Phenotypic Characterisation of *Clostridium difficile* PCR Ribotype 078 and Comparison with PCR Ribotypes 027 and 002

Phenotypic Characterisation of *Clostridium difficile* PCR Ribotype 078 and Comparison with PCR Ribotypes 027 and 002

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Abstract

Clostridium difficile is an anaerobic, Gram-positive bacterium which resides in the gut of animals and humans. There are over 600 different polymerase chain reaction (PCR) ribotypes of *C. difficile*, some of which are pathogenic. Despite 5% of healthy humans having *C. difficile* within their normal gut microflora, this organism can cause illness in the elderly and immunocompromised patients. Symptoms of *C. difficile* infection (CDI) range from mild diarrhoea to death, and are due to two toxins (toxin A and toxin B) that the bacterium produces. Treatment for CDI includes the use of antibiotics, such as metronidazole and vancomycin, however some antibiotics, such as fluoroquinolones, can cause CDI, as they can disrupt the normal gut flora. *C. difficile* also produces biofilms which protect the bacteria within from antibiotic therapy. This study evaluated three genetically distinct *C. difficile* groups, PCR ribotypes 078, 027 and 002. All these PCR ribotypes cause disease, however PCR ribotypes 078 and 027 have been stated to be hypervirulent strains, therefore causing more severe illness. This study compared: the growth rate and pattern; cytotoxin production; susceptibility to a range of antimicrobials; biofilm production; viable and spore counts; and antimicrobial susceptibility for the three PCR ribotype groups to determine if any of these factors might contribute to the enhanced virulence status of PCR ribotype 078. These assays were completed by conducting: batch culture growth curves; cytotoxin production assays; antimicrobial susceptibility tests (agar dilution methods); a standard 96-well microtitre plate biofilm assay for biofilm quantification; and the Calgary Biofilm device (CBD) to assess biofilm formation and susceptibilities to metronidazole and vancomycin. This study found PCR ribotype 078 had higher average absorbance readings (biomass) than the PCR ribotypes 027 and 002 at the peak of growth (8 hours of incubation in an anaerobic cabinet): average OD_{600} readings for the PCR ribotype 078 group were 3.70 whereas the PCR ribotype 027 group had an OD_{600} of 2.82 and PCR ribotypes 002 3.26. PCR ribotype 002 had significantly the highest average maximum specific growth rate (μ_{max}) (0.73 h^{-1}) whereas PCR ribotype 078 had the lowest average μ_{max} (0.53 h^{-1}) ($P \leq 0.001$). PCR ribotype 078 also had significantly higher cytotoxin production than PCR ribotypes 027 and 002, with PCR ribotype 078 median cytotoxin titres of 3 $\log_{10}$ relative units (RU) in 72 hour cultures, whereas PCR ribotypes 027 and 002 median titres were 1 RU ($P \leq 0.001$). All the strains in each PCR ribotype group were susceptible to metronidazole and vancomycin. PCR ribotype 078 strains were susceptible to most of the antimicrobials used in this study, for example the vancomycin and metronidazole geometric mean minimum inhibitory concentration (MIC)s for PCR ribotypes 078 were vancomycin: 0.57 mg/L and metronidazole: 0.08 mg/L. The results for vancomycin were similar to the other two PCR ribotypes ($P=0.79$) whereas the metronidazole result were significantly different ($P \leq 0.001$) (PCR ribotypes 027: vancomycin: 0.53 mg/L and metronidazole: 1.37 mg/L; PCR ribotype 002: vancomycin: 0.53 mg/L and metronidazole: 0.18 mg/L). PCR ribotype 078 average biofilm production significantly increased over three (0.12 OD_{590}) to six (0.28 OD_{590}) days whereas the average for the PCR ribotype 002 group did increase but not significantly (three days: 0.07 OD_{590} and six days: 0.09 OD_{590}), however biofilm production by PCR ribotype 027 strains decreased (three days: 0.11 OD_{590} and six days: 0.08 OD_{590}). PCR 078 demonstrated the lowest biofilm total viable counts (5.17 $\log_{10}$ colony forming units (CFU)/ml) and spore counts (4.58 $\log_{10}$ CFU/ml) using a 96-well microtitre plate after six days of growth compared to the other two PCR ribotypes in which total viable counts were 5.87 $\log_{10}$/5.70 $\log_{10}$ CFU/ml and spore counts were 5.32 $\log_{10}$/5.27 $\log_{10}$ CFU/ml for the PCR ribotype 027/002 groups respectively ($P \leq 0.001$). The biofilm susceptibility testing results showed PCR ribotype 078 geometric mean biofilm MIC (bMIC) and minimum biofilm eradication

concentrations (MBEC) for vancomycin (0.50 mg/L and 0.57 mg/L respectively) and metronidazole (0.50 mg/L and 0.55 mg/L respectively) had similar results to those of PCR ribotypes 027 (vancomycin: bMIC 0.50 mg/L and MBEC 0.50 mg/L and metronidazole: bMIC 0.50 mg/L and MBEC 0.66 mg/L) and 002 (vancomycin: bMIC 2 mg/L and MBEC 2 mg/L and metronidazole: bMIC 4 mg/L and MBEC 2 mg/L). Total viable counts and spore counts on static CBD were <100 CFU/peg for all PCR ribotypes. This study also demonstrated that agitating the CBD in a four day growth period facilitated more extensive biofilm formation compared to static CBD assays. This study has demonstrated differences in growth (planktonic and biofilm) and cytotoxin production between the three *C. difficile* PCR ribotype groups assessed. These results could influence the behaviour and pathogenesis of PCR ribotype 078 in CDI if these results translated into the *in vivo* setting. Further studies are required in order to assess the reproducibility of these data in a larger cohort of isolates of the ribotypes studied, and in isolates obtained from varied hosts (human and animal) and environmental settings.

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To Absent Family and Friends.

Abbreviations List

Abbreviations	Meaning
μ_{max}	Maximum specific growth rate
A domain	Activity domain
A site	Aminoacyl-tRNA site
A549 cell line	Human lung adenocarcinoma cell line
agr	Accessory gene regulator
AIM	Agar incorporation method
AMP	Antimicrobial peptides
B domain	Binding domain
BHIS broth	Brain Heart Infusion broth with supplements
bMIC	Biofilm minimum inhibitory concentration
bp	Base-pair
BSAC	The British Society for Antimicrobial Chemotherapy
BSH	Bile salt hydrolase
C domain	Cutting domain
Caco-2	Human intestinal epithelial cell line
CBD	Calgary biofilm device
CDI	*Clostridium difficile* infections
c-di-GMP	3',5'-cyclic diguanylic acid
CDRN	*Clostridium difficile* Ribotyping Network
CDT	*Clostridium difficile* transferase
CF	Cystic fibrosis
cfr	Chloramphenicol-florfenicol resistance
CFU	Colony forming unit
CHO cells	Chinese hamster ovary cells
Cip	Ciprofloxacin
Clind	Clindamycin
CLSI	Clinical and Laboratory Standards Institute
Conc	Concentration
CPE	Cytopathic effect
Cwp	Cell wall protein
D domain	Delivery domain
DH	Department of Health
DMEM	Dulbecco's Modified Eagles Medium with supplements
E site	Exit site
ECOFF values	Epidemiological cut-off values
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
erm	Erythromycin resistance methyltransferase
Ery	Erythromycin
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FAO	The Food and Agriculture Organization of the United Nations
FliC	Flagellin protein
FliD	Flagellar cap protein
FMT	Faecal microbiota transplantation

fur	Ferric uptake regulator
GDH	Glutamate dehydrogenase
glu	Glutamate dehydrogenase
gyr	DNA gyrase
HBSS-trypsin-EDTA	Hanks Balanced Salt Solution supplemented with trypsin EDTA
hemN	Oxygen-independent coproporphyrinogen III oxidase
HPA	Health protection agency
HT29	Human colon carcinoma cells
I	Intermediate
IF	Initiation factors
Ig	Immunoglobulin
IMS	Industrial methylated spirit
Lin	Linezolid
LSR	Lipolysis-stimulated lipoprotein receptors
lux	Luciferase
MBEC	Minimum biofilm eradication concentrations
MDR	Multidrug resistance
mef	Macrolide efflux
Met	Metronidazole
MIC	Minimum inhibitory concentration
MLVA	Multilocus variable number tandem repeat analysis
Mox	Moxifloxacin
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NAAT	Nucleic acid amplification test
nim	Nitro-imidazole reductase
OD	Optical density
ORF	Open reading frame
P site	Peptidyl-trna site
PaLoc	Pathogenicity locus
par	Pseudoautosomal
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PHE	Public Health England
PMC	Pseudomembranous colitis
QRDR	Quinolone resistance-determining region
R	Resistant
RCM	Reinforced clostridial medium
ROS	Reactive oxygen species
RT	PCR ribotype
RU	Relative units
S	Susceptible
SC	Spore count
SCFA	Short chain fatty acids
SE	Standard error
S-layer	Surface layer
Tcd	*Clostridium difficile* Toxin

TcdA	*Clostridium difficile* Toxin A
TcdB	*Clostridium difficile* Toxin B
Tet	Tetracycline
Van	Vancomycin
VC	Viable count
Vol	Volume
WHO	World Health Organization

Contents

1. Introduction

1.1. Background

Clostridium difficile is a Gram-positive, anaerobic bacterium (Figure 1.1) (Hall and O'Toole, 1935). *C. difficile* can be identified by its rod shape and by using polymerase chain reaction (PCR) to find the *gluD* gene which encodes for the enzyme glutamate dehydrogenase (GDH) which is unique to *C. difficile* (Goorhuis *et al.*, 2008). There are now over 600 genetic variants of *C. difficile*, which can be differentiated using PCR ribotyping. These PCR ribotypes vary in virulence, susceptibly to antibiotics and the host/environment from which they are commonly isolated, such as within the gut of humans and other animals (including pigs and horses) and soil (Baverud *et al.*, 2003; Janezic *et al.*, 2012; O'Neill *et al.*, 1996). Approximately 5% of healthy adults and about 50% of new born children, have *C. difficile* in their normal gut flora with no symptoms (Donelli *et al.*, 2012; Nelson *et al.*, 2011; Tsutsumi *et al.*, 2014). Despite this, *C. difficile* infection (CDI) still occurs, most commonly in the elderly (>65 years old), as the likelihood of contracting CDI increases by about 2% for every additional year after 18 years old, also in immunocompromised individuals, such as patients undergoing chemotherapy, and in patients who are taking certain antibiotics, such as clindamycin, ciprofloxacin and aminopenicillins (Best *et al.*, 2012; Borgmann *et al.*, 2010; Debast *et al.*, 2014; Lawley *et al.*, 2009; Loo *et al.*, 2011; O'Connor *et al.*, 2004; Tsutsumi *et al.*, 2014). Due to highly resistant spores, transmission of *C. difficile* is hard to control, this results in epidemics in the UK and around the world (Lawley *et al.*, 2009). CDI patients, in England, can stay in hospitals for up to 21 days longer than other patients and costs approximately £7 000 per case (Pereira *et al.*, 2014; Wilcox, 2013). *C. difficile* has two major virulence factors which causes CDI, toxin A and toxin B, which result in a range of symptoms, from mild diarrhoea to death, depending on the patient and PCR ribotype (Kuehne *et al.*, 2010; NHS, 2012; Rupnik *et al.*, 2005; Shen, 2012). This review will look at: a brief history of the discovery of *C. difficile*, the characteristics which define different PCR ribotypes with an emphasis on two so called 'hypervirulent' PCR ribotypes, PCR ribotype 078 and 027, which causes severe illness, as well as a virulent PCR ribotype, PCR ribotype 002, how *C. difficile* infects the host, how different treatments and the host's body work to stop infections and how hospitals prevent the spread of CDI (Goorhuis *et al.*, 2008; Tagashira *et al.*, 2013).

1.1.1. History of *C. difficile*

In 1935 *C. difficile* was identified and described in the intestinal contents of new-born babies as part of the gut microflora (Hall and O'Toole, 1935; Jones *et al.*, 2013). Hall and O'Toole (1935) described this bacterium as being slow-growing and having *"less striking physiologic properties"* compared to *Kopfchenbacterien*. Subsequently, the bacterium was named *Bacillus difficilis* due to the difficulties encountered in isolating and studying the bacterium. In the 1970s, *B. difficilis* was reclassified *Clostridium difficile* (Goudarzi *et al.*, 2014; Kelly and LaMont, 2008). Then in 1978 *C. difficile* was found to be a cause of a proportion of cases of antimicrobial associated diarrhoea and colitis, and most cases of pseudomembranous colitis (PMC) (Freeman *et al.*, 2010; Heinlen and Ballard, 2010). Molecular analysis of all the toxins produced by *C. difficile* began in the

1980s, at which time the genes were sequenced, defined and regulations were studied (Rupnik *et al.*, 2005). It has now been proposed, by Yutin and Galperin (2013) that *C. difficile* be reclassified further to *Peptoclostridium difficile* due to finding similarities between *C. difficile* 16S rRNA-based phylogeny and DNA gyrase (GyrB) to that of the genus *Peptostreptococci.*

1.1.2. *C. difficile* Epidemiology

PCR is used to sub-categorise *C. difficile* into different genotypic groups (PCR ribotypes). The process involves using primers to amplify the spacer region between the 16S and 23S rRNA genes. The spacer region was discovered to differ in length for different ribotypes (Bidet *et al.*, 1999; Gürtler, 1993). This has helped to discover over 600 PCR ribotypes of *C. difficile*. The *C. difficile* Ribotyping Network (CDRN) within Public Health England (PHE) (formally Health Protection Agency (HPA)), uses multi-locus variable repeat analysis (MLVA) to further differentiate *C. difficile* isolates within the same PCR ribotype, and this helps to determine the prevalence of different PCR ribotypes in the UK (PHE and CDRN, 2014). Two of major ribotypes of interest are PCR ribotype 027 and 078, which have previously both been designated as hypervirulent, which are suggested to cause more severe CDI (Goorhuis *et al.*, 2008; Janezic *et al.*, 2012; Stabler *et al.*, 2009).

PHE has been collecting data on the incidence of CDI from laboratories in England and Wales since 1990,and in Northern Ireland since 2001 (HPA, 2013; PHE, 2014). The data collected includes the number of toxin-positive faecal samples in a range of age groups, in a given year, for both sexes (PHE, 2014). In 1990 there was a total of 1194 positive samples in England and Wales: within the decade there was a 20-fold increase (20527 samples). When Northern Ireland was included in 2001 the number of cases increased to 22352 samples (PHE, 2014). In 2007 the number of positive samples more than doubled to 57228 (PHE, 2014). After 2007 the number of positive samples decreased, to 13547 samples, in 2013, where 40.38% were male and 58.40% were female, and over half (57.19%) of the positive sample were from the 75 and over age group (PHE, 2014). The prevalence of different ribotypes has changed between 2007 and 2013, for example PCR ribotype 002 prevalence has increased overall since 2007 (30.8% to 7.69%), however it has decreased between 2012 (10%) and 2013 (7.69%). PCR ribotype 078 has also increased in prevalence since 2007 (2.31%) to 2013 (14.62%), however, PCR ribotype 027 has decreased overall since 2007 to 2013 (63.85% to 5.38%), but between 2012 (3.85%) and 2013 (5.38%) there was a slight increase in prevalence (PHE and CDRN, 2014). In the rest of Europe 8% of CDI was the result of PCR ribotype 078, whereas, only 5% of CDI was due to PCR ribotype 027 (Kurka *et al.*, 2014).

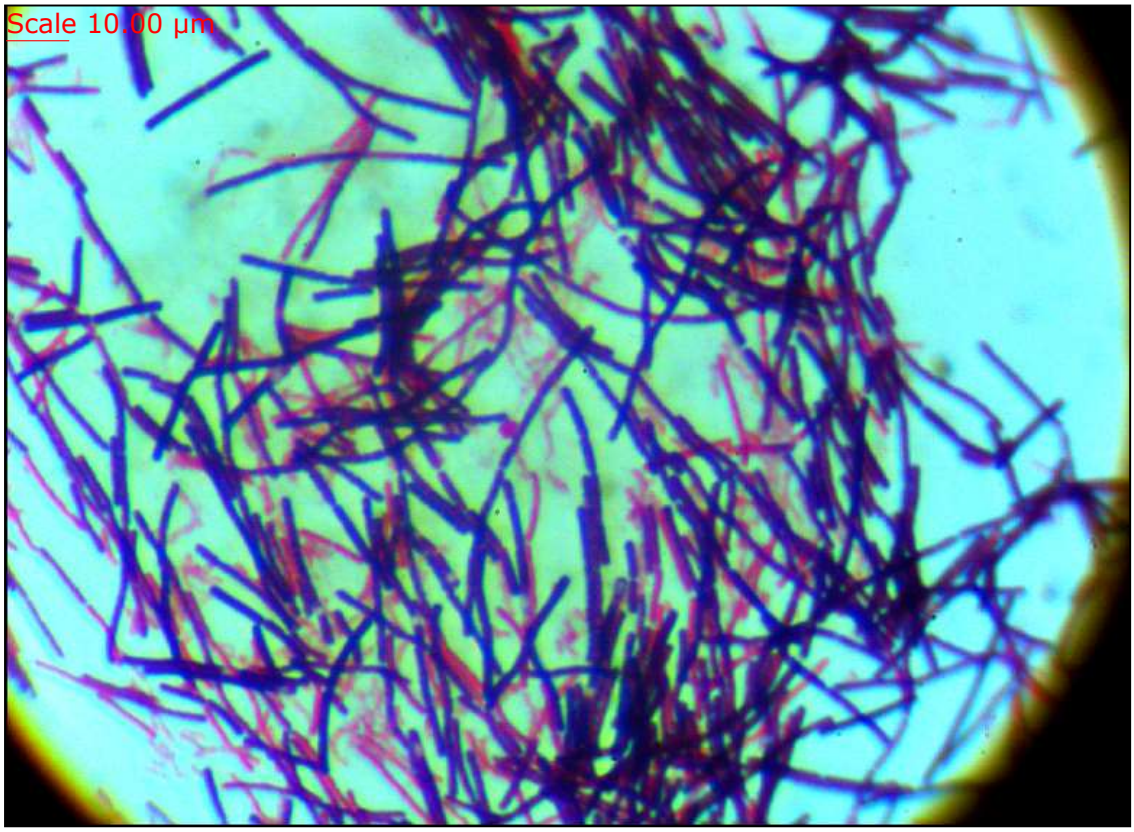

Figure 1.1. Gram stain of *C. difficile* (x1000 magnification) observed under a light microscope. Showing the Gram-positive (purple) rod shaped *C. difficile*.

PCR ribotype 027 was first isolated in Quebec, Canada, in 2003, since then outbreaks of CDI caused by PCR ribotype 027 have spread to Europe and Asia (Stabler *et al.*, 2009). In the UK, the first outbreak was at Stoke Mandeville hospital, Buckinghamshire, in 2004/05 (Brazier, 2012; Stabler *et al.*, 2009). The hypervirulent status of PCR ribotype 027 was thought to be due to a mutation within the *tcdC* gene, which was a putative negative regulator for toxin production (MacCannell *et al.*, 2006; Murray *et al.*, 2009). MacCannell *et al.* (2006) were the first to report the 18-base pair (bp) *tcdC* deletion. This study retrieved PCR ribotype 027 isolates and used PCR to identify the genes associated with virulence: *tcdA* (encodes for toxin A), *tcdB* (encodes for toxin B), *cdtA*, *cdtB* (both encode *C. difficile* transferase (CDT) and the erythromycin resistance methyltransferase *(ermB)* genes in all strains. They also amplified and sequenced the *tcdC* gene. MacCannell *et al.* (2006) found that all strains in the study had *tcdA*, *tcdB*, *cdtA*, *cdtB* and *ermB* genes, and an 18-bp deletion within the 330 to 347 positions of the *tcdC* gene. The *tcdC* gene also had another frameshift mutation, which was undocumented before this study, at position 117. These mutations were hypothesised by MacCannell *et al.* (2006) to contribute to the reported hypervirulence of PCR ribotype 027 as it causes the PCR ribotype to produce toxin in its exponential phase of growth (MacCannell *et al.*, 2006). However, there are still uncertainties on how the mutation in the *tcdC* gene affects toxin production in PCR ribotype 027. For example, Warny *et al.* (2005) found that PCR ribotype 027 has elevated levels of toxin A and toxin B, whereas Freeman *et al.* (2007) found PCR ribotype 027 produces cytotoxin for longer, than PCR ribotype 001. Cartman *et al.* (2012) used a strain of PCR ribotypes 027 (R20291) and manipulated the *tcdC* gene to ascertain if the gene has an effect on toxin production. The study found no differences in toxin production between any of the strains, with wild-type *tcdC* or with introduced mutations. However, Deakin *et al.* (2012) then found the increase in toxin production could be due to a mutation within the *spo0A* gene and pondered if the gene negativity regulates toxin production.

PCR ribotype 078 is another, hypervirulent PCR ribotype which has been shown to be an increasing cause of CDI in humans (Goorhuis *et al.*, 2008; Janezic *et al.*, 2012). Goorhuis *et al.* (2008) defined PCR ribotype 078 as hypervirulent due to its increase in prevalence in humans as well as its similarities to PCR ribotype 027, which has be proven to be hypervirulent (Valiente *et al.*, 2012). Goorhuis *et al.* (2008) had come to this conclusion by conducting a three year study collecting human isolates from hospitals, care homes and other healthcare facilities in The Netherlands and Europe, as well as isolates collected from pigs. *C. difficile* was identified by PCR and the strains were further studied, via PCR ribotyping and toxinotyping, to find the presence of *tcdA*, *tcdB* and the *cdt* genes and to find mutations in *tcdC* (Bakker *et al.*, 2012; Goorhuis *et al.*, 2008). Antimicrobial susceptibility testing was also conducted to find how resistant the isolates were to moxifloxacin, ciprofloxacin, clindamycin and erythromycin, and MLVA was performed to assess the genetic relatedness between isolates. The authors found that all of the isolates studied contained all four genes (*tcdA*, *tcdB* and both the *cdt* genes) and a 39-bp deletion in *tcdC*. The susceptibility tests of the PCR ribotype 078 isolates (41 human and 8 pig) revealed that 57% were susceptible (MICs <4 mg/L) to clindamycin, 78% were resistant to erythromycin (≥4 mg/L) and 14% of the isolates contained the *ermB* gene which encodes for resistance to other antibiotics, such as erythromycin (Corso *et al.*, 2009; Spigaglia *et al.*, 2005). With the fluoroquinolones, 94% of *C. difficile* were resistant (MICs ≥4 mg/L) to

ciprofloxacin and 88% were resistant to moxifloxacin. There was no significant difference between pig and human isolates in susceptibility to the antibiotics evaluated in this study. The MLVA analysis demonstrated that all the isolates were genetically related.Goorhuis *et al.* (2008) also gave questionnaires to CDI patients infected with different PCR ribotypes and 715 were returned; 57 were infected with PCR ribotype 078, 129 patients were infected with PCR ribotype 027 and the rest of the patients were infected with other PCR ribotypes other than PCR ribotype 078 and 027. The questionnaire responses were compared for each PCR ribotype and the authors reported that the patients infected with PCR ribotype 078 were younger and their symptoms were just as severe as PCR ribotype 027. The questionnaire results also suggested that PCR ribotype 078 infected patients, in comparison to patients with other strains of *C. difficile*, were less likely to have other diseases and more frequently had received fluoroquinolone therapy. This study concluded that due to the severity and similarities to PCR ribotype 027, PCR ribotype 078 was a hypervirulent strain. However the study did not state what facets constitute a hypervirulent strain and did not test the magnitude of toxin production (Goorhuis *et al.*, 2008; Shen, 2012).The study also did not assess the susceptibility to antimicrobials which are used to treat CDI, such as vancomycin and metronidazole (Debast *et al.*, 2014).

1.1.3. *C. difficile* Infection

The main symptom of CDI is diarrhoea, which is defined as: 1) passing three or more stools per day which correspond to types five to seven on the Bristol stool chart, or 2) passing more loose stools per day than is normal for an individual. Diarrhoea can occur up to two months after antibiotic treatment for another condition (Debast *et al.*, 2014; WHO, 2013; Wilcox, 2003). The diarrhoea usually has a foul odour and may contain (although rare) blood (Bartlett and Gerding, 2008; Bennett *et al.*, 1984). In more severe cases of CDI PMC can occur, this is identified by the visualisation of yellow/white plaques (pseudomembranes) along the colon wall, which are detectible via colonoscopy (or sigmoidoscopy), or histopathology (Debast *et al.*, 2014; Johal *et al.*, 2004; Wilcox, 2003). The mortality rate of CDI patients is 17% after 30 days from the start of infection (Wilcox, 2013). This could be due to the development of toxic megacolon when the patient also has PMC, this condition is rare but noticeable by the dilation of the colon (Autenrieth and Baumgart, 2012; Wilcox, 2003). Despite treatment there is about a 20% chance of CDI recurring within one to eight weeks, but has been known to take place up to 12 weeks after antibiotic treatment is completed for an initial episode. CDI recurrence may be due to persistence of *C. difficile* spores which are resistant to antibiotics, and the normal gut microflora not being able to grow back to normal after CDI to restore colonisation resistance (Bakken *et al.*, 2011; Debast *et al.*, 2014; Im *et al.*, 2011).

1.1.4. *C. difficile* Sporulation and Germination

Sporulation occurs when the environment changes so the *C. difficile* cannot survive, such as the introduction of antibiotics or a limitation in nutrients (Joshi *et al.*, 2012; Oka *et al.*, 2012). Spores are excreted in the faeces of an infected person and are easily transmitted between patients this is due to their resistance to aerobic conditions and many disinfectants (Deakin *et al.*, 2012; Heeg *et al.*, 2012; Lawley *et al.*, 2009; Paredes-Sabja and Sarker, 2011; Wheeldon *et al.*, 2008a).The environmental stresses cause sensor kinases to phosphorylate the

proteins involved in sporulation, one of which is encoded by the *spo0A* gene (Deakin *et al.*, 2012; Saujet *et al.*, 2011; Underwood *et al.*, 2009). Sporulation starts when a mother cell divides and then engulfs the spore which matures inside the cell until the cell denatures. The spore lays dormant until it is exposed to bile salts within the colon (Figure 1.2.) (Higgins and Dworkin, 2012; Paredes-Sabja *et al.*, 2014; Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2008a). The spore goes through different phases, including the dark-phase where the spore becomes less resistant to environmental changes such as antibiotics and heat, the spore grows out into a vegetative cell (Figure 1.2.) (Liu *et al.*, 2014; Nerandzic and Donskey, 2013). Merrigan *et al.* (2010) found that hypervirulent strains of *C. difficile* produce more spores (after 48 hours) than non-hypervirulent strains. One gene which is recognised as a contributor in this process is the *spo0A* gene. This initiates sporulation by activing RNA polymerase sigma factors needed for sporulation to occur (Paredes-Sabja *et al.*, 2014). Deakin *et al.* (2012) found sporulation was decreased in *spo0A* mutant strains of 630Δerm and R20291 (compared to wild-type strains), and that complementation of the *spo0A* into the mutant restored the wild-type phenotype.

Germination is the transition from a phase bright spore into a phase dark spore. This occurs when the spore is in the ideal environment for vegetative *C. difficile* to survive (Figure 1.2.) (Setlow, 2003; Sorg and Sonenshein, 2008). Germination has been studied in other *Clostridium* species, such as *C. perfringens*, however due to differences in germination triggers it is difficult to predict how germination is induced in *C. difficile* spores (Paredes-Sabja and Sarker, 2011; Paredes-Sabja *et al.*, 2014). For example, Paredes-Sabja and Sarker (2011) found *C. difficile* spores do not germinate when incubated with epithelial cells (*in vitro*) whereas *C. perfringens* spores did. This may be due to *C. difficile* spores having different germination receptors than spores of other *Clostridium* species (Ramirez *et al.*, 2010). Known germination triggers of *C. difficile* spores are primary bile salts, such as sodium taurocholate (Sorg and Sonenshein, 2008). Sorg and Sonenshein (2008) treated *C. difficile* spores with 0.1 % sodium taurocholate in supplemented Brain Heart Infusion solution, serially diluted then cultured onto agar, their results showed a mean colony forming unit (CFU) recovery of 1.27%. Wheeldon *et al.* (2008a) found the optimal concentrations of sodium taurocholate for germination were between 0.1 and 100 mmol/L. Germination is also induced by secondary bile salts, such as deoxycholate (Sorg and Sonenshein, 2008). Deoxycholate has also been found to induce germination by Sorg and Sonenshein (2008), using the same methods as the sodium taurocholate. The study showed a mean CFU recovery of 1.48% with deoxycholate, which was similar to the recovery of CFUs with sodium taurocholate in the same study. When Sorg and Sonenshein (2008) exposed vegetative cells to deoxycholate, it was found that growth was inhibited.

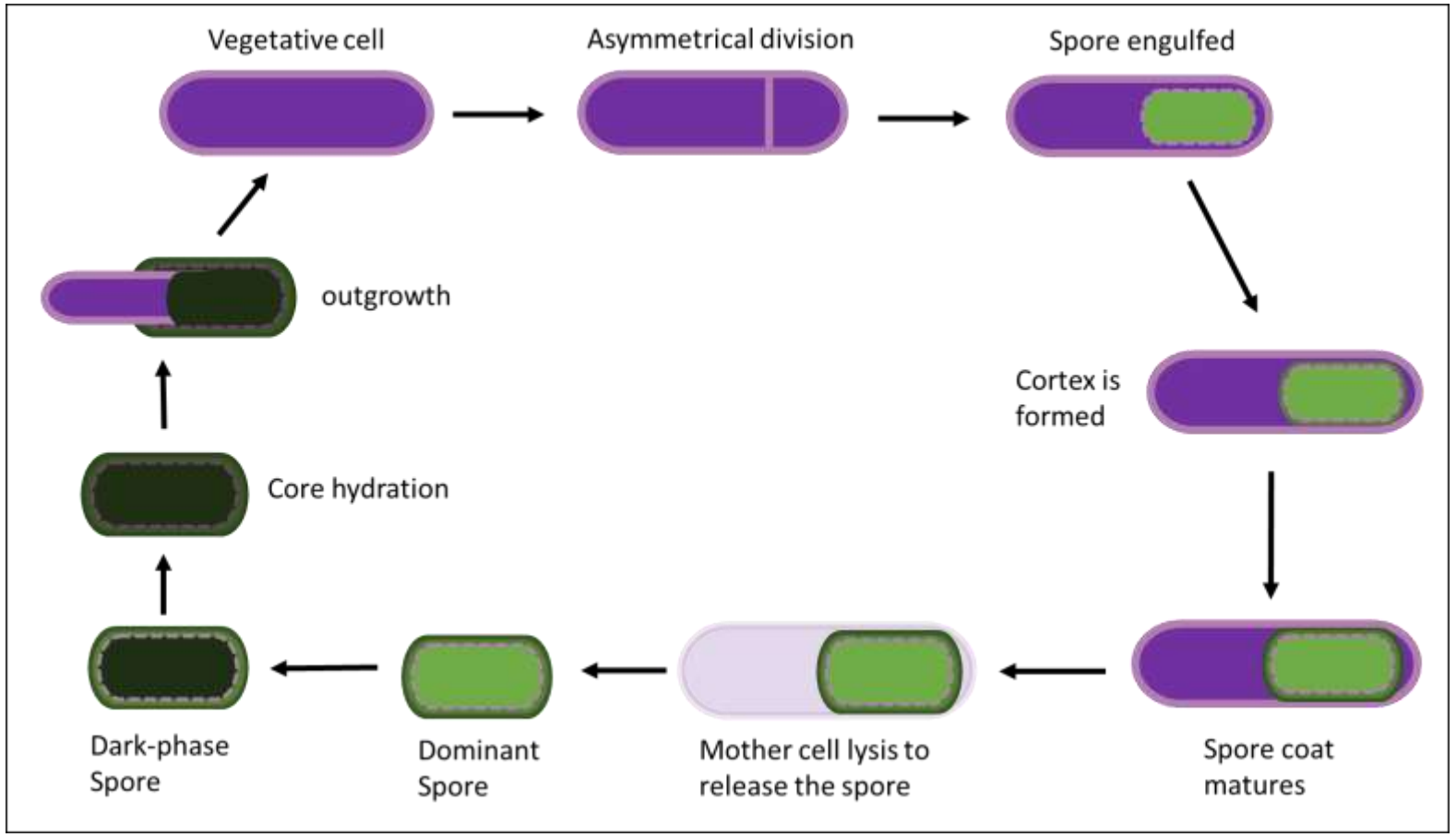

Figure 1.2. The cycle of sporulation from vegetative cell to spore, when environmental conditions are not preferable to the bacterial cell, and back to vegetative cell, when exposed to bile salts. (Based on figures presented in Higgins and Dworkin (2012) and Liu *et al.* (2014)).

1.1.5. The Toxins of *C. difficile*

The symptoms of CDI are caused by two toxins, toxin A and toxin B, which are the major virulence factors of *C. difficile* and are produced by PCR ribotype 078, 027 and 002 (Carter *et al.*, 2010; Kuehne *et al.*, 2010; Lyras *et al.*, 2009; Rupnik *et al.*, 2005; Shen, 2012; Tagashira *et al.*, 2013). These toxins are glucosyltransferases, which catalyse glycosylation of the Rho/Rac family of GTPases within gut epithelial cells, this in turn causes the actin cytoskeleton to become unregulated and consequently the cell structure is adversely affected (the cells become rounded), cell-cell junctions are lost due to a disruption in signalling and the end result is cell death (Kuehne *et al.*, 2010; Liu *et al.*, 2014; Lyras *et al.*, 2009; Pruitt *et al.*, 2010; Shen, 2012). Two studies used similar methods to determine which toxin was more active however the results from these studies were contradictory (Kuehne *et al.*, 2010; Lyras *et al.*, 2009). Lyras *et al.* (2009) used human colon carcinoma (HT29) cells and African green monkey kidney (Vero) cells in cytotoxicity assays and exposed them to different toxins produced by *C. difficile* whose genes were mutated to stop them being functional (Kuehne *et al.*, 2010). The *C. difficile* strains used, either had the toxin A gene (*tcdA* gene) mutated, so toxin A was not produced (A-B+), or the toxin B gene (*tcdB* gene) mutated, so toxin B was not produced (A+B-), or a wild-type parent PCR ribotype which possessed both fully functional toxin genes (Lyras *et al.*, 2009). Lyras *et al.* (2009) stated that the A+B- strain, produced more toxin A than the wild-type. Toxin A, from the A+B- strain, was found to be less toxic to the HT29 than the toxin A from the wild-type strain, and the toxin B, from the A-B+ strain, was less toxic than the toxin B from the wild-type strain. However the toxin B (A-B+) was just as toxic to the Vero cells as the wild-type strains toxin A, and the toxin A (A+B-) was less toxic to Vero cells than the toxin B (A-B+) and the wild-type strain. Hamster models were used to see which toxin was more active *in vivo*, by colonising the hamsters with the different toxin variants of *C. difficile*. The researchers found that the strain which only produced toxin B (A-B+) maintained a virulent phenotype of the wild-type strain, however the strain producing just toxin A (A+B-) was virulent, thus suggesting the *C. difficile* does not need both toxins to be virulent (Lyras *et al.*, 2009). Conversely, Kuehne *et al.* (2010) used a parental strain (A+B+), a strain which only produced toxin A (A^+B^-), a strain which only produce toxin B (A-B+) and a strain with a double-toxin mutation (A-B-). The study showed that the A-B- strain culture supernatant had no effect on the HT29 cells and Vero cells. The A-B+ toxin preparation had a reduced effect on both cell lines and the A+B- preparation had an increased effect on both cell types, however the results were not significant. The study went on to use hamsters which the authors infected with all the *C. difficile* strains, and all hamsters except those infected with the A-B- *C. difficile* strain showed symptoms, suggesting that both toxins are needed for *C. difficile* virulence (Kuehne *et al.*, 2010).

Some *C. difficile* PCR ribotypes produce a third toxin, a *C. difficile* transferase (CDT), which has been proposed to aid in the colonisation and adhesion of *C. difficile* to the epithelial cells on the gut wall (Carman *et al.*, 2011; Geric *et al.*, 2006; Schwan *et al.*, 2009). CDT is a binary toxin, composed of two unlinked molecules designated CDTa and CDTb. CDTb (encoded by the *cdtB* gene) binds to lipolysis-stimulated lipoprotein receptors (LSR) on the host cell which causes them to cluster and create lipid rafts which helps form a pre-pore. CDTa (encoded by the *cdtA* gene) is the enzyme component of the binary toxin and binds to the pre-pore and is up taken into the cell via endocytosis. Acidification, an influx of

hydrogen ions, of the endosome induces the release of the CDTa into the cytosol. The polymerisation of the cellular actin is inhibited by this process, which leads to a disrupted actin cytoskeleton and the formation of long microtubule protrusions through the cell membrane, increasing the adherence and potentially colonisation of *C. difficile* (Aktories *et al.*, 2012; Carman *et al.*, 2011; Carter *et al.*, 2007; Geric *et al.*, 2006; McDonald *et al.*, 2005; Papatheodorou *et al.*, 2013; Rupnik *et al.*, 2003; Shen, 2012). *C. difficile* strains expressing CDT alone are not virulent. Geric *et al.* (2006) conducted a study to determine the likely contribution of CDT to *C. difficile* virulence. The study used hamsters and gave them clindamycin to disrupt the gut bacterial flora. On the fifth day after the administration of clindamycin, the hamsters were colonised with one of four strains of *C. difficile*, three were toxin A and toxin B negative and CDT positive (A-B-CDT+) and one strain was toxin A/B positive and CDT negative (A+B+CDT-), which was the positive control. The study found the hamsters which were colonised with the A-B-CDT+ strains remained colonised for the rest of the study (27 days, after clindamycin administration) with no symptoms, whereas, the control hamsters died the day after the colonisation of the A+B+CDT- *C. difficile* strain. Geric *et al.* (2006) concluded the CDT does not cause disease on its own, and hypothesised CDT may play another role in the virulence of strains which produce toxin A and B. This alternate role was studied by Schwan *et al.* (2009), who inoculated human intestinal epithelial cell line (Caco-2) with CDT. The study found when the Caco-2 cells were inoculated with CDT, the toxin induced the production of microtubule protrusions along the cell boarder, which created a mesh after two hours of being exposed to CDT. The study then inoculated the Caco-2 cells with CDT then, after an hour, introduced a *C. difficile* strain which produced only toxin A and toxin B. After 90 minutes the cell culture was washed and an antibody, which detected *C. difficile* surface proteins and was used to see if any *C. difficile* cells remained (Schwan *et al.*, 2009). The study found that *C. difficile* remained in the cell culture, therefore, Schwan *et al.* (2009) concluded that CDT induced microtubules which may aid in the adhesion of the *C. difficile* to the gut wall.

1.1.6. Diagnosis of CDI

As *C. difficile* is not always the cause of diarrhoea after antibiotic use, a two or three stage algorithm is performed to diagnose CDI (Beaugerie and Petit, 2004; Debast *et al.*, 2014; Wilcox, 2003). This algorithm is low cost and has a quick turnaround time however the tests involved have not been standardised and are not sensitive when used separately (Debast *et al.*, 2014; Kvach *et al.*, 2010). The algorithm involves conducting an enzyme immunoassay to detect the presence of GDH in a stool sample (Debast *et al.*, 2014; Kim *et al.*, 2014). This antigen, unique to *C. difficile*, indicates the presence of *C. difficile* but not if the strain is toxigenic for toxin A and B (Debast *et al.*, 2014; Goorhuis *et al.*, 2008; Kim *et al.*, 2014). If the sample is positive for GDH, the next stage is to test for toxins, this is done by performing cytotoxin assays on the faecal samples this confirms or refutes the presence of toxin A and B (Debast *et al.*, 2014; Kvach *et al.*, 2010; Quinn *et al.*, 2010). Another method to diagnose CDI, if samples are GDH positive, is to perform a nucleic acid amplification test (NAAT), these tests are more sensitive than the toxin assays and work by using PCR to detect the *tcdB* gene within the samples (Debast *et al.*, 2014; Gould *et al.*, 2013). Gould *et al.* (2013) found that when diagnostic laboratories switched from using cytotoxin assays to NAAT the percentage of CDI incidence increased by 24%.

1.1.7. The Immune Response to *C. difficile*

The gut has innate mechanisms to keep bacterial homeostasis and defend against pathogenic bacteria, involving a mucus layer, epithelial cells and immune cells (Kurashima *et al.*, 2013). The mucus layer contains antimicrobial peptides (AMP) and immunoglobulin (Ig) A antibodies (Kurashima *et al.*, 2013). Below the mucus layer are epithelial cells, such as goblet and Paneth cells, which produce the AMPs and transport IgA via transcytosis through the columnar epithelial cell from the immune cells (Johal *et al.*, 2004; Kurashima *et al.*, 2013; Zasloff, 2002). The immune cells include mast cells, which support the production of IgA, innate lymphoid cells, which instigate the inflammatory response by AMP production and regulates tissue repair, as well as plasma cells which produce IgA antibodies (Kurashima *et al.*, 2013; Shapiro-Shelef *et al.*, 2003). IgA antibodies are the frontline defence if any pathogen is present within the gut, and in agreement with this, Olson *et al.* (2013) demonstrated IgA to be effective against *C. difficile* toxin A. They found this by inoculating HT29 cells with toxin A and IgA to see how the antibodies affected the toxins uptake in the cells. They found that IgA not only decreased the uptake of toxins into the HT29 cells but also decreased the effects of toxin A on the permeability of the HT29 cells (Olson *et al.*, 2013). Additionally, IgA opsonises bacteria to aid removal from the gut (Bollinger *et al.*, 2003). IgG and IgM antibodies are produced by B lymphocytes and these antibodies are also important in the immune response against toxins A and B (Kyne *et al.*, 2000; McDonnell *et al.*, 1990; Qiu *et al.*, 2003; Torres *et al.*, 1995).

1.1.8. Colonisation Resistance Against CDI

The normal gut microflora is an important defence against CDI, so-called colonisation resistance (Nelson *et al.*, 2011). There are approximately 10^{11} bacteria/g of faeces within the human colon, including the genera *Escherichia*, *Streptococcus* and *Clostridium* (Ridlon *et al.*, 2006). If *C. difficile* is introduced, through the transmission of highly resistant spores, the stable resident bacteria can inhibit *C. difficile* from overgrowing (Taur and Pamer, 2014). Once the spores have reached the colon, germination is triggered by the exposure to primary bile salts, such as sodium taurocholate, which are produced by the liver (Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2008a). In a healthy colon, without antibiotics, vegetative *C. difficile* cells are inhibited due to the production of the secondary bile salt deoxycholate, which is converted from taurocholic acid by bacteria within the gut, such as *Bifidobacterium*, *Streptococcus* and *Clostridium* species (not including *C. difficile*) (Grill *et al.*, 1995; Sorg and Sonenshein, 2008; Taur and Pamer, 2014). *Bifidobacterium* species produce bile salt hydrolase (BSH), these enzymes hydrolyse the amide bonds of bile salts, which are created when cholesterol conjugates to either glycine or taurine in the liver, to produce secondary bile salt acids and amino acids (Grill *et al.*, 1995; Sorg and Sonenshein, 2008; Taranto *et al.*, 1999). The gut bacteria, including *C. difficile*, also help to reinforce the host's gut defences, by the production of short chain fatty acids (SCFA), one example is butyrate (Pryde *et al.*, 2002). Butyrate, like other SCFA, has been found to help in increasing tight junction protein expression, this decreases epithelial lining permeability, it also has anti-inflammatory properties and increases mucin and AMP production (Antharam *et al.*, 2013; Muñoz-Tamayo *et al.*, 2011; Pryde *et al.*, 2002). Butyrate-producing bacteria, such as *C. difficile* and *C. acetobutylicum*, have been found to convert acetoacetyl-CoA to acetyl-CoA and butyrate using butyryl-CoA:acetate-CoA transferase (Duncan *et al.*, 2002;

Muñoz-Tamayo *et al.*, 2011; Pryde *et al.*, 2002). *Roseburia intestinalis* and *R. inulinivorans* use β-fructofuranosidase to produce butyrate using oligosaccharides and polysaccharides (Scott *et al.*, 2011; Shahinas *et al.*, 2012). When antibiotics are given to a patient they can suppress these microbes in the gut, which decreases deoxycholate production this promotes the vegetative *C. difficile* cells to grow causing an infection (Nelson *et al.*, 2011; Shahinas *et al.*, 2012; Taur and Pamer, 2014).

1.1.9. Treatment of CDI

There are three main antibiotics that are used as treatments for CDI, metronidazole and vancomycin, which have been used for more than 30 years and are the first-line treatments for CDI, along with fidaxomicin which is a new antimicrobial agent recently licensed for the treatment of CDI (Debast *et al.*, 2014; Johnson and Wilcox, 2012; Wu *et al.*, 2013). Metronidazole is a nitroimidazole compound which has a major role in treating anaerobic bacterial infections (Menendez *et al.*, 2002). The drug is usually administered in the form of a 450 mg oral tablet three times a day for 10 days for the first recurrence of mild or moderate cases of CDI (Debast *et al.*, 2014; Freeman *et al.*, 1997). Metronidazole has been found to be mutagenic to bacterial DNA and causes the DNA to breakdown by denaturing its helical structure (Edwards, 1980; Menendez *et al.*, 2002). Vancomycin is a glycopeptide compound which is produced by *Amycolatopsis orientalis* and is used to treat Gram-positive bacterial infections and enterocolitis which is caused by *C. difficile* (Losey *et al.*, 2001; Nagarajan, 1991). This drug is administered orally, 125 mg four times a day for 10 days for non-severe, severe and re-occurring CDI (Al-Nassir *et al.*, 2008; Debast *et al.*, 2014). Vancomycin inhibits the maintenance of the bacterial cell wall by inhibiting peptidoglycan synthesis (Nagarajan, 1991; Yim *et al.*, 2014). Fidaxomicin is a narrow spectrum macrocyclic antibiotic which inhibits transcription by binding to DNA-dependent RNA polymerase to inhibit RNA synthesis in the bacteria (Babakhani *et al.*, 2013; Johnson and Wilcox, 2012; Louie *et al.*, 2011). This antibiotic is taken orally as a 200 mg tablet twice a day for 10 days for initial severe and non-severe CDI as well as recurrent infection (Debast *et al.*, 2014). In *C. difficile* it was found that fidaxomicin can inhibit spore formation, but also may potentially reduce the spread of CDI to other patients (Babakhani *et al.*, 2013). Additionally, Babakhani *et al.* (2013) also found that fidaxomicin inhibits transcription of the toxin A and B genes. Fidaxomicin has been found to be eight times more effective than vancomycin against *C. difficile in vitro*, and reduces the chance the recurrence of CDI (Babakhani *et al.*, 2013; Louie *et al.*, 2011).

Other non-antibiotic based treatments for CDI exist, including faecal microbiota transplantation (FMT) and other probiotic therapies, and the use of these treatments depend on severity of illness, the PCR ribotype, the patient, and whether a CDI episode is a recurrence or not (Debast *et al.*, 2014). FMT involves restoring normal gut microflora by transplanting healthy stool, via faecal enema or nasogastric administration, which can re-establish normal gut microflora along with normal gut function (Bakken *et al.*, 2011). It is hypothesised that bacteria, introduced from the healthy faeces, could convert taurocholic acid to choline which is then converted to deoxycholate, by other bacteria, which inhibits *C. difficile* vegetative cell growth (Sorg and Sonenshein, 2008; Taur and Pamer, 2014). FMT was first used as a treatment for CDI in 1983 and since then there has been a success rate of 96% in treating CDI (Bakken *et al.*, 2011). Despite this apparent

efficacy, FMT remains controversial as pathogens could be transferred as well as microbes which influence other conditions, such as obesity, autoimmune diseases and metabolic syndromes (Taur and Pamer, 2014). Studies have been performed to find the bacteria which are involved in these processes so to make the treatment safer and easier to administer (Taur and Pamer, 2014). Shahinas *et al.* (2012) conducted a study to see which bacterial species are present in the gut after FMT treatment. Stool samples were taken from CDI patients before FMT was conducted (but after vancomycin treatment), and then 14 days after successful treatment more samples were acquired and frozen for 24 hours. The V5-V6 regions of the bacterial 16S rRNA were analysed to find the bacterial taxa present within the stool sample before and after treatment. The study found low diversity but high abundance of the taxa present, such as *Proteobacteria*, in the pre-treatment samples, there was also a low numbers of *Lachnospiraceae*, which if not present in the gut is thought to prolong CDI (Shahinas *et al.*, 2012; Taur and Pamer, 2014). After successful treatment samples had higher diversity and more *Firmicutes*, *Bacteroidetes* and *Bacteroides* species as well as increased populations of *R. intestinalis* and *R. inulinivorans*. These bacteria could be linked to treatment of CDI via FMT due to the normalisation of gut microflora and the production of bile salts, which inhibits *C. difficile* growth, and butyrate which has been linked with tissue repair and development (Shahinas *et al.*, 2012; Taur and Pamer, 2014).

Probiotics are a possible prevention strategy for CDI (including recurrence) and are given in some cases with antibiotics (Debast *et al.*, 2014; McFarland, 2009). Probiotics are defined by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), as *"Live microorganisms which when administered in adequate amounts confer a health benefit on the host"* (WHO and FAO, 2001). Like FMT, probiotics are used to help re-establish the microflora within the gut after antibiotic treatment (McFarland, 2009). This prevention method involves using living bacteria, such as *Lactobacillus*, *Clostridium*, *Streptococcus* and *Bifidobacterium* species, and fungal species, such as *Saccharomyces boulardii* (Debast *et al.*, 2014; Johnson *et al.*, 2012). As this treatment involves different organisms there are different modes of action (McFarland, 2009). For example, *S. boulardii* is a yeast species isolated from fruit, like lychees, which produces a protease which have been shown, *in vitro*, to degrade the *C. difficile* toxins and inhibits the toxins from binding to the gut wall receptors (Johnson *et al.*, 2012). It has also been suggested to inhibit the adherence of *C. difficile* cells *in vitro*, for example Tasteyre *et al.* (2002) found that *S. boulardii* reduced the adherence of *C. difficile* to Vero cells by 34% (compared to the control). Trejo *et al.* (2006) found that several *Bifidobacterium* species, including *B. pseudolongum*, *B. longum* and *B. bifidum*, produced lactic acid and acetic acid. These supernatants were found to inhibit growth of all the *C. difficile* strains (*in vitro*) as well as decrease the adhesion of some strains of *C. difficile* used (Trejo *et al.*, 2006). However, there are some disadvantages to using probiotics, for example there is an increased risk to immunocompromised patients as infection with the probiotic organism could occur (Debast *et al.*, 2014; McFarland, 2009; Wada *et al.*, 2010). Riquelme *et al.* (2003) reported two case studies where the patients had conditions which resulted in immunodeficiency, both patients contracted CDI so were given *S. boulardii* as treatment. In both cases treatment had to be ceased, as the *S. boulardii* caused a fever and was found in blood cultures taken from the two patients (Riquelme *et al.*, 2003).

1.1.10. Antimicrobial Susceptibilities of *C. difficile*

Antimicrobial resistance is quickly becoming a major problem in the world, and if new treatments for bacterial infections are not discovered soon antibiotics will become obsolete (WHO, 2014). Some *C. difficile* PCR ribotypes have developed resistance to certain groups of antibiotics, this resistance can result into an increased risk of CDI in patients as well as a recurrence of CDI (McDonald *et al.*, 2005; Nelson *et al.*, 2011). As antibiotics used to treat other infections could inhibit the gut microflora allowing the resistance *C. difficile* to thrive (Nelson *et al.*, 2011). Resistance to metronidazole and vancomycin is seldom seen in *C. difficile*, however, susceptibly to metronidazole has be found, by Chong *et al.* (2014), to be reducing in strains of PCR ribotype 027, *in vitro*. Additionally, Leeds *et al.* (2013) found some strains of *C. difficile* have developed reduced susceptibly to vancomycin and fidaxomicin, *in vitro*. Resistance to fluoroquinolones has also developed in certain PCR ribotypes, such as PCR ribotype 078 and 027, this has led to an increase in prevalence of CDI in patients receiving fluoroquinolone treatment (Goorhuis *et al.*, 2008; He *et al.*, 2013; Solomon *et al.*, 2011; Vardakas *et al.*, 2012).

1.1.11. Infection Control Procedures for *C. difficile*

NHS hospitals operate strict protocols in the control and prevention of CDI. If a patient is suspected of having CDI then the 'SIGHT' mnemonic protocol is used (Appendix 1.1). All unnecessary antibiotics and drugs, which cause diarrhoea, should be ceased, the patient is kept in isolation, and their fluid, nutrition and electrolyte replacement is reviewed daily until the diarrhoea has stopped for 48 hours. The patient's hospital room should be cleaned with chlorine-containing cleaning agents daily and any bedding treated as contaminated (DH and HPA, 2008).

One possible prevention measure to reduce the spread of *C. difficile* is the use of copper. The antimicrobial properties of copper have been known of since the time of the ancient Egyptians, at about 2600 BC, who used the metal to sterilise chest wounds and drinking water. Since then the benefits of using bronze or brass door knobs in hospitals and using copper vessels to carry drinking water in developing countries have been documented (Grass *et al.*, 2011). The contact with the copper is what triggers the killing of bacteria, as the copper dissolves the ions produced damage the cell wall, then catalysis the production of reactive oxygen species (ROS), in the form of hydroxyl radicals, which causes damage to the DNA, in turn causes the bacterial cell to die (Grass *et al.*, 2011; Hong *et al.*, 2012; Molteni *et al.*, 2010). As *C. difficile* has highly resistant spores to normal cleaning procedures, it is hard to control the spread of infection, so using copper could potentially help (Weaver *et al.*, 2008). Weaver *et al.* (2008) inoculated copper, copper alloy and stainless steel (as a control) coupons with *C. difficile* vegetative cells and spores and spores alone. The coupons were incubated at room temperature in aerobic conditions for a range of time points. Once incubated the cultures were transferred into phosphate buffer solution (PBS) so they could be spread onto agar plates, which were incubated anaerobically and the concentration of *C. difficile* CFUs were counted. This study found that the initial cell death occurred in the first three hours, this was believed to be due to the aerobic conditions (glass coupons had the same results). However, the coupons with higher copper content (including 100% copper) had higher death rates with both the vegetative and spores and

spore cultures alone (Weaver *et al.*, 2008). Wheeldon *et al.* (2008b) also compared the effect of copper and stainless steel on *C. difficile* vegetative cells and spores from two strains (PCR ribotype 027 and NCTC 11204). However, the vegetative cell tests were conducted in anaerobic conditions and the spore experiments were performed in aerobic conditions. D/E neutralizer was added to neutralise the copper at 30 minute intervals (for three hours). The spores were then suspended in molten Fastidious Anaerobe Agar (supplemented with sodium taurocholate) and left to stand at room temperature, whereas the vegetative cells were inoculated onto Wilkins Chalgren agar and incubated anaerobically at 37°C. Some of the spores were then inoculated again onto copper after being exposed to the sodium taurocholate and then cultured onto Wilkins Chalgren agar. This study found that copper did have a reduced number of vegetative cells after 30 minutes. Contrastingly, Wheeldon *et al.* (2008b) found copper had no effect to *C. difficile* spores not exposed to sodium taurocholate, however, the spores, from both strains, which were exposed to the bile salt had a lower number of viable cells on the copper than the stainless steel after 60 minutes. This suggests that the copper has antimicrobial effects on germinating spores. These results can be translated into real-world applications as spores which have been through the body have been exposed to bile salts which aids germination, therefore if copper surfaces were used in hospitals it is likely to reduce the spread of *C. difficile* (Wheeldon *et al.*, 2008a; Wheeldon *et al.*, 2008b).

1.2. Aim and Objectives

The main aim of this study is to characterise *in vitro* factors that may contribute to the virulence of PCR ribotype 078 and comparator strains of PCR ribotype 027 and 002.

- Growth and toxin production for each PCR ribotype (over 72 hours) will be compered by producing batch culture growth curves and acquiring toxin samples for Vero cell cytotoxicity assays.

- The susceptibility for each PCR ribotype to eight antimicrobial agents will be tested using the agar incorporation method (AIM) to produce minimum inhibitory concentration (MIC).

- The biofilm growth (over six days) for each PCR ribotype will be measured using 96-well microtitre plates assay.

- The biofilm MIC (bMIC) and minimum biofilm eradication concentration (MBEC) for each PCR ribotype will be investigated using Calgary biofilm device (CBD) assay for metronidazole and vancomycin.

- Viable count and spore count within biofilms for each PCR ribotype will be retrieved using 96-well microtitre plates (over six days) and CBD assay (over four days)

1.3. General Methods

1.3.1. Preparation of *C. difficile* strains

Human clinical *Clostridium difficile* strains of PCR ribotypes 078, 027 and 002, as well as, two control strains: E4 (PCR ribotype 010) and ATCC 700057 (VPI 11186) (PCR ribotype 038), were assessed in all studies. All clinical strains were isolated from the Leeds Teaching Hospitals NHS Trust and supplied courtesy of Professor Mark Wilcox, who retrieved them from multiple areas in the UK (Table 1.2).

C. difficile strains were initially cultured on Brazier's agar (CM0601, Oxoid, Basingstoke) supplemented with 5 mg/L lysozyme (62971-10G-F, Sigma-Aldrich, Gillingham) and 2% defibrinated horse blood (SR0050, Oxoid, Basingstoke), lysed with saponin (47036-50G-F, Sigma-Aldrich, Gillingham), which were incubated in an anaerobic cabinet at 37°C for two days. Once the colonies had grown, they were inoculated onto pre-reduced Columbia agar with 5% whole defibrinated horse blood and incubated in the anaerobic cabinet at 37°C for seven to 10 days for sporulation to occur. Once spores were produced they were harvested and mixed into a 50% ethanol 50% saline solution ($\sim 10^6$ spores/ml). The solution was left at room temperature for an hour to kill any vegetative cells and leave the spores in solution; then the spore solution was then stored at 4°C (Freeman *et al.*, 2003).

When needed, 10-20 µl of spore solution was inoculated on Brazier's agar (with supplements) for one to three days in an anaerobic cabinet at 37°C until the spores had germinated and grown into colony forming units (CFU).

1.3.2. Statistical analysis

All statistical analysis was conducted using IBM® SPSS® Statistics Version 22/23. Data were tested for normality using the 'Explore tool' and the Shapiro-Wilk test results were interpreted. If the data was normally distributed a one-way ANOVA test was conducted using the 'one-way ANOVA tool' or a two-way ANOVA test was conducted using the 'Univariate tool'. If the results were not normally distributed a Kruskal-Wallis test was conducted using 'K Independent Samples…' tool. A significant difference was considered as a P-value of ≤ 0.05.

Table 1.1. *C. difficile* isolates used in this study, with PCR ribotype, year of isolation and location of isolation.

Isolate Number	Strain Number	PCR Ribotype	Year of Isolation	Location of Isolation
1	24	"078"	2012	Leeds
2	31	"078"	2012	Derriford
3	32	"078"	2012	Derriford
4	45	"078"	2012	Cumberland
5	46	"078"	2012	Cumberland
6	47	"078"	2012	Cumberland
7	48	"078"	2012	Cumberland
8	59	"078"	2012	Sheffield
9	60	"078"	2012	Sheffield
10	61	"078"	2012	Southampton
11	62	"078"	2012	HPA SE
12	9	"027"	2012	Bradford
13	10	"027"	2012	Bradford
14	11	"027"	2012	Bradford
15	12	"027"	2012	Bradford
16	19	"027"	2012	Bath
17	20	"027"	2012	Bath
18	35	"027"	2012	Leeds
19	36	"027"	2012	Leeds
20	37	"027"	2012	Airedale
21	38	"027"	2012	Bradford
22	41	"027"	2012	Barnsley
23	1	"002"	2012	Ireland
24	4	"002"	2012	Ireland
25	5	"002"	2012	Ireland
26	6	"002"	2012	Ireland
27	39	"002"	2012	Cumberland
28	40	"002"	2012	Cumberland
29	53	"002"	2012	Leeds
30	54	"002"	2012	Leeds
31	66	"002"	2012	Harrogate
32	68	"002"	2012	Harrogate
33	71	"002"	2012	Maidstone
Control Strains				
E4	110	"010"	-	-
ATCC 700057 (VPI 11186)	111	"038"	-	-

2. Comparing the Growth Rate and Cytotoxin Production Rate of *Clostridium difficile* PCR Ribotypes 078, 002 and 027

2.1. Introduction

There are four stages of bacterial growth in batch culture: lag phase, exponential phase, stationary phase and death phase. The duration of these phases may differ between microorganisms but the shape of the curve is likely to be similar. The lag phase is where there is no or little cell division, the exponential phase (also called the log or growth phase) is where cellular reproduction occurs, the stationary phase is where the growth rate and death rate are at equilibrium, and the death phase is where the death of cells exceeds cellular reproduction (Tortora *et al.*, 2004). Bacterial pathogens may express virulence factors at different stages within their growth cycle, indeed even bacteria of the same genus and/or species may be heterogeneous with respect to when toxins are released (Drummond *et al.*, 2003a; Vohra and Poxton, 2011). Consequently, studies assessing heterogeneity of virulence factor expression between members of the same species are important in that they may help to elucidate differences between strains which may have an impact on the pathogenicity/virulence of the organism *in vivo*.

Clostridium difficile is a mesophilic bacterium, therefore, this bacterium survives optimally in moderate-temperature environment and has been reported to have an optimum range of 30°C to 37°C (Karlsson *et al.*, 2003; Rodriguez-Palacios *et al.*, 2012; Tortora *et al.*, 2004). As with all of the *Clostridium* species, *C. difficile* is able to sporulate in response to adverse environmental stimuli (nutrient limitation, detergents, or antibiotics, etc.). Indeed, temperature has been demonstrated to significantly affect *C. difficile* spore germination, with germination rate significantly higher at 37°C than at 20°C (Wheeldon *et al.*, 2008a). Kazamias and Sperry (1995) demonstrated the pH of the culture medium is important in determining *C. difficile* growth, which was optimal in an alkaline medium. Consequently, for an organism like *C. difficile* that inhabits the human colon (pH range 5.7-6.7) the optimal pH for growth may affect the site at which infection and subsequent disease occurs (Fallingborg, 1999).

There are now over 600 PCR ribotypes of *C. difficile*, however only some are toxigenic and contains a 19.6KB pathogenicity locus (PaLoc), while non-toxigenic strains contain a non-coding 115-bp region (Braun *et al.*, 1996; Fluit *et al.*, 1991; Janezic *et al.*, 2012; O'Connor *et al.*, 2009). The PaLoc involves five genes, which produce, regulate or facilitate the release of toxins. The *tcdA* gene encodes toxin A (TcdA), the *tcdB* gene encodes toxin B (TcdB), the *tcdR* gene encodes a sigma factor that positivity regulates toxin production, the *tcdC* gene product putatively negatively regulates toxin production, and the *tcdE* gene product facilitates toxin A and toxin B release out of the cell. However, *C. difficile* also secretes its toxins when cell lysis occurs (Curry *et al.*, 2007; Govind and Dupuy, 2012; Lyras *et al.*, 2009; Matamouros *et al.*, 2007; O'Connor *et al.*, 2009; Olling *et al.*, 2012). The *Spo0A* gene is also involved in toxin productionn but is not part of the PaLoc (Underwood *et al.*, 2009). TcdA and TcdB are the major virulence factors of *C.*

difficile and are both glycosylating toxins comprised of four domains: an activity (A) domain, a cutting (C) domain, a delivery (D) domain and a binding (B) domain (Carter *et al.*, 2010; Curry *et al.*, 2007; Shen, 2012). The toxins enter the host cell by binding to receptors on the cell surface and it is thought that TcdA and TcdB bind to different receptors, which subsequently triggers uptake of the toxins via endocytosis (Shen, 2012). Endosomal acidification then occurs which prompts the translocation of the A domain of the toxins into the cytosol which then binds to and glucosylates Rho GTPases. Consequently, the Rho GTP-GTD switching is disturbed which causes the actin cytoskeleton to disassemble, the cell to become rounded, and apoptosis to occur (Shen, 2012). These toxins are believed to have different functions in CDI, TcdA is thought to be involved more in intestinal damage whereas TcdB induces systemic intoxication and is thought to be more cytotoxic than toxin A (up to 1000-fold) (Aktories, 1997; Qa'Dan *et al.*, 2000). Some strains only produce TcdB, these have both the toxin genes, within the PaLoc, however there is a 5.9 kb deletion within the 3' end of the *tcdA* and *tcdC* genes, this prevents production of the TcdA protein (Drudy *et al.*, 2007; Soehn *et al.*, 1998). Strains which produce toxin B but not toxin A have been involved in case reports (Limaye *et al.*, 2000) and in outbreaks worldwide (van den Berg *et al.*, 2004).

The *tcdR* gene, which encodes for the TcdR protein, positively regulates the production of the two toxins, is auto-activated by different environmental changes, such as temperature or culture medium, during the exponential phase of growth (Karlsson *et al.*, 2003; Matamouros *et al.*, 2007; O'Connor *et al.*, 2009). Contrastingly, TcdC (encoded by the *tcdC* gene) putatively negatively regulates the production of the two toxins by interacting with the *tcdR* gene (Murray *et al.*, 2009). It is uncertain if TcdC does truly negativity regulate toxin production, however when bacterial growth enters stationary phase the expression of transcription of the *tcdC* gene stops, and toxin production increases (Matamouros *et al.*, 2007). The *tcdE* gene encodes for the holin-like membrane protein, TcdE, which aids the secretion of toxin A and B out of the bacterial cell, however, unlike the *tcdE* gene in other bacteria, such as *Escherichia coli*, the *C. difficile tcdE* gene does not cause cell damage (Govind and Dupuy, 2012; O'Connor *et al.*, 2009). Another locus which could also influence toxin production is the accessory gene regulator (agr) locus, which has been found in *C. difficile* PCR ribotypes, including PCR ribotype 027 (Carter *et al.*, 2014; Martin *et al.*, 2013; Stabler *et al.*, 2009). AgrA was previously found to positively regulate the production of TcdA in PCR ribotype 027 strains (Darkoh *et al.*, 2015; Martin *et al.*, 2013).

As well as being involved in sporulation, the Spo0A protein may also be involved in toxin regulation (Underwood *et al.*, 2009). Underwood *et al.* (2009) found when the *spo0A* gene was inactivated when there was a reduction in toxin production. Whereas, Rosenbusch *et al.* (2012) concluded the Spo0A protein had a negative effect on toxin production. Both used the *C. difficile* 630Δerm strain, with the *spo0A* gene manipulated so it was active or inactive (or over active). Mackin *et al.* (2013) used a wild-type strains of PCR ribotype 027 (M7404 and R20291 strains) and strains with a mutated *spo0A* and, when observed, found an increase in toxin production (both toxin A and B) in the mutant strains.

There are many variables which can affect cytotoxin production, for example: temperature, indeed Onderdonk *et al.* (1979) found, using a diploid human WI-38 cells cytotoxin assay, that an increase in temperature from 37°C to 45°C elevated

cytotoxin production 1000-fold, however, Lei and Bochner (2013) compared *C. difficile* cytotoxin production between 22°C and 42°C, using a cytotoxin assay (using HT29, human lung adenocarcinoma (A549), Vero, and Chinese hamster ovary (CHO)-k1 cell lines), and observed that cytotoxin production was highest at 37°C. Interestingly, this may be because the *tcdR* gene auto-activates at 37°C (Karlsson *et al.*, 2003). Additionally, culture medium pH is another variable which may influence cytotoxin production. Onderdonk *et al.* (1979) demonstrated that a difference between pH 4 and pH 8 did not affect *C. difficile* cytotoxin production, however, Barth *et al.* (2001) reported that pH does influence the uptake of TcdB into cells. Barth *et al.* (2001) demonstrated that in pH 4.5 medium uptake of TcdB into CHO cells was greater when compared to a pH 7.5 medium.

2.2. Aims and Objectives

The aim of this experiment is to characterise and compare growth rate and cytotoxin production by *C. difficile* PCR ribotypes 078, 027 and 002.

- This will be achieved by performing batch cultures of 11 *C. difficile* strains of each PCR ribotype and recording their growth profiles over a 48 hour period and also calculating maximum specific growth rates (μ_{max}) between 2 to 6 hours.

- Toxin samples will be semi-quantified at 2 hour intervals over 72 hours using a Vero cell cytotoxicity assay.

2.3. Materials and Methods

2.3.1. *C. difficile* strains

All strains were prepared using the methods stated in Section 1.2.1.

The batch culture growth curves and μ_{max} experiment (Section 2.3.3.) used all 11 human clinical strains of each PCR ribotypes 078, 027 and 002 in triplicate.

The Vero cell cytotoxicity assays (Section 2.3.4.) incorporated four human clinical strains of each PCR ribotypes 078, 027 and 002 in duplicate.

2.3.2. *C. difficile* Batch Culture Preparation

C. difficile colonies were swabbed from the Braziers agar plates into pre-reduced Brain Heart Infusion broth (CM1135, Oxoid, Basingstoke) supplemented with 0.1% (w/v) L-cysteine hydrochloride (C1276-50G, Sigma-Aldrich, Gillingham) and 0.5% (w/v) yeast extract (92144-500G-F, Sigma-Aldrich, Gillingham) (BHIS broth) (Dawson *et al.*, 2012), and left to grow overnight in an anaerobic cabinet at 37°C. The overnight culture was inoculated into fresh BHIS, to give a standardised starting OD_{600} of 0.1.

2.3.3. Preparation of Batch Culture Growth Curves and Maximum Specific Growth Rate Calculation

Batch cultures were prepared using methods in Section 2.3.2. Samples were aseptically removed from each of the batch cultures, to record the OD_{600} at 2, 4,

5, 6, 24, 48 and 72 hours. To ensure accuracy, if the OD_{600} was above 0.700 the sample was diluted with sterile BHIS to ensure that readings fell within the linear range for the spectrophotometer (CE2021, Cecil, 2000 series) and the dilution factor accounted for when calculating the actual OD_{600}. μ_{max} (h^{-1}) was calculated from the exponential phase (between 2 and 6 hours) of *C. difficile* growth by determining the gradient (trend line) of the biomass natural log (ln(biomass)) versus time (hours) plot in triplicate.

2.3.4. Vero Cell Cytotoxicity Assay

2.3.4.1. Preparation of Growth for *C. difficile* Cytotoxin Production

To investigate the cytotoxin production profiles, *C. difficile* PCR ribotypes were prepared using methods in Section 2.3.2. The batch cultures were assayed at two hour intervals between 2 and 24 hours and the OD_{600} was also determined at each interval. Samples were also taken at 48 and 72 hours (from Section 2.3.3.). All cytotoxin samples were centrifuged (13000 rpm for 10 minutes) and the supernatants was removed and stored at 4°C for retrospective cytotoxin testing. All assays were performed in duplicate.

2.3.4.2. Vero Cell Preparation

Vero cells (84113001, African Green Monkey Kidney Cells, PHE culture collections) were retrieved from liquid nitrogen and slowly thawed out in 5% CO_2 at 37°C. The thawed Vero cells were cultured in 25 cm^3 flasks containing Dulbecco's Modified Eagles Medium (D6546-Sigma-Aldrich, Gillingham), supplemented with 10% (v/v) new-born calf serum (N4637-Sigma-Aldrich, Gillingham), 1% (v/v) antibiotic/antimycotic solution (A5955, penicillin 100 U/ml, 100 mg/L streptomycin, 0.25 mg/L amphotericin, Sigma-Aldrich, Gillingham) and 1% (v/v) L-glutamine (G7513-Sigma-Aldrich, Gillingham) (DMEM) and incubated in 5% CO_2 at 37°C until a confluent monolayer was visible when examined under an inverted microscope (Olympus CKx41) (Figure 2.1.A). Once confluent, the Vero cell monolayer was passaged three times before use in the experiment in order that the optimal growth rate could be obtained (Ammerman *et al.*, 2008).

Passaging of Vero cells was conducted by removing DMEM from confluent monolayers and adding 0.5 ml of Hanks Balanced Salt Solution (H9394-Sigma-Aldrich, Gillingham) supplemented with 0.25 g/L trypsin-EDTA (T4174-Sigma-Aldrich, Gillingham) (HBSS-trypsin-EDTA). HBSS-trypsin EDTA was agitated over the monolayer for 30 seconds to remove loosely adherent/dead cells, this was then discarded. A further 3 ml of HBSS-trypsin-EDTA solution was added to the 25 cm^3 flask and incubated in 5% CO_2 for 10 minutes until the monolayer was no longer adherent. Trypsinised Vero cells were diluted into fresh supplemented DMEM such that the resultant cell concentration elicited a confluent monolayer after 24 hours (1:10 dilution) or 48 hours (1:20 dilution) and then were incubated in 5% CO_2 at 37°C until confluent (Figure 2.1.A.).

2.3.4.3. Preparation of Vero Cell Cytotoxicity Assay Microtitre Plates

Trypsinised and diluted Vero cells were aliquoted into 96-well flat bottomed microtitre plates (FB56412, Fisher Scientific,) and incubated in 5% CO_2 at 37°C until a confluent monolayer was visible under the inverted microscope (Figure 2.1.A.). *C. difficile* culture supernatants were serially 10-fold diluted in sterile PBS

in 96-well microtitre plates (FB56412, Fisher Scientific,) to 10^{-5} and 20 µL was inoculated into each appropriate well of the microtitre plate (Figure 2.2.). Additionally, 20 µl of *C. sordellii* antitoxin (PL6508, Pro-Lab, Bromborough) was aliquoted into one well per dilution series (row B) which contained the undiluted *C. difficile* culture supernatant. Microtitre plates were then incubated in 5% CO_2 at 37°C for 24 and 48 hours and examined under the inverted microscope. A positive cytopathic effect (CPE) was determined in microtitre plate wells that demonstrated ≥50% Vero cell are rounding (Figure 2.1 B.). Cytotoxin titres were expressed as $\log_{10}$ relative units (RU) where rounding in the undiluted culture supernatant was scored 1 RU, rounding in the 10^{-1} dilution scored 2 RU etc. Neutralisation of the toxin by the *C. sordellii* antitoxin was required in order to confirm the specificity of the CPE. All assays were performed in triplicate, the median cytotoxin titre for each PCR ribotype was then calculated.

2.3.5. Statistical Analysis

For the cytotoxin assay results the Kruskal-Wallis statistical test was used. The Two-way ANOVA statistical test was used to analyse the difference between the growth for each PCR ribotype and time-point. The μ_{max} results used a one-way ANOVA to test the significance. A P-value of ≤0.05 was considered statistically significant.

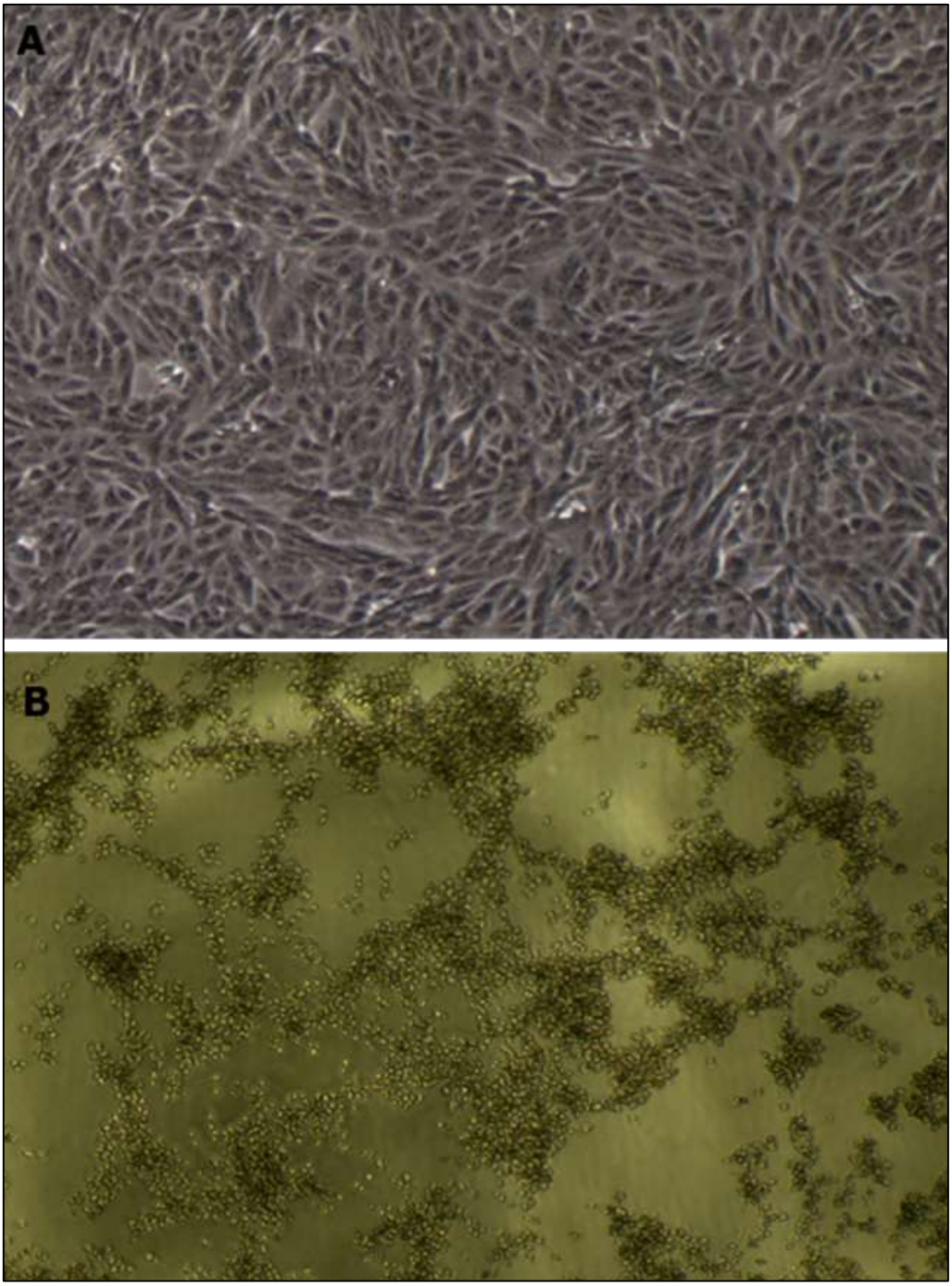

Figure 2.1. Vero cells were grown using supplemented Dulbecco's Modified Eagles Medium (DMEM) then passaged using HBSS-trypsin-EDTA; **A)** Confluent monolayer of Vero cells; **B)** Cytopathic effect caused when *C. difficile* toxin was added (observed at 100X magnification under an inverted microscope).

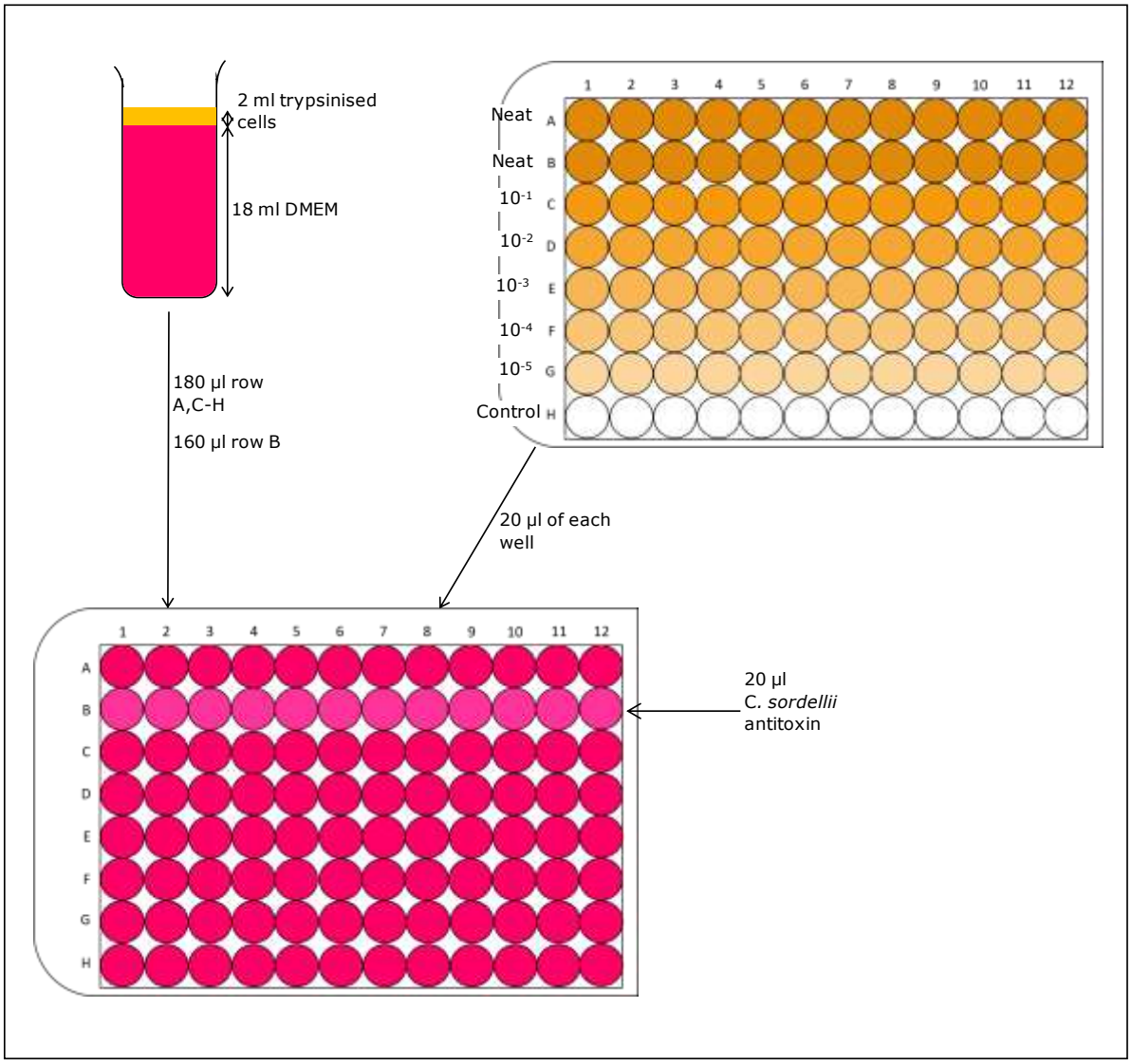

Figure 2.2. Vero cell culture cytotoxicity assay for determining *C. difficile* cytotoxin titres. *C. difficile* toxin was diluted from a 10-fold dilution series into to Vero cells in a 96-well microtitre plate (after the Vero cells had grown in supplemented Dulbecco's Modified Eagles Medium (DMEM)). A dilution with ≥50% cell rounding was classed as 1 titre (relative unit, RU). *C. sordellii* antitoxin was added to row B to neutralise *C. difficile* cytopathic effect.

2.4. Results

2.4.1. Batch Culture Growth Curves and Maximum Specific Growth Rate Calculation

The μ_{max} varied between the PCR ribotypes. PCR ribotype 002 had the highest mean μ_{max} (0.73 h^{-1}) whereas PCR ribotype 078 had the lowest (0.53 h^{-1}). There was a significant difference between all three PCR ribotypes (PCR ribotype 027 μ_{max}: 0.64 h^{-1}) ($P \leq 0.001$) (Appendix 1.2). The range of μ_{max} of strains, within the PCR ribotype groups, overlapped (Appendix 1.5).

Between 0-2 hours PCR ribotype 078 had a significant increase in biomass (0 hours: 0.11 OD_{600}, 2 hours: 0.31 OD_{600}) ($P \leq 0.001$), whereas, PCR ribotype 027 (0 hours: 0.09 $_{OD600}$, 2 hours: 0.11 OD_{600}) ($P=0.15$) and 002 (0 hours: 0.09 OD_{600}, 2 hours: 0.1 OD_{600}) ($P=0.15$) were similar, this suggests that PCR ribotype 078 has a shorter lag phase. The exponential phase (between 2 and 6 hours) showed PCR ribotype 078 had significantly higher average biomass readings at 6 hours (2.44 OD_{600}) than PCR ribotype 027 (1.39 OD_{600}) ($P \leq 0.001$) and 002 (2.10 OD_{600}) ($P \leq 0.001$) (Figure 2.3.). Between 8 and 12 hours could be defined as the death phase, as there was a dramatic decrease in biomass between these two time points for all three PCR ribotypes (Appendix 1.3). After 12 hours the OD_{600} readings plateaued for PCR ribotype 078 (72 hours: 1.33 OD_{600}), whereas PCR ribotype 027 gradually decreased between 12 to 72 hours (0.77 OD_{600}) and PCR ribotype 002 plateaued between 12 to 24 hours (1.82 OD_{600}) then decreased between 24 to 72 hours (1.08 OD_{600}) (Appendix 1.3).

It was observed that the overnight cultures for each PCR ribotype were variable in their OD_{600} readings. PCR ribotype 002 had noticeable higher overnight absorbance readings (average 2.17 OD_{600}) than either PCR ribotype 027 (average 1.79 OD_{600}) and 078 (average 1.85 OD_{600}) (results not shown). It was also observed, in BHIS medium, that PCR ribotype 078 showed more of a biomass pellet and the bottom of a culture tube than PCR ribotype 002 and 027 (Figure 2.4).

2.4.2. *C. difficile* Cytotoxin Production

Some strains of PCR ribotype 078 started to produce detectable cytotoxin between 2 and 24 hours, however most strains produced enough cytotoxin to induce some cell rounding (<50%) but not enough to elicit a titre of 1 RU (Appendix 1.5). At 48 hours the cytotoxin titres of the PCR ribotype 078 group ranged between 1 to 3 RU then increased to between 2 to 3 RU at 72 hour (Appendix 1.5). The PCR ribotype 027 group produced less than 1 RU of toxin between 2 to 10 hours, between 12 and 22 hours some strains produced 1 RU of toxin, then at 24 hours some strains produced more than 1 RU of toxin but not enough to be classed as 2 RU (Appendix 1.3). At 48 hours all PCR ribotype 027 strains produced 1 RU of toxin then increased to producing 1 to 2 RU of toxin after 72 hours. None of the PCR ribotype 002 strains produced detectable cytotoxin before 24 hours, after 24 hours of incubation some strains produced 1 RU, at 48 hours all strains produced 1 RU of toxin, and at 72 hours all strains produced between 1 and 2 RU of toxin (Appendix 1.3. and Appendix 1.5).

At 24 hours, median cytotoxin titres did not differ significantly between PCR ribotype 078 and 027 (1 RU) (P=0.31). PCR ribotype 078 had the largest variability in cytotoxin titres at 24 hours (0 to 2 RU), whereas, PCR ribotype 027 cytotoxin titres were all 1 RU at 48 hours PCR ribotype 078 median cytotoxin titres increased to 2 RU (range 1-3 RU), which was significantly higher than the median cytotoxin produced by PCR ribotype 027 and 002 (both: 1 RU) (P≤0.001).

2.4.3. Comparison of Geographical Location

The geographical location where each strain was isolated did not seem to correlate with growth rate or toxin production. PCR ribotype 078 showed little variation in μ_{max} and toxin production for strains from different locations. The PCR ribotype 027 isolates did have some variation, for example isolates from Bath (strains 19 and 20) had an average μ_{max} of 0.58 h^{-1} whereas the average μ_{max} for Leeds isolates (strains 35 and 36) was 0.69 h^{-1} (Appendix 1.5.). However there was no variation in toxin production, all isolates at all-time points had 1 RU (Appendix 1.5). Some PCR ribotype 002 isolates had similar results, for example the Leeds (strains 53 and 54) average μ_{max} was 0.76 h^{-1} and the Harrogate (strains 66 and 68) average was 0.75 h^{-1}, whereas some showed μ_{max} variation, for example the Cumberland (strains 39 and 40) average was 0.80 h^{-1} (Appendix 1.5). There was no variation between the locations for PCR ribotype 002 toxin production as all strains did not produce toxin at 2 hours and produce a titre of 1 RU at 24 and 72 hours (Appendix 1.5).

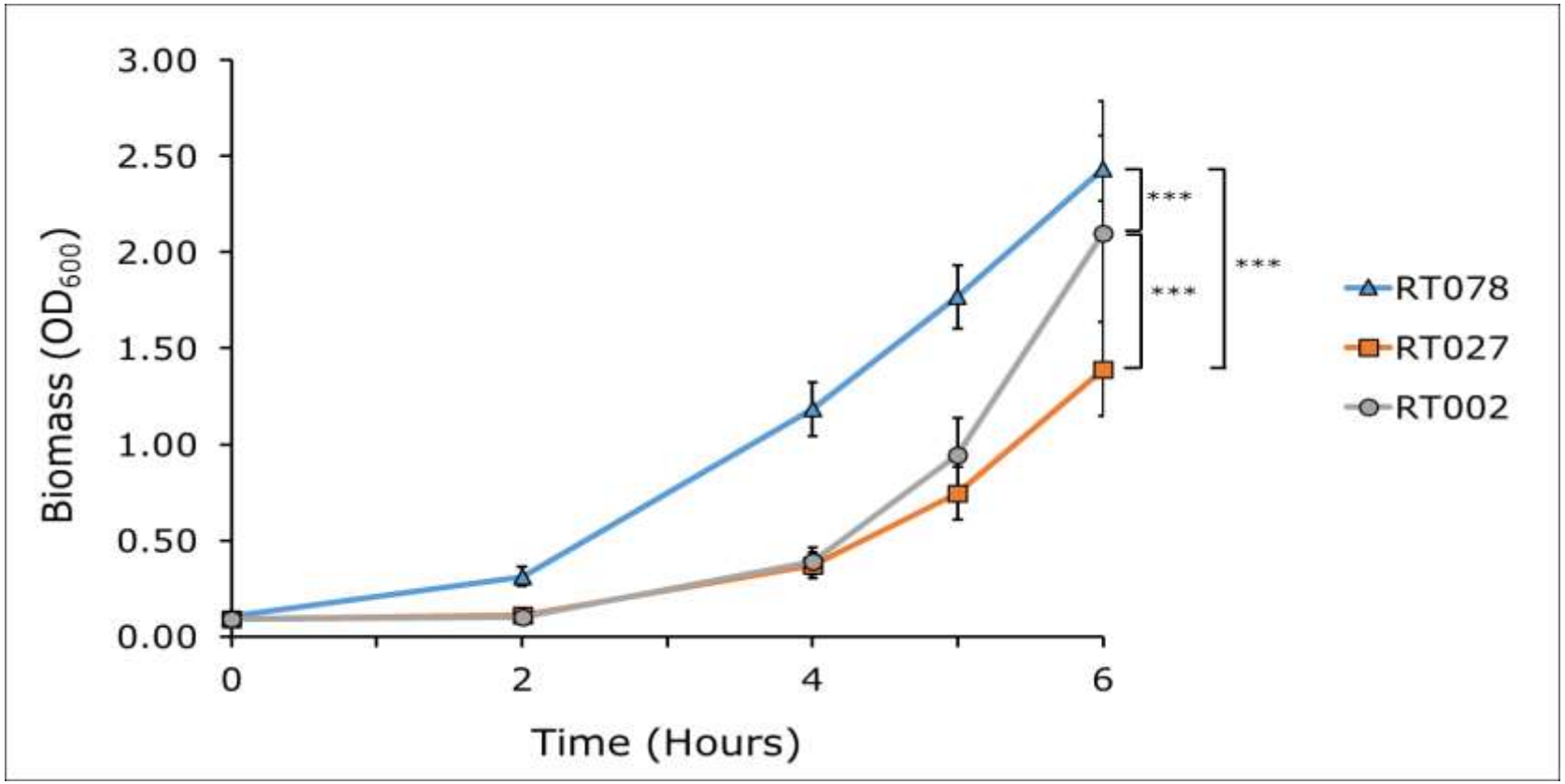

Figure 2.3. Growth curves of *C. difficile* PCR ribotypes (RT) 078, 027, and 002 (mean optical density $(OD)_{600}$ (±SE)) in supplemented Brain Heart Infusion broth over 6 hours in batch culture.

*** statistically significant difference P-value ≤0.001

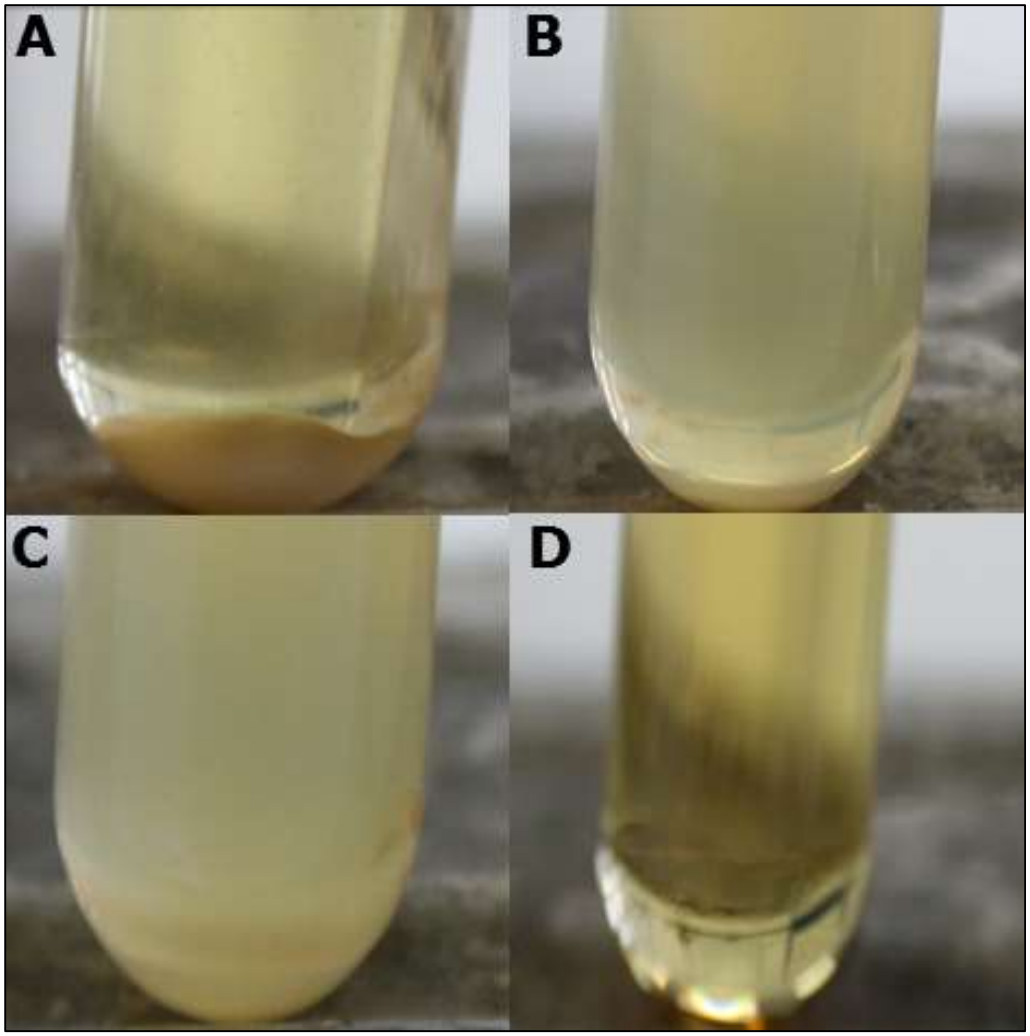

Figure 2.4. *C. difficile* PCR ribotypes cultured in supplemented Brain Heart Infusion broth broth overnight; **A)** PCR ribotype 078; **B)** PCR ribotype 027; **C)** PCR ribotype 002; **D)** blank broth.

2.5. Discussion and Conclusion

Some PCR ribotypes of *C. difficile* are pathogenic and contain the PaLoc. This locus contains five genes, two of which are the *tcdA* and *tcdB* genes, which encode toxin A (TcdA) and toxin B (TcdB) (Janezic *et al.*, 2012; O'Connor *et al.*, 2009). These toxins are the major virulence factors of *C. difficile*, and some PCR ribotypes which produce more toxin, therefore cause more severe illness, are said to be hypervirulent (Curry *et al.*, 2007; Goorhuis *et al.*, 2008; Knetsch *et al.*, 2011; Shen, 2012). Goorhuis *et al.* (2008) classified PCR ribotype 078 as hypervirulent after comparing clinical severity in patients infected with this PCR ribotype to severity observed in patients infected with PCR ribotype 027. However, this study did characterise virulence factors of PCR ribotype 078, for example cytotoxin production (Goorhuis *et al.*, 2008; Janezic *et al.*, 2012; PHE and CDRN, 2014; Shen, 2012). PCR ribotypes 078, 027, and 002 produce both TcdA and TcdB. The present study examined cytotoxin production (Section 2.2.4.) over 72 hours of four clinical isolates of *C. difficile* from each PCR ribotype using a Vero cell cytotoxicity assay (Appendix 1.3). Additionally, growth (for 11 strains per PCR ribotype) characteristics (Section 2.2.2.) over 48 hours were determined and compared between PCR ribotypes by assessing OD_{600} (Figure 2.3.).

The growth curves and μ_{max} varied of all three groups *C. difficile* PCR ribotypes throughout the experimental period of this study. The PCR ribotype 002 had the fastest μ_{max} (0.73 h^{-1}) compared to PCR ribotype 078 (0.53 h^{-1}) and 027 (0.64 h^{-1}) (Appendix 1.2). These results are similar to Carlson Jr *et al.* (2013),who found that PCR ribotype 027 had a slower growth rate to other PCR ribotypes (such as PCR ribotype 014).

Contrastingly, the observations of Moore *et al.* (2013) differed from the present study, for example, PCR ribotype 078 had lower absorbance readings than PCR ribotype 027 in Moore *et al.* (2013), however there was not a substantial difference between them both. Additionally, Moore *et al.* (2013) showed the growth curve of PCR ribotype 078 and 027 as having an exponential phase between approximately eight and 12 hours and a stationary phase between approximately 12 and 16 hours, following which biomass readings decreased. Whereas, in the present study the results for PCR ribotype 078 and 027 suggested the exponential phase was between two and eight hours. After eight hours average OD_{600} of all three PCR ribotype groups decreased and there was no obvious stationary phase. These results may differ from Moore *et al.* (2013), who started the growth curve with $1x10^3$ *C. difficile* spores, whereas the present study diluted a vegetative cell culture, therefore growth had already started when the readings were taken. Similarly to the present study, Vohra and Poxton (2011) showed the exponential phase of PCR ribotype 027, between zero and eight hours, with no noticeable lag phase. The stationary phase was between eight and 16 hours then, after 16 hours, the *C. difficile* entered its death phase. These results may differ, to the present study, because Vohra and Poxton (2011) inoculated spores into anaerobic incubation medium and waited until the OD_{600} reached 1.0 before taking measurements to create the growth curves, the present study used BHIS medium to produce an overnight culture of vegetative cells, which was diluted to produce an OD_{600} of 0.1, therefore it is likely that the *C. difficile*, in Vohra and Poxton (2011), had already reached the exponential phase when the measurements were taken, whereas the growth in the present study may have been delayed.

The cytotoxin production profile (Section 2.2.4.) for the *C. difficile* PCR ribotype 078 group differed to the profile of PCR ribotypes 027 and 002 assessed in this study. PCR ribotype 078 demonstrated increased cytotoxin production over the 72 hour period in this study, which was also observed by Mackin *et al.* (2013), using a Vero cell cytotoxicity assay. Some strains of PCR ribotype 078 in the present study started demonstrate measureable cytotoxin at two hours, however this may be due to residual toxin from the overnight cultures when they were diluted (Appendix 1.5). Contrastingly, PCR ribotypes 027 and 002 cytotoxin titres were lower than PCR ribotype 078 at 48 and 72 hours, and titres increased over the experimental period. Warny *et al.* (2005) suggested that PCR ribotype 027 produced quantitatively more toxins than a comparator group (toxinotype 0), however PCR ribotype 078 (toxinotype V) was not assessed. Vohra and Poxton (2011) used the *C. difficile* TOX A/B II kit to assess toxin production by PCR ribotype 027 over 24 hours. The authors observed substantially increased toxin production over 12 hours for PCR ribotype 027, and then a plateau between 12 and 24 hours. Similarly, in the present study, PCR ribotype 027, cytotoxin was produced before 12 hours (albeit not enough to be classed as 1 RU of toxin), between 12 and 24 hours some of the strains did produce measurable toxin, however at 24 hours all strains produced cytotoxin above the 1 RU quantification limit. Some PCR ribotype 002 strains started to produce cytotoxin at 18 hours (less than 1 RU), and quantifiable levels at 24 hours, as the *C. difficile* biomass plateaued (Appendix 1.3.). This is consistent with Drummond *et al.* (2003b) who showed that strains from different virulent PCR ribotypes followed a similar pattern. However this study used different methods to the present study and only measured TcdA production using a toxin A ELISA. Median cytotoxin titres for PCR ribotype 002 strains were lower at 24 hours to those of PCR ribotypes 078 and 027 and this may be due to the cells producing more toxin as there was not a significant difference between the OD_{600} of the three PCR ribotype groups. Similarly, at 48 hours the OD_{600} of PCR ribotype 078 and 002 were similar, whereas the OD_{600} for PCR ribotype 027 was lower. However, PCR ribotype 078 had higher toxin production than PCR ribotype 027; this may be due to there being more cells to produce the toxin. This could be the same at 72 hours as PCR ribotype 078 had the highest OD_{600} reading compared to PCR ribotype 002 and 027. PCR ribotype 002 started to produce toxin at 48 hours as the OD_{600} reading decreased, this may be due to the higher number of cells dying and breaking down, as the bacterial lysis occurs the toxin stored within was released (Olling *et al.*, 2012). These observations suggest that PCR ribotype 078 strains produce elevated levels of cytotoxin, even when compared to other strains which have been designated hypervirulent (PCR ribotype 027), which may potentially contribute to this PCR ribotypes reported hypervirulence (Goorhuis *et al.*, 2008).

There are other factors which could contribute to the reported elevated virulence of *C. difficile* PCR ribotypes 027 and 078. One such factor is a mutations within the *tcdC* gene, which negatively regulates cytotoxin production (Curry *et al.*, 2007; Matamouros *et al.*, 2007). PCR ribotype 027 possesses a mutated version of this gene, which has an 18-bp deletion and a single base pair deletion in the 117 position, resulting in a frame-shift in the gene which in turn produces a truncated TcdC protein (Curry *et al.*, 2007; de Boer *et al.*, 2010; Dupuy *et al.*, 2008; Murray *et al.*, 2009). Carter *et al.* (2011) reported a wild-type of PCR ribotype 027 started to produce cytotoxin at 24 hours, slightly decreases at 48 hours and then increases at 78 hour (Cartman *et al.*, 2010). Carter *et al.* (2011) also reported when genetically modified PCR ribotype 027 had non-mutated

version of *tcdC,* cytotoxin production was dramatically decreased at all-time points, suggesting the mutated *tcdC* gene does contribute to the control of toxin production. Cartman *et al.* (2012) came to a different conclusion, the study compared wild-type R20291 (which has the deletion in the 117 frameshift and the 18-bp deletion) and wild-type *C. difficile* 630Δerm (PCR ribotypes 012) (which has an intact *tcdC* gene) with four strains of R20291 with different versions of the *tcdC* gene: 1) one with base-pairs 61 to 653 (out of 680) in the open reading frame (ORF) deleted to produce an in-frame deletion; 2) a strain with the single bp deletion in the 117 position repaired; 3) a strain with the 18-bp deletion also repaired; and 4) a strain with the whole ORF replaced with the one in *C. difficile* 630Δerm, and two modified strains of *C. difficile* 630Δerm; 1) one with the base pair 61 to 672 (out of 699) deleted to create an in-frame deletion; and 2) another with a silent base pair in place of the in-frame deletion. The authors concluded there was no difference in cytotoxin production between these strains, regardless of *tcdC* status, therefore there is no link between the mutation in *tcdC* gene and toxin production in PCR ribotype 027 (Curry *et al.*, 2007; de Boer *et al.*, 2010; Dupuy *et al.*, 2008).

PCR ribotype 078 also has a mutation in the *tcdC* gene however it is a 39-bp deletion, but to date there have been no studies done to determine how this TcdC variant modulates cytotoxin production (Cartman *et al.*, 2010; Goorhuis *et al.*, 2008). The mutated *tcdC* of PCR ribotype 078 may potentially elicit less efficient negative regulation of *C. difficile* toxins than the comparative mutated *tcdC* of PCR ribotype 027, and therefore explain the elevated cytotoxin production observed in the present study. In order to prove this hypothesis, a similar study with PCR ribotype 078 which did not have a mutated *tcdC* would need to be performed.

In conclusion, the results of the present study contribute to our knowledge about factors that might enhance the apparent hypervirulent status of PCR ribotype 078. This is due to its increased toxin production compared to the hypervirulent PCR ribotype 027 and the virulent PCR ribotype 002. PCR ribotype 078 was also shown to produce increased peak biomass when compared to PCR ribotype 027 and 002 over an 8 hour period, but growth curves with additional time points after 8 hours would need to be performed in order to assess the reproducibility of these findings and also determine if comparator ribotypes reached the same peak biomass at a period between 8 and 24 hours (Appendix 1.3). Elevated cytotoxin production titres and also cytotoxin production earlier in the growth cycle could contribute to the virulence of *C. difficile* PCR ribotype 078 and elicit more severe disease in patients. The elevated μ_{max} of *C. difficile* PCR ribotype 002 in comparison to the reported hypervirulent ribotypes is interesting and warrants further study, especially since PCR ribotype 002 is now one of the most commonly isolated ribotypes in the UK (Prof. Mark Wilcox – personal communication).

3. Comparing Minimum Inhibitory Concentrations of *Clostridium difficile* PCR Ribotypes 078, 027 and 002

3.1. Introduction

Antimicrobial susceptibility testing is performed to characterise bacterial (and fungal) pathogens *in vitro*, to determine which antimicrobials (and concentrations) could possibly help treat bacterial infections *in vivo*. Indeed, susceptibility testing can also be used to see if antimicrobial resistance has evolved in bacteria which cause disease (Kahlmeter *et al.*, 2003). This study determined the MIC of eight antimicrobials against three *C. difficile* PCR ribotypes using an agar incorporation method (AIM).

Antibiotic properties of organisms have been known about since the Roman and Egyptian eras, as shown by traces of antibiotics found in skeletons from those eras (Aminov, 2010). Even though penicillin is widely thought to be the first antibiotic discovered by Alexander Fleming in 1929, by observing a fungus which "*most closely resembles [*Penicillium*]* rubrum", antibiosis was being observed before this discovery was made (Aminov, 2010; Fleming, 1929). William Roberts, in 1874, noticed *Penicillium glaucum* was not contaminated with bacteria easily. Correspondingly, this was later supported by John Tyndall, who noted that a measurement of broth rarely contained both bacteria and fungi at the same time (Wheat, 2001). Since then microbiologists, such as Fleming, Schmith and Reymann, developed the methods to determine the susceptibly of the organisms to these antimicrobial agents (Fleming, 1929; Wheat, 2001). Fleming (1929) was one of the first to use methods to determine MICs (although the term was not used), using broths containing different concentrations of penicillin and observing how it affected the growth of bacteria, such as *Haemophilus influenzae* (formerly *Bacillus influenzae*). In the 1940's Schmith and Reymann expanded the methods by incorporating sulphapyridine into agar to find the MIC of gonococci (Wheat, 2001). Subsequently, in the 1960s WHO published a report to standardise these methods. At present, in European organisations such as The British Society for Antimicrobial Chemotherapy (BSAC) and The European Committee on Antimicrobial Susceptibility Testing (EUCAST), have standardised different methods, however in 2016 BSAC will support the EUCAST method to acquiring breakpoints (BSAC, 2015; Wheat, 2001). Methods for assessing susceptibility to antimicrobial agents include: ellipsoidal methods (including E-test), disc diffusion testing, broth MIC testing, and AIM. All of these methods have their own advantages: E- testing is easier to use than AIM but may not detect reduced susceptibility to some antimicrobial agents, for example metronidazole, AIM can concurrently test multiple bacteria and identifies the MIC endpoints, disc diffusion methods can be used as a screening test and can be modified easily (Baines *et al.*, 2008; OIE, 2012; Varela *et al.*, 2008). There may be variability in antimicrobial susceptibility results regardless of the method employed whether in the clinical or research settings. For example, Baines *et al.* (2008) compared the MICs acquired from E-testing and AIM and found *C. difficile* isolates had a geometric mean MIC for metronidazole of 9.19 mg/L using AIM and 3.12 mg/L using E-testing (both using Wilkins Chalgren base agar); therefore AIM produce higher results than E-testing (Baines *et al.*, 2008; OIE, 2012). Triple-stage chemostat gut models are

used to replicate the environment within the gut, therefore, it is a good *in vitro* indicator of how *C. difficile* is affected by the incorporation of the antibiotics in a gut environment (*in vivo*) (Baines *et al.*, 2009; Macfarlane *et al.*, 1998). These methods have been used to study antimicrobial resistance in the gut microflora and *C. difficile*. Other methods have been developed to find genetic mechanisms of resistance. One of the advantages of using the genetic methods, such as PCR, is that they can be faster in the detection of resistance markers, so can be used to find an appropriate treatment for emergency cases (Courvalin, 1991).

It is common practice to use antimicrobial breakpoints to determine the susceptibility of bacteria to antimicrobials (Angeby *et al.*, 2012; Jorgensen and Ferraro, 2009). Committees, such as BSAC, EUCAST and the Clinical and Laboratory Standards Institute (CLSI), define these breakpoints (Angeby *et al.*, 2012; BSAC, 2013; CLSI, 2007; EUCAST, 2014; Kahlmeter, 2014). One of the factors which go towards determining the breakpoint is to find the Epidemiological cut-off (ECOFF) values which are determined by compiling the MICs of wild-type strains (defined as strains with no resistance mechanisms (mutational or phenotypically detectible)) from many different sources (Angeby *et al.*, 2012; Kahlmeter, 2014). The data collected goes towards the production of three antimicrobial susceptibility classifications: resistant, intermediate and susceptible. If a strain is deemed resistant to an antibiotic, it is unlikely to be inhibited by a dosage normally given in a clinical setting. An antibiotic-susceptible strain is highly likely to be treated with normal dosage use, whereas, with a strain of intermediate susceptibility it is unclear how successfully the normal dosage of antibiotic will affect the strain (Angeby *et al.*, 2012; Jorgensen and Ferraro, 2009). The disadvantage of using breakpoints is that there is debate of how reliable they are, for example BSAC breakpoints corresponds to the concentrations of antibiotics within the blood, whereas, European committees set their breakpoints lower than the breakpoints set by BSAC (Phillips, 2001). There is a difference of breakpoints around the world, for example, Ferraro (2001) reports there is an eight-fold difference between the MIC breakpoints for cefotaxime and ceftazidime used in USA (≤8 mg/L for both antibiotics) and UK (≤1 mg/L for both antibiotics), this could be the result of varying dosages and methods.

CDI can be contracted by a patient taking antibiotics, which inhibit other bacteria within the gut, allowing C. *difficile* to thrive (Nelson *et al.*, 2011). However, not all antibiotics cause CDI. Studies have been conducted to see which antibiotics maybe suitable for treatment of CDI. For example, in a clinical setting metronidazole and vancomycin are already used as antibiotic treatments for different severities of CDI (Al-Nassir *et al.*, 2008; Freeman *et al.*, 2007).

Metronidazole, a nitroimidazole compound, has been a major treatment for anaerobic bacterial infections, including *C. difficile* infection, since the 1960s (Edwards, 1993; Freeman *et al.*, 1997; Menendez *et al.*, 2002). Metronidazole inhibits bacterial DNA synthesis, to do this, first the compound has to become reduced within the cell. The reduced form of metronidazole oxidises DNA and results in the breakage of the DNA strands and ultimately death of the cell (Edwards, 1993; Menendez *et al.*, 2002; Sigeti *et al.*, 1983). Reduced susceptibility to metronidazole has been shown in strains of *C. difficile* including some strains of PCR ribotype 027, although the mechanism(s) of this reduced susceptibility remain to be determined (Chong *et al.*, 2014). This may be due to a group of nitroimidazole reductase (*nim*) genes, which has been found in a range

of anaerobic bacteria, such as *Bacteroides fragilis*. The *nim* gene product reduces nitrates left behind from the antibiotics into amino acids which are non-toxic to bacterial DNA (Chong *et al.*, 2014; Dubreuil and Odou, 2010). Additionally, some strains of *C. difficile* have been found to produce the RecA protein after metronidazole treatment, which aids in the repairing of DNA (Chong *et al.*, 2014). Other resistance mechanisms could involve mutations within the ferric uptake regulator (*fur*) gene and oxygen-independent coproporphyrinogen III oxidase (*hemN*) gene, which have been found in the genome of PCR ribotype 027 (Lynch *et al.*, 2013; Moura *et al.*, 2014). Lynch *et al.* (2013) found that some metronidazole resistant strains of *C. difficile* have mutations in the *fur* and *hemN* genes.

Vancomycin is used to treat more severe cases of CDI (Al-Nassir *et al.*, 2008). This antibiotic was discovered in 1953 as a glycopeptide compound produced by *Amycolatopsis orientalis* and 5 years later it started to be used to treat Gram-positive bacterial infections. Subsequently, in 1978 vancomycin was used to treat CDI (Freeman *et al.*, 2010; Heinlen and Ballard, 2010; Losey *et al.*, 2001; Nagarajan, 1991; Yim *et al.*, 2014). Vancomycin, like other glycopeptide antibiotics, binds to the terminal D-ala-D-ala residues of new peptidoglycan and its precursor lipid II within the bacterial cell wall. This inhibits peptidoglycan synthesis by isolating the substrate within D,D-transpeptidases which bonds peptidoglycan strands together (Nagarajan, 1991; Yim *et al.*, 2014). Vancomycin also separates the substrates from the enzyme transglycosylases, which transfers a pentapeptide subunit from the lipid II precursor to the cell wall. These two processes weaken the cell wall, affect cell division and results in cell death (Yim *et al.*, 2014). Vancomycin resistance was reported in *C. difficile* in Poland in 1991 and reduced susceptibility has been found to develop in some strains (*in vitro*) (Leeds *et al.*, 2013; Owens *et al.*, 2008). This may be due to a substitution of proline and the addition of leucine in the MurG protein, which is involved in peptidoglycan synthesis (Leeds *et al.*, 2013). Other Gram-positive bacterial species such as *Enterococcus* spp. have a range of *van* genes which produce peptidoglycan precursors with the reduced ability to bind to glycopeptide antibiotics (Chen *et al.*, 2013).

Two antibiotics which are part of the fluoroquinolone class are ciprofloxacin and moxifloxacin. This group of antibiotics are active against both Gram-negative and Gram-positive bacteria by targeting two enzymes involved in DNA replication (Goswami *et al.*, 2006; Hooper, 2000). DNA gyrase is usually targeted in Gram-negative bacteria, the enzyme catalyses the negative supercoiling of DNA, from positively supercoiled DNA to relaxed DNA, then relaxed DNA to negatively supercoiled DNA. Topoisomerase IV is typically targeted in Gram-positive bacteria, this enzyme also catalyses positively supercoiled DNA into relaxed DNA but can also separate linked daughter chromosomes after DNA replication (Collin *et al.*, 2011; Drlica and Zhao, 1997; Hooper, 2000; Rafii *et al.*, 2005; Redgrave *et al.*, 2014; Sriram *et al.*, 2006). Both enzymes have to bind to the DNA to break the strand so the DNA can be relaxed and unlinked, then joined back together during replication. Fluoroquinolones modify the topoisomerase enzymes so the release of the DNA is inhibited (Redgrave *et al.*, 2014). Ciprofloxacin has a second function in Gram-positive bacteria, the antibiotic forms ROS by breaking down the iron regulatory dynamics inside the bacteria and this results in cell death (Goswami *et al.*, 2006; Kohanski *et al.*, 2007). Resistance to fluoroquinolones in *C. difficile* has been associated with mutations within a region of DNA known as the quinolone

resistance-determining region (QRDR), which contains *gyr*A and *gyrB* (which encode for DNA gyrase). This region also contains pseudoautosomal (*par*) C and *par*E (encodes of topoisomerase IV) in other bacteria, such as *E. coli*, however *C. difficile* does not have these genes. This region inhibits the modification of the enzymes by the fluoroquinolones (Dridi *et al.*, 2002; Redgrave *et al.*, 2014; Rodriguez *et al.*, 2014; Solomon *et al.*, 2011). Both moxifloxacin and ciprofloxacin have been associated with increased risk of potentiating CDI (Borgmann *et al.*, 2010; O'Connor *et al.*, 2004).

Bacterial ribosomes are targeted by many antibiotics as they are the main structures in bacterial protein synthesis (Lambert, 2012). The ribosome consists of two subunits, 30S and 50S, to create a 70S structure. Each subunit has two sites: the 30S has aminoacyl-tRNA (A) site and peptidyl-tRNA (P) site, 50S has exit (E) site and P site. These sites help with the binding of tRNA and translocation in the ribosome (Fischer *et al.*, 2010; Selmer *et al.*, 2006). The 30S subunit joins to mRNA, fmet-tRNA, tRNA, methionine, GTP and three initiation factors (IF 1 to 3) to form the initiation complex, this joins to the 50S subunit, to initiate protein synthesis (Bozdogan and Appelbaum, 2004).

Linezolid is an oxazolidinone antibiotic which is used to treat Gram-positive bacterial infections. However, this antimicrobial agent is not currently used to treat *C. difficile* infections (Baines *et al.*, 2011). Linezolid binds to the 50S subunit within the bacterial ribosome, blocking the initiation complex from binding to the 50S subunit. Linezolid is also thought to prevent the formation of the initiation complex, which hinders the development of the 70S complex, therefore, inhibiting proteins synthesis (Bozdogan and Appelbaum, 2004; Diep *et al.*, 2012). Linezolid is used to treat infections where the bacteria are resistant to other antibiotics, such as infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) (Pelaez *et al.*, 2002). Pelaez *et al.* (2002) found linezolid was active against *C. difficile* strains which were resistant to metronidazole or had reduced susceptibility to vancomycin *in vitro*. Resistance to linezolid is rare, despite this, resistance has been found to occur in some strains of *C. difficile* (Ager and Gould, 2012; Baines *et al.*, 2011). In other Gram-positive bacteria, such as *S. aureus*, resistance has been found to occur due to a mutation within the drug target site of the 23S rRNA, part of the 50S subunit, which could be caused by a multidrug resistance (MDR) gene known as the chloramphenicol-florfenicol resistance (*cfr*) gene. The *cfr* gene encodes for an addition of a methyl group to the 23S rRNA which affects the binding of the antibiotics to the site (Ager and Gould, 2012; Chen *et al.*, 2013; Long *et al.*, 2006; Morales *et al.*, 2010; Steitz *et al.*, 2012).

Tetracycline, part of the tetracycline family, is a broad spectrum antibiotic which is produced naturally by *Streptomyces aureofaciens*, *S. rimosus*, and *S. viridofaciens*. This antibiotic is used to treat infections of both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001; Thaker *et al.*, 2010). This antibiotic is a group one tetracycline antibiotic, which means it has lower absorption than other tetracyclines within the body and is administered orally. Between 20 and 60% of the administered tetracycline is excreted in faeces, since tetracyclines can be absorbed by the stomach and duodenum before passing into the colon. This suggests only a small percentage of the antibiotic would get to the colon to be effective against the *C. difficile* (Agwuh and MacGowan, 2006). Like linezolid, tetracycline also binds to and inhibits the bacterial ribosome. However, tetracycline binds to the 30S subunit so the A site cannot bind to it, this inhibits

protein syntheses (Chopra, 2002; Griffin *et al.*, 2011; Thaker *et al.*, 2010). Tetracycline resistance in bacteria may be encoded by a range of genes designated *tet*, for example *tetL* and *tetM*. The *tetL* gene encodes for a membrane-bound efflux protein, which transport tetracycline out of the cell (Chopra and Roberts, 2001). The *tetM* gene encodes for a cytoplasmic ribosome protection protein, TetM, which binds to the A site of the ribosome to inhibit the binding of tetracycline (Burdett, 1996; Chopra and Roberts, 2001). Even through, tetracycline is a broad spectrum antibiotic it has been shown to have a low risk of causing CDI (Brown *et al.*, 2013).

Erythromycin and clindamycin are two antibiotics which are active via a similar mechanism. Erythromycin was discovered in 1952 as a product of *Streptomyces erythreus* and was classified as a member of the macrolide family (Mazzei *et al.*, 1993). Clindamycin is a lincosamide antibiotic, which is a derivative of a naturally occurring antibiotic produced by *S. lincolnensis*, known as lincomycin (Kulczycka-Mierzejewska *et al.*, 2012). Clindamycin is usually used to treat Gram-negative bacteria because it has high solubility in lipids, this allows it to penetrate through the lipid membrane of the bacteria. However, erythromycin is active against some Gram-positive and some Gram-negative bacteria, although it is unable to pass through the cell wall of some Gram-negative Bacilli (Douthwaite, 1992; Peters *et al.*, 1992; Tortora *et al.*, 2004). Both antibiotics bind within the nascent peptide exit tunnel, part of the 50S subunit, this dislocates peptidyl-tRNAs from the ribosome and blocks proteins which are produced by the ribosome (Gamerdinger and Deuerling, 2012; Tenson *et al.*, 2003). Erythromycin and clindamycin resistance is encoded by a range of *erm* genes, one in particular is the *ermB* gene. These genes add methyl groups to the 23S rRNA within the 50S subunit, inhibiting the binding of the erythromycin or clindamycin (Palmieri *et al.*, 2013; Rose *et al.*, 2012; Solomon *et al.*, 2011). Another range of genes, known as the macrolide efflux (*mef*) genes, are also associated with erythromycin resistance in *Clostridium* species, such as *C. perfringens*. This gene encodes for a membrane-bound efflux pump, which activity removes erythromycin from the bacterial cell (Leclercq, 2002; Poole, 2005; Soge *et al.*, 2009). Both the *ermB* and the *mefA* have been linked to transposons which can pass the genes to *C. difficile* strains, therefore it is possible for these genes to be part of the *C. difficile* genome (Soge *et al.*, 2009).

Antibiotic resistance in bacteria is an increasing problem worldwide which may soon make antibiotics obsolete (WHO, 2014). *C. difficile* PCR ribotypes have acquired resistance to some antibiotics, which has resulted in outbreaks across the world (McDonald *et al.*, 2005). Resistance to fluoroquinolones has developed in PCR ribotypes including 002, 027 and 078, and has been linked to CDI outbreaks caused by these PCR ribotypes (Goorhuis *et al.*, 2008; Solomon *et al.*, 2011; Vardakas *et al.*, 2012). For example, Borgmann *et al.* (2010) found a correlation between the increase of ciprofloxacin prescriptions and incidents of CDI. McDonald *et al.* (2005) found the development of fluoroquinolone resistance in PCR ribotype 027 isolates was connected to outbreaks in the USA. Resistance to fluoroquinolones, in animal isolates of PCR ribotype 078, has also resulted in a spread of PCR ribotype 078 in pig farms (Keessen *et al.*, 2013). Wiuff *et al.* (2011) found out of 101 PCR ribotype 002 isolates in Scotland only 1% were resistant to moxifloxacin. Clindamycin resistance also varies between different PCR ribotypes. Indeed, Tenover *et al.* (2012) found different PCR ribotypes isolates in North America with varying resistance to clindamycin. The study found 71.4% of PCR

ribotype 078 isolates, 47.5% of PCR ribotype 027 and 5.9% of PCR ribotype 002 were resistant to clindamycin (Tenover *et al.*, 2012).

3.2. Aim and Objective

To compare susceptibilities of *C. difficile* PCR ribotype groups 078, 027, and 002 to a range of antimicrobial agents.

- An Agar Incorporation Method (AIM) will be used to determine the MICs of eight antimicrobial agents against *C. difficile* PCR ribotypes 078, 027 and 002.

3.3. Materials and Methods

3.3.1. *C. difficile* strains

This experiment used 11 human clinical strains of *C. difficile* PCR ribotypes 078, 027 and 002, along with an internal control strain, E4 (PCR ribotype 010), and ATCC 700057 (VPI 11186) (PCR ribotype 038). The strains were prepared using methods in Section 1.2.1.

3.3.2. MIC Testing using Agar Incorporation Methods

AIM was used to determine the MIC of the eight antibiotics, the methods used were based on methods in O'Connor *et al.* (2008) and Freeman and Wilcox (2001). The antibiotics compared were: metronidazole (M3761-25G, Sigma-Aldrich, Gillingham), vancomycin hydrochloride (V2002-1G, Sigma-Aldrich, Gillingham), linezolid (PF-00184033, Pfizer, Surrey), erythromycin (E6376-25G, Sigma-Aldrich, Gillingham), tetracycline hydrochloride (T3383-25G, Sigma-Aldrich, Gillingham), clindamycin hydrochloride (PF-00344619-01, Pfizer, Surrey), moxifloxacin (06669292-SV0001HC, Bayer, Berkshire) and ciprofloxacin (11939800-SV00019P, Bayer, Berkshire).

A spore suspension of each *C. difficile* strain was inoculated onto supplemented Brazier's agar plates and placed into an anaerobic cabinet at 37°C for 24 hours. Four to five well-separated *C. difficile* CFUs were inoculated into 5 ml of pre-reduced Schaedler's anaerobic broth (CM0497, Oxoid, Basingstoke) and incubated in the anaerobic cabinet at 37°C overnight. Wilkins-Chalgren anaerobe agar (CM0619, Oxoid, Basingstoke) was made (to the manufacturer's instructions) for each concentration of antibiotic and two for control plates, then placed in an autoclave. A 2560 mg/L stock solution was made for each antibiotic, dissolved in distilled water (for erythromycin ethanol was used). The stock solution was then sterilised using a 0.22 µm syringe filter. The stock was used to make a 2-fold dilution series in sterilised distilled water (Table 3.1). Each dilution was mixed into a bottle of molten Wilkins-Chalgren agar (50^{o}C) and poured into sterile Petri dishes.

The agar was left to solidify and then dried in a microbiological safety cabinet for approximately 10 minutes. Each culture was diluted 1:10 in sterile pre-reduced saline solution in a sterile multipoint inoculator (Mast Uri™ Dot, Mast Diagnostics) block, so there was approximately 1x10^{4} CFU/spot. Each plate was inoculated using 1 µL/spot pins, making sure to inoculate one of the anaerobic and aerobic

controls at the start and at the end. In between each set of antibiotics the pins were dipped in ethanol and then flamed. The inoculated agar plates were incubated overnight at 37°C in the appropriate atmosphere. The plates were read and the MIC endpoint was determined as the lowest concentration of antimicrobial where a marked change in growth when compared to the control plates was observed.

Geometric mean MIC values were calculated using the 'geomean' function in Microsoft Office Excel 2013, to show variations between the different PCR ribotypes and antibiotics which may not be seen using the MIC_{50}, MIC_{90} and range (Davies, 1990). The MIC_{50} and MIC_{90} are the concentrations which will inhibit 50% and 90% of the bacteria isolates respectively, and were calculated by ordering the MICs in numerical order then using the equations from Schwarz *et al.* (2010) (below) to find the position of the value used for the MIC_{50} and MIC_{90}:

If number of strains (*n*) is an odd number then the MIC_{50} is the MIC at position X in the ordered MIC list, where X = number of strains (*n* + 1) x 0.5

If *n* is an even number, then X = *n* x 0.5.

For MIC_{90}, X = *n* x 0.9 (next whole number) etc.

3.3.3. Breakpoints

The breakpoints for each antimicrobial agent are shown on Table 3.2. These breakpoints were created by CLSI (2007) which determined breakpoints using the agar dilution method, BSAC (2013) which used disc diffusion method to determine the breakpoints (as *C. difficile* breakpoints were not presented the breakpoints for *Staphylococci* were used) and EUCAST (2014) which used breakpoints based on ECOFF values.

3.3.4. Statistical Analysis

The Kruskal-Wallis statistical test was used to assess the statistical significance of differences in MICs between *C. difficile* strains. A noticeable difference was classed as a more than one doubling dilution difference between MICs.

Table 3.1. The volumes of distilled water and antibiotic used to produce 10-fold dilutions for each antibiotic used in order to produce the desired concentrations of antimicrobials that were incorporated into Wilkins Chalgren agar plates.

Vol of Stock (mL)	Stock Conc (mg/L)	+	Vol H_20 (mL)	=	Resultant Conc (mg/L)	Dilution in Agar	Final Conc
5	2560	+	5	=	1280	10-fold	128
2.5	2560	+	7.5	=	640	10-fold	64
2.5	2560	+	17.5	=	320	10-fold	32
~12mL of 320mg/L stock remaining							
5	320	+	5	=	160	10-fold	16
2.5	320	+	7.5	=	80	10-fold	8
2.5	320	+	17.5	=	40	10-fold	4
~12mL of 40mg/L stock remaining							
5	40	+	5	=	20	10-fold	2
2.5	40	+	7.5	=	10	10-fold	1
2.5	40	+	17.5	=	5	10-fold	0.5
~12mL of 5mg/L stock remaining							
5	5	+	5	=	2.5	10-fold	0.25
2.5	5	+	7.5	=	1.25	10-fold	0.125
2.5	5	+	17.5	=	0.6	10-fold	0.06
~12mL of 0.6mg/L stock remaining							
5	0.6	+	5	=	0.3	10-fold	0.03

Table 3.2. Breakpoints used to determine if the *C. difficile* strain are resistant (R), susceptible (S) or in the intermediate range (I) for metronidazole (Met.), vancomycin (Van.), ciprofloxacin (Cip.), clindamycin (Clind.), erythromycin (Ery.), linezolid (Lin.), moxifloxacin (Mox.) and tetracycline (Tet.). These breakpoints were produced by CLSI (2007) which used the agar dilution method to determine the breakpoints, EUCAST (2014) which based the breakpoints on epidemiological cut-off values and BSAC (2013) which used disc diffusion method to determine the breakpoints (as *C. difficile* breakpoints were not presented the breakpoints for *Staphylococci* were used).

	S	I	R
CLSI			
Met.	≤8	16	≥32
Clind.	≤2	4	≥8
Mox.	≤2	4	≥8
Tet.	≤4	8	≥16
EUCAST			
Van.	≤2	-	>2
BSAC			
Cip.	≤1	-	>1
Ery.	≤1	2	>2
Lin.	≤4	-	>4

3.4. Results

3.4.1. Antimicrobial Susceptibilities: Comparison between PCR Ribotypes

The metronidazole geometric mean MIC for the PCR ribotype 078 group was 0.08 mg/L which was significantly less than the corresponding geometric mean MIC for PCR ribotype 002 strains (0.18 mg/L) (P≤0.01) and 027 (1.37 mg/L) (P≤0.001) strains. The PCR ribotype 027 groups metronidazole geometric mean MIC was significantly greater than that of PCR ribotype 002 group (P≤0.001) (Table 3.3.). The MIC_{50} for PCR ribotype 078 isolates was the lowest (0.125 mg/L) compared to that of the PCR ribotype 027 group (2 mg/L) and PCR ribotype 002 group (0.25 mg/L). The MIC_{90} for PCR ribotype 078 isolates (0.125 mg/L) was lower than PCR ribotype 002 strains (0.25 mg/L) and there was a marked difference between them and the MIC_{90} of PCR ribotype 027 group (4 mg/L). All the strains for each PCR ribotype were susceptible to metronidazole using breakpoints but isolates with MICs ≥2mg/L were above the ECOFF (Figure 3.1. and Appendix 1.5).

The vancomycin results (Table 3.3.) indicated that the geometric mean MICs were not significantly different. There were no substantial differences between vancomycin MIC_{50} (all were 0.5 mg/L) and MIC_{90} (the PCR ribotype 078 strains were 1 mg/L and the PCR ribotype 002 and 027 groups were both 0.5 mg/L). All the strains for each PCR ribotype were susceptible to vancomycin (Figure 3.1. and Appendix 1.5).

The ciprofloxacin geometric mean MICs were significantly lower for PCR ribotype 078 isolates (7.51 mg/L) compared to the PCR ribotype 027 group (68.16 mg/L) (P≤0.001) and the PCR ribotype 002 group (10.29 mg/ml) (P≤0.05). The difference between PCR ribotype 027 and 002 geometric mean MICs for ciprofloxacin were also statistically significant (P≤0.001). The ciprofloxacin MIC_{50} for each PCR ribotype were the same (8 mg/L) and MIC_{90} results for the PCR ribotype 078 strains (8 mg/L) were noticeably lower than the MIC_{90} for the PCR ribotype 027 isolates (>128 mg/L), but there was no marked differences between the PCR ribotype 078 and 002 groups (16 mg/L). All the strains for all the PCR ribotypes were resistant to ciprofloxacin (Figure 3.1. and Appendix 1.5).

The clindamycin results (Table 3.3.) showed PCR ribotype 002 had the significantly highest geometric mean MIC (3.31 mg/L) compared to PCR ribotype 027 (0.44 mg/L) (P≤0.001) and 078 (0.09 mg/L) (P≤0.001), and the highest MIC_{50} and MIC_{90} (both 8 mg/L). PCR ribotype 078 had the lowest geometric mean MIC (0.09 mg/L) (PCR ribotype 027: P≤0.01) and the lowest MIC_{50} (0.06 mg/L) and MIC_{90} (0.25 mg/L). The geometric mean for PCR ribotype 027 was 0.44 mg/L, the MIC_{50} and MIC_{90} were 0.5 mg/L. All PCR ribotype 078 and 027 strains were susceptible to clindamycin, whereas 64% of the PCR ribotype 002 strains were (Figure 3.1. and Appendix 1.5).

The geometric mean MIC results for erythromycin MIC for the PCR ribotype 027 group (128 mg/L) were significantly higher than PCR ribotype 078 (P≤0.001) and PCR ribotype 002 (P≤0.001). The MIC_{50} of the PCR ribotype 027 group was also 128 mg/L and the MIC_{90} was ≥128 mg/L. The geometric mean MICs for the PCR ribotype 078 and 002 groups were similar (P=0.07). All the strains for PCR ribotype 027 and 9% of strains of PCR ribotype 078 were resistant to

erythromycin, All PCR ribotype 002 strains and 91% of the PCR ribotype 078 strains were susceptible (Figure 3.1. and Appendix 1.5).

The geometric mean MIC for linezolid (Table 3.3.) for the PCR ribotype 078 group (0.73 mg/L) was significantly higher than the PCR ribotype 027 group (0.25 mg/L) ($P \leq 0.01$) but was similar to the PCR ribotype 002 group (0.5 mg/L) ($P=0.25$). The PCR ribotype 027 group geometric mean MIC was significantly lower than PCR ribotype 002 group geometric mean ($P \leq 0.05$). There was a marked difference between the MIC_{50} of the PCR ribotype 078 group (1 mg/L) and the PCR ribotype 027 isolates (0.25 mg/L) however there was not a marked difference between the PCR ribotype 002 strains (0.5 mg/L) and the other PCR ribotypes. There was a marked difference between the MIC_{90} for the PCR ribotype 078 strains (2 mg/L) and the MIC_{90} for the PCR ribotype 027 group (0.5 mg/L). All the strains from all PCR ribotypes were susceptible to linezolid (Figure 3.1. and Appendix 1.5).

The results for moxifloxacin (Table 3.3.) showed that the geometric mean MIC for the PCR ribotype 078 group was 1.07 mg/L, which was significantly lower than the geometric mean for the PCR ribotype 027 group (32 mg/L) ($P \leq 0.001$) and PCR ribotype 002 isolates (1.76 mg/L) ($P \leq 0.01$). The results for PCR ribotype 027 and 002 were also significantly different ($P \leq 0.001$). The MIC_{50} results were the same as the MIC_{90} results for each PCR ribotype. The MIC_{50} and MIC_{90} for the PCR ribotype 027 strains (32 mg/L) were the highest, whereas, there was no noticeable difference between the MIC_{50} and MIC_{90} results for the PCR ribotype 078 (1 mg/L) and 002 (2 mg/L) groups. All the PCR ribotype 002 strains and 91% of the PCR ribotype 078 were susceptible to moxifloxacin, 9% of the PCR ribotype 078 and all the PCR ribotype 027 strains were resistant (Figure 3.1. and Appendix 1.5).

The results for tetracycline (Table 3.3.) showed a significant difference between the geometric mean for PCR ribotypes 078 (0.93 mg/L) and 002 (0.06 mg/L) ($P \leq 0.05$) but not between PCR ribotype 027 (0.11 mg/L) and 078 ($P=0.06$) or PCR ribotype 002 and 027 ($P=0.62$). PCR ribotype 078 strains had the highest MIC_{50} and MIC_{90} results (both 8 mg/L). The PCR ribotype 002 isolates also had the same MIC_{50} and MIC_{90} (0.06 mg/L). Whereas, PCR ribotype 027 strains had a MIC_{50} of 0.06 mg/L and the MIC_{90} was 4 mg/L. All the strains for PCR ribotype 027 and 002 were susceptible to tetracycline, 45% of PCR ribotype 078 were also susceptible, whereas 55% were in the intermediate range of susceptibly (Figure 3.1. and Appendix 1.5).

3.4.2. Comparison of Geographical Location

The MIC results for the isolates of PCR ribotypes 078 were variable and seemed to be specific to locations of isolation for metronidazole and vancomycin, for example the Derriford isolates (strains 31 and 32) both had a metronidazole MICs of <0.03 mg/L and vancomycin MICs were both 0.5 mg/L (Appendix 1.5). Whereas, the Sheffield isolates metronidazole MICs were (strain 59) 0.125 mg/L and (strain 60) 0.03 mg/L and vancomycin MICs were both 0.5 mg/L (Appendix 1.5). The PCR ribotype 078 MIC results for the other antibiotics were similar, for example most of the isolates had a MIC of 8 mg/L for ciprofloxacin and 0.06 mg/L for erythromycin (Appendix 1.5). The PCR ribotype 027 isolates MICs showed there was no variability between the strains for some of the antibiotics, such as vancomycin (most strains were 0.5 mg/L), moxifloxacin (all strains 32 mg/L) and tetracycline (all strains >128 mg/L), whereas the metronidazole results showed

some variability but this was not location specific, for example of the four Bradford isolates, two strains (9 and 11) had a MIC of 2 mg/L and the other strain had MIC results of 1 mg/L (12) and 4 mg/L (10) (Appendix 1.5). The PCR ribotype 002 MIC results were not variable, for example the metronidazole MICs most of the MICs were 0.125 mg/L or 0.25 mg/L and the vancomycin results most strains were 0.5 mg/L (Appendix 1.5).

Table 3.3. Geometric mean minimum inhibitory concentration (MIC) (mg/L), MIC_{50} (mg/L), range (mg/L) and MIC_{90} (mg/L) of metronidazole (Met.), vancomycin (Van.), ciprofloxacin (Cip.), clindamycin (Clind.), erythromycin (Ery.), linezolid (Lin.), moxifloxacin (Mox.) and tetracycline (Tet.) for PCR ribotype (RT) 078, 027 and 002 groups (N=11 strains) using an agar incorporation method with Wilkins-Chalgren anaerobe agar and *C. difficile* cultures grown in Schaedler's anaerobic broth ($1x10^4$ CFU/plate).

* defined MIC end-points were difficult to determine due to re-growth at some antimicrobial concentrations for some strains.

Antimicrobial Agents	Geometric mean (mg/L)			MIC_{50} (mg/L)			Range (mg/L)			MIC_{90} (mg/L)		
	RT078	RT027	RT002	RT078	RT027	RT002	RT078	RT027	RT002	RT078	RT027	RT002
Met.	0.08	1.37	0.18	0.125	2	0.25	0.03-0.25	0.5-4	0.125-0.25	0.125	4	0.25
Van.	0.57	0.53	0.53	0.5	0.5	0.5	0.25-1	0.5-1	0.5-1	1	0.5	0.5
Cip.	7.51	68.16	10.29	8	8	8	4-8	16-≥128	8-16	8	≥128	16
Clind.	0.09	0.44	3.31	0.06	0.5	8	0.06-2*	0.06-1	0.5-8	0.25	0.5	8
Ery.	0.50	128.00	0.25	0.25	128	0.25	0.25-≥128	128-≥128	0.25	0.5	≥128	0.25
Lin.	0.73	0.25	0.50	1	0.25	0.5	0.03-2	0.03-0.5*	0.06-2	2	0.5	1
Mox.	1.07	32.00	1.76	1	32	2	0.25-8	32	1-2	1	32	2
Tet.	0.93	0.11	0.06	8	0.06	0.06	0.03-8	0.03-4	0.06	8	4	0.06

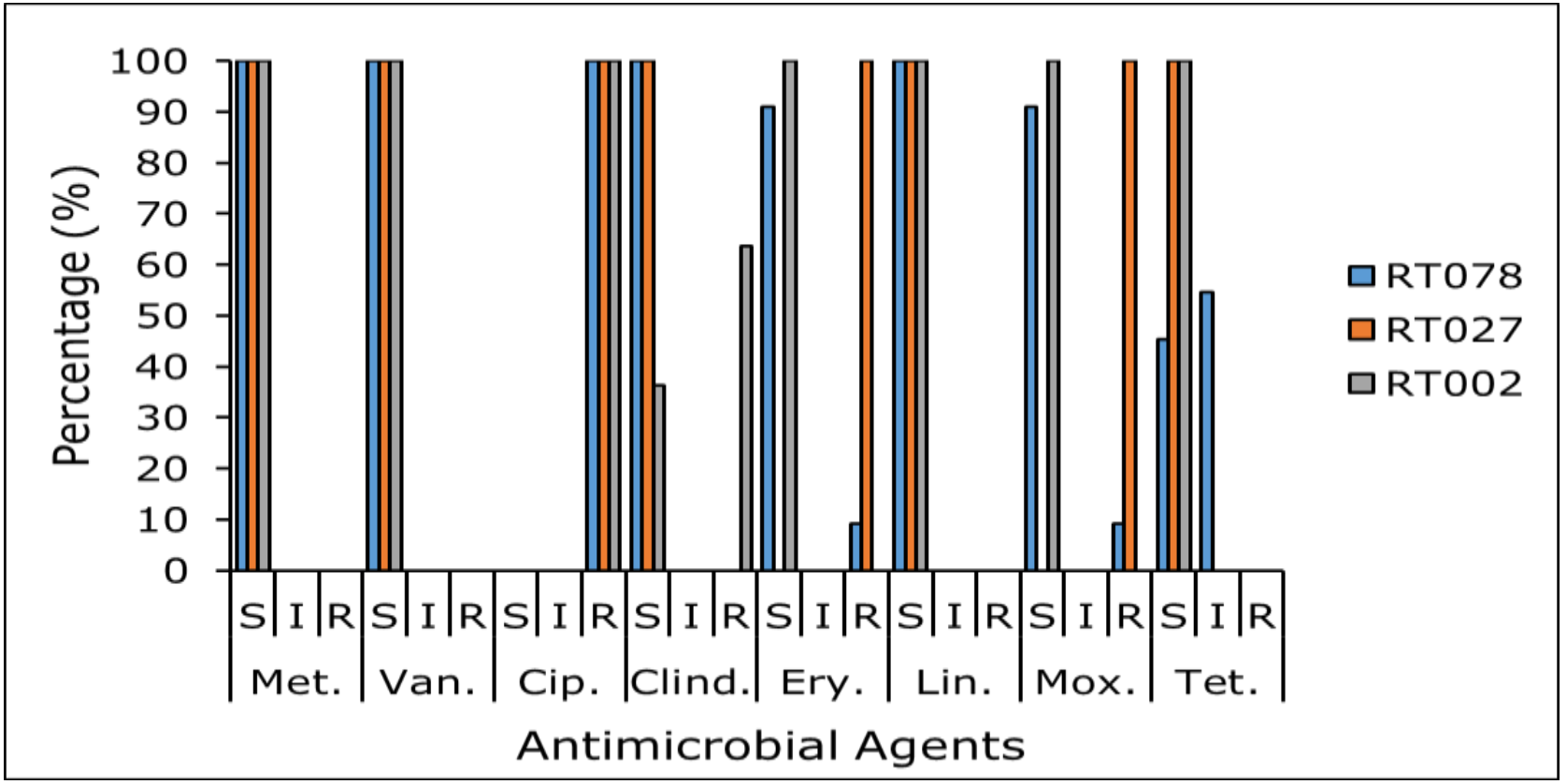

Figure 3.1. The percentage of strains in each PCR ribotype (RT) group (N=11) (RT078, RT027 and RT002), which were in the resistant (R), susceptible (S) or intermediate (I) range when exposed to the antimicrobial agents based on CLSI (2007), EUCAST (2014) and BSAC (2013) breakpoints. Abbreviations: metronidazole (Met.), vancomycin (Van.), ciprofloxacin (Cip.), clindamycin (Clind.), erythromycin (Ery.), linezolid (Lin.), moxifloxacin (Mox.) and tetracycline (Tet.).

3.5. Discussion and Conclusion

Antibiotic properties have been known of and exploited since the Roman and Egyptian eras, since then new antibiotics and ways to determine bacterial resistance have been discovered and developed (Aminov, 2010; Fleming, 1929; Wheat, 2001). To investigate which are the most efficacious antibiotics to use as treatments for bacterial (or fungal) infections, MIC testing and PCR can be used to determine the resistance to antibiotics (Courvalin, 1991; Kahlmeter *et al.*, 2003). CDI can be caused by the use of some antibiotics, such as fluoroquinolones. However, it can also be treated by antibiotics, such as metronidazole or vancomycin, depending on the severity of the condition (Al-Nassir *et al.*, 2008; Borgmann *et al.*, 2010; Menendez *et al.*, 2001). The present study used AIM to characterise and compare the susceptibilities of PCR ribotypes 078, 027 and 002 to eight antibiotics. The breakpoints used to determine the susceptibility to the antibiotics are from BSAC, EUCAST and CLSI, each committee uses different antimicrobial susceptibility testing methods to demine the breakpoints: BSAC (2013) used disc diffusion method (*C. difficile* breakpoints were not found for ciprofloxacin, erythromycin and linezolid so breakpoints for *Staphylococci* were used), EUCAST (2014) used breakpoints based on ECOFF values, whereas CLSI (2007) used the agar dilution method, this method is used in the current study. Therefore, the CLSI breakpoints are more relevant, however they might not always be able to be used as the CLSI have not tested all antibiotics used in the current study.

The geometric mean metronidazole MIC results suggested that there was a substantial difference between the susceptibilities of the PCR ribotype 078 and 027 groups to certain antimicrobial agents. PCR ribotype 078 was more susceptible to metronidazole (0.08 mg/L) than PCR ribotype 027 (1.37 mg/L) but just as susceptible as PCR ribotype 002 (0.18 mg/L) (Table 3.3.). However, this is not consistent with Moura *et al.* (2013) whose results, using AIM, for PCR ribotype 027 (0.125 mg/L to 0.5 mg/L) and PCR ribotype 078 (0.125 mg/L) were very similar. The marked difference in the PCR ribotype 078 range of MICs suggests variations of individual strains (or the culture media used), within the same PCR ribotype, showing varying levels of susceptibly to metronidazole (Table 3.3. and Appendix 1.5). There was a marked difference between the geometric mean metronidazole MICs of the PCR ribotype 078 and 002 groups which suggests there was a noticeable variation between the two PCR ribotypes despite their MIC_{50}, MIC_{90} and ranges being so close (Table 3.3.). These results were less than the breakpoint for metronidazole (≤8 mg/L) set by CLSI (2007), so it can be concluded that all of the strains studied within the PCR ribotype groups studied were susceptible to metronidazole (Appendix 1.5). Bolton and Culshaw (1986) observed metronidazole concentrations within the faecal contents of CDI patients are approximately 9.3 mg/L, however, Freeman *et al.* (2007) observed 7-25% less than this level in an *in vitro* model of the human colon, which was still higher than the MICs of metronidazole for all PCR ribotypes in the present study.

The MIC results for vancomycin suggested there were no differences in susceptibility between the PCR ribotype groups. These results are supported by Debast *et al.* (2013) whose results showed no noticeable difference in susceptibility between PCR ribotype 078 (MIC_{50} 0.5 mg/L and MIC_{90} 1 mg/L) and 027 (MIC_{50} 0.5 mg/L and MIC_{90} 0.5 mg/L) (Table 3.3). Similarly, the results from the present study for PCR ribotype 078 (MIC_{50} 0.5 mg/L and MIC_{90} 1 mg/L) and

027 (MIC_{50} 0.5 mg/L and MIC_{90} 0.5 mg/L) were within the accepted error for MIC testing methods (one doubling dilution) (Table 3.3). As these MIC results are below the breakpoint set by EUCAST (2014) for vancomycin (≤2 mg/L) it can be concluded that all of the strains studied within the PCR ribotype groups in this study were susceptible to vancomycin (Appendix 1.5). Baines *et al.* (2009) found with gut model studies that vancomycin levels within the gut could get to >550 mg/L which is more than enough to be active against the *C. difficile* PCR ribotypes in the present study.

Linezolid geometric mean MIC results were not noticeably different compared to vancomycin and metronidazole. The linezolid MICs against PCR ribotype 027, in the present study, showed that PCR ribotype 027 had a geometric mean MIC of 0.25 mg/L for linezolid compared to 1.37 mg/L for metronidazole and 0.53 mg/L for vancomycin. Similarly, Baines *et al.* (2011) also showed PCR ribotype 027 demonstrated a higher metronidazole geometric mean MICs (1.31 mg/L) compared to linezolid (1.15 mg/L) and vancomycin (0.88 mg/L) which is consistent with the current study (Table 3.3.). The linezolid resistance breakpoint for *Staphylococci* species is >4 mg/L*,* this suggests PCR ribotype 078 (0.73 mg/L), PCR ribotype 027 (0.25 mg/L) and PCR ribotype 002 (0.5 mg/L) were susceptible to linezolid (BSAC, 2013). Lode *et al.* (2001) found the average linezolid faecal concentration from 12 volunteers to be 7.1 mg/kg at four days and 3.0 mg/kg at eight days (dosage of linezolid: two 600 mg tablets a day over seven days) therefore this suggests all three PCR ribotype would be susceptible to gut concentrations of linezolid (if mg/kg are assumed equivalent to mg/L).

In this study erythromycin geometric mean MICs were the highest compared to the other antibiotics. This correlates with the study performed by Mutlu *et al.* (2007) which showed erythromycin MIC_{50} (≥32 mg/L) and MIC_{90} (≥32 mg/L) were highest compared to a range of antibiotics including metronidazole (MIC_{50} 1 mg/L and MIC_{90} 2 mg/L), vancomycin (MIC_{50} 2 mg/L and MIC_{90} 4 mg/L), clindamycin (MIC_{50} 8 mg/L and MIC_{90} 16 mg/L) and tetracycline (MIC_{50} ≥1 mg/L MIC_{90} 2 mg/L) (Table 3.3.). The resistance breakpoints for other Gram-positive bacteria, such as staphylococci, are >2 mg/L for erythromycin (BSAC, 2013). Therefore, the *C. difficile* PCR ribotype 027 strains in this study were resistant to erythromycin (MICs were all 128 mg/L), whereas *C. difficile* PCR ribotype 078 (0.50 mg/L) and 002 (0.25 mg/L) were susceptible to erythromycin (Table 3.3.). *C. difficile* was more susceptible to clindamycin than metronidazole and vancomycin in the present study but not in the study of Mutlu *et al.* (2007). The susceptibility breakpoint for *C. difficile* for clindamycin is ≤2 mg/L, the PCR ribotype 027 (0.44 mg/L), 078 (0.09 mg/L) groups were susceptible to clindamycin, whereas the PCR ribotype 002 (3.31 mg/L) group is in the intermediate category for clindamycin (intermediate: 4 mg/L, resistance: ≥8 mg/L) (Table 3.3.) (CLSI, 2007). Chilton *et al.* (2014) found the concentrations of clindamycin had a peak range of 35.1 to 62.3 mg/L in a simulated model of the colon, suggesting that all ribotypes would be affected by the gut concentrations within the gut. It was also found by Solomon *et al.* (2011) that most PCR ribotype 078 isolates studied did not possess *ermB*, whereas, most of the PCR ribotype 027 isolates studied did have the *ermB* gene, which could be the reason for the difference in the present studies erythromycin results. However, Spigaglia and Mastrantonio (2004) found isolates which were erythromycin resistant but clindamycin susceptible (which is also shown in the PCR ribotype 027 group in present study) did not possess the *ermB* gene. The difference in the PCR ribotype 027 and 078 clindamycin results may be due the

mefA gene, which have been found in other *Clostridium* species, like *C. perfringens* and has been linked to transposons associated with *C. difficile* (Soge *et al.*, 2009). If *C. difficile* strains acquired the *mefA* gene the bacterium would be more resistant to erythromycin but not to clindamycin (Leclercq, 2002). In the study of Mutlu *et al.* (2007) *C. difficile* was shown to be more susceptible to tetracycline than metronidazole and vancomycin, which is consistent with the results of the current study. CLSI (2007) breakpoints for tetracycline are ≤4 mg/L, therefore, all of the strains within all the PCR ribotype groups in the current study were susceptible to tetracycline (PCR ribotypes 078: 0.93 mg/L, PCR ribotype 027: 0.11 mg/L, PCR ribotype 002: 0.06 mg/L) (CLSI, 2007)(Table 3.3). In contrast, Bakker *et al.* (2010) found that the PCR ribotype 078 strains which were resistant to tetracycline contained the *tetM* gene, whereas, Stabler *et al.* (2009) found that PCR ribotype 027 has no tetracycline resistance genes (Bakker *et al.*, 2010; Dong *et al.*, 2014; Stabler *et al.*, 2009). Rashid *et al.* (2013) found the faecal concentrations of doxycycline in 16 volunteers, which is a member of the tetracycline antimicrobial family. The study found an average of 0.98 mg/kg of doxycycline at 16 weeks of taking 40 mg capsules. If this is the case for tetracycline then the concentration is higher than all three PCR ribotypes' geometric mean MICs observed in the present study, so it could have an antimicrobial effect *in vivo*. Conversely, the MIC_{50} suggests that this concentration of tetracycline would not be effective against at least half of the strains, in this study. However, this may not be relevant *in vivo* in CDI as tetracycline has been found, by Brown *et al.* (2013), to have a low risk of causing CDI so may have little or no effect on the rest of the gut microflora.

Compared to ciprofloxacin and moxifloxacin *C. difficile* was more susceptible to vancomycin and metronidazole in the present study, which reflects the report of Bourgault *et al.* (2006). In this study the authors reported that ciprofloxacin (MIC_{50} >128 mg/L, MIC_{90} >128 mg/L) and moxifloxacin (MIC_{50} 64 mg/L, MIC_{90} 64 mg/L) MIC_{50} and MIC_{90} results were higher than the results for metronidazole (MIC_{50} 0.25 mg/L MIC_{90} 0.05 mg/L) and vancomycin (MIC_{50} 1 mg/L; MIC_{90} 1 mg/L). The study also had a higher range for both ciprofloxacin (16 mg/L to >128 mg/L) and moxifloxacin (2 mg/L to >128 mg/L) than metronidazole (≥0.06 mg/L- 1 mg/L) and overlapped with vancomycin MICs (≥0.05 mg/L to 4 mg/L), which is similar to the results in the present study. Bourgault *et al.* (2006) also found that for moxifloxacin, PCR ribotype 027 had higher MIC_{50} (64 mg/L) and MIC_{90} (128 mg/L) results compared to the other PCR ribotypes that were tested and this is consistent with the results in the present study (MIC_{50} 32 mg/L MIC_{90} 32 mg/L) (Table 3.3.). The MIC results for ciprofloxacin and moxifloxacin showed that overall *C. difficile* is more resistant to ciprofloxacin than moxifloxacin which reflects prior studies, however the study of Wilcox *et al.* (2000) did not specify all of the PCR ribotypes that were used. The resistance breakpoint for moxifloxacin (≥8 mg/L) set by CLSI (2007) suggests the PCR ribotype 027 group (geometric mean MIC: 32 mg/L) was resistant to moxifloxacin, whereas, the PCR ribotype 078 group and the PCR ribotype 002 group were susceptible (susceptible category is ≤2 mg/L) (Table 3.3.). Rodriguez *et al.* (2014) also demonstrated resistance to moxifloxacin in PCR ribotype 078 (using E-testing) and resistance was associated with a mutation within the *gryA* gene which encodes for part of DNA gyrase. Additionally, Saxton *et al.* (2009) found PCR ribotype 027 isolates with resistance to moxifloxacin (using agar incorporation method) also had a mutation in the *gryA* gene and a mutation in the *gryB* gene. The resistance breakpoint for *Staphylococci* to ciprofloxacin is >1 mg/L, and if this breakpoint is used for *C. difficile*, the results

of the current study showed that all the PCR ribotypes were resistant to ciprofloxacin (PCR ribotype 027: 68.16 mg/L; PCR ribotype 078: 7.51 mg/L; PCR ribotype 002: 10.29 mg/L) (Table 3.3.) (BSAC, 2013). Saxton *et al.* (2009) found that the mean moxifloxacin concentrations in a simulated model of the colon were 43 mg/L, this suggests that the moxifloxacin levels would be affective against PCR ribotype 002 and 078 but not PCR ribotype 027. Whereas, the gut mean concentrations for ciprofloxacin were 139 mg/L which is much higher than the geometric mean MICs of all PCR ribotypes in the current study (Saxton *et al.*, 2009).

The geographical location of the isolates may have an influence of the metronidazole and vancomycin MIC results for PCR ribotype 078 strains, as there was some variation between the results for different locations of isolation, such as between Derriford and Sheffield isolates (Appendix 1.5). However with PCR ribotype 027 and 002 and other antimicrobials the MIC results may not be affected by the location of isolation as there was little variation between geographical locations (Appendix 1.5).

In conclusion, *C. difficile* PCR ribotype 078 was more susceptible to most of the antibiotics tested than PCR ribotype 027 and 002, including metronidazole and vancomycin, which are common treatments for CDI. PCR ribotype 078 was the only PCR ribotype to be susceptible to all the antibiotics used in this study (Al-Nassir *et al.*, 2008; BSAC, 2013; EUCAST, 2014; Menendez *et al.*, 2001). The results from this study suggest that all the strains from all three PCR ribotypes were susceptible to only three antibiotics: vancomycin, metronidazole and linezolid (Figure 3.1. and Appendix 1.5). The clindamycin showed all the strains in the PCR ribotype 027 and 078 group were susceptible however most of the PCR ribotype 002 strains (64%) were resistant (Figure 3.1. and Appendix 1.5). The results also showed that only 45% of the PCR ribotype 078 were susceptible to tetracycline compared to 100% of the PCR ribotype 027 and 002 strains (Figure 3.1. and Appendix 1.5). As breakpoints are good indicators of whether an antibiotic would be a good treatment it would be a fair conclusion that linezolid (according to these results) would be a good alternative treatment for CDI caused by PCR ribotype 078, 027 and 002 (Angeby *et al.*, 2012; Jorgensen and Ferraro, 2009).

4. Comparing the Biofilm Production and Resistance to Antibiotics of Three PCR Ribotypes of *Clostridium difficile*

4.1. Introduction

Bacteria can grow in two forms, planktonic (individually) or within a self-produced polymeric matrix, called a biofilm (Đapa *et al.*, 2013). Bacterial cells are believed to attach to a surface and subsequently replicate into micro-colonies which mature by producing an extracellular polymeric substance (EPS) matrix. The bacteria also continue to multiply and produce separate colonies within the biofilm (Conibear *et al.*, 2009; Davey and O'Toole, 2000; Semenyuk *et al.*, 2014). The EPS matrix may comprise of extracellular DNA (eDNA), proteins and polysaccharides (Hall-Stoodley and Stoodley, 2009; Semenyuk *et al.*, 2014). Biofilms are the most common form of microbial growth and can contain more than one species of bacteria, they can also contain species of algae, protozoa and fungi (Burmolle *et al.*, 2014). Biofilms are believed to protect the bacteria to environmental changes, such as oxygen stress, dehydration, and they may increase the resistance to antibiotics by up to a 1000-fold more compared to planktonic cells (Burmolle *et al.*, 2014; Charlebois *et al.*, 2014; Đapa *et al.*, 2013; Mah and O'Toole, 2001). Additionally, biofilms can grow on organic surfaces, such as teeth in the form of dental plaque, and within the lungs of patients with cystic fibrosis (CF). Growth can also occur on inorganic surfaces, for example catheters and stents (Hoiby *et al.*, 2011). As a result of this property it is thought that biofilms are associated with 65-80% of nosocomial infections and the resulting healthcare costs have been estimated at >$1 billion annually (Mah and O'Toole, 2001; Sawhney and Berry, 2009).

Within the human body, macrophages are a first line of defence against bacterial infections. Macrophages use phagocytosis to eliminate pathogenic microbes, for example bacteria, and this involves binding to the bacteria using the CD64 receptors on the macrophage membrane (Hernández-Jiménez *et al.*, 2013). Once bound by the macrophage, a bacterium is engulfed into the cell in a phagosome, in which the pathogen is destroyed via endosomal and lysosomal fusion, which involves the release of toxins and enzymes which breakdown the pathogenic components (Flannagan *et al.*, 2012; Hernández-Jiménez *et al.*, 2013). Biofilms protect the bacteria from this process, this may be shown by the differential expression of CD64 receptors (Hernández-Jiménez *et al.*, 2013; Jefferson, 2004). Hernández-Jiménez *et al.* (2013) observed that macrophages preferentially attacked *E. coli* planktonic cells over those within a biofilm, this was shown by an increase in expression of CD64 receptors, on the macrophage cell membrane, in the presence of the planktonic cells, whereas, there was no elevation in the presence of bacteria in a biofilm (Hernández-Jiménez *et al.*, 2013).

Biofilms have been shown to reduce the susceptibility of bacteria to antibiotics. There are several hypotheses as to why this is the case, one is thought to be due to the inability of the antibiotic to penetrate the biofilm or that antibiotics may demonstrate a slow diffusion rate through the biofilm (Jefferson *et al.*, 2005). Jefferson *et al.* (2005) found vancomycin took approximately 60 minutes to bind to bacteria in the deeper layers of a *S. aureus* biofilm compared to taking

approximately five minutes to bind to planktonic cells of the same organism. Consequently, some antibiotics could become inactivated more rapidly than they could penetrate through the biofilm, due to microbial resistance gene products, binding to charged polymers within the biofilm, or interactions with antibiotic-degrading enzymes and/or dead cells (Anderl *et al.*, 2000; Hall-Stoodley and Stoodley, 2009; Stewart and Costerton, 2001). Another hypothesis is due to differences in physiological properties of the bacterial cells, for example the growth rate of the cells or age of the biofilm (Anderl *et al.*, 2000). Duguid *et al.* (1992) found *S. epidermidis* biofilms increased in susceptibly to ciprofloxacin as the μ_{max} within the biofilm increased, owing to more targets (DNA gyrase and Topoisomerase IV) for the ciprofloxacin to modify (Drlica and Zhao, 1997). Additionally, Anwar and Costerton (1990) concluded the older a *Pseudomonas aeruginosa* biofilm the more resistant to antibiotics (tobramycin and piperacillin) the bacteria became, this may be due to the biofilm becoming thicker or the growth rate of the bacteria within the biofilm slowing down (Anwar *et al.*, 1992; Monzón *et al.*, 2001). Another factor that may help to explain reduced antimicrobial activity against microbial biofilms is the atmospheric characteristics of the biofilm as the oxygen gradients can differ throughout the layers of a biofilm, so much so that it creates anaerobic areas in deeper layers. These areas produce acidic waste and decrease the pH, which may inhibit the action of the antibiotics (Stewart and Costerton, 2001). Varying nutrient levels, within a biofilm, may also contribute to slow bacterial growth or complete cessation of growth, this therefore also increases the resistance to antibiotics (Mah and O'Toole, 2001). Multispecies biofilms have been found to have greater resistance to antibiotics than single species biofilms (Burmolle *et al.*, 2014; Lopes *et al.*, 2012). For example, Lopes *et al.* (2012) found bacteria which are isolated from CF patients, such as *Dolosigranulum pigrum* and *Inquilinus limosus*, demonstrated higher MBEC, to certain antibiotics, when cultured within a biofilm containing *P. aeruginosa*, a common pathogen in patients with CF, than in their respective individual single species biofilms.

C. difficile has been found to penetrate the mucus layer within the gut so it can adhere to colonic epithelial cells and produce a biofilm on the gut wall within mammals, such as mice (Crowther *et al.*, 2014; Reynolds *et al.*, 2011; Twine *et al.*, 2009). Additionally, Crowther *et al.* (2014) used an *in vitro* gut model to find that a biofilm (containing *C. difficile* and other gut bacteria) was the more dominant structure over planktonic cells. Some *C. difficile* ribotypes have flagella at the surface of the cell, which may aid in the adherence to the gut wall, the bacterium divides and then starts the production of the EPS matrix (Stephens, 2002; Tasteyre *et al.*, 2001; Twine *et al.*, 2009). Tasteyre *et al.* (2001) found that flagellated strains for *C. difficile* bound to mouse cecum more than non-flagellated strains. The flagella are made of proteins including the flagellar cap (FliD) and flagellin (FliC) proteins, which both aid in the adherence of the *C. difficile* to the mucus layer on the gut cell wall, FliD also aids in the attachment of the cells (Baban *et al.*, 2013; Barketi-Klai *et al.*, 2014; Tasteyre *et al.*, 2001). The *agr* quorum sensing locus has been found by Martin *et al.* (2013) to increase the production of flagella in PCR ribotype 027 strains. Pili are another important component factor in the ability of certain bacterial species to produce biofilms, for example *H. influenzae*. The genes which encode for the pili, the Type IV pilin biogenesis components, in other biofilm-producing clostridia, for example *C. perfringens*, have also been found in *C. difficile* (Murphy and Kirkham, 2002; Piepenbrink *et al.*, 2014; Varga *et al.*, 2008). As well as pili, *C. difficile* has also

been found to produce the second messenger 3',5'-cyclic diguanylic acid (c-di-GMP) and Purcell *et al.* (2012) found that when c-di-GMP is in high levels the bacteria are clumped together and produce long thin fibres similar to pili. *C. difficile* also possesses a crystalline surface layer (S-layer) which aids in the adherence to the gut wall (Cerquetti *et al.*, 2000; Đapa *et al.*, 2013; Đapa and Unnikrishnan, 2013). One protein needed to for the maturation of the S-layer is cell wall protein (Cwp) 84 (Đapa *et al.*, 2013; Đapa and Unnikrishnan, 2013; Hammond *et al.*, 2014). Đapa *et al.* (2013) found the *C. difficile* with a mutation in the *cwp84* gene did not form a biofilm as well as the wild-type *C. difficile*.

As well as the aforementioned properties needed for biofilm production, there is an essential process, known as quorum sensing, which is also needed for *C. difficile* to form a biofilm (Đapa *et al.*, 2013; Đapa and Unnikrishnan, 2013). Quorum sensing is a cell to cell signalling process, usually using chemicals, which is required for almost all bacteria to communicate (not just for biofilm production)(Miller and Bassler, 2001). In *C. difficile* the LuxS protein plays a role in this process. Đapa *et al.* (2013) found that *C. difficile* strains with a mutation in the *luxS* gene could not form a monolayer, let alone a biofilm. (Đapa *et al.*, 2013; Đapa and Unnikrishnan, 2013; Hammond *et al.*, 2014).

Spores and toxins are also produced in a *C. difficile* biofilm structure (Figure 4.1.) (Crowther *et al.*, 2014; Semenyuk *et al.*, 2014). Semenyuk *et al.* (2014) showed that *C. difficile* biofilms form after 24 hours and cell debris starts to be detected. After three days of growth the biofilm contains vegetative cells, spores and dead cells, whereas after six days there were more spores than viable cells. It was discovered that cell growth continued within the biofilm at 24 hours, additionally, toxin was also produced at 24 hours, in the three day biofilms toxin was detected in all samples in the study. There was a significant increase in toxin levels in the six day biofilms. When the biofilm is mature (in *P. aeruginosa* biofilms) bacterial cell are dispersed to start the cycle again. The spores produced in a *C. difficile* biofilm may also be part of this process of biofilm dispersal and are a potential factor in the recurrence of CDI (Crowther *et al.*, 2014; Stoodley *et al.*, 2002).

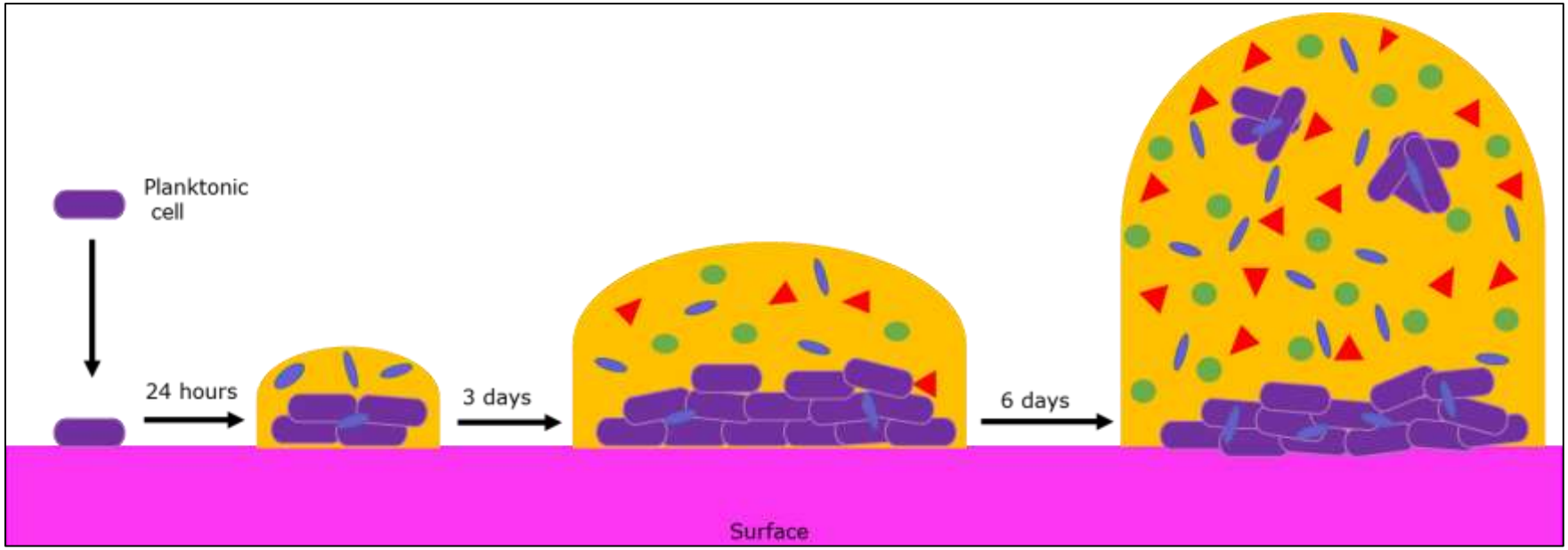

Figure 4.1. Proposed *C. difficile* ( ) biofilm production over six days. *C. difficile* attaches to the surface, multiplies and starts to produce extracellular polymeric substances ( ) before 24 hours. Spores ( ), toxin ( ) and cell debris ( ) are detected after three days. After six days there are more spores, toxin and cell debris than viable cells and new colonies are formed. (Based on Semenyuk *et al.* (2014)).

4.2. Aims and Objectives

The aims of this study are to compare the biofilm production of three *C. difficile* PCR ribotype groups (PCR ribotype 078, 027 and 002). Furthermore, the susceptibility of the *C. difficile* biofilms to metronidazole and vancomycin will also be assessed.

- This will be achieved by producing batch cultures of 10 *C. difficile* strains of each PCR ribotype group then aliquoting the cultures into 96-well microtitre plates and allowing the biofilm to grow over three and six days. The biofilms will be stained with crystal violet and the absorbance determined.

- The susceptibility of biofilms to antimicrobial agents will be assessed by growing biofilms of each strain using the Calgary Biofilm Device (CBD) and placing the biofilm into culture media with different concentrations of metronidazole or vancomycin.

4.3. Materials and Methods

4.3.1. *C. difficile* strains

The strains in each of the experiments were prepared using methods in Section 1.2.1.

The biofilm growth (Section 4.3.2.), biofilm minimum inhibitory concentration (bMIC), and Minimum Biofilm Eradication Concentrations (MBEC) (Section 4.3.4.) experiments all used 10 *C. difficile* strains each from PCR ribotype 078, 027 and 002 groups in triplicate.

In the 96-well microtitre plate biofilm assay experiment (Section 4.3.3) 11 *C. difficile* strains each from PCR ribotypes 078, 027 and 002 were used in triplicate.

In the assessment of biofilm total viable counts and spore counts using Calgary Biofilm Device (CBD) Assay (Section 4.3.5.) three strains from each *C. difficile* PCR ribotype were evaluated (PCR ribotype 078, 027 and 002) in triplicate.

All experiments used the control strains, E4 (PCR ribotype 010) and ATCC 700057 (VPI 11186) (PCR ribotype 038) in triplicate.

4.3.2. Preparation of Biofilm Growth Assay

The methods in this study adapt the methods of Dawson *et al.* (2012). The present study used 96-well microtitre plates (FB56412, Fisher Scientific) whereas Dawson *et al.* (2012) used 24-well microtitre plates (Nunc).

A 1:10 dilution of an overnight culture of each *C. difficile* isolate was aseptically aliquoted into BHIS broth in two 96-well microtitre plates (FB56412, Fisher Scientific) as displayed in Figure 4.2. The 96-well microtitre plates were wrapped in Parafilm® M (PM-992) to reduce evaporation of liquid and incubated at 37°C for three and six days respectively in an anaerobic cabinet. BHIS was removed from each well and disposed of the wells were then washed three times with sterile PBS. Subsequently, 200 µl of 1% (w/v) crystal violet (Sigma-C6158-100g) diluted

in 95% industrial methylated spirit (IMS) (Fisher-M14450/17) was aliquoted into each well and left at room temperature for 30 minutes to stain the contents of the well. The wells were then washed gently five times with PBS and then 200 µl of methanol (Fisher- M/4000 PC17) was aliquoted into each well and left for 15 minutes at room temperature. The absorbance of the 96-well microtitre plate wells was read at 590 nm (using a microtitre plate spectrometer (Original multiskan EX- Thermo Scientific - 51118170)) using the stained control BHIS well as the blank. This process was repeated on the sixth day of growth. Biofilm biomass (via OD_{590}) was measured in triplicate and the average was calculated.

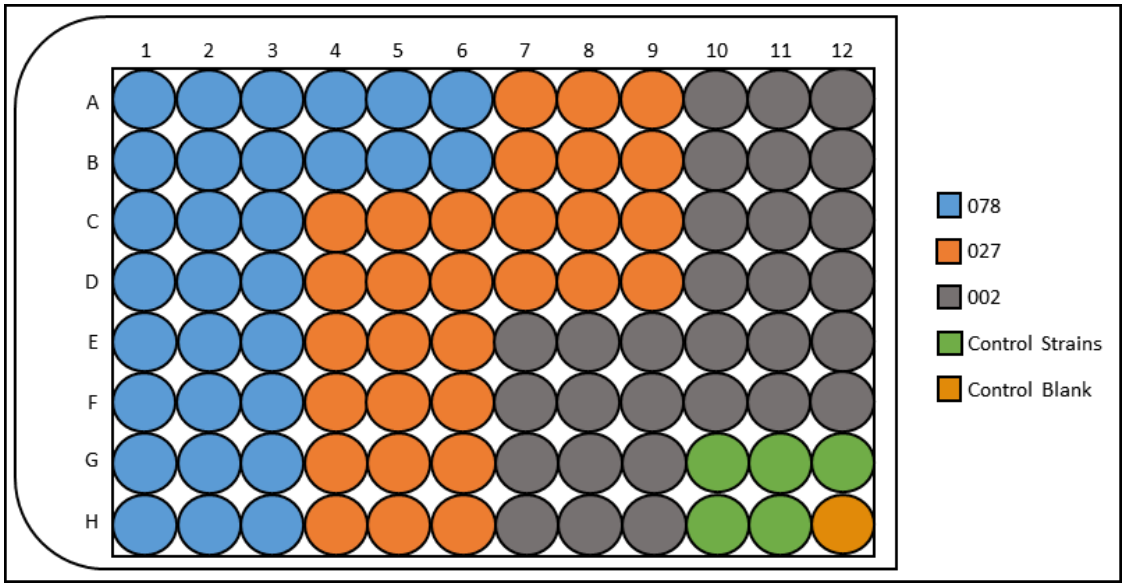

Figure 4.2. The arrangement of *C. difficile* strains in the 96-well microtitre plate. So each strain in triplicate.

4.3.3. Preparation of Biofilm Total Viable and Spore Counts using a 96-Well Microtitre Plate Biofilm Assay

A 1:10 dilution in BHIS of an overnight culture (prepared from Section 1.2.) of each *C. difficile* isolate was aseptically aliquoted into two 96-well microtitre plates (FB56412, Fisher Scientific). The 96-well microtitre plates were wrapped in Parafilm® M (PM-992) to reduce evaporation of liquid and then incubated at 37°C for three and six days in independent experiments, in an anaerobic cabinet. An additional microtitre plate was prepared to assess biofilm at the zero hour time point. BHIS was carefully removed from each well and disposed of, the wells were then washed twice with sterile pre-reduced PBS. The biofilm was disrupted by adding 100 µl PBS into the wells and agitating at 1300 rpm for 10 minutes using an orbital shaker (Obris personal plate Shaker, Mikura, W, Sussex, Serial number: 1959). A 10-fold dilution series was produced of the suspension in pre-reduced PBS (Neat to 10^{-5}). Each dilution (20 µl) was spread onto Brazier's agar. The 96-well microtitre plate was placed into a hot cabinet at 65°C for 20 minutes to kill of vegetative cells in order that spore counts could be determined. An initial comparison of the effectiveness of heat-inactivation of vegetative *C. difficile* compared to alcohol-shocking *C. difficile* cultures showed that both methods were effective and that spore counts were almost identical (data not shown), therefore heat inactivation methods were used in this study. Each spore dilution (20 µl) was spread on Brazier's agar (with supplements). The plates were incubated for 48 hours in an anaerobic cabinet at 37°C. The dilution with between 20 to 200 CFUs (where possible) was counted. Once counted, CFU/ml was calculated using the equation below, in triplicate, and viable counts were expressed as $\log_{10}$ CFU/ml:

$$CFU\ per\ ml = (number\ of\ CFUs\ \times 1/n) \times 50$$

(n = the dilution as a decimal in which CFUs were counted)

4.3.4. Biofilm Minimum Inhibitory Concentration and Minimum Biofilm Eradication Concentrations

This study modified methods of Ooi *et al.* (2010) who studied *S. aureus* biofilms and a range of antibiotics and the cultures were grown with Mueller-Hinton broth and agar. Conversely, in the present study *C. difficile* was cultured in BHIS and on Braziers agar and metronidazole (M3761-25G, Sigma-Aldrich, Gillingham) and vancomycin hydrochloride (V2002-1G, Sigma-Aldrich, Gillingham) were the antimicrobials evaluated.

An overnight culture of each *C. difficile* strain was aliquoted 1:10 into fresh BHIS in 96-well microtitre plate (167008, Thermo Scientific) in the same arrangement as in Figure 4.2. A CBD lid (445497, Thermo Scientific) was placed onto the 96-well microtitre plate in place of the lid. The microtitre plate was then wrapped in Parafilm® M and incubated in the anaerobic cabinet at 37°C for four days (statically) to allow biofilms to form. An experimental duration of four days was selected, since prior research by Semenyuk *et al.* (2014) indicated that after six days *C. difficile* biofilms were dominated by spores. The metronidazole (M3761-25G, Sigma-Aldrich, Gillingham) and vancomycin hydrochloride (V2002-1G, Sigma-Aldrich, Gillingham) 1024 mg/L solutions (30.72 mg of antibiotic powder in 30 ml of sterile distilled water) were prepared in BHIS as described in Figure 4.3. Each concentration (512 mg/L to 0.5 mg/L) was aliquoted into microtitre plates (one each concentration), 200 µl per well, and left in an anaerobic cabinet to pre-

reduce overnight. On the fourth day of growth each CBD was transferred to a microtitre plate with antibiotics, and incubated in an anaerobic cabinet at 37°C overnight. OD_{590} was determined from the initial 96-well microtitre plate using the blank of BHIS. Subsequently, the CBDs were transferred to sterile microtitre plates containing 200 µl of fresh pre-reduced BHIS and incubated in an anaerobic cabinet at 37°C overnight, the absorbance was then read at 590 nm.

The bMIC and MBEC were determined by interpreting the absorbance readings of the BHIS with antibiotics (bMIC) and the BHIS after antibiotic treatment (MBEC) for each triplicate of each strain. The blank BHIS reading was taken subtracted from each of the biofilm readings and the average of the triplicates was determined. The bMIC was defined as: the lowest concentration of antimicrobial that prevents planktonic growth in wells containing antimicrobials and biofilms. The MBEC was defined as: the lowest antimicrobial concentration that prevents planktonic growth when biofilms, attached to CBD lids, were transferred into fresh BHIS broth with no antimicrobials. As the blank BHIS absorbance readings varied due to staining of residual medium, *C. difficile* growth was classified an OD_{590} of more than 0.08. Geometric mean bMIC and MBEC values were calculated (results <0.5 mg/L were classed as 0.5 mg/L to work out the geometric mean) and the MIC ranges were also found.

4.3.5. Preparation of Biofilm Total Viable and Spore Counts using Calgary Biofilm Device (CBD) Assay

An overnight culture of each *C. difficile* strain was aliquoted 1:10 into fresh pre-reduced BHIS into five 96-well microtitre plates (167008, Thermo Scientific). A CBD (445497, Thermo Scientific) was placed onto the 96-well microtitre plates in place of the lid. The microtitre plates were then wrapped in Parafilm® M and incubated in the anaerobic cabinet at 37°C for two and four days, one was placed on a microplate shaker at 1300 rpm for four days, one microtitre plate was tested the same day to obtain the day zero result. After the growth period, the pegs on the CBD were snapped off using sterile pliers, and placed individually into 1 ml of pre-reduced PBS, then vortexed at 25 Hz for 10 seconds. *C. difficile* total viable counts and spore counts were determined as described above in Section 4.3.3.

4.3.6. Statistical Analysis

The biofilm growth results (Section 4.4.1.) were assessed for normality of distribution and homogeneity of variance using SPSS and analysed using the ANOVA statistical test. A P-value of ≤0.05 was considered statistically significant.

The bMIC and MBEC results (Section 4.2.4) and the viable and spore count data in 96-well microtitre plates (Section 4.4.2.) and on BCD (Section 4.4.4.) were assessed for normality of distribution and homogeneity of variance using SPSS and analysed using the Kruskal-Wallis statistical test. A P-value of ≤0.05 was considered statistically significant.

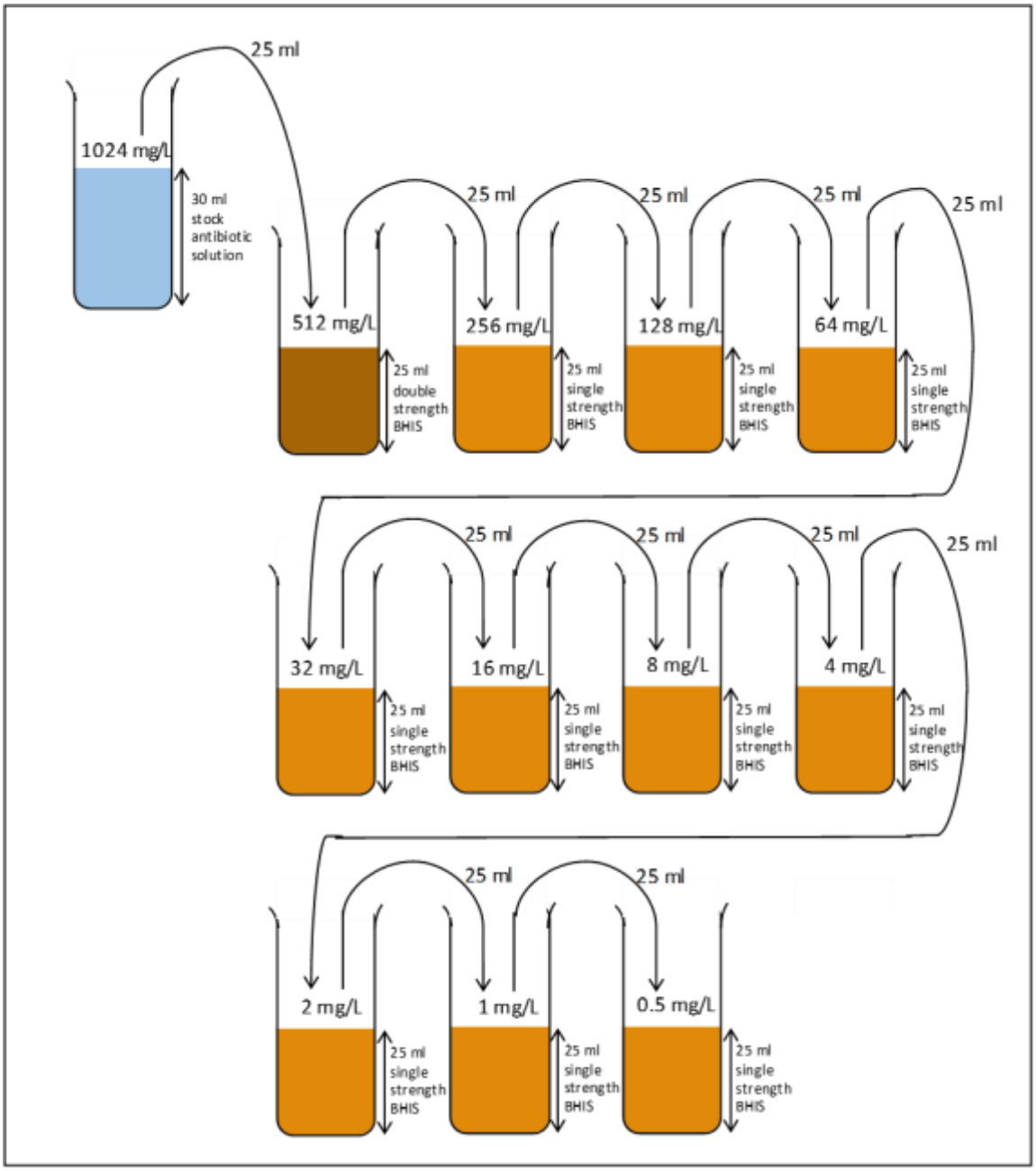

Figure 4.3. The preparation of 1024 mg/L antibiotic solutions was diluted in supplemented Brain Heart Infusion broth with supplements (BHIS).

4.4. Results

4.4.1. Biofilm Growth

Production of biofilm biomass was measured at three and six days using a crystal violet staining assay. All PCR ribotype 078 strains demonstrated a wide range of average absorbance readings (OD_{590} 0.06 to 0.22) at three days of growth, whereas the PCR ribotype 027 group (OD_{590} 0.09 to 0.16) and 002 (OD_{590} 0.06 to 0.08) readings were similar. After six days of growth the PCR ribotype 078 had the widest range (OD_{590} 0.16 to 0.56), PCR ribotype 027 (OD_{590} 0.04 to 0.14) and 002 (OD_{590} 0.03 to 0.12) readings were similar (Appendix 1.5.). The PCR ribotype 078 group had the highest average biofilm biomass at three days (OD_{590} 0.12), however it was not significantly higher than PCR ribotype 027 average group (OD_{590} 0.11) (P=0.54), whereas it was significantly higher than PCR ribotype 002 (OD_{590} 0.07) (P≤0.05). At six days of growth PCR ribotype 078 (OD_{590} 0.28) had a significantly higher biofilm growth than PCR ribotype 027 (OD_{590} 0.08) (P≤0.001) and PCR ribotype 002 (OD_{590} 0.08) (P≤0.001) PCR ribotype 078 strains had a significant increase between three days and six days (P≤0.001), the biggest increase in biofilm biomass. The PCR ribotype 002 strains had the lowest biofilm biomass out of the three PCR ribotypes, however there were not significantly different to the results for PCR ribotype 027 (day three: P=0.80, day six: P=0.84). The increase for PCR ribotype 002 between three and six days was not significant (P=0.58). The results for the PCR ribotype 027 group shows an insignificant decrease in biofilm biomass between three and six days (P=0.30) (Figure 4.4.). Interestingly, it was observed that *C. difficile* PCR ribotype 078 cultures aggregated substantially in 5 ml volumes of BHIS broth in prior experiments, whereas PCR ribotypes 027 had little aggregate and 002 cultures did not aggregate (Figure 2.4).

4.4.2. Biofilm Total Viable Count and Spore Count in a 96-Well Microtitre Plate Biofilm Assay.

The average total viable count within a biofilm for all the PCR ribotypes had a significant increase between zero and three days of growth (Figure 4.5). PCR ribotype 078 total viable counts were 2.71 $\log_{10}$ CFU/ml at day zero and increased to 5.47 $\log_{10}$ CFU/ml at day three (P≤0.001) then at day six the total viable count was 5.17 $\log_{10}$ CFU/ml (P≤0.001), resulting in a 1.91 $\log_{10}$-fold increase overall. The spore counts for PCR ribotype 078 increased from 1.72 $\log_{10}$ CFU/ml at day zero to 3.96 $\log_{10}$ CFU/ml on day three (P≤0.001) and then to 4.58 $\log_{10}$ CFU/ml after six days of growth (P≤0.001), so there was a 2.65 $\log_{10}$-fold increase overall. The viable count was significantly higher than the spore count at every time point (day zero: 1.57 $\log_{10}$-fold; day three: 1.16 $\log_{10}$-fold; day six: 1.13 $\log_{10}$-fold) (P≤0.001) (Appendix 1.4).

PCR ribotype 027 total viable counts increased from 2.76 $\log_{10}$ CFU/ml to 4.94 $\log_{10}$ CFU/ml (P≤0.001) over three days then increased to 5.87 $\log_{10}$ CFU/ml (P≤0.001) at day six (Figure 4.5). Overall, there was a 2.13 $\log_{10}$-fold increase. There was an increase from 0.85 $\log_{10}$ CFU/ml to 4.27 $\log_{10}$ CFU/ml over three days (P≤0.001) for spore count and then a further increase to 5.32 $\log_{10}$ CFU/ml after six days of biofilm growth (P≤0.001). At all the time points the viable count was significantly higher than the spore count (P≤0.001) (Appendix 1.4).

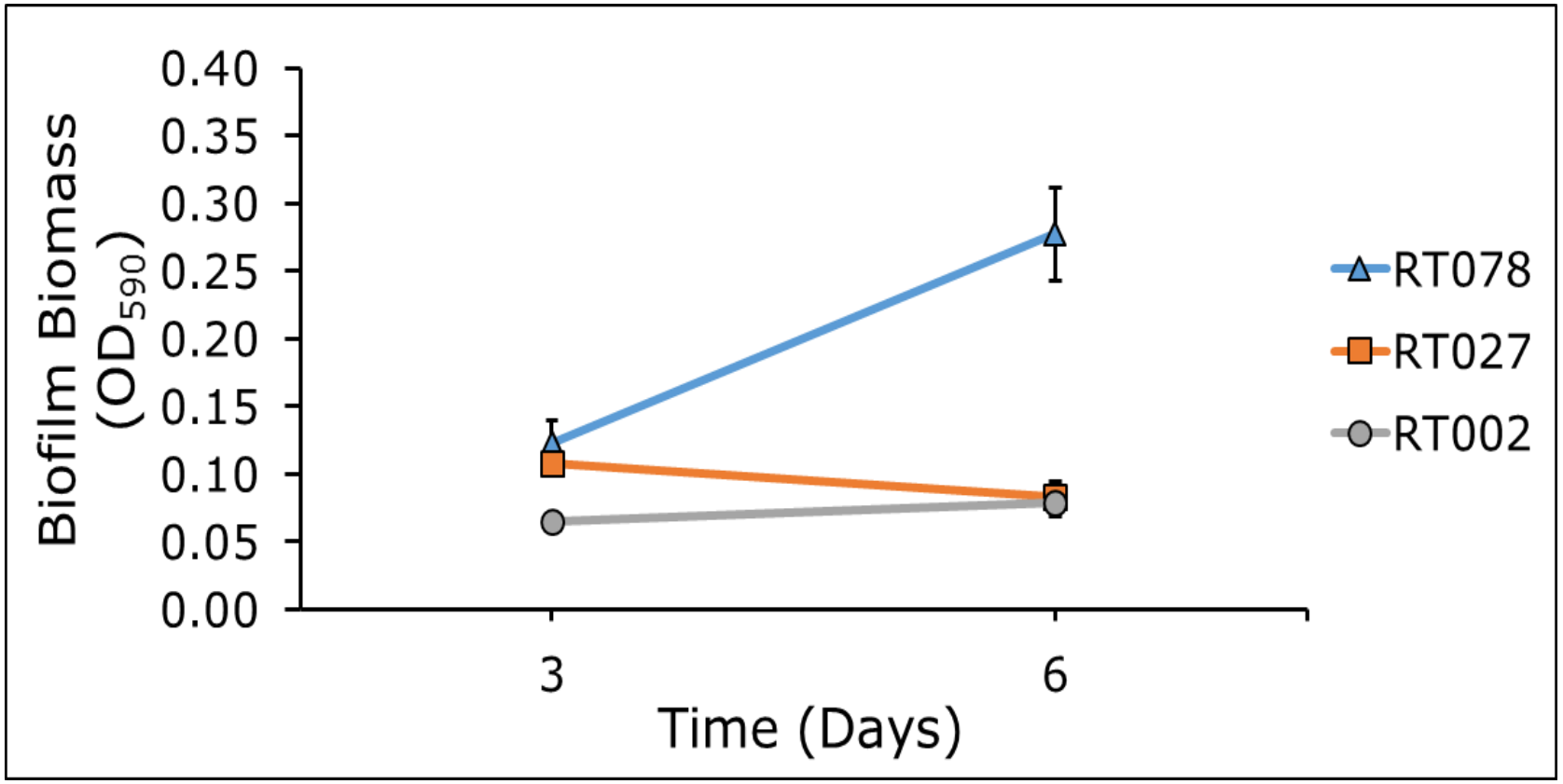

Figure 4.4. Mean biofilm biomass (optical density (OD_{590}) (±SE)) measured at day three and six of biofilm growth, for *C. difficile* PCR ribotypes (RT) 078, 027 and 002 (N=11 strains of each PCR ribotype). Biofilms were grown in BHIS broth and stained with crystal violet to determine the biomass.

* statistically significant difference P-value ≤0.05
*** statistically significant difference P-value ≤0.001

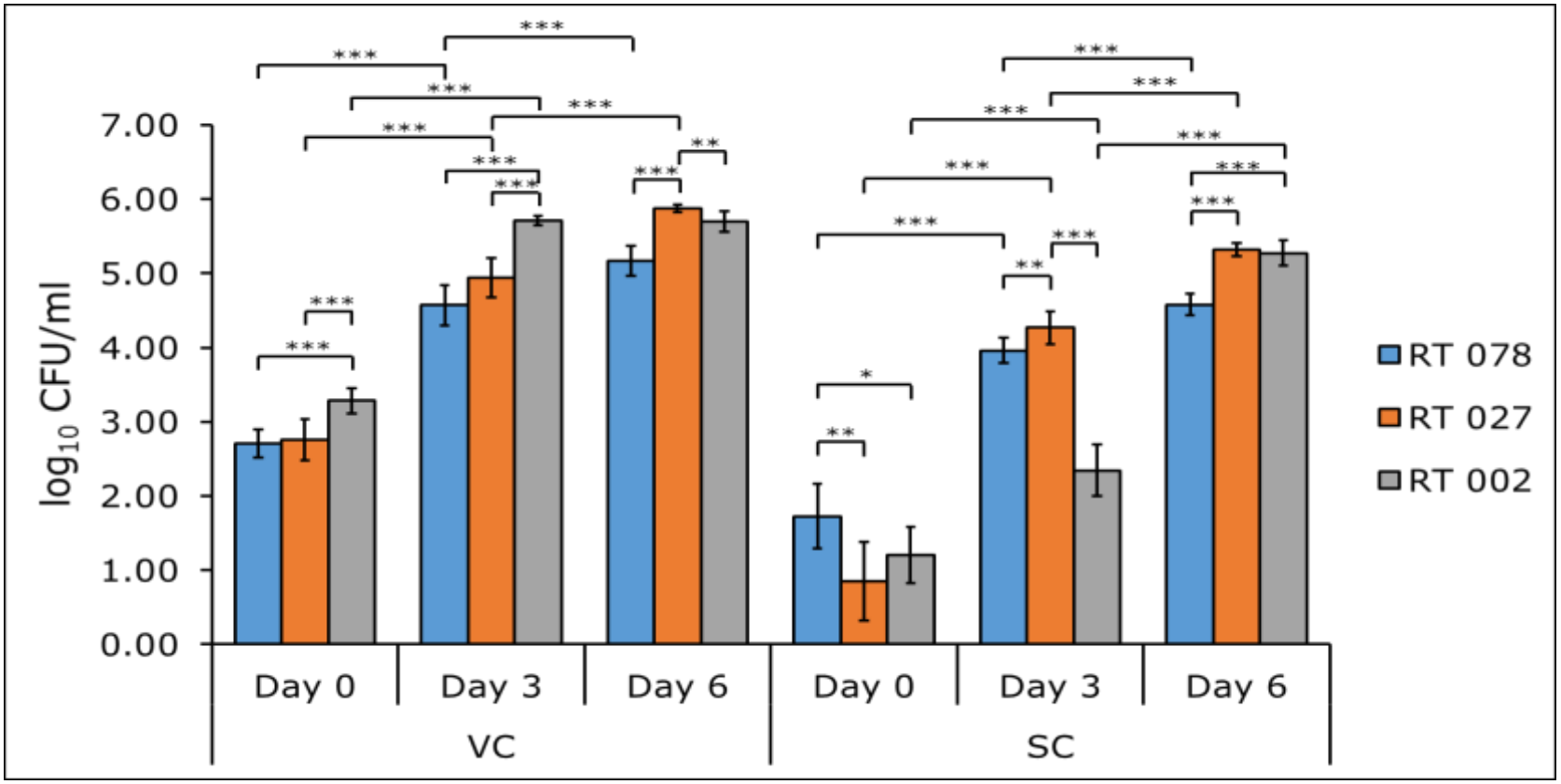

Figure. 4.5. Average total viable count (VC) and spore count (SC) (log_{10} colony forming units (CFU)/ml (±SE)) in biofilms formed in a 96-well microtitre plate after zero, three and six days growth, for PCR ribotype (RT) 078, 027 and 002 (N=11 strains of each PCR ribotype) in BHIS broth.

* statistically significant difference P-value ≤0.05
** statistically significant difference P-value ≤0.01
*** statistically significant difference P-value ≤0.001

The PCR ribotype 002 biofilm total viable counts increased from 3.28 log_{10} CFU/ml at day zero to 5.71 log_{10} CFU/ml at day three ($P \leq 0.001$), then remained constant until day six ($P=0.95$). Overall, there was a 2.42 log_{10}-fold increase between day zero and day six. PCR ribotype 002 spore counts were 1.20 log_{10} CFU/ml at day zero and increased to 2.34 log_{10} CFU/ml ($P \leq 0.001$) and 5.27 log_{10} CFU/ml after three and six days respectively ($P \leq 0.001$) (Figure 4.5.). After six days of growth the spore count increased 4.07 log_{10}-fold. The spore count was significantly lower at every time point than the total viable count ($P \leq 0.001$) (Appendix 1.4).

There was significantly higher total viable cell counts from biofilms in the PCR ribotype 002 biofilm at day zero (Figure 4.5.) than PCR ribotype 078 (1.21 log_{10}-fold) ($P \leq 0.001$) and 027 (1.19 log_{10}-fold) ($P \leq 0.001$) however there were similar total viable counts between PCR ribotypes 078 and 027 ($P=0.91$). The spore counts from biofilms at day zero for PCR ribotype 078 were significantly higher (2.03 log_{10}-fold) than a spore counts from PCR ribotype 027 ($P \leq 0.01$) and 002 ($P \leq 0.05$) biofilms. At day three (Figure 4.5.) of biofilm growth, PCR ribotype 002 had total viable counts were significantly higher than corresponding counts from PCR ribotype 078 (1.16 log_{10}-fold) ($P \leq 0.001$) and 027 (1.25 log_{10}-fold) ($P \leq 0.001$) biofilms. Conversely, the spore counts for PCR ribotype 027 were significantly more than PCR ribotype 002 (1.71 log_{10}-fold) ($P \leq 0.001$) and 078 (1.02 log_{10}-fold) ($P \leq 0.05$), as were the spore counts for PCR ribotype 078 compared to PCR ribotype 002 biofilm (1.69 log_{10}-fold difference) ($P \leq 0.001$).

At day six (Figure 4.5.) PCR ribotype 027 had the highest total viable count from biofilm which was significantly greater than the corresponding count from PCR ribotype 078 ($P \leq 0.001$) and 002 ($P \leq 0.01$). There was no significant difference between the spore counts for PCR ribotype 002 and 027 at day six, however PCR ribotype 027 ($P \leq 0.001$) and PCR ribotype 002 ($P \leq 0.001$) demonstrated significantly higher spore counts than those observed in PCR ribotype 078 biofilms.

4.4.3. Comparing Biofilm Growth, Total Viable Counts and Spore Count in a 96-Well Microtitre Plate Biofilm Assay

The results for PCR ribotype 078 showed viable count (4.57 log_{10} CFU/ml to 5.17 log_{10} CFU/ml; $P \leq 0.001$), spore count (3.96 log_{10} CFU/ml to 4.58 log_{10} CFU/ml; $P \leq 0.001$) and OD_{590} (0.12 OD_{590} to 0.28 OD_{590}; $P \leq 0.001$) increases between three to six days. This is not true for PCR ribotype 027 which showed an increase in viable count (4.94 log_{10} CFU/ml to 5.87 log_{10} CFU/ml; $P \leq 0.001$) and spore (4.02 log_{10} CFU/ml to 5.32 log_{10} CFU/ml; $P \leq 0.001$) counts but absorbance results plateaued between three and six days (0.12 OD_{590} to 0.08 OD_{590}; $P=0.30$). PCR ribotype 002 results showed an increase in spore count (2.34 log_{10} CFU/ml to 5.27 log_{10} CFU/ml; $P \leq 0.001$), whereas the total viable count (5.71 log_{10} CFU/ml to 5.70 log_{10} CFU/ml; $P=0.96$) and absorbance (0.07 OD_{590} to 0.08 OD_{590}; $P=0.58$) plateaued over six days (Figure 4.5. and Appendix 1.4). The viable count was significantly higher than spore count for all PCR ribotypes at all-time points ($P \leq 0.001$) (Appendix 1.4).

4.4.4. Biofilm Minimum Inhibition Concentration and Minimum Biofilm Eradication Concentrations

There was no difference between all of the PCR ribotypes bMIC and MBEC results for both antibiotics (Table 4.1). The same is true for the differences between the bMIC and MBEC for both antibiotics for all the PCR ribotypes (Table 4.1).

4.4.5. Comparing bMIC, MBEC and MIC

There was a significant difference between the planktonic MIC and bMIC and planktonic MIC and MBEC for metronidazole for all the PCR ribotypes. The geometric mean planktonic metronidazole MIC for PCR ribotype 078 (0.08 mg/L) was significantly lower than the bMIC (0.05 mg/ml) ($P \leq 0.001$) and MBEC (0.57 mg/L) geometric mean ($P \leq 0.001$). This is also true for the results for PCR ribotype 002 (MIC: 0.18 mg/L) (bMIC: $P \leq 0.001$; MBEC: $P \leq 0.001$). Whereas, the PCR ribotype 027 planktonic MIC (1.37 mg/L) was significantly higher than the bMIC ($P \leq 0.001$) and MBEC ($P \leq 0.05$). The vancomycin MIC results for all PCR ribotypes (PCR ribotype 078: 0.57 mg/L; 027: 0.53 mg/L; 002: 0.53 mg/L) were similar to the bMIC results (PCR ribotype 078: P= 0.30; 027: P=0.34; 002: P=0.88) and MBEC results (PCR ribotype 078: P=0.74; 027: P=0.34; 002: P=0.07) (Table 3.3. and Table 4.1.).

4.4.6. Biofilm Total Viable Count and Spore Count in a Static Calgary Biofilm Device Assay

PCR ribotype 078 total viable counts and spore counts results stayed constant throughout the experimental period (Figure 4.6.). There was not a significant difference between viable count and spore count for PCR ribotype 078 at each time point (day zero: P=0.15; day two: P=0.50; day four: P=0.07) (Figure 4.6.). The viable count results for PCR ribotype 027 also remained constant however there was a significant increase in spore count as the experiment progressed ($P \leq 0.001$) (Figure 4.6.). There was no significant difference between viable count and spore count at any time point for the PCR ribotype 027 results (Figure 4.6). PCR ribotype 002 total viable counts and spore counts increased significantly over the course of the experiment ($P \leq 0.05$) (Figure. 4.6.).There was not a significant difference between the PCR ribotype 002 spore count and viable count at any of the days tested (Figure 4.6.).

4.4.7. Static versus Agitated Calgary Biofilm Device Biofilm Assays

Agitation of the CBD significantly increased formation of biofilms for all *C. difficile* PCR ribotypes, including total viable counts and spore counts (PCR ribotype 078 and 002: viable count and spore count: $P \leq 0.01$, PCR ribotype 027: viable count and spore count: $P \leq 0.001$) (Figure 4.7).

Table 4.1. The geometric mean and range for biofilm minimum inhibitory concentration (bMIC) and minimum biofilm eradication concentration (MBEC) for PCR ribotype (RT) 078, 027 and 002 groups (N=11 strains of each PCR ribotype) for vancomycin (Van.) and metronidazole (Met). Determined using a Calgary Biofilm Device over four days in supplemented Brain Heart Infusion broth.

	Met.			
	bMIC		MBEC	
	Geometric mean (mg/L)	Range (mg/L)	Geometric mean (mg/L)	Range (mg/L)
RT078	0.50	<0.50-0.50	0.55	<0.50-1.00
RT027	0.50	<0.50-0.50	0.66	<0.50-1.00
RT002	0.57	<0.50-2.00	0.57	<0.50-1.00
	Van.			
	bMIC		MBEC	
	Geometric mean (mg/L)	Range (mg/L)	Geometric mean (mg/L)	Range (mg/L)
RT078	0.50	<0.50-0.50	0.57	<0.50-2.00
RT027	0.50	<0.50-0.50	0.50	<0.50-0.50
RT002	0.54	<0.50-1.00	0.73	<0.50-2.00

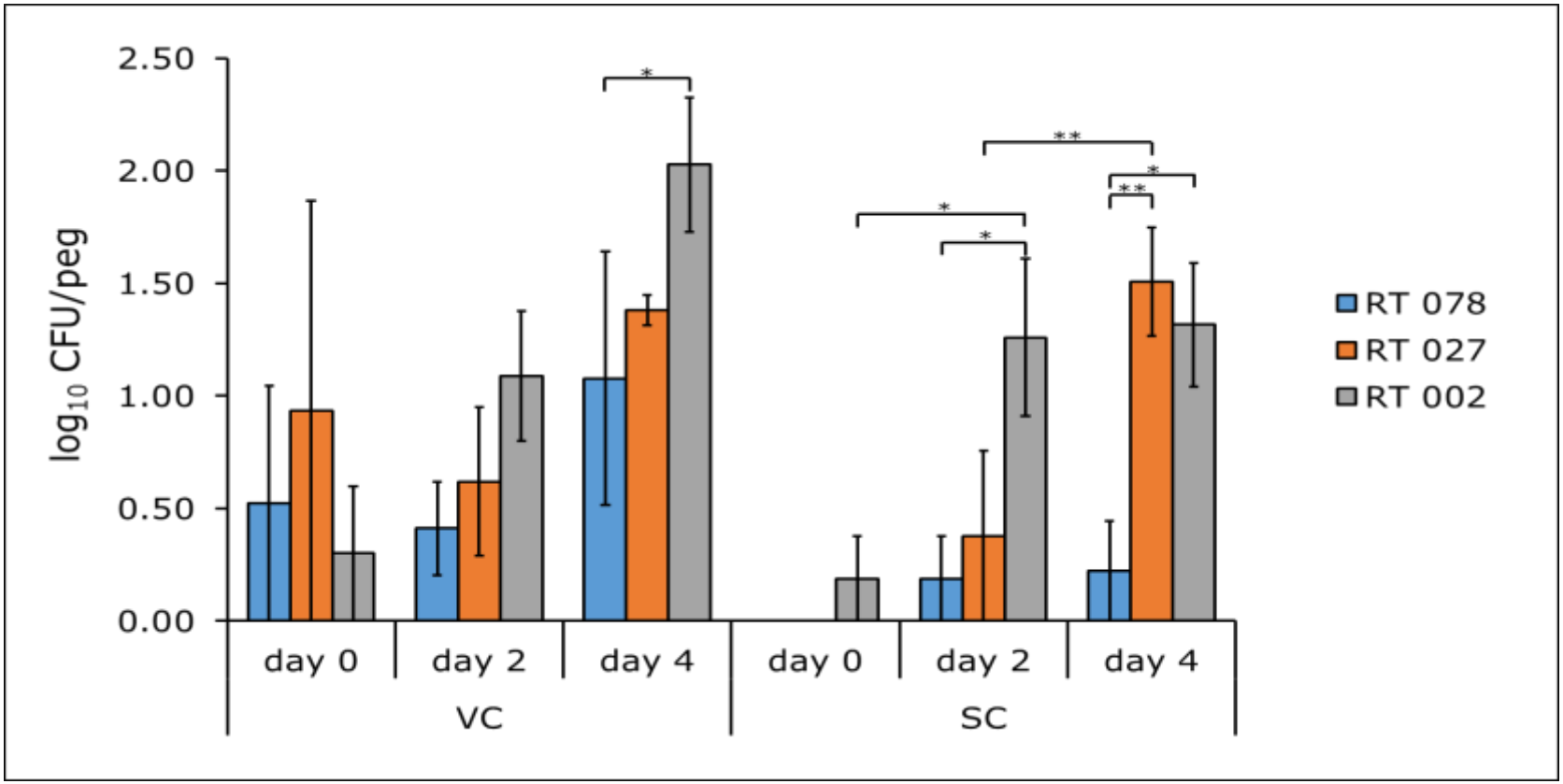

Figure. 4.6. Average Viable count (VC) and Spore count (SC) (log_{10} colony forming unit (CFU)peg (±SE)) in biofilms formed on a static Calgary Biofilm Device at zero, two and four days of growth, for PCR ribotype (RT) 078, 027 and 002 in supplemented Brain Heart Infusion broth.

* statistically significant difference P-value ≤0.05
** statistically significant difference P-value ≤0.01

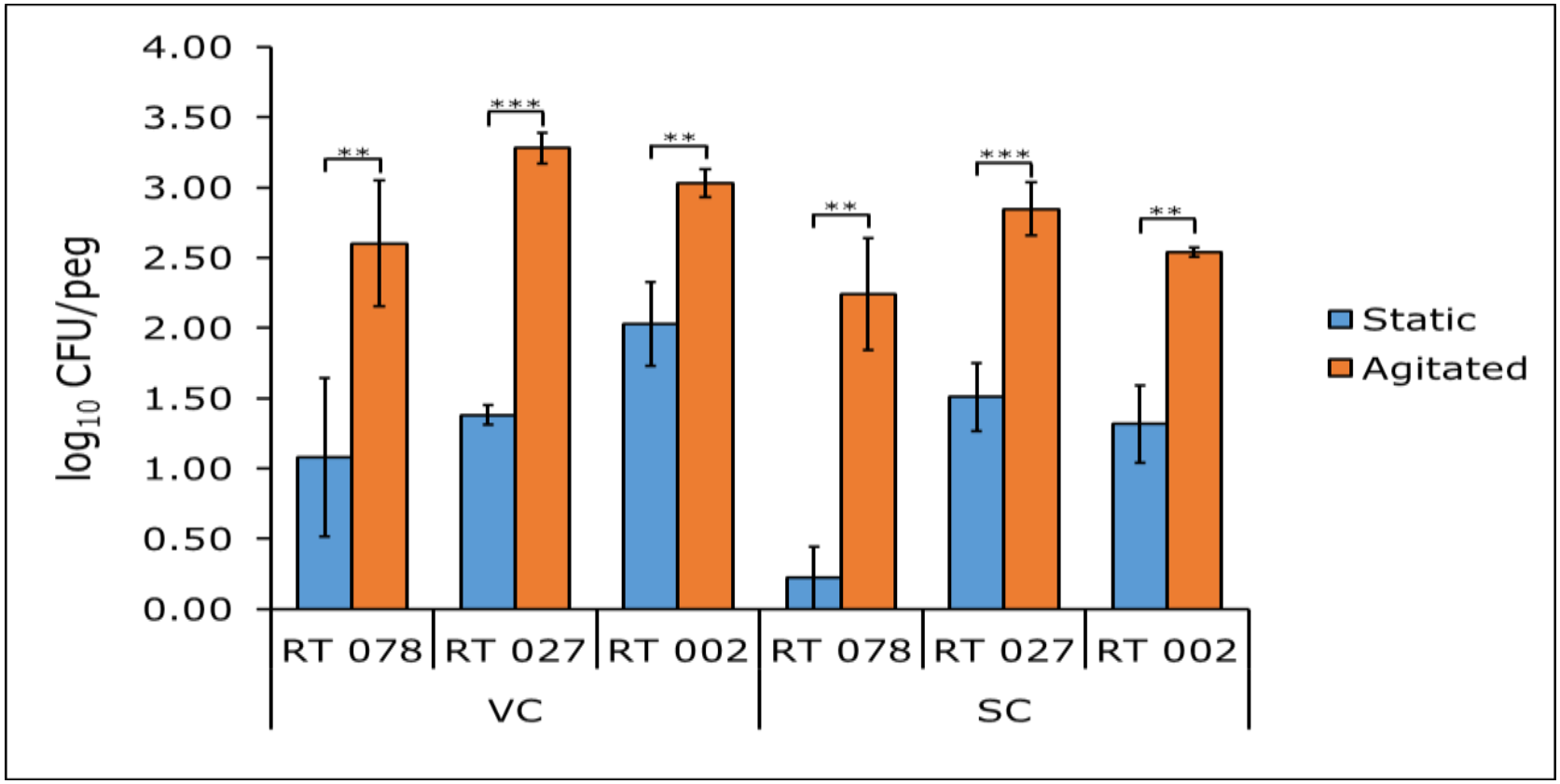

Figure 4.7. Total viable count (VC) and spore count (SC) (log_{10} colony forming unit (CFU)/peg (±SE)) in biofilms formed on a static or agitated (1300 rpm) Calgary Biofilm Device over four days of growth, for PCR ribotype (RT) 078, 027 and 002 in supplemented Brain Heart Infusion broth.

** statistically significant difference P-value ≤0.01
*** statistically significant difference P-value ≤0.001

4.4.8. Comparison of Geographical Location

The biofilm growth results for the PCR ribotype 078 group had no substantial variation between locations The bMIC and MBEC showed no substantial variation between locations of isolation as most the results for all three PCR ribotypes for both metronidazole and vancomycin were ≤0.5 mg/L (Appendix 1.5).

4.5. Discussion and Conclusion

Biofilms are the most common state for bacteria, as they give better protection to the bacteria within, from changes in the surrounding environment such as antibiotic therapy (Đapa *et al.*, 2013). The aims of this study were to determine if differences existed in biofilm production between three *C. difficile* PCR ribotype groups (PCR ribotype 078, 027 and 002) and also to determine if differences existed in biofilm susceptibility to metronidazole and vancomycin between these PCR ribotype groups. This was done by growing the biofilm over three and six days, then staining them with crystal violet, once washed, the staining was leeched out with methanol and the absorbance was read. The susceptibility of biofilms to metronidazole and vancomycin was also determined by growing biofilms for four days on a CBD, then placing the CBD into a concentration of antibiotics overnight, the absorbance was read of the antibiotic solution to find the bMIC. The CBD lid was placed into fresh BHIS overnight and the absorbance was read to find the MBEC.

PCR ribotypes 078 and 002 both showed an increase in biofilm mean biomass between three and six days of growth (only PCR ribotype 078 was significant), which is consistent with Lipovsek *et al.* (2013) who found that all PCR ribotypes they studied (including PCR ribotypes 078 and 027) had the potential to produce EPS matrix. However, Lipovsek *et al.* (2013) also demonstrated, that after two days of growth there was no EPS matrix present, whereas, after a five day growth period there was. Whereas, the results from the present study, for PCR ribotype 027, there was an insignificant decrease in the absorbance readings between three and six days, however Đapa *et al.* (2013) found a significant decrease in biofilm biomass, of a PCR ribotype 027 strain (R20291), between 24 hours and three days of growth and a noticeable decrease between three days and five days. It is reasonable to assume that a decrease could have occurred between five and six days, which were observed in the present study. Hammond *et al.* (2014) also found PCR ribotype 027 had a decrease in biofilm biomass between 24 and 48 hours, it could be assumed that the trend would continue over six days. This study used the same process as the present study, however Hammond *et al.* (2014) used Reinforced Clostridial Medium broth and glutaraldehyde solution to fix the biofilm. Interestingly, *C. difficile* maybe inefficient in adhering to a 96-well microtitre plate compared to other anaerobic bacteria, Donelli *et al.* (2012) described *C. difficile* as moderately adhering to the bottom of a 96-well microtitre plates compared to other Gram-positive anaerobes, such as: *C. perfringens* and *Finegoldia magna*, and Gram-negative anaerobes, such as: *B. fragilis* and *Prevotella intermedia*.

Biofilms were grown for three and six days for all the isolates, to find the total viable counts and spore counts within the biofilms. The viable counts for all PCR ribotypes significantly increased between three and six days (Figure 4.5). Contrastingly, (Dawson *et al.*, 2012) observed more living cells within a three day

old biofilm than within a six day old biofilm. The study also used a similar method to the present study and observed less vegetative cells in six day biofilms than three day biofilms, and the spore counts increased over six days, which confirms the results found in the present study (Dawson *et al.*, 2012). PCR ribotype 027 biofilms increased in viable counts and spore counts over three days of growth this is supported by Crowther *et al.* (2013) which also found an increase in both counts when a biofilm was grown in BHI broth. Growth of *C. difficile* in a 96-well microtitre plates compared to larger 24-well microtitre plates or larger 24-well microtitre plates or batch cultures in universal bottles, may potentially have affected growth dynamics and subsequent biofilm production. However, due to time constraints and difficulties in methods, such as constantly removing the microtitre trays from the anaerobic cabinet, which could have a considerable effect on *C. difficile* growth, this study was unable to conduct such experiments.

All viable counts results were significantly higher than the spore count results for all PCR ribotypes at every time point in the 96-well microtitre plates. This suggests there was a persistent population of vegetative cells throughout the experimental period. The day three results are supported by Semenyuk *et al.* (2014) who found more vegetative cells than spores within a three day old biofilm for all the strains in their study (including two strains of PCR ribotype 027). The present studies day six results are different than the results found in Semenyuk *et al.* (2014), whose results showed more spores than vegetative cells in a day six biofilm. The present study's results are also contradicted by Dawson *et al.* (2012), who found that there were more vegetative cells than spores in a biofilm produced by a PCR ribotype 027 (R20291) and strain 630Δerm at three days of growth. Whereas, at six days of growth there are more spores than vegetative cells for R20291 biofilm, this is not the case for 630Δerm biofilm. These results may differ due to differences in the methods used, for example Dawson *et al.* (2012) grew the biofilms in BHIS broth in tissue culture flasks, once diluted in PBS the cells and spores were spread on BHIS (supplemented with taurocholate), whereas in the present study the biofilms were grown in 96-well microtitre plates and grown on Brazier's agar.

There was not a significant difference between the total viable counts in biofilms produced by PCR ribotype 027 and 078 however PCR ribotype 002 demonstrated significantly more viable cells than both PCR ribotype 027 and 078 at day three. However, at day six PCR ribotype 027 had significantly more than the other two PCR ribotypes and PCR ribotype 078 had the lowest total viable count. (Dawson *et al.*, 2012) found strain R20291 had significantly higher viable counts than strain 630Δerm after both three and six days of biofilm growth. The differences maybe explained due to in the methods as described previously.

PCR ribotype 027 and 078 spore counts were significantly higher, at day three of growth than PCR ribotype 002 counts, and also after six days of biofilm growth. PCR ribotypes 027 and 002 sporulated to a significantly greater degree than PCR ribotype 078. Burns *et al.* (2011) also found cultures for PCR ribotype 027 strains had a significantly higher spore count than the that of non-PCR ribotype 027 strains after two days of growth and higher spore counts after five days, although they did not reach statistical significance. The present study found at six days, PCR ribotype 002 had a significantly higher spore count than PCR ribotype 078, this is supported by Cheng *et al.* (2011), who found that PCR ribotype 002 isolates, from patients, had a higher frequency of sporulation than non-PCR ribotype 002 strains, however, this study differed substantially from the present study in the

methodology of determining the viable counts. Semenyuk *et al.* (2014) demonstrated no difference between the sporulation of PCR ribotype 027 within biofilms compared to biofilms of a non-PCR ribotype 027 strain, however substantial differences in the methods utilised (culture media and spore assay protocol) may contribute to the difference in results.

Crystal violet (CV) staining was utilised to estimate total biofilm biomass, but this method stains both the EPS matrix and bacterial cells (dead or alive) but not spores (Kozuka and Tochikubo, 1991; Kwasny and Opperman, 2010; Pantanella *et al.*, 2013). In a PCR ribotype 027 biofilm, total viable and spore counts increased whereas the biomass plateaued this suggests sporulation was occurring within the biofilm, as the spores being produced do not take up the CV, and is possible additional EPS matrix was not being produced in the latter stages of biofilm formation (Appendix 1.4). PCR ribotype 002 biofilms showed an increase in spore count and a plateau in total viable count and biomass, this suggests again, sporulation was occurring and additional EPS matrix may not have been produced in the latter stages of biofilm formation (Appendix 1.4). Đapa *et al.* (2013) showed different results to the present studies results, the study produced graphs showing the absorbance (A_{570}) and viable counts (CFU/ml) for strain 630Δerm and strain R20291; both graphs showed the viable count results decreasing as the biomass plateaued between three and five days, there was not a difference in methods used in this study or the present study so the difference in results could be due to the strains used. However, the results of Dawson *et al.* (2012) are consistent with the results produced by PCR ribotype 078 in the present study, the paper showed an increase in viable and spore count and biomass between three and six day of biofilm growth, in strain 630Δerm and R20291 (Appendix 1.4). However, it cannot be stated definitively if elevated EPS matrix was being produced in PCR ribotype 078 biofilms as the total viable counts and spore counts from biofilms were collected from different experiments. Further studies are required to confirm these data.

The present study showed that there was not a significant difference between any of the vancomycin biofilm MIC results (Table 4.1.). This suggests PCR ribotype does not influence *C. difficile* susceptibility in a biofilm state when exposed to vancomycin. When compared to other Gram-positive bacteria, such as *Enterococcus faecium* and *S. aureus*, the *C. difficile* MBEC results, in the current study, are comparatively low (Holmberg and Rasmussen, 2014; Meije *et al.*, 2014). Holmberg and Rasmussen (2014) found *E. faecium* MBEC results, when exposed to vancomycin, ranged from 256 mg/L to 512 mg/L. However, Meije *et al.* (2014) found the MBEC for two strains of *S. aureus* (methicillin-susceptible *S. aureus* (MSSA) and MRSA) were more than 2000 mg/L when exposed to vancomycin. When comparing the MBEC and bMIC to the planktonic cell MICs (Table 3.2), the planktonic vancomycin MICs for each PCR ribotype were similar to the biofilm MIC results (Table 3.2 and Table 4.1). These observations contradict those of Antunes *et al.* (2011) whose study showed that the MIC for vancomycin against some *Staphylococcus* species was significantly lower than the MBEC produced. The current study are also different to the observations of Soriano *et al.* (2009), which found the MIC for vancomycin against the Gram-positive bacterium *Corynebacterium urealyticum* was approximately 22 times less than the MBEC (Soriano *et al.*, 2009; Soriano *et al.*, 1995). The difference in methods and bacterial species used in the current study to find the bMIC and BMEC (Table 4.1.) and MIC (Table 3.3.) could contribute to the differences in results and also there

is little *C. difficile* biofilm susceptibility work in the published literature to compare to. Additionally, *C. difficile* may be a comparatively weaker biofilm forming organism than these other bacterial species therefore less antimicrobial may be required to kill biofilms containing less biomass.

The metronidazole bMIC and MBEC results were similar for all the PCR ribotypes (Table 4.1.). Other anaerobic bacterial biofilm, such as those produced by *Gardnerella vaginalis* are affected by metronidazole treatment. For example, McMillan *et al.* (2011) found that 6 mg/L of metronidazole made holes in *G. vaginalis* biofilms and Swidsinski *et al.* (2008) found treatment with metronidazole temporarily suppressed *G. vaginalis* biofilms. When compared to the planktonic MICs, the bMIC and MBEC for PCR ribotype 078 and 002 were significantly less than the MICs, whereas the PCR ribotype 027 MICs were significantly higher, than the bMIC and MBEC results (Table 4.1. and Table 3.3.). This is, partly, supported by Wright *et al.* (1997) who found the anaerobic bacteria *Porphyromonas gingivalis* had a lower planktonic MIC (0.375 mg/L) compared to its bMIC (20 mg/L) for metronidazole (Diaz *et al.*, 2006). This was also true in Larsen (2002), which found *P. gingivalis* (ATCC 33277) had a bMIC of 4 mg/L, whereas, the planktonic MIC was 1 mg/L. The difference between the methods employed in the current study and the study of Larsen (2002) could contribute to the differences in results for the bMIC and BMEC (Table 4.1.) and the MIC (Table 3.3.).

Differences within the results for biofilm growth of each isolate within all three PCR ribotypes seem to not be influenced by location of isolation (Appendix 1.5). The MBEC and bMIC results showed no variation between the individual isolates within each PCR ribotype group (Appendix 1.5).

The Calgary biofilm device (CBD) has not been used previously to assess *C. difficile* biofilm formation and antimicrobial susceptibilities. The observations that some *C. difficile* PCR ribotypes grow as pellets of growth at the bottom of cultures tubes (for example PCR ribotype 078) (Figure 2.4.), suggested that the CBD may not be an optimal method for the study of biofilm susceptibilities in *C. difficile.* In order to address the potential lack of motility of certain *C. difficile* PCR ribotypes, the similar planktonic and biofilm MICs demonstrated previously (see Table 3.2 and Table 4.1), a study was undertaken to quantify biofilms on CBD pegs in static and agitated CBD experiments. The results of the present study indicated that total viable count and spore counts on a static CBD over four days of growth for all PCR ribotypes remained constant. Ali *et al.* (2006) found a significant increase in biofilm production between 24 and 48 hours, when *E. coil* and *Listeria innocua* were grown on CBDs, they also found after two days of growth both bacteria (5.78 $\log_{10}$ count/peg ±0.064) had noticeably more viable cells than were observed at day two in the present study (1.63 $\log_{10}$ CFU/peg±0.64). There was also an increase in biofilm production (over 25 hours) when Ceri *et al.* (1999) grew *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) on CBDs. The μ_{max} of the *E. coli* (planktonic μ_{max}= 1.73 h^{-1}), *L. innocua* (planktonic μ_{max} =0.78 h^{-1}) and *P. aeruginosa* (planktonic μ_{max}= 0.74 h^{-1}) could have an influence on biofilm production, as the faster the growth the sooner biofilm could be produced, however the average growth rate for *C. difficile* (0.63 h^{-1}) from this study, was lower than the growth rates of the other bacteria (Cox, 2004; Folsom *et al.*, 2010; Shama *et al.*, 2005).

Spores are resistant to antibiotics so if there are more spores in a biofilm, the biofilm may be able to survive antimicrobial therapy (Oka *et al.*, 2012). PCR ribotype 078 had significantly more spores in its biofilm (on the CBD) compared to PCR ribotypes 027 and 002, however their geometric means for the bMICs and MBECs (for vancomycin and metronidazole) were similar. The similarities in the results may be due to the number of vegetative cells within a biofilm, as there was no significant difference between the viable count for PCR ribotypes 078 and the other two PCR ribotypes. There was a significant difference between the biofilm spore counts which suggested varying numbers of vegetative cells in the biofilm. Therefore, other factors may contribute to similar bMIC and MBEC results such as the planktonic antibiotic susceptibility. As the planktonic susceptibility results were similar to the biofilm susceptibility results (Table 3.2 and Table. 4.1).

In conclusion, PCR ribotype seemingly does have an effect on the biofilm production; it would appear that the hypervirulent PCR ribotypes 078 and 027 produce more biofilm at three days of growth then the PCR ribotype 002. After six days of biofilm growth, PCR ribotype 078 biofilms further increased, in contrast to PCR ribotype 002 whereas, PCR ribotype 027 biofilm production decreased (although insignificantly). At six days, the viable count and spore count studies demonstrated that *C. difficile* biofilms were composed of a large proportion of spores, which would not stain with crystal violet, therefore this may explain why OD_{590} did not increase for some strains at day six. The assessment of other methods for quantifying *C. difficile* biomass may explain this phenomenon further. Additionally, all the bMIC and MBEC were similar regardless of PCR ribotype, which conflicts with some prior studies and warrants further study to confirm these findings and improve the reproducibility of the results obtained. It is possible that the CBD is not a suitable experimental system in which to study *C. difficile* biofilms, due to culture settling and the bottom of the microtitre plate wells (Figure 2.4.), and no prior studies in *C. difficile* using this model exist in the literature. However, the present study shows that a *C. difficile* biofilm will grow on a CBD and will grow better if the microtitre plate is agitated during the growth period (Figure 4.7). Due to biofilms being the natural state for most bacteria and *C. difficile* being able to produce biofilms within the gut (of mice), these results suggest that biofilms produced by PCR ribotype 078 in CDI patients could cause the infection to last longer, as the PCR ribotypes biofilm production increases between three and six days (*in vitro*) (Crowther *et al.*, 2014; Jefferson *et al.*, 2005; Semenyuk *et al.*, 2014). Thicker biofilms contain more antibiotic-resistant spores, which also increases the likelihood of recurrence of CDI. Thicker *C. difficile* PCR ribotype 078 biofilms may be more difficult to eradicate with antibiotics due to reduced drug penetration compared to the biofilms of PCR ribotype 027 and 002.

5. Main Discussion and Conclusions

C. difficile is an anaerobic, Gram-positive spore-forming bacterium which resides in many different environments (Baverud *et al.*, 2003; Lawley *et al.*, 2009). The species is classified into over 600 different PCR ribotypes. The present study compared three common PCR ribotype groups which were isolated from clinical cases of CDI in the UK (Table 1.1.), and are often isolated from the intestinal tracts of humans and animals (Janezic *et al.*, 2012). This study aimed to compare the growth characteristics, cytotoxin production, antimicrobial susceptibilities, and biofilm growth and antimicrobial susceptibilities between these three PCR ribotype groups.

PCR ribotype 078, 027 and 002 all cause disease by producing two toxins (toxin A and toxin B). These toxins can cause a spectrum of disease including diarrhoea, PMC and even death (Debast *et al.*, 2014; Shen, 2012). CDI occurs when the normal gut microflora becomes compromised by treatments, such as chemotherapy, and most commonly antibiotic therapy, which inhibits the other bacteria in the gut and in turn causes *C. difficile* to thrive (Debast *et al.*, 2014; Loo *et al.*, 2011). Treatments for CDI include two antibiotics, metronidazole and vancomycin, depending on the severity of the illness. PCR ribotype 078 has incrementally increased in prevalence in humans from 2007 to 2013 and has been classed, as hypervirulent by Goorhuis *et al.* (2008), as has PCR ribotype 027 (PHE and CDRN, 2014). Both PCR ribotypes have been hypothesised to produce more toxin as a consequence of mutations within the PaLoc, and in turn, cause more severe illness than other virulent PCR ribotypes (Goorhuis *et al.*, 2008; MacCannell *et al.*, 2006). Hypervirulent PCR ribotypes are also thought to be more resistant to certain classes of antibiotics, therefore if these antibiotics are used for other illnesses, they are more likely to cause CDI. For example, resistance to fluoroquinolones has been observed in PCR ribotypes 078 and 027 (He *et al.*, 2013; Keessen *et al.*, 2013; McDonald *et al.*, 2005).

Toxin production is considered to be the major factor of virulence in *C. difficile* and hypervirulent PCR ribotypes are thought to produce quantitatively more toxin therefore cause more severe disease (Curry *et al.*, 2007; Goorhuis *et al.*, 2008; Knetsch *et al.*, 2011; Shen, 2012; Warny *et al.*, 2005). The results of the present study were interesting in that cytotoxin production by PCR ribotype 078 group was elevated in comparison to PCR ribotype 002, and more interestingly the reported hyper-toxin producing PCR ribotype 027 (Warny *et al.*, 2005). This could be due to the 39-bp mutation within *tcdC* which has been suggested to negatively regulates toxin production (Cartman *et al.*, 2012; Curry *et al.*, 2007; Knetsch *et al.*, 2011), although contradictory evidence does exist for the role of *tcdC*. It may be that slightly higher OD_{600} readings for the PCR ribotype 078 group (up to six hours) may have contributed to this to a small degree but at the latter time points where cytotoxin expression was observed at higher levels (24 and 48 hours), OD_{600} readings were similar between ribotypes. Low-level cytotoxin production was observed sooner in the PCR ribotype 078 group than in the comparator groups, therefore this may have contributed to the eventual elevated cytotoxin titres in a cumulative fashion. Further studies assessing the level of transcriptional activity of the PaLoc genes may help to determine if these observations were indeed a consequence of true earlier cytotoxin production or an artefact of *in vitro* experimentation. These results suggest that PCR ribotype 078 could cause illness

sooner if these growth characteristics were observed *in vivo*, and with elevated cytotoxin production, CDI of greater severity is a possibility, although prior studies have failed to conclusively link toxin levels in faeces of CDI patients with severity of disease (Akerlund *et al.*, 2006; Cartman *et al.*, 2012; Curry *et al.*, 2007; Knetsch *et al.*, 2011).

Antimicrobial resistance in *C. difficile* could potentially contribute to a patient contracting CDI (Nelson *et al.*, 2011). In the present study PCR ribotype 078, was susceptible to most of the antibiotics used in this study including the two antibiotics used as treatments for CDI; metronidazole and vancomycin. The present study showed PCR ribotype 078 was more susceptible to metronidazole (breakpoint ≤8 mg/L; Geometric mean MIC: 0.08 mg/L) than PCR ribotype 027 (Geometric mean MIC: 1.37 mg/L) but not substantially different in susceptibility compared to PCR ribotype 002 (Geometric mean MIC: 0.18 mg/L) (CLSI, 2007). Even though these results are not consistent with Moura *et al.* (2013), who used AIM (using Schaedler's broth and Wilkins-Chalgren agar) to demonstrate that PCR ribotype 078 (0.125 mg/L) had very similar metronidazole MIC results to PCR ribotype 027 (0.125 mg/L to 0.5 mg/L), as well as other virulent strains, such as PCR ribotype 001 (0.125 mg/L to 4 mg/L). Despite its hypervirulent status, PCR ribotype 078 remains, potentially, treatable with metronidazole if simple antimicrobial susceptibility data are considered alone. A similar scenario was observed for the vancomycin (breakpoint ≤2 mg/L) geometric mean MIC results, (PCR ribotype 078: 0.57 mg/L; PCR ribotype 027 and 002: 0.53 mg/L) which are similar to the results reported by Debast *et al.* (2013) whose results showed no noticeable difference in susceptibility for the PCR ribotype 078 or 027, therefore these results, suggest vancomycin could treat CDI caused by PCR ribotype 078 just as well as if it were caused by PCR ribotype 027 or 002 (Table 3.3.) (EUCAST, 2014). The clindamycin (susceptibility breakpoint: ≤2 mg/L) geometric mean MIC for PCR ribotype 078 (0.09 mg/L) was the lowest out of the three PCR ribotype groups assessed in this study (PCR ribotype 027: 0.44 mg/L; PCR ribotype 002: 3.31 mg/L), concluding PCR ribotype 002 are less susceptible to clindamycin (CLSI, 2007). PCR ribotype 078 had a lower geometric mean MIC to erythromycin (0.5 mg/L) than PCR ribotype 027 (128 mg/L) and similar results to PCR ribotype 002 (0.25 mg/L) (breakpoint: >2 mg/L) (BSAC, 2013). Therefore PCR ribotype 078 is more susceptible to these antibiotics than the other PCR ribotypes. The linezolid (breakpoint: >4 mg/L) geometric mean MIC for the PCR ribotype 078 group (0.73 mg/L) was noticeably higher than PCR ribotype 027 (0.25 mg/L) and 002 (0.5 mg/L). This is the same for the tetracycline (breakpoint: ≤4 mg/L) results (PCR ribotype 078: 0.93 mg/L; PCR ribotype 027: 0.11 mg/L; PCR ribotype 002: 0.06 mg/L), this could be explained by the tetracycline resistance gene, *tetM*, which PCR ribotype 078 does possess, whereas PCR ribotype 027 does not (Bakker *et al.*, 2010; CLSI, 2007; Stabler *et al.*, 2009). Therefore these results show PCR ribotype 078 is relatively less susceptible to linezolid and tetracycline than the other two PCR ribotypes. This could result in PCR ribotype 078 CDI potentially occurring earlier in the course of therapy with these agents, before the gut microflora had recovered, than for *C. difficile* strains which were more susceptible. Additionally, PCR ribotype 078 was more susceptible to ciprofloxacin (7.51 mg/L) and moxifloxacin (1.07 mg/L) than the other two PCR ribotypes (PCR ribotype 027: ciprofloxacin (68.16 mg/L) and moxifloxacin (32 mg/L); PCR ribotype 002: ciprofloxacin (10.29 mg/L) and moxifloxacin (1.76 mg/L)). Previous studies have found both PCR ribotype 078 and 027 may possess mutations in *gyrA* and/or *gyrB* which have been linked to fluoroquinolone resistance (O'Connor *et al.*, 2009;

Rodriguez *et al.*, 2014; Saxton *et al.*, 2009). These results suggest, PCR ribotypes 078 and 027 (and 002) are both resistant to ciprofloxacin (breakpoint: >1 mg/L) but only PCR ribotype 027 was resistant to moxifloxacin (breakpoint: ≥8 mg/L) and therefore may have increased risk of causing CDI in moxifloxacin-treated patients (BSAC, 2013; CLSI, 2007). It can be concluded by these results that antibiotic susceptibly cannot be determined by PCR ribotype, therefore could not contribute to the hypervirulent status of PCR ribotype 078, contradictory to previous studies, such as Goorhuis *et al.* (2008), who contributed fluoroquinolone resistance to PCR ribotype 078 and 027 hypervirulent status, as all strains in all PCR ribotypes were resistant to ciprofloxacin therefore resistance to this antibiotic could increase in a clinical setting. Regular screening for resistance will need to be performed so resistance to other antibiotics in clinical setting can be monitored and changes can be made where possible.

Biofilms are the most common state for most bacteria, and *C. difficile* produces biofilms within the gastrointestinal tracts of mice and also *in vitro* in simple and complex biofilm models, which have been found to increase the antibiotic resistance of the bacteria within (Burmolle *et al.*, 2014; Crowther *et al.*, 2014; Dawson *et al.*, 2012; Jefferson *et al.*, 2005). Biofilms are thought to potentially contribute to the recurrence of CDI, as a mature *C. difficile* biofilm contains highly resistant spores, which are dispersed in the later stage of biofilm production (Semenyuk *et al.*, 2014). The present study found that PCR ribotype 078 had increased biofilm production between three and six days, compared to PCR ribotype 027 which decreased and PCR ribotype 002 where minimal biofilm production was observed. The effects of the antibiotics metronidazole and vancomycin were assessed in their ability to inhibit *C. difficile* biofilms using a CBD. The results of the present study were difficult to interpret as the bMIC were low and comparable to the MBEC. Further testing demonstrated that agitating the CBD throughout the growth period, increased viable and spore count. It is possible that agitation of the microtitre CBD plates may facilitate better mixing and more robust biofilm formation and therefore bMIC and MBEC that reflect similar studies in other organisms (Jefferson *et al.*, 2005). Given the apparent greater degree of biofilm formation by PCR ribotype 078, if these observations translated to the *in vivo* situation, it could be more difficult to treat these biofilms than those of other PCR ribotypes, such as PCR ribotype 027 and 002. PCR ribotype 078 biofilms may potentially produce more EPS matrix, therefore be thicker and could be more resistant to antibiotic treatment. (Crowther *et al.*, 2014; Semenyuk *et al.*, 2014; Stoodley *et al.*, 2002). Experiments with the CBD in pure culture are a simplification of *in vivo* biofilms within the gastrointestinal tract where complex biofilms of multiple bacterial species exist (Crowther *et al.*, 2014), therefore results from simple pure culture biofilms must be interpreted with some caution. Despite this, differences under the same experimental conditions were observed for the biofilms of the *C. difficile* groups assayed in this study which warrant further study to assess their significance and reproducibility.

The location of strains appears to have little or no effect on the results for all the properties investigated in this study (Appendix 1.5). However, it is hard to demine using this study due to the small number of strains from each location, to further investigate if location of isolation does influence MIC or biofilm production (for examples) more isolates from different locations would have to be studied. This will also determine similarities between different regions and counties.

In conclusion, in this study that assessed the phenotype of the hypervirulent *C. difficile* PCR ribotype 078 it was observed that a group of PCR ribotype 078 *C. difficile* isolates produced elevated cytotoxin titres and potentially began producing low-level cytotoxin earlier in the growth cycle, even when compared to the hypervirulent PCR ribotype 027. Furthermore, increased biofilm production by PCR ribotype 078 was also observed which could potentially contribute to greater difficulty in eradicating *C. difficile in vivo* if these results were translated in patients. The antibiotics, metronidazole and vancomycin, used at present in hospitals around UK, were effective against planktonic cells and bacteria within a biofilm, during *in vitro* experiments. Resistance to fluoroquinolones in PCR ribotypes 078 and 002, such as ciprofloxacin, could be the result of fluoroquinolone selective pressure observed in the clinical setting as a consequence of their increasing prevalence in the UK (PHE and CDRN, 2014).

This study adds to the literature published on the hypervirulent PCR ribotype of *C. difficile.* Goorhuis *et al.* (2008) previously studied PCR ribotype 078 investigated the PCR ribotype 078 PaLoc and observed the presence of intact *tcdA* and *tcdB* genes and a mutation in *tcdC*, however the study did not investigate the production of the toxins compared to other PCR ribotypes. The study of Goorhuis *et al.* (2008) also compared patients with CDI, caused by PCR ribotype 078 and 027, to see if there were similar characteristics, such as age, severity of illness and mortality, and found that patients with PCR ribotype 078 induced-CDI were younger but had similar severity of symptoms and mortality rates to PCR ribotype 027. The present study investigated potential virulence-enhancing traits of *C. difficile*, such as cytotoxin production, and compared two hypervirulent PCR ribotypes and a comparator strain (PCR ribotype 002). The present study also compared the properties which could contribute to more severe illness and CDI recurrence in patients such as growth characteristics, biofilm production, and biofilm/planktonic cell susceptibilities to antimicrobial agents. More studies are required in order to evaluate PCR ribotype 078 further, in more gut-reflective *in vitro* models or in animal models of CDI.

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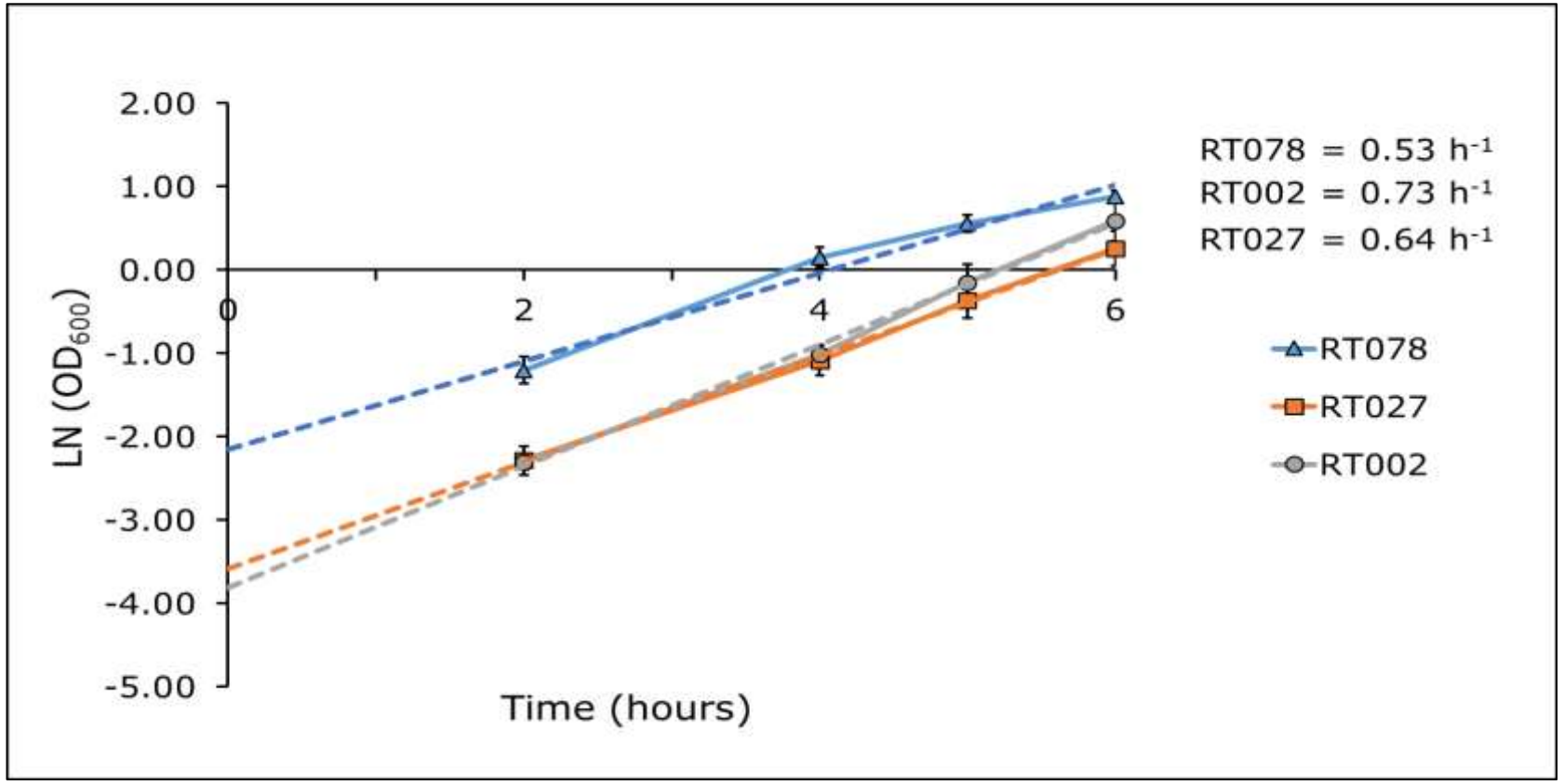
Appendices

Appendix 1.1. The 'SIGHT' protocol is used in hospitals when a patient is suspected of having *C. difficile* infection (DH and HPA, 2008).

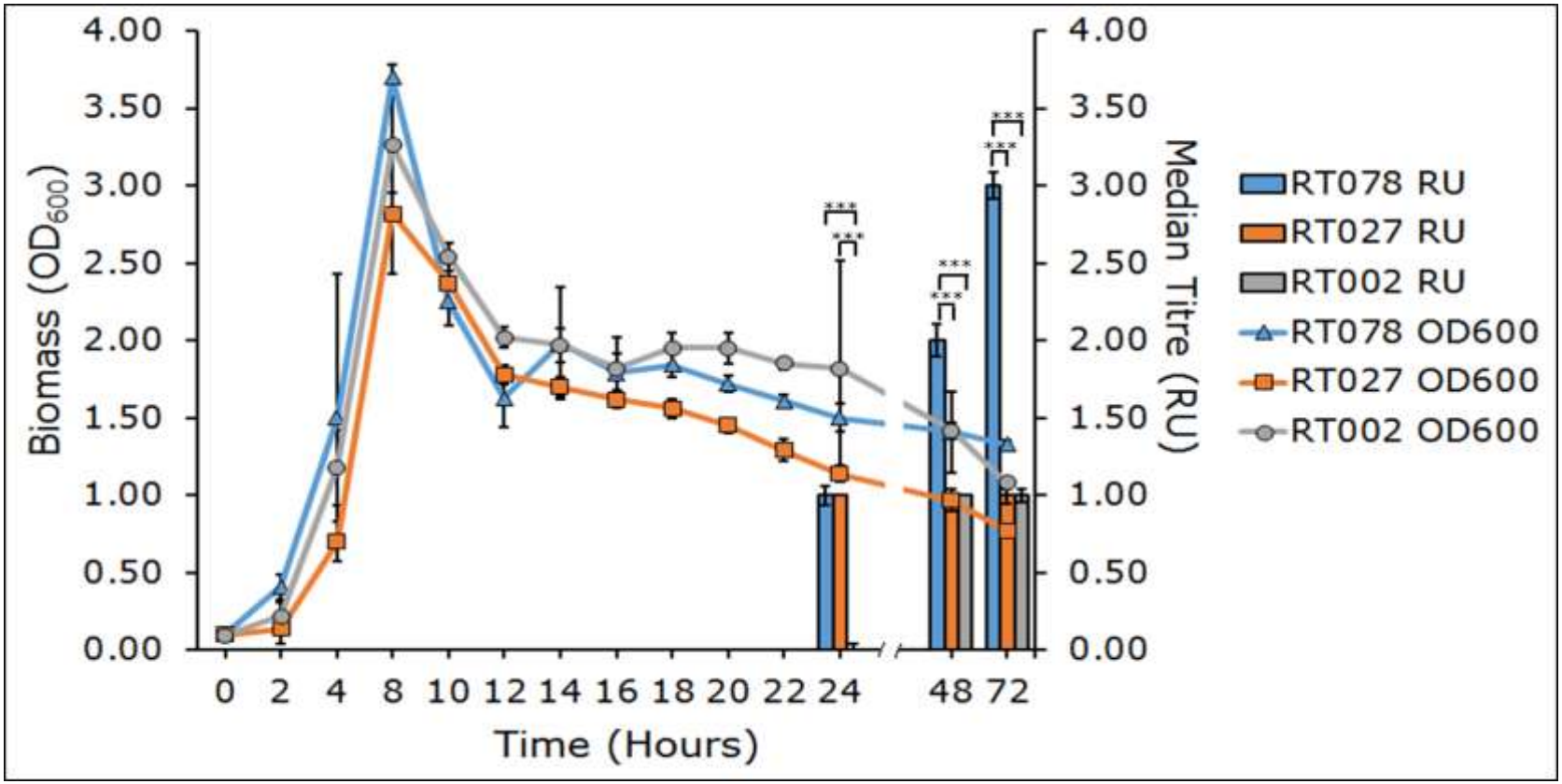
S	Suspect that a case may be infective where there is no clear alternative cause for diarrhoea.
I	Isolate the patient and consult with the infection control team (ICT) while determining the cause of the diarrhoea.
G	Gloves and aprons must be used for all contacts with the patient and their environment.
H	Hand washing with soap and water should be carried out before and after each contact with the patient and the patient's environment.
T	Test the stool for toxin, by sending a specimen immediately.

Appendix 1.2. The natural logarithm (LN) of mean OD_{600} (±SE) in batch culture growth in supplemented Brain Heart Infusion broth of *C. difficile* PCR ribotypes (RT) 078, 027and 002 (N=11 of each PCR ribotype) between 2 and 6 hours. Showing the maximum specific growth rate (h^{-1}) for each PCR ribotype.



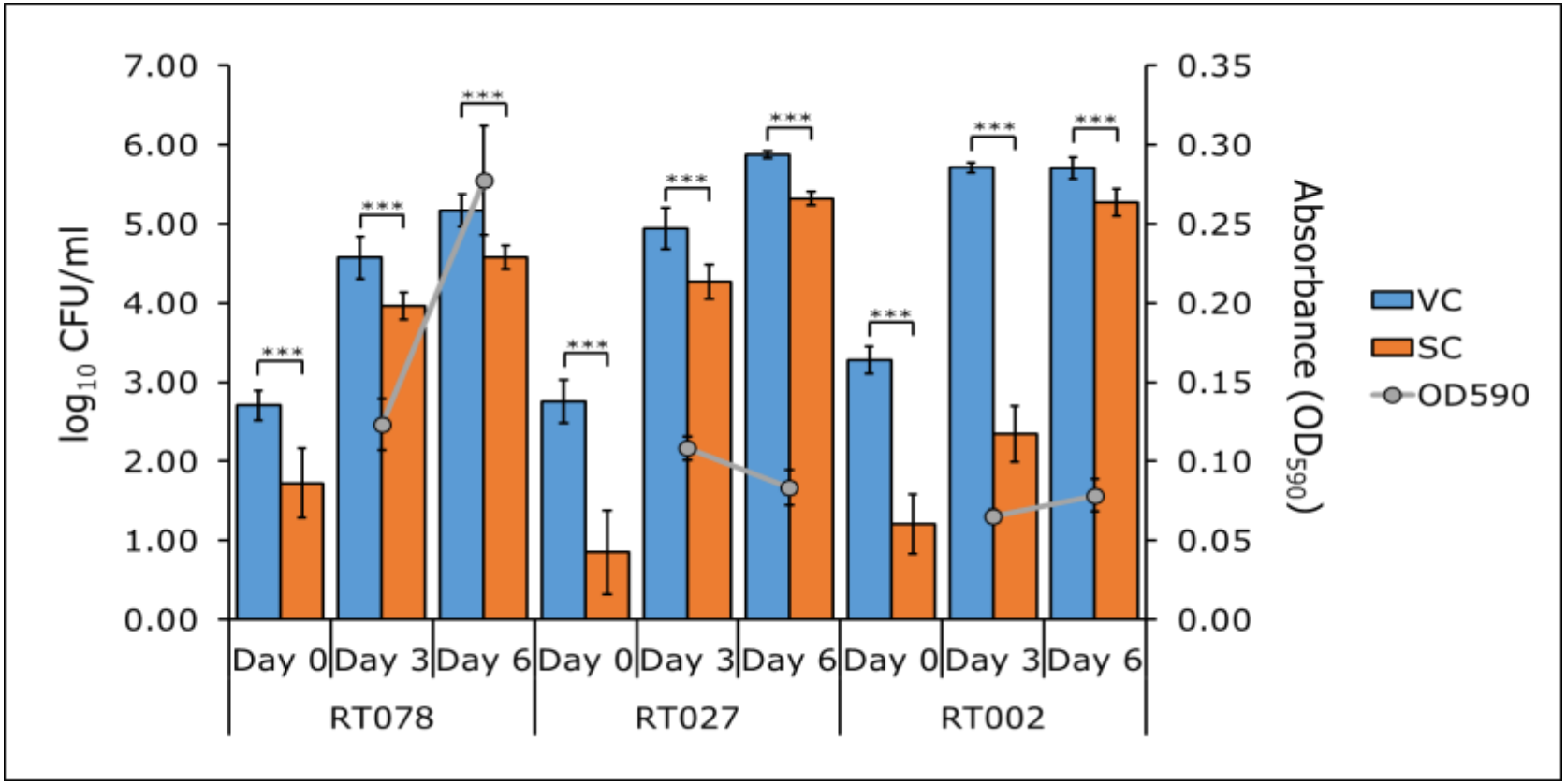
Appendix 1.3. Biomass (optical density $(OD)_{600}$ (±SE)) and median cytotoxin titre (relative units, RU (±SE)) for PCR ribotypes (RT) 078, 027 and 002 (N=11 strains of each PCR ribotype) grown in supplemented Brain Heart Infusion broth.

*** statistically significant difference P-value ≤0.001



Appendix 1.3. Biomass (optical density $(OD)_{600}$(±SE)) and median cytotoxin titre (relative units, RU (±SE)) for PCR ribotypes (RT) 078, 027 and 002 (N=11 strains of each PCR ribotype) grown in supplemented Brain Heart Infusion broth.

*** statistically significant difference P-value ≤0.001



Appendix 1.5. Results for each individual isolate for PCR ribotype (RT) 078, 027 and 002, including the two control strains (E4 and ATCC 700057) as well as strain number and location of isolation. The results are for specific growth rate (μ_{max}) (h^{-1}), median cytotoxin production (relative units, RU) at 24, 48 and 72 hours (h), minimum inhibitory concentration (MIC) (mg/L) for metronidazole (Met.), vancomycin (Van.), ciprofloxacin (Cip.), clindamycin (Clind.), erythromycin (Ery.), linezolid (Lin.), moxifloxacin (Mox.) and tetracycline (Tet.), as well as biofilm MIC (bMIC) and minimum biofilm eradication concentration (MBEC) (mg/L) for Met. and Van. and biofilm average biofilm growth (OD_{590}) at 3 and 6 days (D) of growth.

Strain Number	Location of Isolation	μ_{max} (h^{-1})	Median Toxin Production (RU)			MIC (mg/L)								bMIC (mg/L)		MBEC (mg/L)		Biofilm Growth (OD_{590})	
RT078			**24h**	**48h**	**72h**	**Met.**	**Van.**	**Cip.**	**Mox.**	**Clind.**	**Lin.**	**Tet.**	**Ery.**	**Met.**	**Van.**	**Met.**	**Van.**	**D3**	**D6**
24	Leeds	0.51	1	1	3	0.125	1	8	1	8	2	0.25	0.06	<0.5	0.5	NR	0.5	0.11	0.56
31	Derriford	0.55	1	2	3	<0.03	0.5	8	1	8	1	0.25	0.06	NR	0.5	0.5	0.5	0.10	0.28
32	Derriford	0.44	1	1	3	<0.03	0.5	4	1	0.5	0.03	0.25	0.06	<0.5	0.5	<0.5	0.5	0.08	0.20
45	Cumberland	0.49	1	2	3	0.03	0.25	8	1	0.03	1	>128	0.06	<0.5	<0.5	1.0	2	0.12	0.29
46	Cumberland	0.48	1	2	3	0.125	0.5	8	1	8	0.5	0.25	0.06	<0.5	0.5	<0.5	0.5	0.12	0.30
47	Cumberland	0.54	1	2	2	0.25	1	8	0.25	8	1	0.25	0.06	<0.5	<0.5	<0.5	0.5	0.11	0.24
48	Cumberland	0.56	1	2	2	0.125	0.5	8	1	8	0.5	0.25	0.06	0.5	0.5	<0.5	<0.5	0.06	0.28
59	Sheffield	0.51	1	2	3	0.125	0.5	8	1	0.03	2	0.5	0.06	<0.5	<0.5	NR	<0.5	0.13	0.23
60	Sheffield	0.59	1	2	2	0.03	0.5	8	8	8	1	0.5	2	<0.5	0.5	<0.5	0.5	0.22	0.24
61	Southampton	0.62	1	2	2	0.125	1	8	1	0.03	1	0.25	0.06	<0.5	<0.5	0.5	<0.5	0.20	0.16
62	HPA SE	0.53	1	2	3	0.125	0.5	8	1	0.125	1	0.25	0.25	NR	NR	NR	NR	NR	NR

Strain Number	Location of Isolation	μ_{max} (h^{-1})	Median Toxin Production (RU)			MIC (mg/L)								bMIC (mg/L)		MBEC (mg/L)		Biofilm Growth (OD_{590})	
RT027			**24h**	**48h**	**72h**	**Met.**	**Van.**	**Cip.**	**Mox.**	**Clind.**	**Lin.**	**Tet.**	**Ery.**	**Met.**	**Van.**	**Met.**	**Van.**	**D3**	**D6**
9	Bradford	0.54	1	1	1	2	0.5	128	32	0.03	0.06	>128	0.5	0.5	<0.5	1.0	<0.5	0.16	0.14
10	Bradford	0.57	1	1	1	4	1	>128	32	0.03	0.25	>128	0.5	0.5	<0.5	0.5	0.5	0.10	0.06
11	Bradford	0.67	1	1	1	2	0.5	>128	32	0.06	0.25	>128	0.5	<0.5	<0.5	<0.5	0.5	0.10	0.13
12	Bradford	0.65	1	1	1	1	0.5	64	32	0.06	<0.03	>128	0.5	0.5	<0.5	<0.5	<0.5	0.14	0.11
19	Bath	0.60	1	1	1	4	0.5	>128	32	0.06	0.5	>128	0.5	<0.5	<0.5	<0.5	<0.5	0.09	0.11
20	Bath	0.56	1	1	1	2	0.5	128	32	0.06	0.25	>128	0.5	<0.5	<0.5	<0.5	<0.5	0.09	0.06
35	Leeds	0.69	1	1	1	0.5	0.5	16	32	4	0.5	>128	0.5	0.5	0.5	0.5	0.5	0.09	0.04
36	Leeds	0.69	1	1	1	1	0.5	16	32	4	0.5	>128	0.5	0.5	<0.5	1.0	0.5	0.10	0.08
37	Airedale	0.72	1	1	1	0.5	0.5	64	32	0.06	0.5	>128	0.5	0.5	<0.5	1.0	<0.5	0.10	0.06
38	Bradford	0.74	1	1	1	2	0.5	64	32	0.03	0.25	128	0.06	0.5	<0.5	1.0	0.5	0.11	0.05
41	Barnsley	0.58	1	1	1	0.5	0.5	64	32	0.06	0.5	>128	1	NR	NR	NR	NR	NR	NR

Strain Number	Location of Isolation	μ_{max} (h^{-1})	Median Toxin Production (RU)			MIC (mg/L)								bMIC (mg/L)		MBEC (mg/L)		Biofilm Growth (OD_{590})	
RT002			24h	48h	72h	Met.	Van.	Cip.	Mox.	Clind.	Lin.	Tet.	Ery.	Met.	Van.	Met.	Van.	D3	D6
1	Ireland	0.73	0	1	1	0.125	0.5	8	2	0.06	0.5	0.25	8	<0.5	0.5	<0.5	0.5	0.09	0.03
4	Ireland	0.68	0	1	1	0.25	0.5	8	1	0.06	2	0.25	8	<0.5	<0.5	0.5	<0.5	0.08	0.06
5	Ireland	0.64	0	1	1	0.25	0.5	8	2	0.06	1	0.25	8	2.0	0.5	0.5	1	0.07	0.06
6	Ireland	0.76	0	1	1	0.125	0.5	8	2	0.06	1	0.25	8	0.5	<0.5	0.5	1	0.06	0.03
39	Cumberland	0.77	0	1	1	0.125	0.5	16	2	0.06	1	0.25	8	<0.5	0.5	0.5	1	0.06	0.12
40	Cumberland	0.80	0	1	1	0.25	0.5	16	2	0.06	0.06	0.25	0.5	0.5	0.5	1.0	0.5	0.04	0.09
53	Leeds	0.69	0	1	1	0.25	1	16	2	0.06	0.5	0.25	8	0.5	1	1.0	0.5	0.07	0.11
54	Leeds	0.76	0	1	1	0.25	0.5	16	2	0.06	0.5	0.25	1	0.5	NR	<0.5	NR	0.06	0.10
66	Harrogate	0.74	0	1	1	0.125	0.5	8	1	0.06	0.5	0.25	1	<0.5	0.5	0.5	0.5	0.07	0.08
68	Harrogate	0.75	0	1	1	0.125	0.5	8	2	0.06	1	0.25	8	<0.5	0.5	0.5	2	0.06	0.10
71	Maidstone	0.67	0	1	1	0.25	0.5	8	2	0.06	0.06	0.25	0.5	NR	NR	NR	NR	NR	NR

Strain Number		μ_{max} (h^{-1})	Median Toxin Production (RU)			MIC (mg/L)								bMIC (mg/L)		MBEC (mg/L)		Biofilm Growth (OD_{590})	
Control strains			24h	48h	72h	Met.	Van.	Cip.	Mox.	Clind.	Lin.	Tet.	Ery.	Met.	Van.	Met.	Van.	D3	D6
110	E4 (RT010)	NR	NR	NR	NR	0.25	0.5	8	2	32	1	0.25	0.25	NR	NR	NR	NR	0.07	0.17
111	ATCC 700057 (VPI 11186) (RT038)	NR	NR	NR	NR	8	0.5	32	32	0.06	0.03	>128	128	NR	NR	NR	NR	0.17	0.06

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