	3	4	2	1
199	Peptide chain release factor 1	prfA	42 kDa	Q180Y2	2	1	2	4	1	3

200	Peptide chain release factor 3	PrfC	60 kDa	A0A0A6PWC7	4	8	0	0	0	0
201	Peptidyl-tRNA hydrolase	pth	21 kDa	Q181A2	7	2	1	5	8	3
202	Peptidylprolyl isomerase	ppiB	19 kDa	Q18D70	0	0	0	0	2	3
203	phosphate propanoyltransferase	pduL	20 kDa	Q183B8	5	6	4	2	2	2
204	Phosphoenolpyruvate-protein phosphotranferase.	ptsI	63 kDa	Q183J1	4	3	2	0	3	4
205	Phosphoglycerate kinase	pgk	43 kDa	PGK_PEPD6	1	2	6	5	4	4
206	Phosphoribosylformylglycinamidine cyclo-ligase	PurG	39 kDa	Q18CW2	5	4	0	0	2	7
207	Polyribonucleotide nucleotidyltransferase	pnp	78 kDa	Q18BI4	3	2	3	4	4	5
208	Proline racemase	CD630_32370	36 kDa	PRAC_CLODI	0	0	3	3	5	4
209	Proline--tRNA ligase	proS1	49 kDa	Q18CD2	2	1	2	4	1	2
210	Protein RecA	recA	38 kDa	Q18BJ4	1	4	3	2	1	5
211	pyridoxamine 5'-phosphate oxidase family protein	CD630_02790	15 kDa	Q18D22	4	5	0	0	3	7
212	Pyruvate kinase (PK) (fragment)	pyk	63 kDa	Q180P2	1	2	6	5	4	4
213	Pyruvate-flavodoxin oxidoreductase	nifj	129 kDa	Q183B6	7	8	2	8	3	4
214	Redox-sensing transcriptional repressor Rex	rex	23 kDa	REX_PEPD6	2	1	2	4	1	3
215	S-adenosylmethionine synthase	metK	43 kDa	Q18CL7	5	1	2	4	2	2
216	Serine hydroxymethyltransferase	glyA	46 kDa	GLYA_PEPD6	1	2	6	5	4	4
217	Serine--tRNA ligase	serS	49 kDa	Q18C97	4	5	3	3	3	7
218	Short-chain-enoyl-CoA hydratase (Fragment)	crt	17 kDa	CRT_CLODI	4	2	7	1	3	4
219	Sugar ABC transporter substrate-binding protein		23 kDa	WP_009888534.1	3	2	0	0	2	2
220	Tellurium resistance protein terD	terD	21 kDa	Q186I7	5	6	4	2	2	2
221	Tellurium resistance protein terD1	terD1	22 kDa	Q186J1	5	1	2	4	2	2
222	Tellurium resistance protein terD2	terD2	23 kDa	Q186J0	2	3	2	5	1	3
223	thioredoxin family protein		6 kDa	EQE71157.1	4	1	0	0	3	2
224	Threonine--tRNA ligase	thrS	69 kDa	Q189B8	1	2	6	5	4	4
225	Transaldolase	tal	37 kDa	TAL_PEPD6	5	1	7	2	3	6
226	Transcriptional regulator, LysR family	CD630_26750	35 kDa	Q183A9	2	3	1	2	4	2

227	Transcriptional regulator, LytR family	CD630_06180	17 kDa	Q189F8	4	1	2	1	3	4
228	Transcriptional Regulator, marR family protein	effr	22 kDa	Q187W6	4	6	6	2	8	12
229	Transketolase	tkt	29 kDa	A0A0H3N3X0	2	2	0	0	4	3
230	Translation initiation factor IF-2	infB	70 kDa	Q18BH4	4	4	3	2	2	2
231	Triosephosphate isomerase	tpI	27kDa	Q181T6	5	2	0	0	4	5
232	tRNA (guanine-N(1)-)-methyltransferase	trmD	27 kDa	Q18BC2	4	3	3	1	2	2
233	tRNA modification GTPase	mnmE	51 kDa	Q181S7	2	4	4	3	2	4
234	tRNA-2-methylthio-N(6)-dimethylallyladenosine synthase	miaB	55 kDa	Q187U6	1	4	5	3	2	4
235	tRNA-specific 2-thiouridylase MnmA	mnmA	41 kDa	Q18BE2	7	4	2	3	3	4
236	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	murA	45 kDa	Q18CL1	9	4	4	3	2	4
237	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	murG	40 kDa	Q182Y6	0	0	2	4	4	3
238	UDP-N-acetylmuramate--L-alanine ligase	murC	56 kDa	Q181B7	5	3	2	1	2	4
239	UDP-N-acetylmuramoylalanine--D-glutamate ligase	murD	50 kDa	Q182Y7	0	0	5	1	7	11
240	UvrABC system protein A	uvrA	105 kDa	Q180Q9	3	2	3	1	3	4
241	UvrABC system protein B	uvrB	76 kDa	Q180R0	4	4	3	2	2	2
242	UvrABC system protein C	uvrC	70 kDa	Q180Q8	4	2	1	2	3	4
243	Valine--tRNA ligase	valS	103 kDa	Q180A1	3	4	2	2	0	0
244	V-type ATP synthase alpha chain	atpA	66 kDa	VATA_PEPD6	2	1	4	4	4	3
245	V-type ATP synthase beta chain	atpB	51 kDa	VATB_PEPD6	1	2	0	4	2	2
246	XRE family transcriptional regulator		22 kDa	WP_021370312.1	2	4	0	0	0	0
247	hypothetical protein BER30_000919	BER30_000919	12 kDa	OMK44041.1	4	4	4	1	0	0
Uncharacterised and Hypothetical proteins										
248	hypothetical protein BM529_15905	BM529_15905	11 kDa	OJT73147.1	0	0	0	0	2	3
249	hypothetical protein QOI_2046	QOI_2046	10kDa	EQI11398.1	2	3	0	0	5	3
250	Uncharacterized protein	CD630_02380	13 kDa	Q18CX8	2	1	2	3	0	0

6.4 DISCUSSION

Due to the increasing incidence of *Clostridium difficile* infection (CDI), several characterisation methodologies have been adopted to understand the detailed adaptive mechanisms of *C. difficile.* One such method is LC-MS/MS analysis of the *C. difficile* proteome, which has been reported in several studies (Boetzkes *et al*., 2012; Chen *et al*., 2013; Chilton,*et al*., 2014; Chong *et al*., 2014; Jain *et al*., 2011, Jain *et al*., 2010; Lawley *et al*., 2009; Maaß *et al*., 2018; Moura *et al*., 2013). These studies highlighted several proteins associated with the adaptation of *C. difficile* within the gut environment. However, none of these studies investigated the proteome of *C. difficile* ribotype 002. Here, the whole-cell proteome of three CD002 strains from different geographical locations and time-lineages were investigated using the LC-MS/MS mass spectrometry approach, to see if any differences existed between strains.

C. difficile infection (CDI) is largely dependent on a range of virulence factors, including toxins, adherence factors, and motility factors. In the present study, the main virulence factors, toxin A and B were identified in the proteome of all investigated CD002 strains. Approximately, 17 - 23% of *C. difficile* strains are capable of producing a third toxin, known as the binary toxin (Eckert *et al*., 2015). The exact role of binary toxin in *C. difficile* pathogenesis is still largely unknown, however, a number of studies have indicated that strains with this toxin cause severe CDI (Gerding *et al*., 2014). In the present study, no binary toxins were detected in any of the investigated strains, in line with the absence of binary toxins in CD002 reported in other studies (Eckert *et al*., 2015; Stubbs *et al*., 2000). Additionally, a global regulatory protein, CodY, which is found in many Gram-positive bacteria that monitors the nutrient sufficiency of the environment and regulates genes expression depending on nutrient availability was detected in all CD002 (Nawrocki *et al*., 2016). CodY binds to the DNA in the presence of GTP

and branched-chain amino acids to represses the transcription of genes including those involved in *C. difficile* toxin synthesis, and more recently it has been demonstrated to negatively regulate sporulation in two *C. difficile* ribotypes (012 and 027) (Nawrocki *et al*., 2016). All three strains were grown under the same conditions, the presence of CodY in the proteome can be attributed to stress and nutrient depletion in the media, as strains were continuously incubated for 64h on Columbia blood agar prior to proteome extraction.

C. difficile spores are produced under stress conditions and are critical for transmission and establishment of an infection. The ability of spores to persist in the gut is attributed to their structure, which is made up of the exosporium, coat, cortex, membrane and core (Barra-Carrasco *et al*., 2013). Recently, Theriot and colleagues demonstrated how spores within the small intestine of mice were able to germinate in the absence of antibiotic disruption(Theriot *et al*., 2016). Suggesting that other factors exist on the spore surface that may facilitate host colonisation. Proteomic and bioinformatics studies of the *C. difficile* spore have revealed that many proteins are included in the spore, however, the assignment of function to these proteins is still ongoing (Lawley *et al*., 2009; Phetcharaburanin *et al*., 2014; Pizarro-Guajardo *et al*., 2018). The most extensively studied are *C .difficile* spore coat proteins, cotA, cotB, cotCB, cotD, cotE and sodA (Hong *et al*., 2017; Permpoonpattana *et al*., 2013, 2011; Pizarro-Guajardo *et al*., 2018). Indeed, CotE, a member of peroxiredoxin family, was recently demonstrated to interact with intestinal mucin promoting its degradation (prior to germination), contributing to colonisation and disease progression(Hong *et al*., 2017). Additionally, CotE expression increased the severity of CDI in a hamster model as animals dosed with spores lacking CotE exhibited significantly delayed symptoms of CDI, thus emphasising the contributory role of this protein to CDI (Hong *et al*., 2017).

In the present study, several spore-associated proteins were detected by LC-MS/MS analysis, including proteins associated with spore morphogenesis (sspA, sspB, spoIIIJ, spoIIGA, spoIVA, spoIIAB, spoVE & spoVG). Interestingly, CotE was identified in recent strains 71 (UK 2011-13) and 137 (Non-UK 2012-147) and absent in strain 174, belonging to the older lineage of strains (UK 2007-8). Whether the failure to detect this spore protein (CotE) in strain 174 means that it was not expressed or was degraded during the sample preparation process is unknown. Consequently, further investigation into the reproducibility of CotE expression in only recent CD002 strains is warranted, before speculating that the expression of certain spore proteins such as CotE in recent CD002 isolates may have facilitated the prevalence of this ribotype in recent years. Strains expressing CotE may succeed better in gut adherence, making a mechanical clearance from the gut lumen more difficult, thus spores persistence and re-infection occur. Contrastingly, it is noteworthy that only three strains were investigated in the present study and therefore may not be wholly reflective of the spore proteins of other isolates within the recent lineages.

The adhesion of intestinal pathogens to the host gastrointestinal tract is a crucial aspect of host colonisation, as it prevents them from being mechanically cleared (Hong *et al*., 2017). Several cell surface proteins coordinate adhesion of the bacterial cell into the gut, however, the mechanism is yet to be elucidated. Several cell surface proteins have been identified as been associated with *C. difficile* adherence. Some of these adhesins include cell wall surface proteins, surface layer proteins, and flagella. In the present study, several cell surface proteins (cwp20, cwp22, cwp66, slpA (77kDa) & cell wall binding repeat 2 family protein), previously demonstrated to contribute to adherence and colonisation, were identified in all strains. However, cell wall binding protein 2 (cwp2) was only expressed by recent CD002 strains

(strain 71 and 137). A mutant of this protein was recently demonstrated to impair adhesion of *C. difficile* to mammalian cells *in vitro* (Bradshaw *et al*., 2017) and it has previously been reported as being present in the spore coat (Lawley *et al*., 2009)and during high toxin production (Mukherjee *et al*., 2002). Despite the absence of this protein in Strain 174 (UK 2007-8), in contrast to recent lineages (71 & 137), we cannot negate the fact that strains lacking the expression of this protein may be prolific in facilitating the production of other virulence-associated factors such as adhesion, toxin production and sporulation. Bradshaw and colleagues demonstrated how a mutant of this protein produced more toxin A(Bradshaw *et al*., 2017), as a result, the expression of this protein with recent lineages may not be a factor contributing to the recent prevalence of CD002.

C. difficile strains are known to possess flagella for motility, however, their role in *C. difficile* adherence and colonisation, appears to be contentious and strain-dependent (Baban *et al*., 2013; Dingle *et al*., 2011; Stevenson *et al*., 2015; Tasteyre *et al*., 2002). The two best characterised *C. difficile* flagellar proteins are FliC, the major flagellin structural monomer and FliD, the cap protein. Mutations in FliC and FliD have been shown to increase adherence to intestinal epithelial cells in some strains(Baban *et al*., 2013) and toxin gene expression in *C. difficile* 630 strain (Stevenson *et al*., 2015). However, Dingle *et al* have shown that mutation in FliC and FLiD leads to lack of expression but does not affect adherence to intestinal cells(Dingle *et al*., 2011).In the present study, seven flagellar associated proteins were identified, with FliC and FliD being expressed by all investigated CD002 strains. Certain flagella proteins (fliW, flgC & flhA) involved in the assembly of *C. difficile* flagellum were expressed only by recent strains (71 and 137), and one putative flagellar core protein was expressed by only UK strains (71 & 174). Whether the expression of these flagellar proteins in recent CD002

strains has contributed to their prevalence is unknown. As a result, further characterisation of these proteins may be warranted to ascertain the contributory role of this protein in recent CD002 prevalence.

Comparative proteomic analysis using LC-MS/MS analysis of the whole-cell proteome of three CD002 strains revealed similarities and differences that suggest minimal adaptation changes may have occurred over time. Additionally, the possibility that some proteins may have been degraded during the sample preparation process, suggests that some important proteins and differences between strains may have been missed during this analysis. As a result, further characterisation of the differences highlighted here is warranted as well as comparative genomic analysis studies, incorporating strains investigated in this study and even more recently isolated strains.

7 GENERAL DISCUSSIONS AND CONCLUSIONS

Clostridium difficile (*C. difficile*) is the leading cause of healthcare-associated infections (HAIs) and an important public health threat (Planche & Karunaharan, 2017). *C. difficile* has been associated with substantial morbidity and mortality among individuals of all ages worldwide, even in the absence of the traditionally recognised risk factors (e.g., prior antimicrobial therapy, elderly and hospitalised patients) (Lessa *et al*., 2015). Efforts to reduce *Clostridium difficile* infections (CDI) (through antimicrobial stewardship programs, infection control measures and alternative preventative measures) have been successful, as demonstrated by UK CDI rates per 100,0000 population, which have fallen from 100.3 in 2007/08 to 24 in 2017/18 (Public Health England, 2018). Despite this success, the epidemiology of CDI is constantly evolving and is characterised by the emergence of strains that cause severe disease. In addition, there has been an increase in the number of community-associated CDI cases, and a suggestion that animal and environmental reservoirs exist (Fawley *et al*., 2016; Rabold *et al*., 2018). The UK prevalence of *C. difficile* PCR ribotype 027 (NAP1) recently declined while other ribotypes emerged, including ribotype 002 (CD002). CD002 is also responsible for CDI in many countries across Europe, North America, Asia and Australia. The aim of this research was to identify phenotypic characteristics that may have contributed to the increased CD002 prevalence in the UK, by investigating the differences that exist between CD002 from different lineages (UK 2007-8, UK 2011-12 and Non-UK 2012-14) of CD002.

Antimicrobial resistance in *C. difficile* has been shown to drive epidemiological change, and the emergence and expansion of new strains types(He *et al*., 2013). As part of this study, we sought to determine the antimicrobial susceptibility and resistance patterns of clinical isolates of CD002 using the agar incorporation method. The majority of CD002 isolates from different

time lineages were susceptible to many of the antimicrobial agents studied, including current CDI therapies; metronidazole, vancomycin and fidaxomicin. Consistent with previous studies, resistance to fluoroquinolones (particularly ciprofloxacin, MIC ≥ 8mg/L) was observed in all lineages of CD002. Investigations into the presence of resistance determinants against fluoroquinolones and rifamycins revealed previously reported amino acid substitutions in *gryA* (Thr-82-lle) and *rpoB* (Arg505Lys) genes. (Curry *et al*., 2009; O'Connor *et al*., 2008; Spigaglia *et al*., 2011; Spigaglia, 2016). Additionally, novel amino acid substitutions in these genes; *gryA* (Arg-98-Thr, and Asp-103-His) and *rpoB* (Leu509Phe) were identified. Furthermore, a high proportion of isolates in all CD002 lineages were reduced susceptible (≥ 1mg/L) to beta-lactams (penicillin and ampicillin), but negative for β-lactamase activity assay. The overall data generated indicated common differences in the antimicrobial susceptibility and resistance patterns between different lineages of CD002. Therefore, it is unlikely that changes in antimicrobial resistance patterns might be a driver for the emergence of CD002 in the UK and in Europe.

During the course of CDI, *C. difficile* sporulates and releases spores into the colonic environment. Recurrences due to relapses or re-infection has been reported in CDI (Barbut *et al*., 2000; Marsh *et al*., 2012; Oka *et al*., 2012), and it is suggested that *C. difficile* spores have mechanisms to efficiently persist in the host colonic environment, and therefore establish an infection following cessation of antimicrobial therapy. CD002 has been associated with increased sporulation frequency (Cheng *et al*., 2011). Thus, the sporulation capacities of CD002 from different lineages was investigated in the present study. Interestingly, greater sporulation capacities after 24h were demonstrated by recent CD002 (UK 2011-13 & Non-UK 2012-14), in comparison to UK 2007-8. Additionally, the ability of purified CD002 spores to

adhere to human adenocarcinoma cells (Caco-2) at different stages of cell development was investigated.CD002 spores adhered more strongly to differentiated Caco-2 cells than undifferentiated Caco-2 cells, but the difference between lineages was not significant. Based on these findings, we hypothesise that recent CD002 strains are able to sporulate rapidly and persist within the colonic environment through spore surface properties that aid their adherence to intestinal epithelial cells. As a result, mechanical clearance is less efficient, consequently leading to a relapse of infection when the gut conditions become favourable. The possibility that these findings can be replicated *in vivo*, remains to be determined.

Biofilms are thought to contribute to intestinal colonisation and recurrent CDI(Baines *et al*., 2005; Crowther *et al*., 2014a; Plaza-Garrido *et al*., 2015; Soavelomandroso *et al*., 2017). Previous studies report the abundance of highly resistant spores harboured within *C. difficile* biofilms, which are dispersed in the later stages of biofilm development(Crowther *et al*., 2014a; Dawson *et al*., 2012; Plaza-Garrido *et al*., 2015; Semenyuk *et al*., 2014). In the present study, biofilm formation was investigated *in vitro* using a simple microtitre plate assay, and recent isolates of CD002 (UK 2011-13 & Non-UK 2012-14) produced more biofilms compared to older isolates (UK 2007-8). The biofilm biomass correlated with the spore abundance in mature biofilms that was observed in the different lineages. These findings suggest that mucosal-associated biofilms may harbour CD002 spores, and thus allow spores to persist in the colon. This further supports the aforementioned hypothesis that spores of recent isolates may persist in the colonic environment and therefore contribute to recurrent CDI.

The main virulence factors produced by *C. difficile* are two large clostridial toxins. Although controversial, Warny *et al* (2005) indicated that hypervirulent PCR ribotype 027 (toxinotype

III) produced quantitatively more of toxins A and B than a comparator toxinotype 0 group and therefore concluded that was why 027 caused more severe disease. Additionally, Baines *et al* (2008) reported an earlier (after 1 day) release of toxins by hypervirulent PCR ribotype 027 strains, in comparison to other *C. difficile* strains. In the present study, no significant difference existed in the cytotoxin production between different lineages of CD002. Interestingly, detectable toxins were produced earlier in the growth cycle (at 12h) by the majority of CD002 strains, this was also observed with a ribotype 078 strain. Conversely, detectable toxins (1RU) were detected after 24h of in a ribotype 027 strain. This suggests that strains of this ribotype could cause infections more readily, however, whether these characteristics are replicated *in vivo,* remains to be determined

Another interesting observation was the higher maximum specific growth rate (μ_{max} range 0.76 - 0.92 h^{-1}) demonstrated by more recent CD002 isolates (UK 2011-13 &Non-UK 2011-12) compared to the older group of isolates (UK 2007-8) (0.69 h^{-1}), and hypervirulent ribotypes, 027 and 078 (0.67 h^{-1}, and 0.36 h^{-1})reported by Keighley *et al* (2015). This suggests recent CD002 may form vegetative cells faster, produce toxin earlier, sporulates rapidly, and therefore be better at causing disease and being transmitted or surviving therapy. Subsequently, the nutrient utilisation profile and competition for glucose in a single-stage fermenter by selected strains were investigated. Indeed, the findings in the present study indicated that strains of the recent lineages utilise more nutrient sources in comparison to strains of the UK 2007-8 lineage. This suggests that recent strains have more options of adaptability in response to nutrient availability within the gut, and this could select for their emergence. However, since recent studies revealed that *C. difficile* is an ecological generalist rather than a specialist (Jenior *et al*., 2017), strains with an expanded metabolic potential will

only be prolific in a favourable gut environment. In addition, CD002 strains utilised five carbon sources (D-ribose, L-xylose, L- arabinose, dihydroxy acetone, D-Arabinose and 2- deoxy- D-ribose) which were not utilised by hypervirulent *C. difficile* strains (RT 078 and RT 027), whether the ability to utilise these substrates facilitates the emergence of CD002 remains to be determined. In the competition study, strains of different lineages, in two out of the three competing pairs, appeared to co-exist together rather than outcompete each other. Interestingly, a recent UK 2011-13 strain outcompeted a recent Non-UK 2012-14. Further investigations are required to clarify the significance of this observation for CD002 *in vivo*

Analysis of the whole-cell proteome of one selected strain per lineage by LC-MS, revealed CotE, a spore coat protein only in recent CD002 isolates (71 (UK 2011-13) & 157(Non-UK 2011-12). CotE was recently reported to aid intestinal colonisation and severity of disease in an animal model (Hong *et al*., 2017). This finding may explain why strains of this ribotype have been implicated in severe forms of CDI recently (Dauby *et al*., 2017; Wong *et al*., 2016). Further analysis is required to ascertain if the observations made in the present study are as a consequence of CotE being absent or defective in the investigated UK 2007-8 CD002 strain (174) or an artefact of sample preparation. Furthermore, comparative analysis of the whole proteome of three strains from the different lineages shows many similarities between strains that suggest a minimal adaptation of the proteome in CD002 has occurred over time. However, since only three strains were investigated, this may not be fully reflective of other CD002 strains. As a result, comparative proteomic analysis of the more CD002 strains is required before firm conclusions can be made.

In conclusion, this study has enhanced our understanding of the potential factors that may have contributed to the recent emergence of strains of ribotype 002 in the UK. Although the

antimicrobial susceptibility patterns between lineages did not differ substantially, resistance to fluoroquinolones and reduced susceptibility to beta-lactams (such as penicillin and ampicillin) demonstrated by CD002, may have occurred as a result of selective pressure due to the continued use of these antimicrobial agents in the clinical setting. As a result, we cannot rule out the likely possibility that these antimicrobial agents could be facilitating the prevalence of this ribotype in recent years. Additionally, the more recent isolates (Both UK and Non-UK) formed more biofilms, sporulated more rapidly, had a higher μ_{max}, and a more expanded nutrient utilisation profile than the older UK isolates. These characteristics were found to be more prominent among strains originating from outside of the UK. Furthermore, CD002 strains were found to produce toxins earlier in their growth cycle, and selected strains were able to utilise certain substrates that had not been utilised by strains of the hypervirulent ribotypes (027 & 078). The increased sporulation, higher μ_{max}, greater biofilm formation, abundance of spores in mature biofilms, and the utilisation of several nutrient substrates, demonstrated by recent *C. difficile* PCR ribotype 002, suggests that the may have a competitive advantage over other ribotypes, therefore increasing their prevalence in recent years. However, whether these factors, have a greater *in vivo* implication for this ribotype, remains to be determined.

7.1 FURTHER WORK

While significant progress has been made in characterising isolates of CD002 and identifying novel factors that may have contributed to the recent emergence of this ribotype, there are still major gaps that remain to be explored. In order to build on the findings from the present

study, the following studies should be considered in more gut –reflective *in vitro* models or animal models of CDI;

1. Examine the phenotypic characteristics of more recent isolates of CD002 in comparison to other emerging ribotypes (such as 005, 014/020 and 015), in order to establish if the factors identified in the present study gives CD002 a greater phenotypic advantage over other ribotypes.
2. Investigate the CD002 spore structure in comparison to other emerging ribotypes to determine the role of exosporium, BcLA proteins and spore coat proteins, in adherence to intestinal epithelial cells.
3. Investigate the role of trehalose (which was recently postulated to have an impact on the emergence of ribotypes 027, 078 and 017) and carbon sources that were only utilised by CD002 in the present study, on the emergence of CD002 and other emerging ribotypes (005, 014/020 and 015)
4. Whole-genome sequencing of historic and recent isolates of CD002 to investigate mutational events within the genome that may have occurred over time to facilitate the prevalence of these strains.

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APPENDICES

a. Appendix 1

1. Liquid media and buffers

All buffers and liquid media used were prepared according to the manufacturer's instructions.

I. **Schaedlers Anaerobic Broth**-CM0497, Oxoid

Formulation	g/L
Tryptone soya broth	10.0
Special peptone	5.0
Yeast extract powder	5.0
Glucose	5.0
Cysteine HCL	0.4
Haemin	0.01
Tris Buffer	0.75

II. **Brain Heart Infusion Broth -**CM1135, Oxoid

Formulation	g/L
Brain Infusion solids	12.5
Beef heart infusion solid	5.0
Preoteose peptone	10.0
Glucose	2.0
Sodium Chloride	5.0
Di-Sodium phosphate	2.5

III. **Phosphate Buffered Saline**- P4417, Sigma

Depending on the required volume for use, one tablet was dissolved in 200 mL of water and sterilised prior to use.

2. Solid Media

I. **Brazier's CCEY agar-** LAB160, LABM

Formulation	**g/L**
Peptone Mix	23.0
Sodium Chloride	5.0
Soluble Starch	1.0
Agar	12.0
Sodium bicarbonate	0.4
Glucose	1.0
Sodium pyruvate	1.0
Cysteine HCl	0.5
Haemin	0.01
Vitamin K	0.001
L-arginine	1.0
Soluble pyrophosphate	0.25
Sodium succinate	0.5
Cholic acid	1.0
p-Hydroxyphenylacetic acid	1.0

II. **Schaedlers anaerobe agar-** CM0437, Oxoid

Formulation	**g/L**
Tryptone soya broth	10.0
Special peptone	5.0
Yeast extract	5.0
Glucose	5.0
Cysteine HCL	0.4
Haemin HCL	0.01
Tris Buffer	0.75
Agar	13.5

III. **Columbia blood agar** - CM0331, Oxoid

Formulation	**g/L**
Special peptone	23.0
Starch	1.0
Sodium Chloride	5.0
Agar	0.01

3. Media solution Used in the Cell culture

I. For the culture of Vero cells, Dulbecco's Modified Eagle's medium (DMEM) (D6546, Sigma) was used. This medium was supplied in sterile 500mL bottles and stored at 4°C.

Formulation	**g/L**	**Formulation**	**g/L**
Calcium Chloride	0.265	L-lysine HCl	0.146
Iron (III) Nitrate	0.0001	L-methionine	0.03
Potassium Chloride	0.4	L-serine	0.042
Magnesium sulphate	0.09767	L-threonine	0.095
Sodium Chloride	6.4	L-tryptophan	0.016
Sodium hydrogen carbonate	3.7	L-tyrosine	0.10379
Sodium dihydrogen phosphate	0.109	L-Valine	0.094
Phenol Red Na	0.015	D-pantothenate ½ ca	4
Pyruvate Na	0.11	Choline chloride	0.004
D-glucose	4.5	Folic acid	0.004
L-arginine HCl	0.084	myo-Inositol	0.0072
L-cysteine	0.0626	Niacinamide	0.004
L-glutamine	0.584	Pyridoxine HCl	0.004
Glycine	0.03	Riboflavin	0.0004
L-histidine HCl.H_2O	0.042	Thiamine HCl	0.004
L-isoleucine	0.105		
L-leucine	0.105		

II. Eagles Minimal medium was used to culture Caco-2 cells (EMEM) (P04-08050, Pan Biotech). This medium was supplied in sterile 500mL bottles and stored at 4°C

Components	**mg/L**	**Components**	**mg/L**
Calcium chloride x $2H_2O$	264.92	Nicotinamide	1.00
Magnesium sulfate	97.67	Pyridoxal x HCl	1.00
Potassium chloride	400.00	Riboflavin	0.10
Sodium chloride	6800.00	Thiamine x HCl	1.00
Sodium dihydrogen phosphate x H_2O	140.00	$NaHCO_3$	2,200.00
D (+)-Glucose	1000.00		
Phenol red	10.00		
L-Arginine x HCl	126.00		
L-Cystine	24.00		
L-Glutamine	0.0		
L-Histidine x HCl x H_2O	42.00		
L-Isoleucine	52.00		
L-Leucine	52.00		
L-Lysine x HCl	72.50		
L-Methionine	15.00		
L-Phenylalanine	32.00		
L-Threonine	48.00		
L-Tryptophan	10.00		
L-Tyrosine	36.00		
L-Valine	46.00		
D-Calcium pantothenate	1.00		
Choline chloride	1.00		
Folic acid	1.00		
myo-Inositol	2.00		

b. Appendix 2

Table 1. The relative hydrophobicity of all CD002 isolates.

UK 2007-8	**Relative Hydrophobicity (RH) %**
160	26.7
161	8.9
162	35.4
163	18.9
165	52.6
166	21.3
167	57.4
168	16.5
169	48.7
170	31.5
171	76.9
172	67.8
173	26.3
174	31.0
Mean	37.1
UK 2011-13	**Relative Hydrophobicity (RH) %**
39	59.9
40	63.7
53	48.6
54	52.7
66	63.6
68	51.0
71	53.3
144	30.7
145	71.4
146	53.1

147	68.3
148	67.1
150	56.6
151	43.9
164	47.9
154	68.8
156	48.2
157	83.6
158	51.7
159	60.9
Mean	**57.3**
Non- UK 2011-12	**Relative Hydrophobicity (RH) %**
1	23.9
4	52.5
6	47.5
126	64.1
127	56.3
128	40.2
129	61.1
130	62.4
131	60.6
132	55.6
133	79.9
135	72.4
136	63.3
137	2.2
138	22.9
139	152.0
140	56.4
141	38.9

142	22.6
143	26.0
155	17.2
Mean	**51.3**