CHARACTERISATION OF REDUCED SUSCEPTIBILITY TO METRONIDAZOLE IN EPIDEMIC *CLOSTRIDIUM DIFFICILE* CLINICAL ISOLATES

Submitted to the University of Hertfordshire in partial fulfilment of the requirement of the degree of PhD

By

Olufunmilayo Ayus Mohammed

Principal Supervisor: Dr Simon Baines

Supervisors - Professor Mark Wilcox, Dr Kate Graeme-Cook

School of Life and Medical Sciences, University of Hertfordshire, UK.

July 2017

ABSTRACT

Reduced susceptibility in some *C. difficile* isolates has been observed to metronidazole, a frontline antibiotic for *C. difficile* infection. Therefore, this study was performed to characterize reduced metronidazole susceptibility in UK *C. difficile* isolates, using phenotypic and genotypic approaches. This research focused on strains of three UK *C. difficile* ribotypes 027, 001, 106 including metronidazole reduced susceptible (CDRM) and metronidazole susceptible (CDSM) *C. difficile* strains, and 2 control strains from ribotype 010 (CDRM) and 038 (CDSM). An agar incorporation method (AIM) was used to determine the susceptibility and heterogeneity of minimum inhibitory concentrations (MICs). Ribotype 001 CDRM strains was observed for metronidazole to have the highest MIC_{90} (8mg/L) and the highest MIC hetereogeneity distributed across 2 MICs was observed in ribotype 106 at 22% and 11% for metronidazole and vancomycin respectively. All strains were susceptible to vancomycin at MIC $\leq$ 2mg/L which reflected in the high area under the curve (AUC) results. Population analysis profiling (PAP) incorporating total viable counting on antibiotic-containing agar plates was also employed and ribotype 001 had the lowest AUC (2.32) for metronidazole. Serial passage experiment (5 passages) with and without sub-inhibitory concentrations of metronidazole was performed using AIM and decline in MIC_{50} from 4mg/L – 0.5mg/L after 5 passages without subinhibitory concentrations of metronidazole was observed. Nitroreductase activity was also evaluated using a spectrophotometric assay and only 3 CDRM strains showed detectable nitroreductase activity at very low levels. Subsequently, metronidazole uptake capacity was determined by employing a microbiological bioassay and AIM was used to evaluate the effect of hemin on the CDRM phenotype using both Wilkins Chalgren and Brucella Agar +/- 5mg/L hemin. Hemin in Brucella agar significantly increased metronidazole MIC (P>0.05) for ribotype 001 and 027 only. The metronidazole uptake assay indicated that the CDRM phenotype was not related to deficiencies in drug reduction and uptake. It does not however eliminate possibility of metronidazole being reduced to its non-toxic amino radical by *nim* genes. Then analysis was done on the whole genome of two *C. difficile* reduced susceptible strain, E4 and a clinical strain of ribotype 001 ribotype, compared with *C. difficile* 630 (CDSM). Whole genome analysis showed single nucleotide polymorphisms (SNPs) in *nimB, glyC, nifJ thiH* and *hemN* genes, linked to electron transfer, activation, oxidative stress and DNA repair which could predispose to CDRM phenotype as they are linked with metronidazole mechanism of action. The SNP detected in *nimB* gene

could activate the conversion of metronidazole to its non-toxic amino radical thus contributing to the CDRM phenotype.

This study has provided the focal areas that has the potential for predisposing to metronidazole reduced susceptibility. Which are factors affecting DNA repair, oxidative stress and metronidazole reduction. Which is of significance due to the CDRM phenotype compounded by the heterogeneity and stability profile that can give false susceptibility results in clinical settings. Which complicates therapy with metronidazole due to its poor colon penetration

Dedication

I dedicate this report to GOD ALMIGHTY, MY ALL IN ALL, MY KING OF KINGS, KING OF GLORY, THE WONDERFUL. I also dedicate it to my lovely parents Mr & Mrs G.J. Mohammed.

Acknowledgement

I give GOD ALMIGHTY ALL THE GLORY, HONOUR AND WORSHIP, ONE MILLION AND ONE PERCENT. I THANK GOD FOR DIVINE PROVISION, FINANCIALLY, HEALTHWISE AND EMOTIONALLY. GOD SAW ME THROUGH THE PhD. MY TEACHER, MY ALL IN ALL, MY WONDERFUL COUNSELLOR, THE REDEEMER, THE FAITHFUL, OMNIPOTENT, OMNIPRESENCE AND OMINISCIENCE.

I thank my parents Mr and Mrs Mohammed and siblings: Success, Lumy, Queen, Tope and Emma for all their love, patience and support. I also thank my principal supervisor Dr Simon Baines and my other supervisors Professor Mark Wilcox, Dr Kate Graeme-Cook, as well as all UH staff for all input and assistance. I appreciate all RCCG members, friends and research colleagues that have encouraged me.

I specially thank my mum, Mrs Jibike Mohammed, my angel for funding me all through my PhD with millions, GOD bless you mum. I thank my Dad for all His financial support through my academics and upbringing. My parents are amazing. Thank You GOD ALMIGHTY.

Contents

ABSTRACT ii

Dedication iv

Acknowledgement v

Contents

Table of figures ....xiii – xvi

List of Tables........xvii- xviii

Abbreviations xix

1.0 LITERATURE REVIEW/INTRODUCTION 1

1.1 Risk Factors 1

1.2 Community acquired CDI 2

1.3 *C. difficile* Life cycle 3

1.4 Clinical manifestations of CDI 6

1.5 Diagnosis of CDI 7

1.6 CDI Recurrence 8

1.7 Virulence Factors 9

1.71 Toxin A and Toxin B (TcdA and TcdB) 9

1.72 CDT toxin 11

1.73 Quorum sensing 13

1.74 Colonisation Factors 13

1.75 Biofilm: 13

1.76 Sporulation 14

1.77 Membrane vesicles 15

1.8 Epidemiology in *C. difficile* 15

1.9 Molecular Epidemiology of CDI........ 16

1.91 *C. difficile* infection decline in UK 18

1.92 *C. difficile* in Africa 19

1.10 Therapy 21

1.10.1 Antimicrobial resistance in *C. difficile* 21

1.10.2 Metronidazole 21

1.10.3. Vancomycin 23

1.10.4 Other therapeutic measures: 24

1.11 Aims of project 31

1.12 Main objectives 31

1.13 Laboratory experiments 31
2.0 Antimicrobial Susceptibility Testing of UK *C. difficile* ribotypes 027, 001, 001/072, 106 ... 32
2.1 Introduction 32
2.11 Antimicrobial Susceptibility in *Clostridium difficile* isolates 32
2.12 Aims 34
2.13 Objectives 34
2.2 Method 35
2.21 *C. difficile* isolates 35
2.22 Agar incorporation MIC testing 35
2.3 Results 37
2.4 Discussion 40
3.0 Heterogeneity in susceptibility to Metronidazole and Vancomycin in UK *C. difficile* isolates. ..43
3.1 INTRODUCTION 43
3.11 Aims 44
3.12 Objectives 44
3.2 Method 45
3.3 Results 46
3.4 Discussion 58
4.0 Population analysis profiling 61
4.1 Introduction 61
4.11 Aims 61
4.12 Objective 61
4.2 Method 62
4.3 Results 64
4.4 Discussion 69
5.0 Stability and mutability of *C. difficile* with reduced susceptibility to metronidazole 72
5.1 Introduction 72
5.1.2 Development of resistant mutant microorganisms on continuous exposure to antibiotics. 73
5.1.3 Development of resistant anaerobes on exposure to antibiotics 74
5.1. 4 Serial passage experiments with *Clostridium difficile* 74
5.1.5 Aim 76
5.1.6 Objectives 76
5.2 Method 77
5.3 Results 79

5.4 Discussion 84
6.0 Nitroreductase assay 86
6.1 Introduction 86
6.11 Nitroreductase in anaerobic organisms 86
6. 1.2 Aim 90
6.1.3. Objectives 90
6.2 Method 91
6.3 Results 92
6.4 Discussion 93
7.0 *Nim* Genes: A Potential Mechanism of Reduced Susceptibility to Metronidazole in *C. difficile* .. 95
7.1 Introduction 95
7.1.1 Presence of *Nim* Genes in Other Microorganisms 96
7.1.2 Aim 97
7.1.3 Objectives 97
7.2 Method 98
7.3 Results 99
7.4 Discussion 100
8.0 Metronidazole Uptake as a factor contributing to reduced susceptibility 102
8.1 Introduction 102
8.1.1 Aim 102
8.1.2 Objectives 103
8.2 Method.. 103
8.3 RESULTS.. 105
8.4 Discussion 108
9.0 Effect of Hemin in The Susceptibility Level of *C. difficile* Strains to Metronidazole......... 110
9.1 Introduction 110
9.2 Method 111
9.3 Result 112
9.4 Discussion 115
10.0 Whole genome sequencing *Clostridium difficile* 118
10.1 Introduction 118
10.1.1 Aim 120
10.1.2 Objectives.. 120
10.2 Method 121

10.3 Results 125
10. 4 Discussion 134
Conclusion 137
Reflective feedback 140
References 141
Appendices 171

TABLE OF FIGURES

Figure 1. 1 *C. difficile* life cycle in the gut from faecal oral transmission to CDI onset and sporulation in the gut ------- 5

Figure 1.2 Colon lining in a healthy and *C. difficile* infected condition ------- 7

Figure1.3 *C. difficile* toxins and related open reading frames ------- 9

Figure 1.4 Structure of *C. difficile* toxin A ------- 10

Figure 1.5 Structure of *C. difficile* toxin B ------- 10

Figure 1. 6 Mechanism of activation of metronidazole ------- 22

Figure 1.7 Mechanism of action and structure of vancomycin ------ 23

Figure 2. 1 MIC_{50} (mg/L) for metronidazole and vancomycin against *C. difficile*. ---- 39

Figure 2. 2 MIC_{90} (mg/L) for metronidazole and vancomycin against *C. difficile*. ---- 39

Figure 3. 1 Metronidazole MICs (mg/L) of ribotype 001 showing heterogeneity ----- 47

Figure 3. 2 Vancomycin MICs (mg/L) of ribotype 001 showing heterogeneity. ----- 48

Figure 3. 3 Metronidazole MICs (mg/L) of ribotype 027 showing heterogeneity. ---- 50

Figure 3. 4 Vancomycin MICs (mg/L) of ribotype 027 showing heterogeneity. ---- 51

Figure 3. 5 Metronidazole MICs (mg/L) of ribotype 106 showing heterogeneity. --- 53

Figure 3. 6 Vancomycin MICs (mg/L) of ribotype 106 showing heterogeneity. ---- 54

Figure 4. 1 Population analysis profiling (PAP) of two CDRM (80 and 86) and one CDSM (114) ribotype 001 strains using doubling concentrations of metronidazole (mg/L) in Oxoid Wilkin's Chalgren agar. ----- 64

Figure 4. 2 Population analysis profiling (PAP) of two CDRM (95 and 99) and one CDSM (12) ribotype 027 strains using doubling concentrations of metronidazole (mg/L) in Oxoid Wilkin's Chalgren agar. ----- 64

Figure 4. 3 Population analysis profiling (PAP) of two CDRM (108 and 109) and one CDSM (120) ribotype 106 strains using doubling concentrations of metronidazole (mg/L) in Oxoid Wilkin's Chalgren agar. ----- 65

Figure 4. 4 Population analysis profiling (PAP) of *C. difficile* control strain E4 (ribotype 010) strains using doubling concentrations of metronidazole and vancomycin (mg/L) in Oxoid Wilkin's Chalgren agar. ----- 65

Figure 4. 5 Population analysis profiling (PAP) of two CDRM (80 and 86) and one CDSM (114) ribotype 001 strains using doubling concentrations of vancomycin (mg/L) in Oxoid Wilkin's Chalgren agar. ----- 66

Figure 4. 6 Population analysis profiling (PAP) of two CDRM (95 and 99) and one CDSM (12) ribotype 027 strains using doubling concentrations of vancomycin (mg/L) in Oxoid Wilkin's Chalgren agar. ---- 66

Figure 4. 7 Population analysis profiling (PAP) of two CDRM (108 and 109) and one CDSM (120) ribotype 106 strains using doubling concentrations of vancomycin (mg/L) in Oxoid Wilkin's Chalgren agar. ---- 67

Figure 5. 1. MICs of metronidazole against control *C. difficile* strain E4 (ribotype 010 coded as 110) before and after five serial passages (110sp) & 110CTL, MIC determined from spore stocks without serial passaging ----- 81

Figure 5. 2 MICs of vancomycin against control *C. difficile* strain E4 (ribotype 010 coded as 110) before and after five serial passages in drug-free medium and control (CTL) 110, MIC determined from spore stocks without serial passaging. ----- 82

Figure 5. 3 Geometric mean MICs of CDRM ribotype 001 strains exposed to sub-inhibitory MICs of metronidazole during five serial passages ----- 82

Figure 5. 4 Geometric mean MICs of CDRM ribotype 106 strains exposed to sub-inhibitory MICs of metronidazole during five serial passages ------ 83

Figure 7. 1 PCR amplification of *C. difficile* strains and *nimB* positive control *BF8*.The first well contained 100 base pair ladder, other wells contained *C. difficile* strains except the last 2 wells that contained the positive control *BF8* ------ 99

Figure 8. 1 Decline in OD600 of *C. difficile* strains exposed to metronidazole (10mg/L) in an uptake assay. ------ 105

Figure 8. 2 Decline in total viable counts (log10-cfu/mL) of *C. difficile* strains exposed to metronidazole (10mg/L) in an uptake assay. ------ 106

Figure 9. 1 Geometric mean metronidazole (MET) MICs (mg/L, ±SE) against *Clostridium difficile* with reduced susceptibility to metronidazole from PCR ribotypes 001 (N=7), 027 (N=5), and 106 (N=4) on Oxoid Wilkin's-Chalgren agar ±5 mg/L additional hemin. ------ 113

Figure 9. 2 Geometric mean metronidazole (MET) MICs (mg/L, ±SE) against *Clostridium difficile* with reduced susceptibility to metronidazole from PCR ribotypes 001 (N=7), 027 (N=5), and 106 (N=4) on Brucella blood agar ±5 mg/L additional hemin. - ----114

Figure 10. 1 Purified high molecular weight DNA from *C. difficile* strains 110 (A, ribotype 010) and 80 (B, ribotype 001) visualised on an agarose gel (1% w/V) alongside a lambda DNA high molecular ladder (11873943, Fisher Scientific, Loughborough, UK) the X lane are DNA in tubes not sent for sequencing. --- 125

Figure 10. 2 Alignment of Oxygen independent coproporphyrinogen III oxidase gene of sample 110 with same gene in CD630 detecting point mutations --- 129

Figure 10. 3 Alignment of Exodeoxyribonuclease gene of sample 110 with same gene in CD630 detecting point mutations --- 129

Figure 10. 4 Alignment of Gyrase A gene of sample 110 with same gene in CD630 detecting point mutations --- 129

Figure 10. 5 Alignment of Endonuclease IV gene of sample 110 with same gene in CD630 detecting point mutations --- 129

Figure 10. 6 Alignment of 5-nitroimidazole reductase gene of sample 110 with same gene in CD630 detecting point mutations --- 130

Figure 10. 7 Alignment of Gyrase A gene of sample 110 with same gene in CD7032989 detecting point mutations --- 130

Figure 10. 8 Alignment of Pyruvate flavodoxin oxidoreductase showing mutations gene of sample 80 with same gene in CD630 detecting point mutations --- 132

Figure 10. 9 Alignment of Toxin A first point mutation gene of sample 80 with same gene in CD630 detecting point mutations ---- 133

Figure 10. 10 Alignment of Toxin A second point mutation gene of sample 80 with same gene in CD630 detecting point mutations ---- 133

LIST OF TABLES

Table 1.1 Molecular epidemiology showing the distribution of *C. difficile* ribotypes in different countries --- 17

Table 2. 1 Antimicrobial susceptibilities of CDRM strains (mg/L) using AIM with Oxoid Wilkin's Chalgren agar, with inocula raised in Oxoid Schaedler's anaerobe broth --- 37

Table 2. 2 Antimicrobial susceptibilities of CDSM (mg/L) strains using AIM with Oxoid Wilkin's Chalgren agar, with inocula raised in Oxoid Schaedler's anaerobe broth. ---38

Table 3. 1 Metronidazole and vancomycin MIC (mg/L) ranges for 3 *C. difficile* ribotypes isolates' colonies tested individual purified colony forming units (13-17 cfu). --- 46

Table 3.2 Number of *C. difficile* strains (CDRM and CDSM separated) heterogeneous in susceptibility to metronidazole and vancomycin --- 55

Table 3.3 Number of *C. difficile* strains (combination of CDRM and CDSM) heterogeneous in susceptibility to metronidazole and vancomycin. - ---- 56

Table 3.4 Total number of strains analysed (including CDSM strains) in this research highlighting those with heterogeneity MIC distribution across two MICs or more in their susceptibility to metronidazole and vancomycin. - --- 57

Table 4.1 Selected strains based on metronidazole susceptibility profile and their respective MICs detected in chapter 2, to be used for PAP test - --- 62

Table 4. 2 Area under the curve (AUC) data for the 3 *C. difficile* ribotypes analysed for PAP test, for metronidazole and vancomycin --- 67

Table 5.1. MICs of metronidazole and vancomycin against clinical *C. difficile* from ribotypes 027, 106, and 001 before serial passage and after five serial passages in drug-free Oxoid Schaedler's anaerobe broth. ---- 80

Table 6. 1 Nitroreductase (Nit) activity demonstrated by *Clostridium difficile* strains from ribotypes 001, 027, 106 using a spectrophotometry based PNBA reduction assay at 540nm. - ---- 92

Table 8. 1 Mean metronidazole (MTZ) concentrations detected in cell-free cultures of *C. difficile* strains exposed to MTZ (10 mg/L); using a microbiological bioassay. *only one replicate demonstrated detectable MTZ ----107

Table 10. 1 Gene targets assessed using bioinformatics tools for *C. difficile* PCR ribotype 001 (strain 80) and ribotype 010 (strain 110, E4) with reduced susceptibility to metronidazole. ----- 123-124

Table 10. 2 DNA yield and purity for DNA preparations from *C .difficile* PCR ribotypes 001 and 010, strains 80 and 110. ----125

Table 10.3: Gene analysis for sample 110 with reference genome, identifying percentage similarity and identifying SNPs ---- 126 -128

Table 10.3: Gene analysis for sample 80 with reference genome, identifying percentage similarity and identifying SNPs ---- 131

LIST OF ABBREVIATIONS

Antibiotic associated diarrhea	AAD
Antimicrobial susceptibility testing	AST
Agar dilution method	ADM
An agar incorporation method	AIM
Antimicrobial susceptibility testing	AST
Area under the curve	AUC
Analysis of variance	ANOVA
C. difficile infection	CDI
Clinical Laboratory Standard Institute	CLSI
Colony forming units	CFU
Community acquired CDI	CA-CDI
Enzyme –Linked fluorescence immunoassay	ELFA
Epidemiological cut-off	ECOFF
Enzyme immunoassays	EIA
European committee on antimicrobial suscepitibility testing	EUCAST
Ferric uptake regulator	FUR
Glutamate dehydrogenase	GDH
Hetero-resistant *S. aureus*	HVRSA
High performance liquid chromatography	HPLC
Hospital acquired CDI	HA-CDI
Lactobacillus casei Shirota	LcS
National center for biotechnology information	NCBI
Basic local alignment search tool	BLAST

Membrane vesicles	MVs
Methicillin resistant *S. aureus*	MRSA
Metronidazole	MET/MTZ
Metronidazole Reduced susceptible *C. difficile* strains	CDRM
Metronidazole susceptible *C. difficile* strains	CDSM
Minimum inhibitory concentrations	MIC
Non-toxigenic *C. difficile*	NTCD
Nitroimidazole resistance genes	*nim*
Number	n
Optical density	OD
Polymerase chain reaction	PCR
Pyruvate oxidoreductase	POR
Population analysis profiling	PAP
4- nitrobenzoic acid	PNBA
4 aminobenzoic acid	PABA
Single nucleotide polymorphisms	SNPs
Single nucleotide variants	SNV
United Kingdom	UK
United States	US
Vancomycin	VAN
Whole genome sequencing	WGS

1.0 LITERATURE REVIEW/INTRODUCTION

Clostridium difficile a spore forming, Gram positive anaerobic bacterium, has been observed to be the most prevalent infectious cause of antibiotic associated diarrhoea (AAD) (Mutlu *et al*., 2007, Lynch *et al*., 2013).

Though *C. difficile* infection is caused through ingestion of *C. difficile* spores acquired from within or outside hospital environment (Shaughnessy *et al.,* 2011), the bacterium can be referred to as a nosocomial pathogen because it is more prevalent in the hospitalized situation. This is due to the fact that in hospitals antibiotics are used to treat infections, thus facilitating *C. difficile* colonisation (Bartlett, 1992, Fawley, 2005, Lynch *et al*., 2013) and leading to *C. difficile* infection (CDI). Since *C. difficile* spores are unaffected by the stomach acidic environment, these spores germinate and outgrow into vegetative cells in the small intestine (Lessa *et al,* 2012). *C. difficile* has also been isolated in animals where it is observed as a pathogen or a commensal. As a result, transmission of *C. difficile* from animals to humans and vice versa is possible (Janezic et al, 2012). There are over 400 PCR *C. difficile* ribotypes that have been identified, and different ribotypes are prevalent in different countries and areas (Janezic et al., 2012). *C. difficile* was first identified as *Bacillus difficillis* by Hall and O'Toole 1935 who encountered the organism in the intestinal contents of neonates. *B. difficilis* was observed to be a pathogen in animals, and such infection could result in mortality. Hall and O'Toole linked pathogenicity in the organism to toxin production (Baines*,* 2006).

1.1 Risk Factors

There are certain factors associated with *C. difficile* infection termed risk factors. These factors are old age or increasing age, long hospital stay, gastrointestinal procedures that do not involve surgery, chronic diseased situations, long exposure to antibiotics and use of varieties of antibiotics (Bignardi, 1998). Chalmers *et al.*, 2016 associated community acquired pneumonia (CAP) with CDI by examining 1883 CAP patients in UK, 61 CDI cases were identified from 365 CAP patients that developed diarrhoea. The CDI risk factors was studied, and it was identified that, long hospital stay predisposed patients to CDI than the class of antibiotic administered. Same CDI risk was observed for all broad spectrum antibiotics used and it was also observed that results from 2005-2010 showed that the replacement of cephalosporins with amoxicillin/clavulanic acid has led to increase in CDI defeating the purpose for the replacement. Thus it was concluded that switching antibiotic classes may have the same effect in CDI decline as shortening treatment duration, and hospital stay (Chalmers *et al.,* 2016).

Age has been observed to be a risk factor for CDI especially those of 65 years and above, also age is a risk factor for recurrence and CDI severity (Louie *et al.,* 2013). A 2013 study observed

that these adverse development actually commences at a much younger age of 40 years, with increasing severity as age increases (Louie *et al.,* 2013). The study consisted of 999 participants who tested positive for CDI, from Canada, US, and Europe, the efficacy of vancomycin and fidaxomicin were as well examined in this study. It was observed that increasing age resulted in decrease of the effectiveness of the therapeutics used, irrespective of type, vancomycin or fidaxomicin (Louie *et al.,* 2013).

Contaminated food, close association with infected individuals and zoonotic transmission have been listed to be potential risk factors for community acquired CDI (Lee and Cohen 2013). Though any antibiotic can lead to CDI but a few have been observed to incite CDI much more. These are fluoroquinolones, Clindamycin and cephalosporin (Slimmings and Riley, 2014).

1.2 Community acquired CDI

C. difficile was historically considered predominantly as a nosocomial infection, however in recent years, research has demonstrated that CDI occurs equally in the community setting. So, other than hospital acquired CDI (HA-CDI), there is also community acquired CDI. Community acquired CDI can be described as the onset of CDI occurring not more than 48 hours after being hospitalised or a case where by the CDI symptoms were observed while still in the community (McDonald et al 2007). Transmission of *C. difficile* spores is usually from faeces of infected patients to the uninfected individuals via ingestion. The spores of *C. difficile* make it possible for CDI to be acquired even outside the hospital because it can survive on surfaces for an increased length of time, thus it can be picked by healthy individuals and ingested in the absence of proper hygiene (Lee and Cohen, 2013). Though the advancement from colonisation with *C. difficile* to the infection is determined by the disruption of normal intestinal microflora by antibiotics and immune compromised cases, so the bacteria will grow and produce toxins which will negatively affect individuals in the absence of antibodies against the toxins of *C. difficile* (Lee and Cohen, 2013). Since antibiotics are used also outside hospitals, community acquired CDI (CA-CDI) is also on the increase (Lee and Cohen, 2013), a 2017 US report has highlighted that though the community was earlier observed as being at a reduced risk of having CDI, CA-CDI acquisition were being noted (Kim and Zhu, 2017). This report has identified a case of a 45 year old woman without prior antimicrobial exposure, having diarrhoea, who was laboratory diagnosed of CDI (Kim and Zhu, 2017). Also, those with CA-CDI have been observed to have milder symptoms in comparison with those with HA-CDI (Lee and Cohen, 2013). Some CA-CDI do sometimes become severe and it has been reported that reoccurrence rate of this infection is similar to that of HA-CDI. However due to differences in research reports this has not been resolved. *C. difficile* has been isolated from a wide range

of animals including pets such as dogs, though zoonotic cause of CDI in humans is yet to be established (Lee and Cohen, 2013). Food sources of CDI is also been observed and there has been cases of *C. difficile* in retail beef which could be as a result of the resistance of spores to heat. Other than meat, *C. difficile* has been isolated in some vegetables, diary and fish produce. Though *C. difficile* transmission via contaminated food is a potential source of acquisition of the organism, it is still yet to be established definitively as a route of transmission (Lee and Cohen, 2013). CA-CDI clinical manifestations is similar to HA-CDI though as earlier mentioned, rates of severe symptoms in CA-CDI are lower than HA-CDI, for instance fulminant colitis is rare in CA-CDI. To prevent CA-CDI, antibiotics should only be used as prescribed or when required (Lee and Cohen, 2013).

A model was made in the United States (US) to examine transmission routes from different CDI sources: community, hospital with and without symptoms, as well as from long term care facilities (LTCF) (Durham *et al.,* 2016). It was observed that compared to hospitalised patients with CDI similar cases in wards with LTCF had a 27% transmission rate, while similar cases in community had <0.1% (Durham *et al.,* 2016). This report also recorded that under isolation and with effecting control measures against infection, individuals without symptoms transfer at a lower rate (15 times) than those with symptoms (Durham *et al.,* 2016).

1.3 *C. difficile* Life cycle

C. difficile is a spore producing microbe, so though the vegetative cells cannot withstand the aerobic environment outside the host and acidity of the stomach, the spores can withstand both conditions. Thus, in the case of an infection via a faecal oral route, *C. difficile* spores may be acquired from animal, human or environmental sources as illustrated in Figure 1.1 (Deakin *et al*, 2012). The spores are easily transported through the stomach environment into the duodenum where they may become metabolically active depending on the nutrients in the environment such as taurocholate and glycine (Sorg and Sonenshein, 2008). Additionally, the spores can attach itself to inert surfaces and also the intestinal wall via an exosporial membrane (Paredes-Sabja and Sarker, 2012). Though the functional role of the exosporium is not well understood, but experiments by Panessa-Warren and colleagues have indicated that the exosporial membranes of both *C. difficile* and *C. sporogenes* have projections facilitating their adhesion to agar surfaces and also to colonic epithelial cells (Panessa-Warren *et al.,* 1997; Panessa-Warren *et al.,* 1999).

The clearing of the intestinal microbiota mostly due to antibiotic therapy is a factor that aids the colonisation by *C. difficile* (Buffie *et al.*, 2015). The attachment of *C. difficile* to colon walls is aided by surface layer proteins and mucolytic enzymes that degrade mucosa of the colon for

example cell surface proteins/cell wall proteins (Cwp84). These two factors are induced by subinhibitory concentrations of ampicillin and penicillin in the environment (Deneve *et al*., 2008). It was reported that ampicillin and clindamycin influenced the elevation of the expression of Cwp84 from 2-27 folds and 2-41 fold respectively (Deneve et al., 2008). A 2-10 fold elevation of expression of SlpA and other colonisation factors Cwp66, Fbp68, was observed to ampicillin. To clindamycin, Cwp66 and SlpA elevation was less than 7 fold and Fbp68 was 1-11 (Deneve et al., 2008).

Then the host immune response is activated differentially based on the virulence factors produced by *C. difficile* for example surface layer proteins (SLP) activate the immune response via toll like receptor 4(TLR4) (Ryan *et al,* 2011). Lysozyme and cationic antimicrobial peptides (Ho *et al.,* 2014) are usually the first means of defence to be produced against *C. difficile* (Smit *et al.,* 2016). These defence mechanisms are resisted by *C. difficile* via different mechanisms such as the modulation of cell charge, thus giving *C. difficile* a charge that is net positive which lowers the effect of antibiotics (Mc Bride & Sonenshein., 2011) and the extracytoplasmic function sigma factor Csfv which aids lysozyme resistance in *C. difficile* (Ho *et al*., 2014). With increase in *C. difficile* cells, each cell produces a signal until it gets to a threshold which stimulates toxin production that causes CDI. This is also the stationary phase where sporulation occurs as a result of the SpoA gene, which has also been linked as a negative regulator to toxin synthesis (Deakin *et al*., 2012)

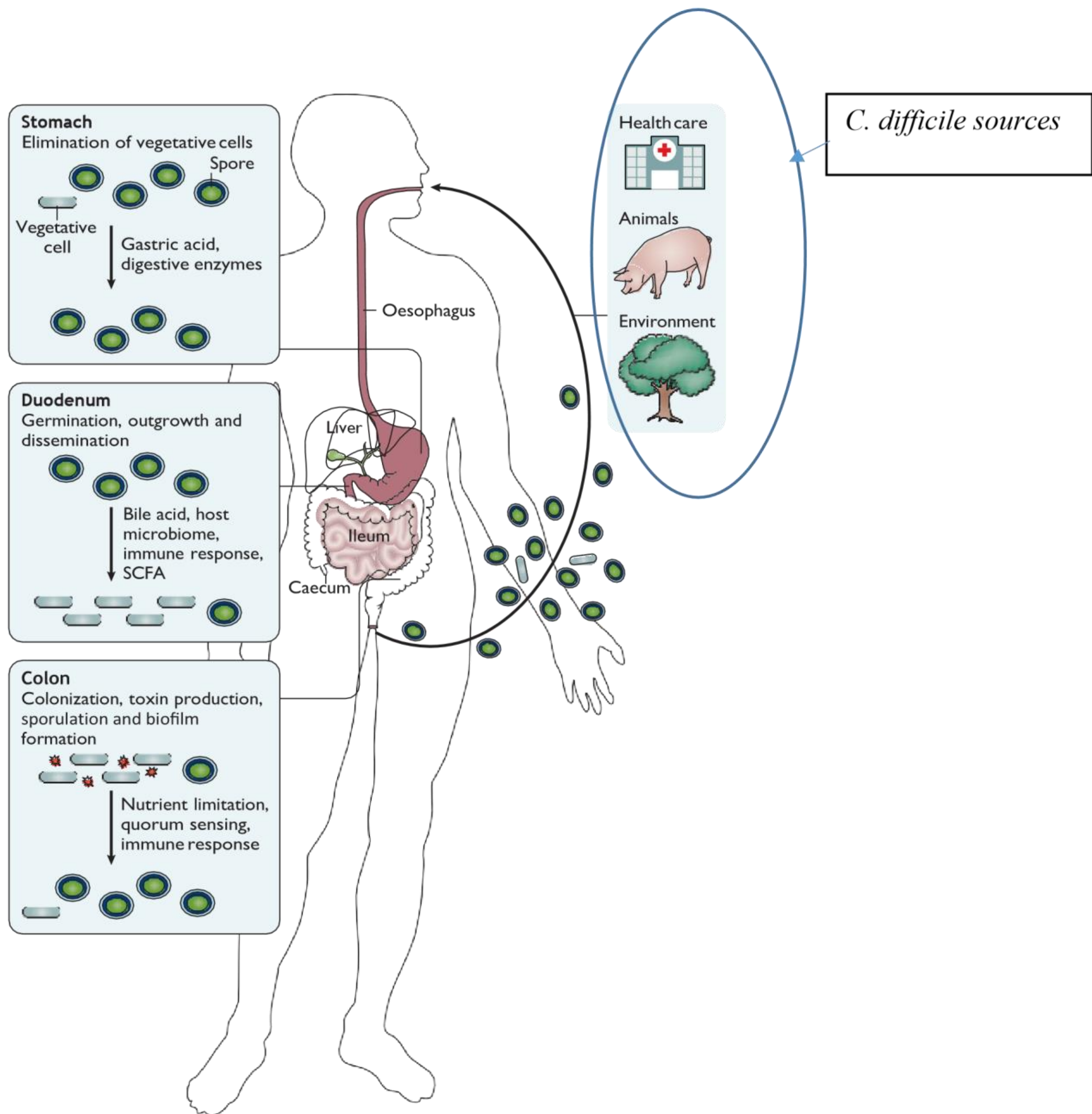

Figure 1. 1 *C. difficile* life cycle in the gut from faecal oral transmission to CDI onset and sporulation in the gut (adapted from Smith *et al.,* 2016)

1.4 Clinical manifestations of CDI

Asymptomatic carriers are individuals that are infected with *C. difficile* but do not have diarrhoea. Carriers can be healthy or hospitalised patients however it has been reported that the potential to be a carrier increases with more hospital exposure (Goudarzi *et al,* 2014). Symptomatic carriers have varying levels of complication from mild diarrhoea, paralytic ileus, pseudomembranous colitis and fulminant colitis.

Antibiotic associated diarrhoea (AAD) can be caused by a number of pathogens such as *Staphylococcus aureus* (Gravet *et al*, 1999), and *Clostridium prefringens* (Modi and Wilcox, 2001) but most AAD with colitis as a feature, is caused by *C. difficile*. In CDI, the diarrhoea commences during antibiotic therapy or up until 8 weeks after the end of antibiotic therapy. Most patients with mild to moderate CDI only have slight cramps in abdominal region along with diarrhoea as a symptom with about 10 bowel movements daily. The stool would have specific bad odour and could be watery, soft or mucoid. Low levels of serum albumin caused by albumin leakage, leucocytosis, cramps and fever are also observed in CDI of moderate severity. Fever, cramps, detection of leucocytosis in faeces, as well as, occult blood (Sunenshine, 2006) and an enlarged colon indicate the presence of colitis (Bartlett and Gerding, 2008) in severe CDI.

The pathophysiology of diarrhoea involves the bowel undertaking a balanced absorptive and secretory processes that on interruption leads to diarrhoea (Whyte & Jenkins; 2012). There are osmotic and secretory diarrhoea. Osmotic diarrhoea occurs when there is a high level of osmotically active particles in the lumen thus increasing the fluid moving into bowel lumen beyond the ability of the gut to absorb the fluid (Whyte & Jenkins; 2012). Secretory diarrhoea is when there is an initiation of excessive secretion of fluid from the bowel mucosa by the presence of an abnormality or a toxin (Whyte & Jenkins; 2012). The fluid production is beyond the capacity of the bowel to absorb, resulting to diarrhoea. Thus the damage of the intestinal mucosa that occurs during colonisation of *C. difficile* or by the *C. difficile* toxins affect the gut ability to absorb fluid which causes diarrhoea. Diarrhoea lasting for over 4 days has been observed to mostly be caused by *C. difficile* (Gerding & Bartlett, 2008)

Paralytic ileus which may progress to toxic megacolon is another feature of severe CDI along with vomiting, lethargy, shock, renal failure and abdominal pains. Paralytic ileus may prevent the passage of stools and so induce constipation. (Gerding & Bartlett, 2008).

Also a complication of CDI is pseudomembranous colitis, a disease of the intestinal mucosal characterised by individual obvious multiple yellow plaques containing a pseudo membrane of mucus debris and swollen cells. It seeps out overlying groups of partially damaged glands and each plaque is obviously discrete using an adjacent plaque mucosa. Cell death may eventually take place causing the plaques to amass (Price & Davies, 1977). This is caused by toxin A and B produced by *C. difficile* that activate the immune system's signal transduction pathways and damage cell cytoskeleton (Surawicz & McFarland *et al.*, 1999).

More still is fulminant colitis, this type of colitis has a very low prevalence as it is observed in just 3% of CDI patients. It involves most of the seriously adverse clinical conditions such as megacolon, long term ileus, perforation and mortality (Goudarzi *et al*, 2014).

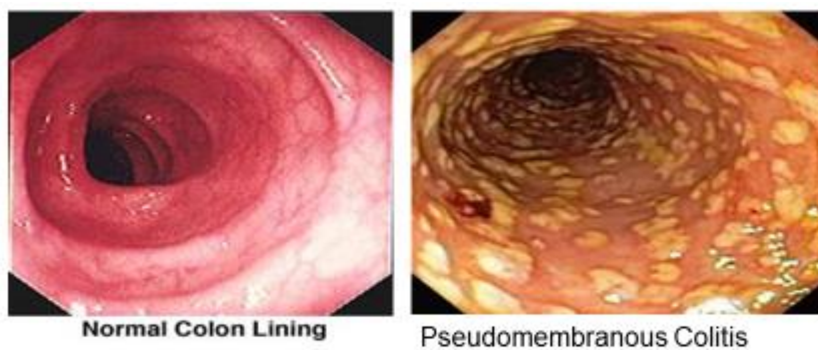

Figure 1.2 Colon lining in a healthy and *C. difficile* infected condition (Fusco; nd, Carrion *et al;* 2010)

1.5 Diagnosis of CDI

There are a number of ways to diagnose CDI such as stool culture, enzyme immunoassay (EIA), cell cytotoxicity assays, endoscopy and computerised tomography scanning. Laboratory diagnosis is chosen rather than CT, radiography, due to their lack of specificity and sensitivity and cost.

Cell toxicity is usually the preferred assay due to its high sensitivity but it is technically demanding and takes more than 24hrs to get a positive result (Gerding and Bartlett 2008). Cell culture neutralization assay is noted to be the standard for toxigenic *C. difficile* identification though it takes a long time of about 1-3 days and its labour intensive (Carroll, 2011, Stamper *et al,* 2009). False positives has been observed and its sensitivity is less than 90% (Carroll, 2011).

Stool culture is next in sensitivity but it also takes above 24hrs and has a low toxin specificity which is required to detect asymptomatic CDI. This method thus requires the use of the culture

broth for further test using cytotoxicity testing or EIA to detect toxin. This method is however useful for typing of organisms molecularly, antibiotic susceptibility testing and studying molecular epidemiology of *C. difficile* (Gerding and Bartlett 2008). The preferred media for selection of *C. difficile* from stool samples is cycloserine – cefoxitin – fructose agar (CCFA).

Enzyme immunoassays (EIA) are less sensitive than the above mentioned methods but they are faster and less costly and produce results within hours. This is a test for glutamate dehydrogenase(GDH) enzyme produced by *C. difficile*. Thus indicating the presence of C. *difficile* not necessarily CDI. This method is used to screen stools that will then be tested using EIA for toxin or cytotoxicity (Lyerly *et al*, 1991, Gerding and Bartlett 2008).

Two immunoassays were involved in a 2015 research on the capacity to identify *C. difficile* in stools (Davies *et al*, 2015). Enzyme immunoassay (EIA) commercially available also referred to as Quik Chek-60 and Vidas *C. difficile* GDH automated Enzyme –Linked fluorescence immunoassay (ELFA) were evaluated in this study (Davies *et al*, 2015). Similarity in performance (95%) was observed in the Quik Chek-60 and Vidas *C. difficile* GDH assay. Though the Quik Chek-60 was observed to require lower quantity of sample and was faster, the Vidas *C. difficile* GDH assay had good traceability, was automated and had a greater than 93% sensitivity as a result was reported to be appropriate as an initial test for CDI diagnosis in a 2 stage algorithm (Davies *et al*, 2015).

CDI can also be diagnosed using Nucleic acid amplification diagnostic methods. This type of diagnostic method is also referred to as real time PCR and known to target *C. difficile* toxin B. In 2011 four types were reported to be in use in the US amongst which the first to be FDA approved was the BD –GeneOhm™ Cdiff assay. It had a quick turnaround time of 2hours and requires manual extraction. It has a specificity and sensitivity of over 94% and 84% respectively (Carroll, 2011).

Furthermore Endoscopic diagnosis can be employed for diagnosing CDI but it is only used in an emergency when no stool sample is available and there is an ileus or negative results were obtained for other test and *C. difficile* is highly suspected (Avila *et al.,* 2016)

1.6 CDI Recurrence

Recurrence of CDI is a subsequent incidence of the disease after the initial episode in the same patient has been successfully treated. Recurrent CDI can be as a result of reinfection with a genotypically distinct *C. difficile* strain, or relapse of infection due to the same PCR ribotype that caused the initial episode. Recurrent CDI is a challenging problem in hospitals which could be as a result of prolonged use of antibiotics (Choi *et al.,* 2011) and CDI recurrence rates have

been observed to range from 5% to 20% (Hedge *et al.,* 2008). Amongst 14,472 CDI Medicare patients that survived therapy, recurrence was observed in 4775 patients (Zilberberg, *et al,* 2017). A report analysed the recurrence prevention capacity in metronidazole and vancomycin. No difference between antibiotics in recurrence rates but significant reduction in the mortality risk of 30 days, was observed with vancomycin than metronidazole (Stevens *et al*, 2017).

The risk factors for CDI such as age and antibiotic exposure are same for its recurrence (Shanon-lowe *et al* 2010).The growth of the microbial flora in the colon could be affected by the antibiotic used to treat the initial *C. difficile* case thus resulting to a second *C. difficile* case (Shanon-lowe et al 2010).

1.7 Virulence Factors

1.71 Toxin A and Toxin B (TcdA and TcdB)

Toxigenic *C. difficile* may produce two major toxins which act as glycosyltransferases, toxin A (308 kDa) and toxin B (270 kDa). TcdA and TcdB are part of the Clostridial glucosylating toxin family and inhibit the action of Rho, Rac and Cdc42 which are of the GTPases family within colonic epithelial cells (Voth and Ballard, 2005). Cellular activities such as actin cytoskeleton organisation are controlled by the Rho GTPase family.

C. difficile possess a pathogenicity locus on its chromosome on which both toxin genes are encoded. The toxin are approximately 66% similar and the genes have a low G+C content compared to the rest of the *C. difficile* genome. The similarity between toxins can be seen in N and C terminals (Voth and Ballard, 2005).

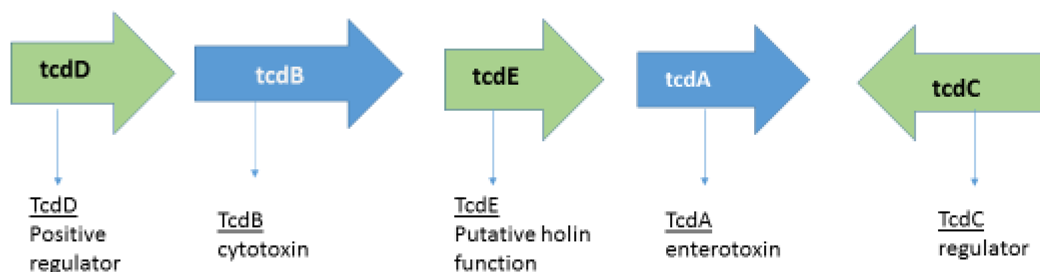

Figure1.3 *C. difficile* toxins and related open reading frames (adapted from Voth and Ballard, 2005)

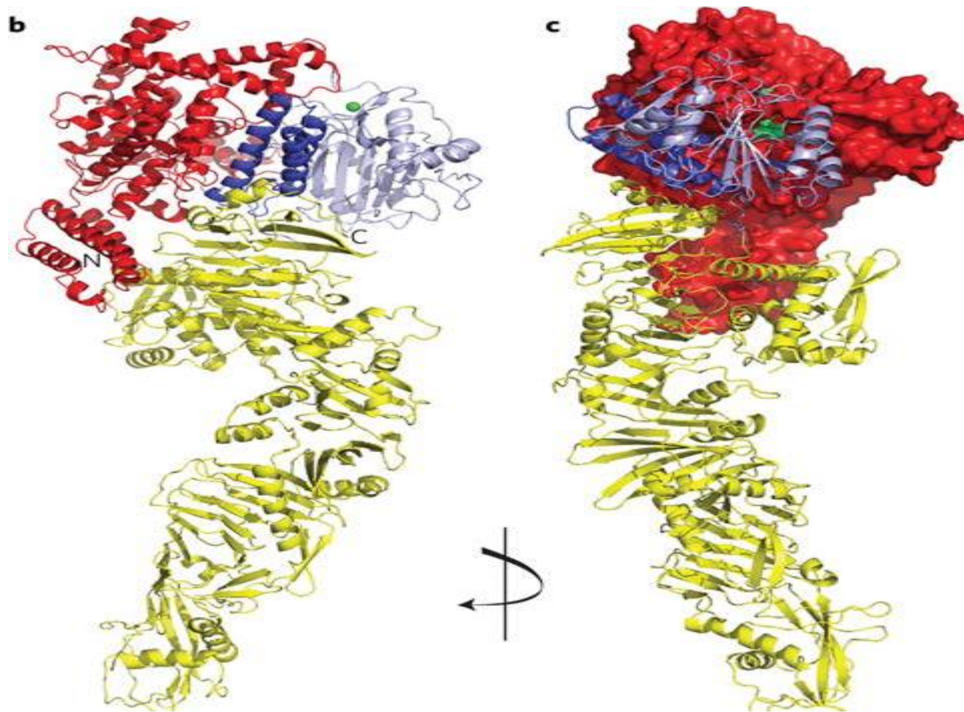

Figure 1.4a Structure of *C. difficile* toxin A (Chumbler *et al*, 2016) with the red portion as glucosyltransferase domain (GTD), the white coded the CROPS domain, the autoprotease domain (APD) coded as purple, yellow coded the delivery domain. Same structure in C is rotated 90 degrees (Chumbler *et al*, 2016)

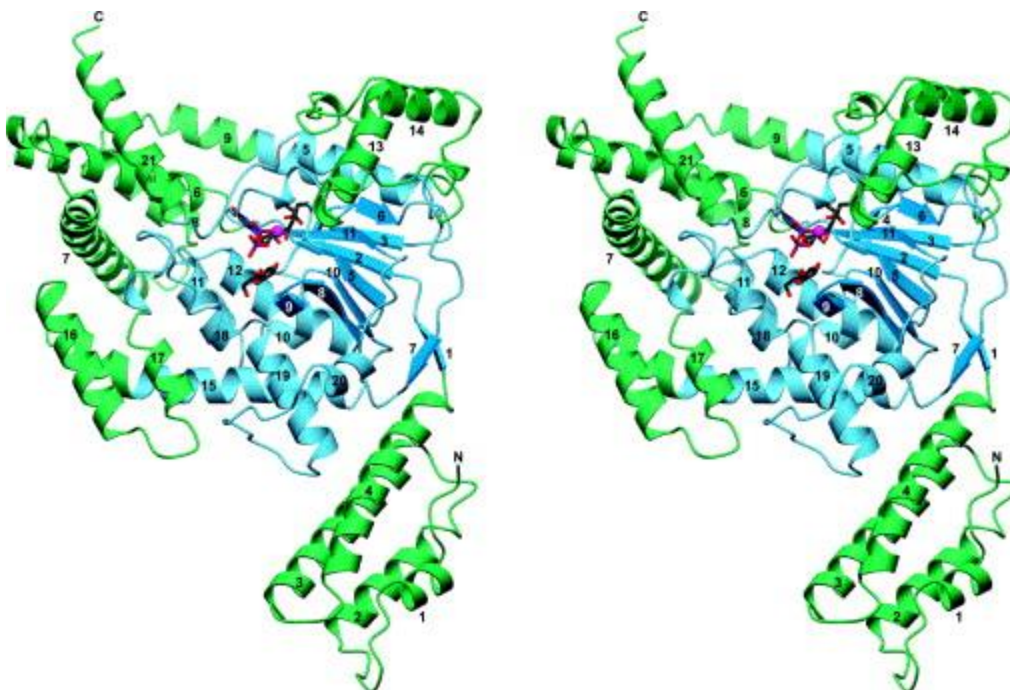

Figure 1.5 Structure of *C. difficile* toxin B (Reinert *et al*, 2005) magneta colour code is the active center, blue is the glycosyl typA while the toxinB additions are green

On the pathogenicity locus both toxin genes are separated by a 1350 nucleotide sequence and on the same locus there are 3 other open reading frames that might aid the regulation of toxin removal from the cell or toxin synthesis. Both genes of the toxins are transcribed in the same direction. The *tcdC* gene is also present in the pathogenic locus and is expressed in the early exponential phase, its gene transcription is in the opposite direction to *tcdA* and *tcdB* and is situated downstream of *tcdA* (Voth and Ballard, 2005). TcdC has been suggested to be a negative regulator to the production of these two toxins because the onset of stationary phase leads to a decrease in the production of TcdC and increase in production of the two toxins. However there has been some dispute on the role of TcdC, it was reported that TcdC in *C. difficile* 630Δerm did not significantly regulate toxin expression (Bakker *et al*, 2012). One major positive regulator of both toxins is TcdD whose gene is found upstream of *tcdB*. TcdD is a sigma factor whose response is dependent on environmental factors (Hundsberger *et al.*, 1997, Voth and Ballard, 2005). TcdE has been reported to possibly promote toxin release through *C. difficile* cell wall and it is structurally similar to a bacteriophage holin protein. The *tcdE* gene is located between *tcdA* and *tcdB*. Environmental factors also contribute to the toxin production which could be classified under catabolite repression and stress. Subinhibitory levels of some antibiotics such as vancomycin could lead to toxin production, in limiting levels, biotin has also been observed to induce toxin production (Yamakawa *et al*, 1996, Voth and Ballard 2005)

These toxins on release from the cell, modify through glycosylation of the Ras superfamily of small GTPases enterocyte, thus inactivating these proteins, thus hindering important cell signalling pathways. The toxins need an acidified endosome for translocation and access the interior of the cell by receptor mediated endocytosis. On gaining entrance into the cell the inactivation of GTPases involves the monoglucosylation of an active threonine responsible for the binding to GTP thereby causing actin condensation, cell rounding and death (Voth and Ballard, 2005). Recent studies have succeeded in generating mutants for both toxins virulence in single toxin knockouts, meaning both toxins can act independently to cause disease, as demonstrated by Toxin $A^{-}B^{+}$ strains *in vivo* (e.g. ribotypes 078). Kuehne *et al.,* (2010) generated a strain lacking both toxins and the double knockout was avirulent (Kuehne *et al.,* 2010).

1.72 CDT toxin

Perelle *et al* (1997), reported in *C. difficile* CD196 the presence of protein encoded by a binary toxin gene *cdtA* and *cdtB*. This protein was an ADP–ribosyltransferase, was observed to function in initiating actin alterations. Both genes encode for different function in the protein,

cdtA encodes for CDTa which functions as the ADP-ribosyltransferase enzyme, whereas the binding component is carried out by CDTb (encoded by *cdtB*). These genes are similarly transcribed and spaced as a result of the presence of 52 non-coding nucleotides between them in *C. difficile (*Perelle *et al.,* 1997*).*

CDT was observed to cause depolymerisation of microfilament. It induces through cellular protrusions in epithelial cells. Which lengthens between 5- less than 150µm. These actions occur at the same time with adp ribosylation of actin (Gerding *et al.,* 2014). CDT binary toxin gene was identified in human *C. difficile* isolates in Brazil for the first time in a 2015 report (Silva *et al.,* 2015)

Goncalves *et al.,* 2004, observed CDT in 6% of 369 strains analysed. These strains were isolated in Paris from AAD patients in healthcare facilities (Goncalves *et al.,* 2004). Further research carried out by Stubbs *et al.,* (2001) in the UK observed 59 strains out of 170 strains analysed which were positive for CDT. CdtR is a lytTR family response regulator, and was observed to be responsible for maximal production of CDT. The *cdtR, cdtB* & *cdtA* gene locus is referred to as Cdt loc (Carter *et al.,* 2007). The role of *CdtR,* was questioned in another research on 078 ribotype, a mutation was observed in *cdTR* gene which should have prevented the secretion of the binary toxin but this was not the case (Metcalf & Wease, 2010).

The role of CDT in causing disease has been suggested in some research. A report in Australia of a 15 year old hospitalised boy with diarrhoea and other health complications, showed a negative stool test for toxin A and toxin B using the Chek complete rapid membrane enzyme immunoassay kit (Androga *et al.,* 2015). However, a second analysis was performed and *C. difficile* was isolated anaerobically and typed using PCR ribotyping. The strain of *C. difficile* was of ribotypes 033 and was confirmed to be toxin A^- and toxin B^- but was positive for the binary toxin. The presence of RT014/020 known to lack CDT but produce toxins A and B was later also detected using a different test. This findings insinuate that CDT in absence of toxin A&B might be able to cause CDI however this is yet to be confirmed (Androga *et al.,* 2015).

Also, in France toxinotype XI of zoonotic origin only possessing binary toxin genes has been observed in patients with apparent CDI. The presence of toxinotype XI is rare in humans but this could largely be due to it not being noticed since the routine test for *C. difficile* test is a test for toxin A and B. So Cdt^+ only strains could have been classified as not toxigenic (Eckert *et al.,* 2015). This study showed that infection of *C. difficile* strain, positive only to the binary toxin was pathogenic giving an inclination to the severity of the disease elicited by this toxin (Eckert *et al.,* 2015)

Other factors contributing to the virulence of *C. difficile*

1.73 Quorum sensing

The production of toxin in *C. difficile* has also been observed to be facilitated and regulated by quorum sensing. The presence of antimicrobial therapy increases the presence of resistant *C. difficile* due to death of the intestinal microbial flora (Darkoh *et al*, 2015). Thus a thiolactone signal is synthesized by these *C. difficile* as the population increases, the thiolactone signal is a form of quorum sensing done by *C. difficile* to quantify its population in an environment before toxin production. Due to continuous cell increase the thiolactone signal reaches a break point which leads to the transcriptional activation of toxin gene as a result of the activation of a 2 component system Agr C2A2 (Darkoh *et al*, 2015).

1.74 Colonisation Factors

Surface layer proteins (SlpA) has been observed to be a major adherence factor in *C. difficile* to epithelial cells (Merrigan *et al,* 2013). An anaerobic quantitative assay was used to detect the level of attachment to host epithelial cells by vegetative cells of *C. difficile* strains. It was observed that the *C. difficile* isolates analysed which included isolates obtained during CDI outbreaks, have high adherence capability, partially as a result of the presence of SlpA (Merrigan *et al.,* 2013). *C. difficile* surface associated protein Cwp84 protease, observed to be associated to S – layer proteins, was characterised and reported to be an inactive proprotein which can be expressed as an active form after a process of maturation as a result of trypsin action or the reducing environment within the colon (Janoir *et al,* 2007). Cwp84 was reported to be responsible for the degradation of vitronectin and laminin as well as the cleaving of fibronectin, thus aiding toxin diffusion (Janoir *et al,* 2007).

1.75 Biofilm:

Biofilms have been earlier reported to protect bacteria from adverse environments such as antibiotic environments due to therapy. Not much information is known with respect to biofilms produced by anaerobic bacteria, this could be as a result of challenges encountered in creating the required environment for *in vitro* formation of biofilms. *C .difficile* biofilms have also been proposed to have the potential to prevent the effect of cellular immune response during CDI thus contributing to pathogenesis and *C. difficile* recurrence (Dapa & Unnikrsihan 2013). Additionally, biofilms may protect *C. difficile* vegetative cells and spores from antimicrobial agents and therefore facilitate recurrence following the cessation of antimicrobial therapy.

Certain features have been observed to influence *C. difficile* biofilm formation, e.g. glucose was observed to increase biofilm formation in *C. difficile* 630 but not in R20291 (ribotype 027), thus the gut nutritional environment can impact on *C. difficile* biofilm formation. A well-developed S layer was observed to be required for biofilm formation probably due to its role in early stage biofilm formation in holding proteins that aid surface binding. *C. difficile* motility was also observed to be an important factor in biofilm formation, since a flagellin knockout mutant, which is a major protein component of flagella, was observed to be defective in formation of biofilm *in vitro* (Dapa *et al.,* 2013). The data obtained in the research also indicated the involvement of quorum sensing mediated by *lux S in vitro*. Dapa *et al.,* 2013 also speculated that initiation of biofilm formation, sporulation and toxin production could be a role done by *spoOA*.

Biofilms have been demonstrated to possess a gradient of nutrient availability and are often nutrient limited within microcolonies. Therefore, sporulation in *C. difficile* may be initiated within biofilms and several studies have demonstrated the presence of *C. difficile* spores in microtitre plate biofilms and in more complex fermentation vessel models (Dapa *et al.,* 2013, Crowther *et al.,* 2014). Sporulation in *C. difficile* is controlled by the master regulator of sporulation, SpoA, and this regulatory protein has been observed to be involved in biofilm formation. As Biofilm regions can be characterised by depletion in nutrients. SpoA has been observed in *B. subtilis* to influence biofilm matrix formation. Phosphorylated SpoA has been observed to activate biofilm matrix formation as well as sporulation dependent on the SpoA. Biofilm matrix is activated by SpoA-P action on SinI a depressor of matrix formation. This is done by sinI binding to sin R a repressor of matrix formation (Chai *et al*., 2011, Viamakis *et al*., 2013). Thus the switch from matrix formation to sporulation is dependent on SpoA-P levels which if high, turns off matrix and switches on sporulation. Thus this switch is also affected by the number of copies of Sin I – SinR complex in a cell (Viamakis *et al,* 2013).

1.76 Sporulation

Stress conditions such as depletion of nutrients enhance the formation of the dormant form of *C. difficile* commonly referred to as spores (Awad *et al.*, 2014). *C. difficile* sporulation ability enables this pathogen to withstand the stomach acidity and aerobic environment when not inside a host (Smith *et al.*, 2016). Awad *et al.,* 2014 also referred to it as an infectious particle necessary for transmission of the infection. The spore consist of inner and outer coat, with the outer coat made up of four layers approximately 75nm in width, surrounding the inner coat (Driks, 1999). These coats enclose a core that contains the organisms entire genome copy (Awad *et al.*, 2014). *Clostridium difficile* strains of hypervirulent ribotype 027 was observed to

have diverse sporulation features and also have similar sporulation rates to other ribotypes analysed (Burns *et al.,* 2011).

1.77 Membrane vesicles

C. difficile was observed to secrete membrane vesicles (MVs) consisting of 262 proteins. In Caco-2- cells, these MVs stimulated the expression of proinflammatory cytokine genes, also surface associated proteins, flagellin and GroEL proteins were also observed using proteomic analysis. Cytotoxicity in cells of colonic epithelium were observed to be induced by *C. difficile* MVs. However the role of MVs in CDI is yet to be elucidated. (Nicholas *et al.*, 2017)

1.8 Epidemiology in *C. difficile*

The epidemiology of *C. difficile* in Canada was carried out in 1997 and another in 2009. It detected the hospital acquired -CDI (HA-CDI) rates in Canada, these ranged from 20-167 cases /100,000 patient days. The rates were different in different Canadian regions and provinces (Gravel *et al.*, 2009). The other provinces were more than 100% lower, in rate of HA-CDI occurrence compared to central Canada 2.8vs7.0 cases for every 1000 admissions in hospitals (Gravel *et al.*, 2009). However there was not much increase in HA_CDI in this 2009 surveillance compared to that in 1997 but increase in death rates were observed to be linked to HA-CDI four fold higher, than in 1997. These patients were above 60years of age. The limitation of this research though was highlighted to be unmonitored data allocation and specify research to teaching hospitals so not totally representing adult patients in all Canada hospitals (Gravel *et al.*, 2009). Another research has quantified the CDI infections in Canada and cost, which totaled to 37900 CDI cases and cost CAD $281 million. More than 90% was due to rehospitalisation and increased stay in hospitals (Levy *et al.*, 2015).

CDI in US, was observed to increase from an approximate 30-40 cases for a population 100,000 in the 1990's to 61cases in 2003 (McDonald *et al.,* 2006). However lower income areas had a decreased CDI rate in the US (Lucado *et al.*, 2012).

CDI in England was reported to have had an increase from 1990 up until 2007 which was the highest report and a decline was observed in 2008 almost a difference of 20,000 reports decline, which decreased steadily, approximately 10,000 reports were received in 2013 and 2014 (public health England surveillance of *C. difficile*, 2014). Hypervirulent ribotype 027 was observed to decline from April 2007 with over 60% incidence down to less than 10% in March 2013. Emergence of 078, 002, 015, 014/020 and 005 were also observed during this period. Rate of *C. difficile* infection at 2014 was very similar in Male and Female but more prevalent in ages 65yrs and above having 50per 100,000 population with higher incidence observed in

85+ ages (>250 per 100,000 population (public health England surveillance of *C. difficile,* 2014).

1.9 Molecular Epidemiology of CDI

Ribotyping is a taxonomic methodology that involves the use of rDNA restriction fragment sizes to differentiate bacterial species. Ribotyping specifically involves PCR amplification of 16S-23S rDNA spacer regions which was observed to be of variable length which enabled differentiation between strains (Gurtler, 1993). Polymerase Chain Reaction (PCR) ribotyping is used for characterising *C. difficile* isolates (Huang, 2009).

Ribotypes 027, 106 and 001 were reported to be the most prevalent in England in 2008, with prevalence rate 36%, 13% and 7% respectively however a 19% reduction was observed in 027 prevalence between 2007 and 2008 (Freeman *et al.*, 2010). As was reported earlier other ribotypes have emerged in the UK following the decline of hypervirulent ribotype 027.

Table 1.1 Molecular epidemiology showing the distribution of *C. difficile* ribotypes in different countries

Ribotype	Characteristics and Country of prevalence
027	• Hypervirulent, also known as North American pulsed field gel electrophoresis type1 (NAP1), and detected to be of a toxinotype III. • Most common PCR ribotype in the Unites States (Janezic *et al.,* 2012) • 027 was reported to be 75.2% of the isolates analysed from Montreal area Hospital (MacCannell *et al.,* 2006) • A decline in 027 has been observed in the UK (Wilcox *et al.,* 2012). • 027 caused outbreak in Portugal (Oleastro *et al*., 2014) • 027 was reported in 2 outbreaks in West and East Scotland (Kuijper *et al* 2008) • 72 Severe CDI cases in Hesse state in Germany reported 027 as the most prevalent ribotype (Arvand *et al*., 2009)
106	• Ribotype 106 was identified in humans and dogs in Brazil (Silva *et al;* 2015). • Britain *C. difficile* surveillance program in 2007 identified ribotype 106 as one of three major ribotypes to be responsible for 3/4 of the CD1 (Brazier *et al,* 2007) • Ribotypes 106 was the third most prevalent ribotype with a prevalence of 12.8% of the Irish clinical *C. difficile* isolates in 2011 (Solomon *et al;* 2011). • In a Brazillian study, ribotype 106 was the second highest prevalent toxigenic *C. difficile* (Silva *et al;* 2015).
010	• Not toxigenic, that is it lacks the pathogenicity locus on which the genes responsible for toxin production are situated (Lee and Cohen 2013). • It has been identified in humans and the environment (Janezic *et al.,* 2012) • 010 was the first *C. difficile* ribotype to be isolated with reduced susceptibility to metronidazole in UK • An Italian study reported the predominant ribotype in dogs to be ribotype 010 (64% prevalence) (Spigaglia *et al;* 2015).

Ribotype	Characteristics and Country of prevalence
017	• Has also been observed in animals (Janezic *et al.,* 2012). • Ribotype 017 was a dominant strain in Poland (Huang, 2009) • Most isolated in Asia (Collins *et al.,* 2013) • Ribotype 017 in a Korean study was the second most predominant ribotype (Lee *et al.,* 2014).
001	• In 2011, ribotype 001 was the third most prevalent ribotype in England and Northern Ireland. • In Korea *C. difficile* ribotyped 001 had the highest prevalence of 26.1% but a decline was observed from year 2000 (Lee *et al.,* 2014) • Ribotype 001 (11%) was observed to be the second most predominant ribotype in Europe (study involved 19 countries) (Davies *et al.,* 2016)
014/020	• One of the common ribotypes in a Korean study (Lee et al, 2014). • The most predominant toxigenic ribotype in a Brazilian study (Silva *et al;* 2015)
078	• Cuts across species affecting both animals and humans (Debast, 2013) • The third highest prevalent ribotype in the North East Ireland in 2009 (Brabazon *et al;* 2014) • A Brazilian report observed 078 only in foals (Silva *et al*; 2015).
002	• Current most prevalent ribotype in the UK and one of the 5 most prevalent ribotypes in Belgium (Dauby *et al.,* 2017). • Though common in Europe ribotype 002 was not found to have a high occurrence in Brazil (Silva *et al*., 2015)

1.91 *C. difficile* infection decline in UK

C. difficile infection was observed from 2006 to have declined drastically (80%). Research was performed to identify the specific control measure that resulted in the decline (Dingle *et al.,* 2017). Two factors considered were limitations on the use of certain antibiotics and increase in infection control interventions performed in hospitals (Dingle *et al.,* 2017). Data from 1998-2014 on antimicrobial prescription, CDI onsets, and 4045 whole genome sequences of *C. difficile* isolates were obtained (Dingle *et al*, 2017). It was detected that transmission factors were not affected but isolates resistant to fluoroquinolone was decreased thus antimicrobial stewardship had a high contributing impact to decline in *C. difficile* infection (Dingle *et al.*, 2017). Fluoroquinolone-resistant isolates were observed to no longer be present in Oxfordshire *C. difficile* population, thus eliminating the use of antibiotics might cause the resistant *C. difficile* to lose its prevalence (Dingle *et al,* 2017).

1.92 *C. difficile* in Africa

There is very little information available on the prevalence of *C. difficile* in sub–Saharan Africa. A recent study researched the prevalence of *C. difficile* in rural Ghana (Janssen *et al;* 2016). This study was in a combination of symptomatic and non-symptomatic individuals (Janssen *et al;* 2016). The *C. difficile* prevalence was determined to be 5%, and mostly nontoxigenic strains were observed in *C. difficile*–positive diarrhoea patients, however, other pathogens causing diarrhoea were not investigated. Despite some patients being prescribed metronidazole 2 weeks prior to the study, susceptibility to metronidazole and vancomycin was observed (Janssen *et al.,* 2016). Resistance was observed mainly to ciprofloxacin and erythromycin (Janssen *et al.,* 2016). A significant correlation of p=0.042 between the occurrence of malaria and *C. difficile* was observed (Janssen *et al.,* 2016). The most common ribotype isolated was ribotype 084 (Janssen *et al.,* 2016), a ribotype previously observed in a pan-European survey under a clade that was majorly susceptible to metronidazole, vancomycin, and fidaxomicin (Debast *et al.,* 2013). This was the first research to report the presence of *C. difficile* in symptomatic and asymptomatic individuals in Ghana (Janssen *et al.,* 2016).

This study concluded that *C. difficile* was not a major cause of diarrhoea in the setting investigated but indicated the need for further research on the increased prevalence of nontoxigenic strains (Janssen *et al.,* 2016). The low prevalence though was associated to the intake of antibiotics by some participants (Janssen *et al.,* 2016). This could also be linked to

the fact that the prevalent ribotype was non-toxigenic as the use of non-toxigenic *C. difficile* strain, M3 (NTCD-M3) spores as a probiotic therapy has been earlier reported to limit the colonisation of toxigenic *C. difficile* reducing recurrence rates (Gerding *et al.,* 2015).

C. difficile has also been identified and reported for the first time in Tanzania. Of 141 diarrhoea patients *C. difficile* was detected in 6.4% which were mostly children. Participants without diarrhoea had no observed *C. difficile* (Seugendo *et al*., 2015). The occurrence of *C. difficile* was linked to HIV infection as well as exposure to antibiotic and long hospital stay. Ribotyping and susceptibility testing was performed for seven isolates, 3 of which were non toxigenic and 2 toxigenic strains had unknown ribotype. The last 2 isolates were toxigenic of ribotype 045 (Seugendo *et al*., 2015). Susceptibility to frontline antibiotics, metronidazole and vancomycin was observed in the 7 isolates (Seugendo *et al*., 2015).

In Nigeria *C. difficile* was detected and screened in faecal sample of children as well as neonates (Emeruwa & Oguike, 1990). Cytotoxin production was also detected in 14.8% of isolates analysed (Emeruwa & Oguike, 1990). Children that were 12months and less were observed to have the highest cytotoxin titres of 16.7% (Emeruwa & Oguike, 1990). There was a gradual reduction in percentage cytotoxin titres with increasing age, chidren from age 3-5 had no toxin titre detected (Emeruwa & Oguike, 1990). The quality of nutrient consumption was also assayed and it was detected that the lowest level of occurrence cytotoxin positive *C. difficile* isolates were with children given only breast milk (17.5%) (Emeruwa & Oguike, 1990). Nineteen percent of children fed with a mix of formula foods and breast milk were positive for *C. difficile,* while the highest occurrence (50%) was with children given only formula foods (Emeruwa & Oguike, 1990). Further studies have been performed in two hospitals in Nigeria. Adult HIV patients staying in the hospitals, had a CDI prevalence of 43%, while the prevalence among outpatients was 14%. No inclination of the prevalent ribotypes in this region of sub-Saharan Africa was determined (Onwueme *et al.,* 2011).

A South African study characterised *C. difficile* in school children and hospital out patients detecting toxigenic profiles and correlation between various pathologic features. Prevalence was observed in out patients more than school children. Toxin negativity was also a characteristic feature in *C. difficile* isolates from school children while toxin positivity was detected for out-patients hospital isolates. Of the sample analysed 14% *C. difficile* prevalence was observed (Sammie *et al,* 2008). *C. difficile* prevalence were also observed in Zimbabwe soil, chicken and water (Simango, 2006)

1.10 Therapy

1.10.1 Antimicrobial resistance in *C. difficile*

C. difficile has been reported to possess resistance to a number of drugs. These resistant phenotypes could result from mutations on target sites, single nucleotide polymorphisms or transposons like TN5397 (Spigaglia, 2011). To chloramphenicol, *C. difficile* confers resistance using a *catD* gene carried by transposon TN4451/ Tn4453 group. This gene is responsible for inactivating the chloramphenicol. Also, Spigaglia *et al.,* 2011, reported 51 of 82 multiresistant strains which showed resistance to rifampicin and moxifloxacin. This moxifloxacin resistance was observed to be mediated by a change in the amino acid of position 82 from threonine to ileucin in *gyrA*. Again, *C. difficile* may possess resistance to rifampicin with a genomic mutation occurring in the β-subunit of RNA polymerase (RpoB) at positions 488 and 548 (Spigaglia, 2011). Resistance to clindamycin in *C. difficile* isolates has been observed (Feldman *et al,* 2005) and resistance to erythromycin has been seen in many *C. difficile* ribotypes including 001, 027, 017 and 078 ribotypes (Reil *et al.,* 2012). Three hundred and sixteen *C. difficile* strains were analysed in 2011, belonging to eleven ribotypes; 148 were observed to be resistant to a minimum of one of the eight antibiotics used in the study. More than half of the resistant strains showed multidrug resistance (Spigaglia *et al.,* 2011).

Metronidazole and vancomycin are first line antibiotics for *Clostridium difficile* infection (CDI). However increases in MICs of metronidazole have been detected and full resistance in some strains has been reported to metronidazole. The mechanism of resistance is yet to be determined (Spigaglia *et al.,* 2011).

1.10.2 Metronidazole

Mechanism of action

Metronidazole has been used as a drug against microbial infections for over 45 years and can be used against Gram positive and Gram negative bacteria. Metronidazole has been observed to depend on the microbial metabolic pathways (Edwards and Mathison, 1970) for its activation which aids its selective toxicity as metronidazole reduction happens mainly in anaerobic cells (Land and Johnson, 1999).

Metronidazole enters bacterial cells through the process of passive diffusion into the cytoplasm where it is reduced. The reduction of metronidazole intracellularly is by scavenging electrons that should have been accepted by ferredoxin. This is possible because the redox potential of

metronidazole is -0.56V and that of ferredoxin is -0.46V. Thus metronidazole would accept more electrons than ferredoxin since its redox potential is more negative (Edwards and Mathison 1970). The intracellular reduction further aids the diffusion of metronidazole into the cell, within the cell an electron is transferred to the nitrogroup of metronidazole converting it into a nitrosofree radical that is unstable; thus activating the drug (Lofmark 2010). The activated drug is cytotoxic and results in DNA degradation (Lofmark 2010). DNA damage has been reported not to be as a result of binding of nitroimidazole to the DNA, but nitroimidazole causes the oxidation of DNA by scavenging electron from it which results in breakage of DNA strands (Edwards, 1993)

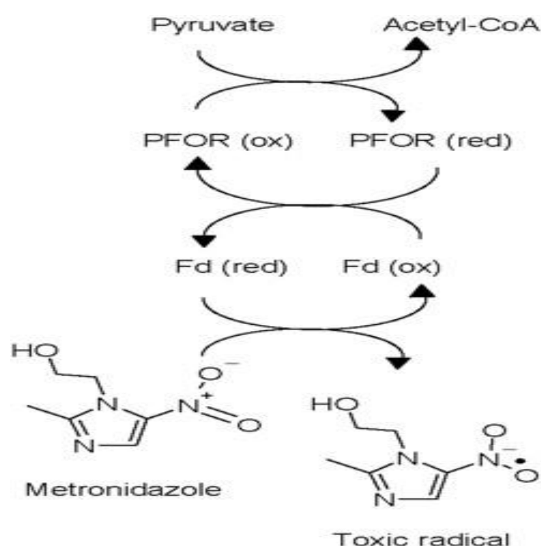

Figure 1. 6 Mechanism of activation of metronidazole (Leiros *et al.,* 2004)

<u>Susceptibility to Metronidazole</u>

Despite the high activity of metronidazole against *C. difficile,* strains of *C. difficile* have been observed to show reduced susceptibility to metronidazole (Baines *et al.,* 2008, Brazier *et al.,* 2001). Reduced susceptibility to metronidazole in *C. difficile* was first identified in 2001 by Brazier *et al,* it was identified to be a nontoxigenic ribotype 010, and the MIC obtained by Etest was 16mg/L (Brazier *et a.l*, 2001). Reduced susceptibility (MIC>2mg/L) has also been identified in ribotype 001 for 24.4% of the isolates tested (Baines *et al,* 2008).

Resistance of metronidazole in *B. fragilis* was seen to be as a result of nitroimidazole resistance genes commonly known as the *nim* genes which confer resistance against nitroimidazole antimicrobial agents by encoding nitroimidazole reductase. These *nim* genes have been seen to confer resistance to metronidazole in *H. pylori*, but none except *nimB* (Chong *et al,* 2014) has been identified in *C. difficile*. A study testing both *C. difficile* isolates with reduced susceptibility and non-reduced susceptibility reported absence of *nim* genes in both groups

(Pelaez *et al.,* 2008). Though it was suggested that the reduced susceptibility could be as a result of reduced uptake of metronidazole by *C. difficile* and reduced nitroreductase activity (Lofmark, 2005).

High level mobility in clinical settings can be as a result of anaerobes causing diseases for example *Bacteroides fragilis* is a disease causing pathogen in the intestines. It has been observed that it has laterally transferred, amongst anaerobic bacteria, genes that are responsible for resistance to antibiotics for example clindamycin and metronidazole. Metronidazole which was used to eradicate most antibiotic resistant *Bacteroides* has now been seen to decline slowly in efficacy as there is an emergence of resistant species (Lofmark, 2005).The therapeutic breakpoint of metronidazole is 32mg/L and anything below is referred to as been susceptible (EUCAST, 2017)

1.10.3. Vancomycin

<u>Mechanism of action</u>

Peptidoglycan synthesis involves more than five steps, the formation of D-alanyl-D-alanine (D-ala-D-ala) being the second step. Vancomycin prevents the formation of peptidoglycan for bacteria cell walls by binding to the C- terminus of D-ala-D-ala thereby hindering the joining of the other precursors. Vancomycin also reacts with target bacteria after the late precursors are translocated to the exterior membrane surface since it does not penetrate the cytoplasm (Courvalin, 2006).

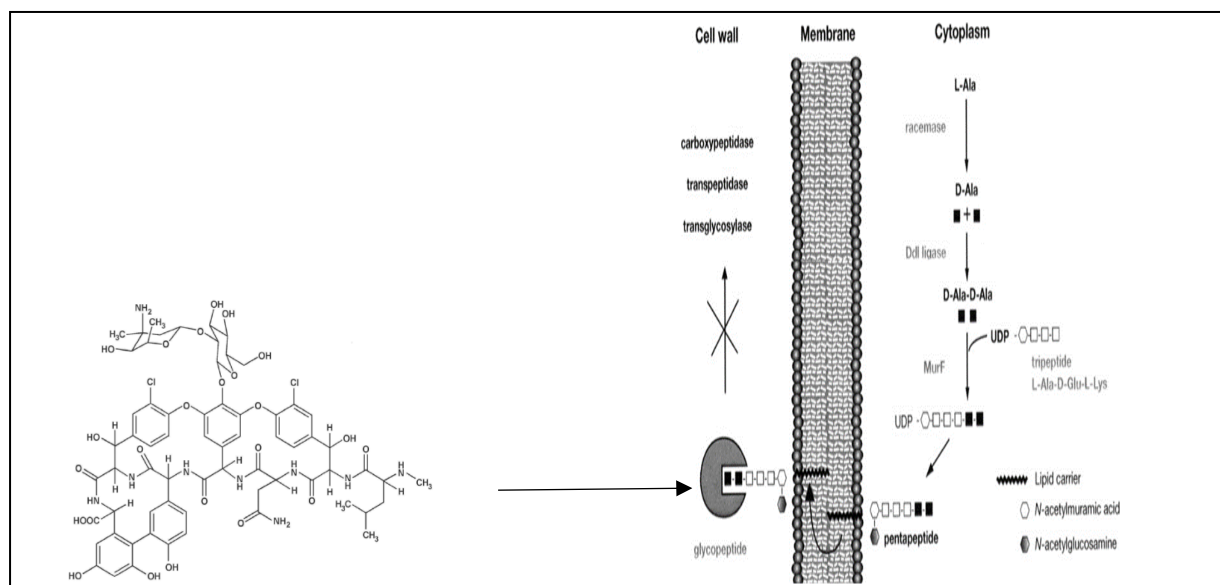

Figure 1.7 Mechanism of action of vancomycin (adapted from Courvalin *et al.,* 2006) and structure of vancomycin (adapted from Vila *et al,* 2007).

Susceptibility to Vancomycin

Vancomycin also is a first line antimicrobial agent used to treat *C. difficile.* The use of vancomycin has been observed to be more effective for chronic CDI compared to metronidazole, both antimicrobials can be used for treatment of mild CDI (Zar *et al*, 2007). However to limit the incidence of vancomycin resistant enterococci (VRE) selection, the use of vancomycin is being reduced newer therapies (e.g. fidaxomicin) are being developed and implemented in the treatment of CDI (Hierholzer *et al*, 1995).

There has been one case of resistance in Poland in 1991 but other cases of reduced susceptibility have been observed, 13 isolates in Spain had high MIC from 8-16mg/l (references Poland and Spain). Mechanism(s) causing these reduced susceptibility were not determined, but in *Enterococcus spp,* vancomycin acquired resistance was seen to be as a result of production of peptidoglycan precursors, which had decreased affinity for glycopeptide antibiotics. This was as a result of the presence of van A, van B, van D, van E, and van G genes (Huang, 2009), so the precursors produced for peptidoglycan synthesis are less susceptible to the action of vancomycin (Courvalin, 2006).

In general the antimicrobial resistance mechanisms such as mutation and acquisition of resistant genetic information in *C. difficile* have been seen to be same in other Gram positive bacteria. As a result this can be applied to ascertain for mechanisms in vancomycin and metronidazole reduced susceptibility that is yet to be determined. An example can be seen in some tetracycline resistance cases are caused by *tetM* gene related to Tn916 whose elements have been seen in some *C. difficile* isolates and which is predominating in other Gram positive bacteria (Huang, 2009).

1.10.4 Other therapeutic measures:

1.10.4.1 Fidaxomicin

Fidaxomicin was prepared for CDI treatment in US and Europe in 2011. This bactericidal antibiotic has been observed to be more active than metronidazole and vancomycin with low MIC's (Mullan, 2014, Goldstein *et al.,* 2011).

Its mechanism of action involves inhibiting RNA polymerase thus inhibiting protein synthesis (Artsimovitch *et al*, 2012) due to decrease in RNA synthesis. Its spectra of activity is higher towards clostridial RNA, though it is also effective against enterococci and staphylococci

(Finegold *et al*, 2004). Yeast are completely resistant to fidaxomicin as well as Gram negative organisms (Beidenbach *et al,* 2010). Therefore the narrow spectrum of activity of fidaxomicin is one of its main advantages, given that it will disrupt less the gut microflora and potentially reduce recurrent infections with *C. difficile*.
Its administration twice daily post antibiotic effect range between 9.5hours -12.5hours and like vancomycin its faecal concentrations is above MIC of *C .difficile* due to its inability to diffuse from the gastrointestinal tract (Babakhani *et al,* 2011)
An elderly CDI patient was unresponsive to intravenous metronidazole and vancomycin administered through a nasogastric tube (Arends; *et al,* 2016). After 10 days, the therapy was stopped and crushed fidaxomicin was given using a nasogastric tube (Arends; *et al,* 2016). This led to a resolution of the disease in one day and a further negative *C. difficile* stool sample. Diarrhoea continued till the tenth day and no recurrence was observed within the in – hospitalstay (Arends *et al,* 2016). Due to the efficacy demonstrated in this case, the use of this form of therapy for chronic cases requires further study (Arends *et al*, 2016).

Clinical studies have demonstrated similar curative rates of CDI using either fidaxomicin-or vancomycin-treated patients (Louie *et al,* 2011). Also comparing fidaxomicin and vancomycin, the clinical trial identified a higher recurrence rate of the non-north American pulsed field type1 strains with vancomycin compared to fidaxomicin previously named OPT 80 (Louie *et al*, 2011). Exposure of CD toxigenic strains (110) from United States was made to 15 antimicrobials these strains included 027 ribotype (n=6) also from Europe and Canada. Five antibiotics were observed to be most active, these were fidaxomicin, rifaximin nitazoxanide, tizoxanide and rifalazil, with MIC 90 ≤ 0.125 mg/L (Hecht *et al*, 2007). Fidaxomicin was also identified to actually eliminate the offending bacteria while vancomycin just hinders the bacteria growth (Babakhani *et al*, 2011).

A *C. difficile* isolate was observed during disease recurrence to have a high MIC of 16mg/L to fidaxomicin following exposure to the antimicrobial, but no mechanism of resistance was determined (Goldstein *et al*., 2011). Reduced susceptibility to fidaxomicin was observed in isolates identified during a serial passage experiment and whole genome sequencing was used to characterise the genotype. Results showed mutation in the CD22120 or *rpoB* in the fidaxomicin reduced susceptible phenotype. The significance of these changes is yet to be fully elucidated (Leeds *et al.,* 2014).

1.10.4.2 Other CDI antimicrobial therapies

Other therapeutic agents are also being developed and researched to increase the options for clinicians. For example surotomycin is a lipopeptide that was observed in an *in vitro* gut model

to decrease the toxin levels and growth of *C. difficile* without the development of reduced susceptible strains (Chilton *et al.,* 2014) Cadazolid is another therapeutic agent in development for treating CDI by inhibiting translation during protein synthesis (Locher *et al.,* 2014), and clinical trial data for this antimicrobial when experimented on 64 healthy men, suggests that it was safe with low systemic exposure (Baldoni *et al,* 2013)

Colectomy: Surgical removal of the colon is recommended in very severe cases showing toxic megacolon and pertoreal signs (Lamontagne *et al.,* 2007). It was reported that patients that were immunocompetent and over 65years had improved health after colectomy (Lamontagne *et al,* 2007).

1.10.5 Biotherapy for CDI

1.10.5.1 Faecal microbiota transplantation (FMT)

Since CDI onset is as a result of the disruption of the colonic microflora of the patient. FMT is a method employed to reinstate the colonic microflora thus limiting the proliferation of *C. difficile* due to presence of competing healthy bacteria. FMT involves the introduction of a faecal suspension from a healthy personnel into a patient's gut. This can be done via enemas, a nasogastric tube and using oral capsules. A study was done involving 19 patients which failed other treatments options, but FMT showed disease resolution from 6 months up until 45years after which reinfection was reported (Rohlke *et al,* 2010)

Amongst the methods of administration, colonoscopy was documented to be preferred by patients (Rohllke *et al.,* 2010). FMT for recurrent CDI has also been reported using the nasogastric method. Ninety percent disease resolution has been published for 100 patients using FMT (Bakken, 2009).

The efficacy of faecal transplantation was determined by administering it 2 days after a 2weeks oral vancomycin was stopped (Hota *et al.,* 2017). It was examined compared to the standard oral vancomycin therapy to resolve CDI therapy. But no significant difference was observed. It should be noted that faecal administration in this study entailed a single administration and was done via enema (Hota *et al.,* 2017).

The use of faecal microbiota transplantation (FMT) was researched on using vancomycin simultaneously on severe and severe/complicated CDI cases. It was reported to have a high recovery rate of 93% (Fischer *et al.,* 2015).

1.10.5.2 Intravenous immunoglobulin

C. difficile toxins which are its major virulence factors may be neutralized using intravenous immunoglobulin (IVIG), thus preventing colitis. IVIG binds and neutralise toxin A as an antitoxin (Shah *et al.,* 2014). A 2014 research study reported IVIG to have been used to successfully treat an elderly patient that was unresponsive to both metronidazole and vancomycin (Shah *et al.,* 2014). This has also been successful administered in severe pseudomembranous colitis (Salcedo *et al.,* 1997, Chandrasakar *et al.,* 2008)

1.10.5.3 Transcutaneous immunization

C. difficile toxin A (CDA) was treated with formalin and administered to mice transcutaneously to induce immune response, alone or with cholera toxin (CT) an immunoadjuvant (Ghose *et al.,* 2007). Anti CDA and Anti CT IgA were observed in stools while in serum, anti-CT immunoglobulin G (IgG), anti-CDA and anti IgA (Ghose *et al.,* 2007). A cell culture assay was used to detect the neutralisation capacity of sera collected from immunized mice (Ghose *et al,* 2007); thus showing the efficacy of transcutaneous immunization using CDA toxoid (Ghose *et al,* 2007). *C. difficile* toxoid vaccine for humans was researched on consisting of toxoids A and B inactivated with formalin. It was experimented on 30 healthy adults and was observed to be safe and immunogenic (Kotloff *et al,* 2001). Toxoid vaccine has also been observed to be useful in recurrent diarrhoea resolution as observed in 3 patients with recurrent CDAD, after the administration of the vaccine, no further recurrence was observed in these patients (Sougioultzis *et al,* 2005)

1.10.5.4 Beta lactamase enzymes

SYN-004, beta- lactamase enzymes was designed as an orally administered therapy to degrade residual β-lactam antibiotics in the gut (Kaleko *et al.,* 2016) to stop further harm on the normal flora in the gut. It was observed to destroy ceftriaxone in the gut of pigs and dogs. SYN-004 has been observed to be a safe therapeutic in a phase 1 trial and its capacity to prevent CDI is being researched in a Phase 2 clinical trial (Kaleko *et al.,* 2016)

1.10.5.5 Toxin binding

An anionic polymer, tolevamer (GT160 -246), was evaluated for its capacity to neutralize *C. difficile* toxin A and B. GT160 -246 was reported to possess no antimicrobial activity thus may have no effect on the normal flora of the intestine. Additionally, invitro test also showed no interference with the other antibiotics (Kurtz *et al*; 2001). The use of this polymer had no

adverse effect with respect to inflammation, fluid accumulation or permeability it was observed to prevent inhibition of protein synthesis and cell rounding caused by toxins (Kurtz *et al.,* 2001). The affinity of GT160-246 to toxin A was suspected to be higher than in toxin B as was detected in vero cells (Kurtz *et al*; 2001). *In vivo* test was unable to give useful information as toxin B receptors is absent in rodents. The test was also done on hamsters as well using metronidazole and cholestyramine (Kurtz *et al*; 2001). Ten percent of animals survived after being administered cholestyramine 100% survived with metronidazole and 80% with GT160 -246 however after 80% survivors after metronidazole withdrawal, died but no death was observed after the administration of GT160 -246 was stopped. GT160 -246 was involved in a phase 1 trial and observed to be of no significant risk to healthy individuals (Kurtz *et al*; 2001) However supporting phase III clinical studies (Lewis and Anderson, 2013), gut model experiments have shown that the cytotoxic effect of *C. difficile* was not affected despite exposure of high concentrations of tolevamer (Baines *et al,* 2009).

1.10.5.6 Non toxigenic *C. difficile* (NTCD)

NTCD has been reported in a number of researches to prevent colonisation of toxigenic *C. difficile* thus limiting the onset of CDI (Wilson *et al*; 1983, Natarajan *et al*, 2013). A study using the hamster model to study the protective capacity of NTCD against CDI onset was performed by Wilson and colleagues, and ninety three percent of animals first exposed to NTCD before toxigenic strains survived compared to the control (21% survival) (Wilson *et al*; 1983). However, no protection was observed when the NTCD and toxigenic strains were given together. The safety profile of NTCD in humans has been investigated by giving healthy adults oral spore suspension of VP20621 strain M3. This NTCD was observed after vancomycin therapy to colonise gut and be tolerated (Viliano *et al*; 2012). The mechanism for this protection is yet to be elucidated, it could possible just be as a result of simply competing for nutrients or for mucosal niche (Natarajan *et al*, 2013). NTCD has also been used to reduce CDI recurrence in patients (Gerding *et al*; 2015). NTCD –M3 spores were administered orally after treatment of CDI patients with metronidazole or vancomycin. Of the 43 patients that received placebo, 30% demonstrated recurrence in CDI but of the 125 patients who received NTCD, only 11% demonstrated recurrent CDI (Gerding *et al*; 2015). A non-toxigenic *C. difficile* strain (Z31, ribotypes 009) was sequenced and analysed in Brazil. An observation of the genome shows it relates to a toxigenic strain ATCC9689, its nontoxigenic characteristics makes it useful as a form of therapy to compete with other toxigenic strains thus preventing the onset of CDI (Pereira *et al.*, 2016). However analysing its sequence was important to detect if it had other features that would enable a successful competition with other toxigenic *Clostridium* strains

(Pereira *et al.*, 2016). Other virulence earlier reported to be necessary for intestinal colonisation such as surface layer protein SlpA and genes coding for flagellar proteins FliC and FliD were also observed. Spo0A necessary for sporulation of these Z31 was also identified as well as *cotA, cotB, cotC, cotD* and *sodA* genes coding for the proteins forming the spore coat. Importantly, none of the genes for toxin A, toxin B and the binary toxin were found (Pereira *et al.*, 2016).

1.10.5.7 Monoclonal Antibodies

Bezlotoxumab and actoxumab are monoclonal human antibodies targeted against toxin A and B for neutralisation of these major *C. difficile* virulence factors. These form of passive immunity were observed for their efficacy in preventing *C. difficile* recurrent infection in 2655 CDI patients (Wilcox *et al*, 2015). While no beneficial use was observed when actoxumab was added to therapy, Bezlotoxumab was detected to prevent recurrence at a higher rate compared to the therapy using placebo, and 38% higher than the use of just the standard of care therapy and no health hazard was observed. More than quarter of the recurrent cases were observed after the standard effective treatment duration of 4 weeks. Twelve weeks duration of recurrent prevention was observed with bezlotoxumab showing its efficacy when administered with standard of care therapy antibiotic (Wilcox *et al,* 2015)

1.10.5.1 Probiotics

Living microbes known to improve the health of another living organism on administration to the later are referred to as probiotics (FA0/WHO, 2002). CDI being an antibiotic associated disease usually correlates with an imbalance of the gut microflora so the use of probiotics has been researched. Most cases of antibiotic-associated diarrhoea result from an osmotic imbalance in the colon, leading to excess luminal carbohydrate and loss of water across the mucosa by osmosis and resultant diarrhoea. Probiotics have been proposed to carry out their mechanism of action by not just replacing the protective coverage of intestinal mucosa but also aiding fermentation of carbohydrate (Hickson 2011) which would hinder diarrhoea downstream. Pilal and Nelson (2008) stated that there is not enough information to base the single use of probiotics in the treatment of CDI (Pillai and Nelson, 2008).

One of the *in vitro* characteristics observed to enable the probiotic effect of an organism is its ability to attach to the intestinal wall, its pH tolerance and the capacity to prevent the proliferation of pathogenic bacteria (Jacobsen*, et al*; 1999). *L. reuteri* DSM12246 was observed to show this antimicrobial feature though did not affect normal flora of the intestine

(Jacobsen, *et al;* 1999). However these features appeared not to be enough to sustain long term colonisation of the *lactobacilli* strains been examined in the gut (Jacobsen, *et al;* 1999). A 2016 systematic review was reported where probiotics in adults between the ages of 18 and 64 years old having AAD, as well as older people. In subjects less than 65 years old, probiotics was observed to have a positive impact in the prevention of AAD but this was not same with those over 65 years old (Jafarnejad *et al;* 2016). The use of probiotics in prevention of CDI was ascertained in another systematic literature review article. These article analysed 7957 patients in 26 randomised control trials. Slightly over 60% reduction in CDI was observed on administration of probiotics. Over 50% of adult and children AAD patients had a positive response to probiotics (Lau and Chamberlain; 2016). The decrease in CDAD (over 55% reduction) was effective with the use of probiotics such as *Lactobacillus, Saccharomyces* (Lau and Chamberlain; 2016).

Live *Lactobacillus casei* Shirota (LcS) contained in a commercially produced drink was examined for its ability to prevent AAD and CDI in spinal cord injury patients. LcS administration was performed during and one week after antibiotic therapy which led to reduced AAD onset (Wong *et al*, 2014)

In summary the onset of *C. difficile* infection can be treated by first ending the medications the patient has been exposed to. All personnel in contact with the patient would be required to make use of disposable medical equipment and practice basic hygiene especially hand washing with soap and water which is the preferred choice above use of sanitizers due to the ability of *C. difficile* to sporulate (Goldstein *et al,* 2015)

As was earlier mentioned metronidazole and vancomycin are the first line medications for treating CDI. In addition the administration of these first line antibiotics is dependent on the severity of the disease and response of patients. Moderate/mild CDI would require oral intravenous metronidazole 500mgq.i.d for 10-14 days (Carmo *et al,* 2015), and 125mg q.i.d of vancomycin administered orally could also be used for this level of severity. A standard vancomycin dose is required if the patient response is not positive after 5-7 days metronidazole use. For severe CDI, 125mg q.i.d- 500mg q.i.d of vancomycin is required. With respect to ileus, megacolon and colonic diversion, vancomycin can be given as a retention enema (Cohen *et al*, 2010). For ileus this can be done in conjunction with intravenous metronidazole (Carmo *et al*, 2015, Ofosu 2016). Fidaxomicin can also be used instead of vancomycin as curative levels have been recorded to be higher in fidaxomicin in comparison to vancomycin (Cornely *et al,* 2014, Carmo *et al,* 2015, Ofosu 2016)

Determination of the characteristics of less susceptible *C. difficile* strains and clear mechanism by which this bacterium becomes less susceptible to metronidazole would enhance research works, thus limiting development of resistance.

1.11 Aims of project

This project aims to characterise reduced metronidazole susceptibility in UK *C. difficile* isolates.

1.12 Main objectives

To isolate current clinical *C. difficile isolates* with reduced susceptibility to metronidazole.

To characterise the reduced susceptibility phenotype using population analysis profiling and antimicrobial susceptibility testing.

To assess the stability, and the potential of further increases in minimum inhibitory concentrations (MICs), of the reduced susceptibility phenotype using serial passaging studies.

To assess the uptake and activation of metronidazole in *C. difficile* strains with reduced susceptibility to metronidazole using antimicrobial bioassay techniques.

To assess nutritional factors (e.g. iron availability) that may contribute to the reduced susceptibility phenotype in *C. difficile* using antimicrobial susceptibility testing techniques.

To determine genetic mechanism(s) of reduced susceptibility to metronidazole.

1.13 Laboratory experiments

This research was focused on strains of three UK *C. difficile* ribotypes: 027, 001 and 106. In total, 37 *C. difficile* strains with metronidazole MICs of ≥4mg/L were studied. These *C. difficile* strains are characterized as *C. difficile* with reduced susceptibility to metronidazole (CDRM) based on comparism to epidemiological cut-off (ECOFF) values for the drug. These CDRM strains were compared to 11 *C. difficile* strains of the same ribotypes which were susceptible to metronidazole (CDSM). Additionally, two experimental control *C. difficile* strains, E4 and ATCC 700057 were also studied. *C. difficile* E4 was the first *C. difficile* strain (non-toxigenic, ribotypes 010 coded as 110) reported with the CDRM phenotype (Brazier *et al,* 2001), while ATCC 700057 is a commercially available *C. difficile* strain (ribotype 038 coded as 111) and is a CDSM strain. *C. difficile* strains in this study are identified using their corresponding University of Hertfordshire freezer position number.

2.0 Antimicrobial Susceptibility Testing of UK *C. difficile* ribotypes 027, 001, 001/072, 106

2.1 INTRODUCTION

Antimicrobial susceptibility testing (AST) is an experiment designed to detect the level of resistance of a certain microbe to an antimicrobial agent. AST is employed in a clinical setting to select the drugs that best combat an offending pathogen. This test could be performed manually for example using agar incorporation methods, gradient agar plate methods, or disc diffusion methods; or it can be achieved with the use of automated instruments (Jorgensen & Ferraro, 2009).

Though disc diffusion tests are not costly and can be used for routine clinical tests, the Clinical Laboratory Standard Institute (CLSI) suggests an agar dilution method as the preferable method for antimicrobial susceptibility testing of *Clostridium difficile*. Agar incorporation methods are convenient for minimum inhibitory concentration (MIC) surveillance and assessment of new antibiotics and also are reproducible (Erikstrup *et al*., 2012).

Research evaluating the antimicrobial susceptibilities of nine *Clostridium difficile* ribotypes against metronidazole using several methods including: E-test, agar dilution method (ADM), an agar incorporation method (AIM) was performed (Moura *et al.,* 2012). It was observed that the ability of AIM to detect reduced metronidazole susceptibility in the test strains transcended that of the other two methods (Moura *et al.,* 2012). Similar research at the University of Leeds in the United Kingdom also demonstrated that AIM was a superior method in detecting reduced metronidazole susceptibilities in *C. difficile* and that both the choice of agar base and the broth ingredients used to raise the inoculum influenced the metronidazole MICs (Baines *et al.,* 2008).

2.11 Antimicrobial Susceptibility in *Clostridium difficile* isolates.

The first line treatments for mild/moderate *Clostridium difficile* infection include metronidazole and vancomycin, therefore routine surveillance of antimicrobial susceptibilities in *C. difficile* is recommended given the few efficacious alternatives that are available. Experiments have been performed to detect susceptibility levels of *Clostridium difficile* to these antibiotics using different methods. Baines *et al.,* (2008) carried out a study with metronidazole whereby MICs were ascertained for different strains using spiral gradient endpoint analysis, Etesting, and AIM. It was also stated that *C. difficile* growth on supplemented Brucella agar and other agars were observed to be a lot less than the reproducible growth observed on Wilkins Chalgren agar during susceptibility testing (Baines *et al.,* 2008). Ribotypes 106 and 027 were susceptible to metronidazole as well as a large quantity of ribotypes 001 strains tested, however reduced susceptibility (Minimum inhibitory concentrrations (MIC) >2mg/L) was observed in

24.4% of ribotype 001 *C. difficile* isolates with the use of agar incorporation method (Baines *et al.,* 2008). *Clostridium difficile* susceptibilities to metronidazole, vancomycin and oritavancin have also been compared using agar incorporation methods and broth macrodilution methods. The use of an agar incorporation method resulted in metronidazole being the most potent antibiotic of the three antibiotics tested against *C. difficile* (O'Connor *et al.,* 2008). Another research tested one hundred and eighty eight C. *difficile* ribotypes for their levels of antimicrobial susceptibility to 30 antibiotics by the use of a broth macrodilution method. The result showed that ribotypes 027 along with 8 other polymerase chain reaction (PCR) ribotypes were multiresistant to the antibiotics tested (Pirs *et al.,* 2013).

The metronidazole MIC range for 415 strains of *C. difficile* in a 7 year study was recorded to be from 0.016-32µg/ml, while the MIC_{50} was 0.5µg/ml and MIC_{90} was 4µg/ml. However, 26 strains out of the 415 strains were metronidazole resistant having MIC ≥32 µg/ml and the clinical breakpoint (CLSI) was 16µg/ml (Pelaez *et al*, 2002). The same numbers of strains were tested against vancomycin and the MIC range was 0.016 to 16µg/ml, MIC_{90} was 2 µg/ml and MIC_{50} was 1µg/ml. Reduced susceptibility to vancomycin was observed in 3.1% of the tested strains but there were no resistant strains (Pelaez *et al*, 2002). The MIC range for 9 *C. difficile* ribotypes were also obtained amongst which 027 ribotype had a range of 0.125-0.5 mg/L, 078 had a range 0.125-0.25 mg/L, 001 was 0.064-1 mg/L, 010 was 0.125-32 mg/L using agar dilution method (ADM). Of the nine ribotypes 2 strains belonging to the PCR ribotype 001 had reduced susceptibility to metronidazole which resulted to 4mg/L when tested with ADM. Ribotypes 027, 078, 001,010 were reported to be metronidazole susceptible and heterogeneous that is the colonies from each ribotype had varying MIC (Moura *et al*, 2012). A pan European study, detected the antibiotic susceptibility levels of common ribotypes of *C. difficile* (Freeman *et al,* 2015). Susceptibility to metronidazole and vancomycin as well as 7 other antimicrobial agents were determined for 953 *C. difficile* strains obtained from 22 countries. Out of the 99 recognized ribotypes identified 027 and 001 were among the four prevalent ribotypes (Freeman *et al*, 2015). Though resistance was observed to some antibiotics like moxifloxacin and clindamycin, no resistance was observed to metronidazole and vancomycin, reduced susceptibility rates detected was low, as MIC_{90} = 2mg/L for both metronidazole and vancomycin (Freeman *et al,* 2015).

2.12 Aims

To determine the antimicrobial susceptibilities of a panel of UK *C. difficile* isolates, some of which have previously shown reduced metronidazole susceptibility.

2.13 Objectives

To use an agar incorporation MIC method to compare the activity of the metronidazole and vancomycin against a panel of *C. difficile* PCR ribotypes 001, 027, and 106 strains.

2.2 METHOD

2.21 *C. difficile* isolates

Thirty seven strains of *C. difficile* from three PCR ribotypes: 027, 001, and 106 were evaluated in this study. These strains were selected because they had earlier been identified with reduced susceptibility to metronidazole (CDRM), with MICs >2mg/L which is the European committee on antimicrobial suscepitibility testing (EUCAST) epidemiological cut-off (ECOFF) for metronidazole (EUCAST, 2017). These 37 strains were compared with 11 *C. difficile* strains of the same ribotypes which were previously demonstrated to be susceptible to metronidazole (CDSM strains) in addition to two control strains, E4 (Brazier *et al.,* 2001) of ribotypes 010 (an environmental isolate, CDRM coded as 110) and ATCC 700057 of ribotype 038 (CDSM strain coded as 111). The clinical strains were all isolated in the United Kingdom and were obtained from Professor Mark Wilcox (Leeds Teaching Hospitals NHS Trust and the University of Leeds). The strains were stored as spores in 50% ethanol which could germinate spontaneously in presence of germinants (e.g. bile salts) contained within the selective agar used

2.22 Agar incorporation MIC testing

The agar incorporation MIC method used in this study was based upon that described by Baines and colleagues (2008), given that this method has been demonstrated previously to be able to detect reduced susceptibility to metronidazole in *C. difficile.* Twenty microliters of spore preparation of each strain was inoculated and streaked out on Brazier's agar (LAB160, Neogen Europe Ltd, Ayr, UK) plate supplemented with 2% (v/v) lysed horse blood (SR0050, Oxoid, Basingstoke, UK) to grow overnight in the anaerobic cabinet (N_2:H_2:CO_2, 80:10:10) (Don Whitley Scientific, UK). Three to four colony forming units of these strains were than swabbed individually using a sterile cotton swab into respective test tubes containing sterile pre-reduced Schaedler's broth (CM0497 Oxoid, Basingstoke, UK) and incubated overnight anaerobically at 37°C.

Four hundred microliters of overnight cultures (approximately 1 x 10^7 cfu/mL) was applied to the surface of pre-dried Wilkins Chalgren agar (CM0619 Oxoid, Basingstoke, UK) plates containing doubling dilutions of antibiotics (metronidazole (M3671, Sigma-Aldrich, Poole UK) and vancomycin (101489773, Sigma-Aldrich, Poole UK)) ranging from 0.03mg/L to 128mg/L using a multipoint inoculator (UriDot, Mast Group Ltd., Bootle, UK). The pins of the multipoint inoculator are manufactured to deliver 1µL of the bacterial inoculum onto the surface of the agar plate (approximately 10^4 cfu), which is in line with the recommended inoculum (CLSI) for anaerobic bacteria when performing AIM susceptibility testing. Metronidazole and vancomycin solutions were prepared, filter sterilized using syringe filters

and diluted to different concentrations using sterile water. Antimicrobial solutions were prepared at ten-fold higher concentrations, then diluted 1:10 in molten (50°C) Wilkin's Chalgren Anaerobe agar and all MICs were determined in duplicate. The susceptibility was confirmed by determining the minimum inhibitory concentrations (MIC) of these antibiotics for the *C. difficile* strains. Geometric mean MIC values were determined for *C. difficile* PCR ribotypes along with MIC_{50} and MIC_{90} values. MIC_{50} is the MIC that inhibited 50% of the *C. difficile* strains studied while MIC_{90} is the MIC that inhibited 90% of the *C. difficile* strains studied. Geomean is a type of mean, it represents all the MIC values of the strains analysed. After inoculation the agar plates were incubated anaerobically at 37°C overnight. Aerobic and anaerobic control plates lacking antibiotics were also inoculated and incubated in the appropriate atmosphere to assess for contamination and as a growth control respectively. The plates were then removed from the anaerobic cabinet and MIC values recorded. The lowest concentration of antimicrobial agent that elicited a mark reduction in visible growth/no growth when compared to anaerobic growth control agar plate was deduced to be the MIC (CLSI) and MIC concentrations for metronidazole greater than 2 mg/L was reported to have reduced susceptibility. Kruskal -Wallis significance testing was performed to detect significance in metronidazole MIC differences between CDRM strains of the ribotypes analysed. Post hoc testing using Dunn's multiple comparism test was also done

2.23 Statistical analysis

Kruskal -Wallis significance testing was performed to detect significance in metronidazole MIC differences between CDRM strains of the ribotypes analysed. Post hoc testing using Dunn's multiple comparism test was also done

2.3 RESULTS

Metronidazole overall geometric mean MICs for *C. difficile* ribotype 001, 027 and 106 were 3.76, 2.72 and 3.18mg/L, respectively (table 1), with ribotype 001 being the highest. The metronidazole MIC range for ribotype 001 was observed to be wider than the other ribotypes analysed. The geometric mean vancomycin MIC for *C. difficile* ribotype 001, 027 and 106 were 0.57mg/L, 0.5mg/L and 0.5mg/L (table 1).

Table 2. 1 Antimicrobial susceptibilities of CDRM strains (mg/L) using AIM with Oxoid Wilkin'sChalgren agar, with inocula raised in Oxoid Schaedler's anaerobe broth

Antibiotics	**PCR Ribotype**	**No of strains tested for each ribotype**	**Range of MIC detected** (mg/L)	**MIC50** (mg/L)	**MIC_{90}** (mg/L)	**Geometric mean**
Metronidazole	001	22	0.125-8	4	8	3.76
	027	9	2-4	2	4	2.72
	106	6	2-4	4	4	3.18
Vancomycin	001	22	0.25-2	0.5	1	0.57
	027	9	0.5	0.5	0.5	0.5
	106	6	0.5	0.5	0.5	0.5

Kruskal -Wallis significance testing was performed to detect significance in metronidazole MIC differences between CDRM strains of ribotypes 001,027, 106. The P value was 0.0187 which was significant and post hoc testing- Dunn's multiple comparism test showed significant difference was between 001 vs 027. However, for vancomycin the MIC differences between ribotypes were not significant as the P-value was 0.944.

The MIC_{50} for metronidazole against all *C. difficile* ribotypes analysed was ≤4mg/L while MIC_{50} for vancomycin against all test C. *difficile* ribotypes analysed were 0.5mg/L (Figure 2.1 and table 2.1). The MIC_{90} of metronidazole for CDRM strains of ribotypes 001 was 8mg/L and in CDRM strains from ribotypes 027 and 106 were 4mg/L, while MIC_{90}s for vancomycin for all *C. difficile* ribotypes analysed were ≤1mg/L (Figure 2.2 and table 1).
The MIC of the control *C. difficile* strains were within the standard range for each strain, e.g. for E4 the metronidazole MIC range expected was 4-16mg/L and for ATCC 700057 was from 0.125- 0.5mg/L (CLSI, 2007). The MIC values of the CDRM strains for metronidazole confirmed most of the strains had the reduced susceptibility phenotype. Amongst the 22 strains of ribotype 001 tested, 20 strains showed reduced susceptibility to metronidazole. While for ribotypes 027 and 106 4 strains of each ribotype were susceptible to metronidazole, out of the 9 and 6 strains respectively that were tested, as indicated in table 2.1.

Table 2. 2 Antimicrobial susceptibilities of CDSM (mg/L) strains using AIM with Oxoid Wilkin's Chalgren agar, with inocula raised in Oxoid Schaedler's anaerobe broth.

Anitbiotics	**Ribotype**	**Range of MIC detected(mg/L)**	**MIC_{50} (mg/L)**	**MIC_{90} (mg/L)**	**Geomean**
METRONIDAZOLE	**001**	0.25 -0.5	0.5	0.5	0.42
	027	1-2	1	2	0.6
	106	0.125-2	1	1	0.63
VANCOMYCIN	**001**	0.5-2	0.5	2	1
	027	0.5-1	0.5	0.5	0.5
	106	0.5-1	0.5	0.5	0.63

No significant difference was observed within ribotypes for both metronidazole and vancomycin for CDSM strains.

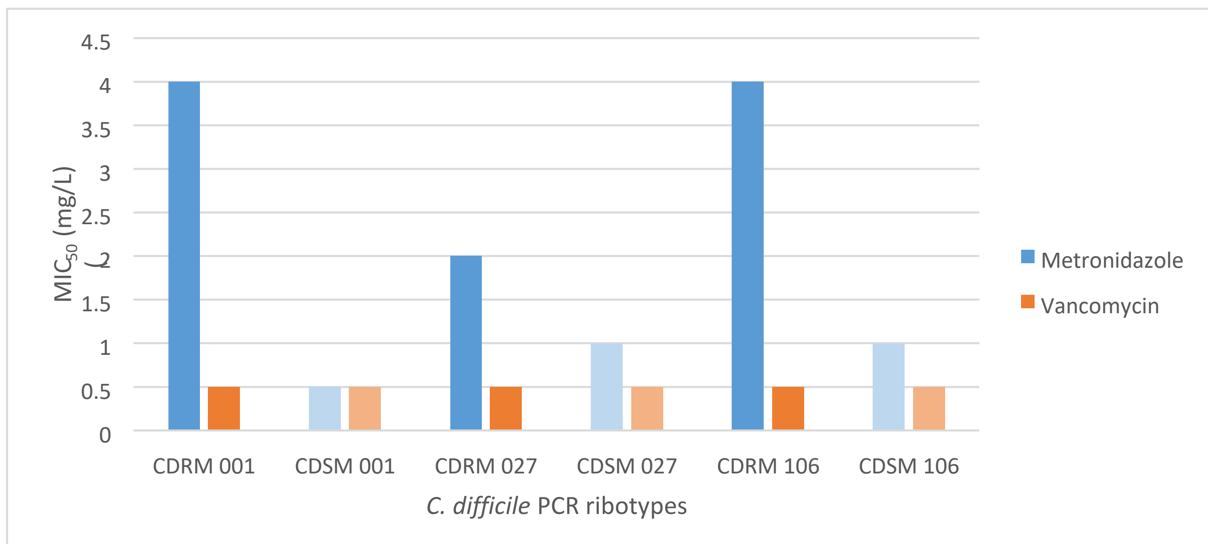

Figure 2. 1 MIC_{50} (mg/L) for metronidazole and vancomycin against *C. difficile*. The ribotypes with the lighter bars indicate the metronidazole susceptible *C. difficile* strains (CDSM) used to compare metronidazole reduced susceptible *C. difficile* strains (CDRM).

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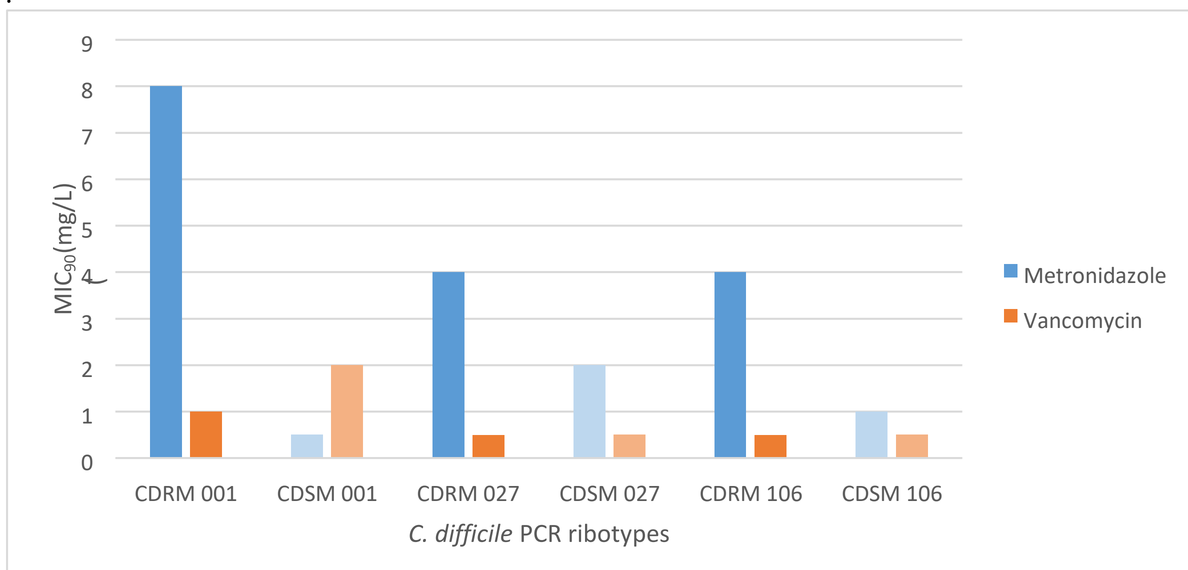

Figure 2. 2 MIC_{90} (mg/L) for metronidazole and vancomycin against *C. difficile*. The ribotypes with the lighter bars indicate the metronidazole susceptible *C. difficile* strains (CDSM) used to compare metronidazole reduced susceptible *C. difficile* strains (CDRM).

All reported CDSM strains were confirmed as susceptible to metronidazole with MIC_{90} $\leq$2mg/L in this experiment. The difference in susceptibility of CDRM and CDSM strains at MIC_{90} was two or more dilutions for metronidazole except 027 with one dilution.

2.4 DISCUSSION

There was a major difference in MIC_{90} between the CDRM and CDSM strains of the 001 ribotype to metronidazole. MIC_{50} of CDRM strains showed that majority the strains of ribotypes 106 and 001 showed reduced susceptibility to metronidazole. But for vancomycin all CDRM and CDSM strains showed same susceptibility level at MIC_{50} = 0.5mg/L. More than 90% of the total strains tested were susceptible to vancomycin at an MIC of less than 2mg/L.

Ribotypes 106 and 027 showed a higher level of susceptibility to metronidazole and vancomycin compared to 001 ribotype. The MIC_{50} and MIC_{90} results were very similar which indicates that within each ribotype the strains had similar response to the antibiotics tested.

Similarly, the past research work by Baines *et al.,* (2008) reported a geometric mean metronidazole MIC for all strains of PCR ribotype 001 (n= 22) to be 5.94mg/L, thus it is in line with this research which observed reduced susceptibility in 20 isolates of the PCR ribotype 001 strains. Ribotype 001 has also been observed to be the most predominant ribotype showing multidrug-resistance to 11 multidrug resistant ribotypes observed in a study that analysed 316 isolates from 14 countries in Europe (Spigaglia *et al,* 2011). These *C. difficile* isolates were obtained from patients in hospitals and 82 of the isolates were identified to be resistant to more than one antibiotic (Spigaglia *et al,* 2011). Though metronidazole and vancomycin were amongst the antibiotics analysed, no reduced susceptibility was observed to metronidazole and vancomycin, but this could be as a result of the Etest method being used (Spigaglia *et al*, 2011). It is worthy of note that detecting MIC is method dependent, using agar incorporation method is the gold standard which has been employed in the present research and the use of Schaedlers broth and Wilkins Chalgren agar for MIC detection maximised the chances of detecting a reduced susceptible phenotype. The therapeutic breakpoint for vancomycin activity on *Clostridium difficile* is 2mg/L and the results for vancomycin showed MICs against the majority of *C. difficile* strains were less than 2mg/L, except 3 strains of *C. difficile* PCR ribotype 001 which were at the breakpoint 2mg/L and one strain of same ribotype that was resistant at an MIC of 4mg/L. This indicates that the strains investigated in this research were susceptible to vancomycin, which is in line with an earlier report which stated that *C. difficile* strains were mostly susceptible to vancomycin and resistance was rare (Freeman *et al,* 2005). Though at low levels, reduced susceptibility to metronidazole and vancomycin has previously been reported in *C. difficile* isolates from a pan European study (Freeman *et al,* 2015). The MIC range being from $\leq$ 0.125-8 for metronidazole having MIC_{90} =2mg/L and $\leq$ 0.125-16 for vancomycin having MIC_{90} =2mg/L (Freeman et al, 2014). Susceptibility to vancomycin was also observed in previously reported CDRM strains of ribotype 001 which displayed a

geometric mean MIC of 2.14mg/L (Baines *et al.,* 2008). The MIC of vancomycin in this research also correlated with the results from a 2002 research that reported a total absence of resistance in the *C. difficile* strains tested to vancomycin (Pelaez *et al.,* 2002).

This research is in line with an additional research, where 330 strains of *C. difficile* were analysed and no strains resistant to vancomycin was observed (Pituch *et al.,* 2011). The difference observed in metronidazole and vancomycin MIC in this research emphasizes the variation in antibiotic activity which has been earlier argued to be same. Based on the MIC_{90} results of each ribotype analysed one can infer that the rate of reduced susceptibility occurrence to metronidazole is more in 001 compared with other ribotypes analysed.

Though reduced susceptibility was observed in this experiment, other than the control strain the highest MIC obtained was 8mg/L it was observed to be slightly lower than metronidazole faecal concentrations which is approximately 9mg/L, while the faecal concentrations of vancomycin is 1000mg/L (Baines and Wilcox, 2015). Though the MIC of these strains might not necessarily reflect the features obtained inside the human gut as MIC testing is an approximation of the likely effects of an antimicrobial on a bacterium, but *in vivo* conditions would be very different: the inoculum density (higher or lower) of *C. difficile* would be different which would affect MIC, spores would be present which would not be affected directly by the antimicrobials so would survive until antimicrobial levels in the gut declined to sub-MIC then they could outgrow. MICs were done in pure culture rather than it being a mixed culture with the gut microflora as it would be *in vivo* so this again might affect *C. difficile* response. Then growth and the levels of the antimicrobial, the growth phase of *C. difficile* might be different *in vivo*, again, this would affect the effects of the antimicrobials (MICs use late log-phase cultures normally which may not reflect CD growth *in vivo*).

Conclusion and future work

This research identified 29 CDRM strains to have reduced susceptibility to metronidazole and one *C. difficile* strains not susceptible to vancomycin. Replicates in these experiments yielded similar results and all control organism MICs were within the appropriate MIC ranges, therefore the MICs can be assumed to be accurate. The reduced susceptibility could be as a result of selection of isolates with reduced susceptibility that used to be heterogeneous by continuous exposure to metronidazole or vancomycin (Baines *et al.,* 2008). It could also be as a result of acquisition/inducing of features causing resistance like *nim* genes for metronidazole and *van A-G* genes for vancomycin (Carlier *et al.,* 1997, Huang, 2009, Chong *et al.,* 2014). All strains including CDRM strains that showed susceptibility to metronidazole will be further

analysed to detect MIC heterogeneity and mechanisms of reduced susceptibility will be assessed. The clinical significance of CDR strains (metronidazole and vancomycin) remains to be definitively demonstrated. This could be achieved by assessing the clinical notes of patients infected with CDRM strains and looking at their clinical outcomes following treatment compared to patients infected with the same ribotypes, at the same time, in the same geographical location. Although this work is beyond the scope of the present study, it would be an important addition to the literature around CDRM.

3.0 Heterogeneity in susceptibility to Metronidazole and Vancomycin in UK *C. difficile* isolates.

3.1 INTRODUCTION

Within any microbial strain it has been identified that there can be physiological variation amongst cells (Lidstrum and Konopka, 2010).These differences might influence the responses of the divergent microbial cells to environmental stimuli such as nutrient availability and antimicrobial compounds. The term heterogeneity indicates differences within a group, therefore within a particular bacterial strain, some colonies might have different minimum inhibitory concentrations (MICs).

Heterogeneity in antimicrobial agent MICs has been observed in some facultatively anaerobic organisms such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) and additionally in microaerophilic pathogens such as *Helicobacter pylori* (*H. pylori*), where heterogeneity in metronidazole MICs was previously identified (Weel *et al*., 1996).Ten percent of the *H. pylori* heterogeneous populations also reflected genetic variation (Weel *et al.,* 1996). *C. difficile* is the most prevalent infectious cause of antibiotic associated diarrhoea (Mutlu et al., 2007, Lynch *et al*., 2013, Debast *et al.,* 2013) and is usually treated with metronidazole, vancomycin, or fidaxomicin, depending on the severity of infection and the status of the patient with regard to recurrent infection. However, not only has *C. difficile* been reported to have reduced susceptibility to metronidazole and vancomycin, heterogeneous MICs to these therapeutic agents have also been reported. Toxigenic *C. difficile* that previously demonstrated resistance to metronidazole were evaluated for metronidazole heteroresistance by Pelaez and colleagues (2008). The authors reported heteroresistance to metronidazole, with MIC ranges in distinct clones (e.g. colony forming units) ranging between 16mg/L - 64mg/L, however all metronidazole-susceptible strains were homogeneous in their MICs to metronidazole (Pelaez *et al*, 2008). Moura *et al* (2012) also reported MIC heterogeneity for metronidazole against *C. difficile* ribotypes 001 and 010 and identified that these ribotypes were more heterogeneous with agar dilution method than with agar incorporation method (Moura *et al.,* 2012). The presence of microbial heterogeneity in susceptibility to antimicrobial agents may lead to incorrect interpretation of susceptibilities, and potentially could impact treatment of infection, if a single colony forming unit was evaluated and was assumed to be representative of the whole bacterial population (Weel *et al*., 1996). Reduced efficacy of administered antibiotics may also be due to MIC heterogeneity in the offending microbial population (Moura *et al.,* 2013). Thus the need for evaluating the heterogeneity in susceptibilities of UK *C. difficile* isolates to metronidazole in comparison to vancomycin is valuable and potentially clinically relevant

3.11 Aims

This research aims to screen for heterogeneity in metronidazole and vancomycin MICs in each of the 48 *C. difficile* strains analyzed.

3.12 Objectives

The objective of this experiment is to purify multiple distinct clones (i.e. colony forming units) of each *C. difficile* strain and determine the MICs of metronidazole and vancomycin using an agar incorporation method.

3.2 METHOD

The heterogeneity test was carried out by determining the MICs for 13-17 single colony forming units per *C. difficile* strain. Numbers of colony forming units (CFU) evaluated differed between strains due to differences in the size and separation of the colonies. Thirty seven metronidazole reduced susceptible *C. difficile* strains (CDRM) belonging to PCR ribotypes 001, 106 and 027 were tested along with two control strains: ATCC 700057and E4 (see 1.14 for details of control strains). The CDRM strains were compared with 11 metronidazole susceptible *C. difficile* strains (CDSM) of ribotypes 001, 106, and 027. The minimum inhibitory concentration (MIC) was detected using an agar incorporation method (refer 2.2) with Wilkins Chalgren agar (WCA) and inocula raised in Schaedler's anaerobe broth (Baines *et al.,* 2008).

Twenty microliters of spore preparation of each strain was inoculated and streaked out on a Braziers agar (LAB160, Neogen Europe Ltd, Ayr, UK) plate supplemented with 2% (v/v) lysed horse blood (SR0050, Oxoid, Basingstoke, UK) to grow overnight in the anaerobic cabinet (N_2:H_2:CO_2, 80:10:10) (Don Whitley Scientific, UK) at 37°C. A range of 13-17 colony forming units were individually removed from the Brazier's agar plates with a sterile cotton swab and inoculated into test tubes containing pre-reduced Schaedler's anaerobe broth and incubated overnight anaerobically at 37°C.

Four hundred microliters of each overnight culture was inoculated into the sterile sample block of the multipoint inoculator and 1µL of each culture was applied to the surface of pre-dried Wilkin's-Chalgren agar (WCA) plates containing doubling dilutions of antibiotics (metronidazole and vancomycin) using a multipoint inoculator (see 2.22). Inoculated agar plated were incubated for 48 hours at 37°C under anaerobic conditions, alongside anaerobic growth control plates and aerobic contamination control plates (no antimicrobial agents were in these control plates). MIC endpoints were determined as in 2.22. Strains were referred to as homogeneous when all clones isolated from that strain had the same MIC. Strains were referred to as heterogeneous when some clones had a different MIC compared to other clones isolated from the same strain.

3.3 RESULTS

The three metronidazole reduced susceptible *C. difficile* strains (CDRM) *ribotype* groups individual clones from single colonies demonstrated metronidazole MICs ranging from 0.125-32mg/L (Table 3.1). CDRM from ribotypes 001 demonstrated the broadest metronidazole MIC range. Vancomycin MICs overall ranged from 0.25-4mg/L for CDRM strains, with the broadest range in MICs noted for the PCR ribotypes 106 (0.5-4mg/L) as can be seen in table 3.1. For all ribotypes it was observed that some metronidazole susceptible *C. difficile* strains (CDSM) had clones that were reduced susceptible to metronidazole at MIC ≥4mg/L (Table 3.1). The range of MIC observed in ribotype 106 antibiotics between CDSM and CDRM were similar with a ±1 dilution. More than other ribotypes, ribotype 027 had a wider metronidazole MIC range in the CDSM strains compared to CDRM.

Table 3. 1 Metronidazole and vancomycin MIC (mg/L) ranges for 3 *C. difficile* ribotypes isolates' colonies tested individual purified colony forming units (13-17 cfu).

***C. difficile* ribotype**	**Antibiotics**	**Range (CDRM)**	**Range(CDSM)**
Ribotype 001	Metronidazole	0.125 – 32mg/L	0.5-4 mg/L
	Vancomycin	1-4mg/L	0.5-4 mg/L
Ribotype 027	Metronidazole	0.5 – 4mg/L	1-16 mg/L
	Vancomycin	0.5 – 2mg/L	0.5-2 mg/L
Ribotype 106	Metronidazole	0.25 – 4mg/L	0.25-4 mg/L
	Vancomycin	0.5 – 4mg/L	0.5-2 mg/L

Fifty percent of ribotype 001 CDRM strains showed heterogeneity in their susceptibility to metronidazole (Figure 3.1). While 75% of the *C. difficile* PCR ribotype 001 CDSM strains showed heterogeneity in their susceptibility to metronidazole. One clone of CDRM tested showed full resistance to metronidazole with an MIC of 32mg/L (Figure 3.1). Amongst the heterogeneous strains of ribotype 001, *C. difficile,* strain 114 (CDSM strain) showed the most heterogeneity, with a total of four MICs observed for the 16 CFU tested (range 0.5-4 mg/L). In

general, *C. difficile* strains that demonstrated heterogeneity in metronidazole MICs elicited two MICs, with the majority of isolates of a particular strain demonstrating one MIC.

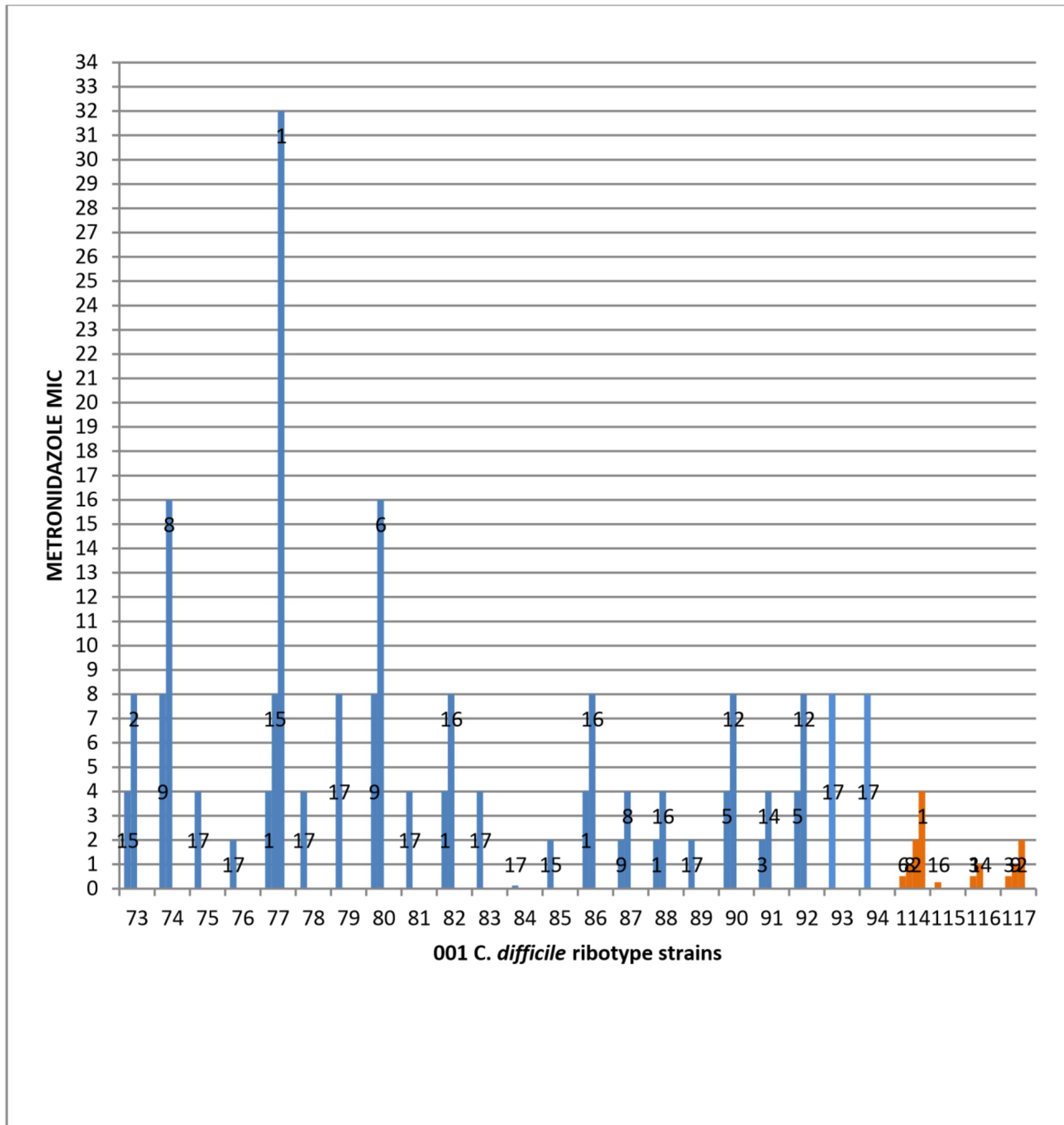

Figure 3. 1 Metronidazole MICs (mg/L) of ribotype 001 showing heterogeneity. Metronidazole reduced susceptible *C. difficile* strains (CDRM) (blue bars) and metronidazole susceptible *C. difficile* strains (CDSM) (orange bars) of individual clones/colony forming units determined using an agar incorporation MIC method. The numbers in the bars show the number of colonies having the MIC.

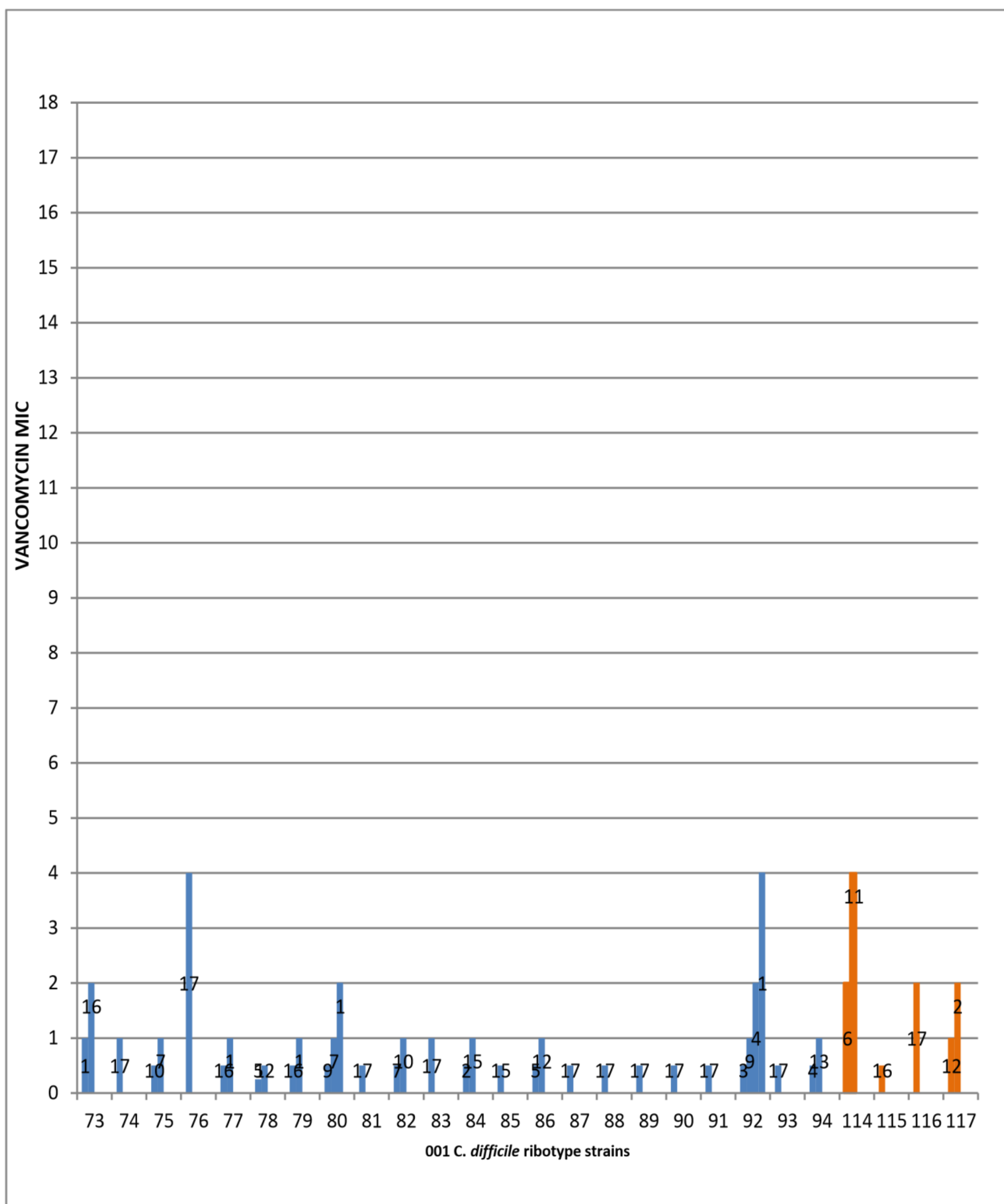

Figure 3. 2 Vancomycin MICs (mg/L) of ribotype 001 showing hetereogeneity. Metronidazole reduced susceptible *C. difficile* strains (CDRM) (blue bars) and metronidazole susceptible *C. difficile* strains (CDSM) (orange bars) of individual clones/colony forming units determined using an agar incorporation MIC method. The numbers in the bars show the number of colonies having the MIC value.

Forty five percent of CDRM 001 ribotype strains showed heterogeneity to vancomycin, while 50% of the susceptible strains also showed heterogeneity to vancomycin. Amongst the heterogeneous strains, MICs from one up to a maximum of four different MICs were observed for one isolate.

Four homogenous CDRM strains (strains 76, 84, 89 and 85), showed susceptibility to metronidazole, though earlier in chapter 2 all four strains showed reduced susceptibility to metronidazole at MIC ≥2mg/L with the exception of strain 84 MIC= 0.125mg/L for susceptibility testing. Strain 84 had the lowest metronidazole MIC of the 001 ribotype strains (Figure 3.1). Most strains were susceptible to vancomycin unlike metronidazole; only three strains had colonies that showed reduced susceptibility to vancomycin, i.e. 2 CDRM strains and 1 CDSM strain (76, 92 and 114 respectively). CDSM strain 114 had 11 colonies with reduced susceptibility to vancomycin. Strain76 a CDRM strain was homogeneous and showed reduced susceptibility to vancomycin but this strain was shown to be susceptible to metronidazole (Figure 3.2).

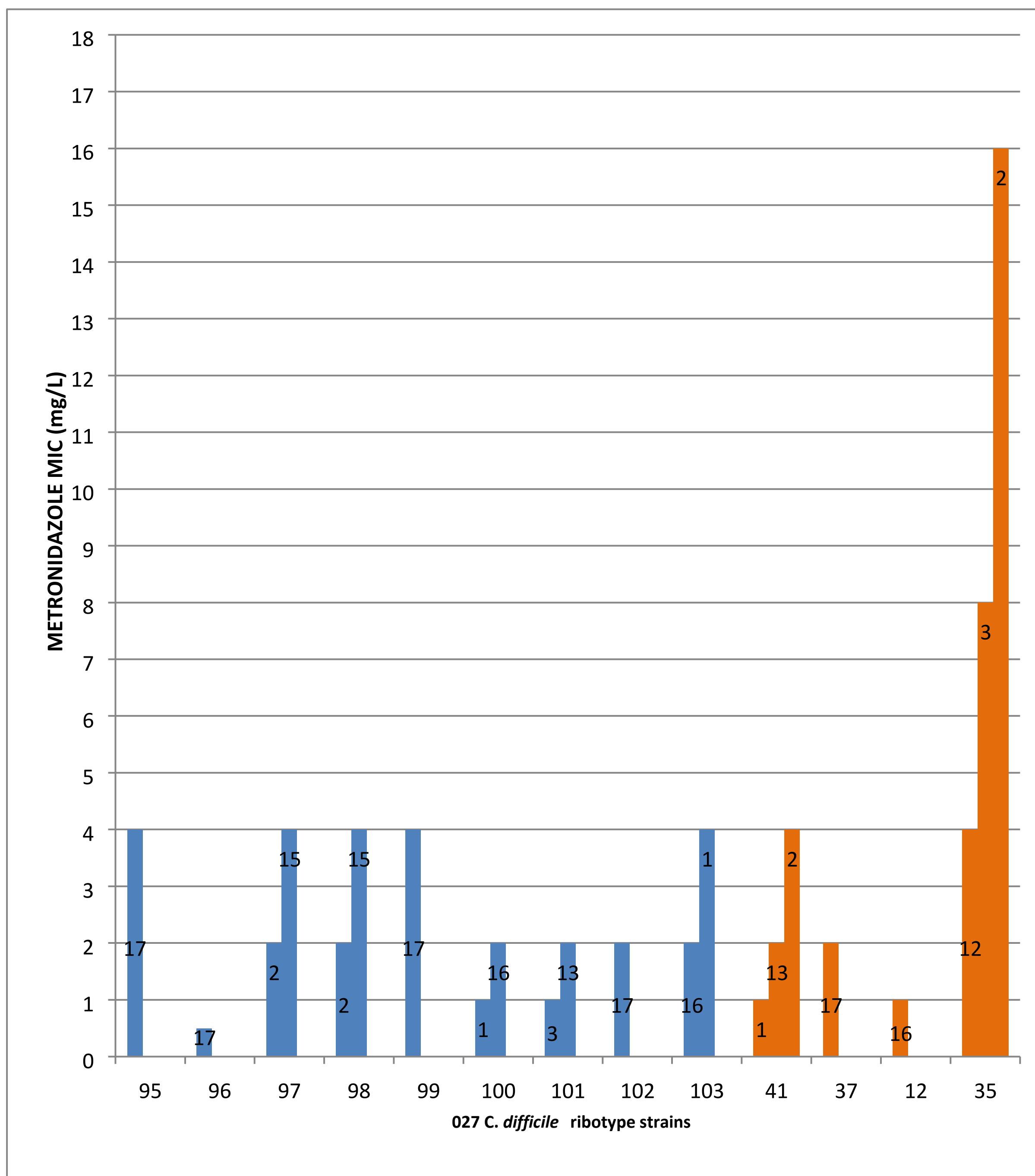

Figure 3. 3 Metronidazole MICs (mg/L) of ribotype 027 showing heterogeneity. Metronidazole reduced susceptible *C. difficile* strains (CDRM) (blue bars) and metronidazole susceptible *C. difficile* strains (CDSM) (orange bars) of individual clones/colony forming units determined using an agar incorporation MIC method. The numbers in the bars show the number of colonies having that MIC

Fifty six percent of 027 *C. difficile* ribotype CDRM strains showed heterogeneity in their susceptibility to metronidazole. While the CDSM strains compared with it had 50% heterogeneity in their susceptibility to metronidazole. A wider range of heterogeneity was observed amongst the CDSM strains, strain 41 (1-4mg/L) and strain 35 (4-16mg/L) than the CDRM strains.

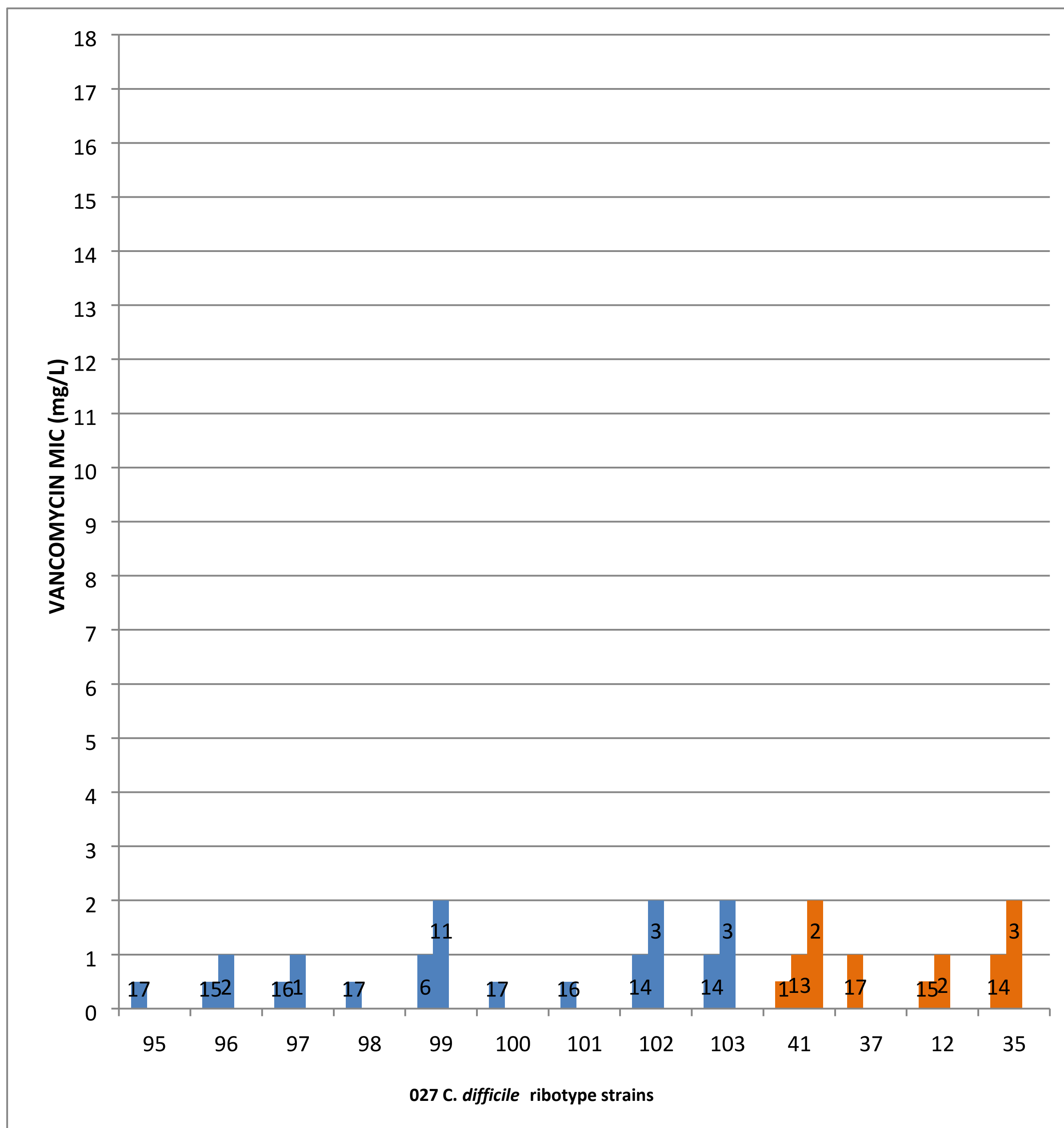

Figure 3. 4 Vancomycin MICs (mg/L) of ribotype 027 showing heterogeneity. Metronidazole susceptible *C. difficile* strains (CDRM) (blue bars) and metronidazole susceptible *C. difficile* strains (CDSM) (orange bars) of individual clones/colony forming units determined using an agar incorporation MIC method. The numbers in the bars show the number of colonies having that MIC.

Fifty six percent of ribotype 027 CDRM showed heterogeneity in their susceptibility to vancomycin. Correspondingly, CDSM strains demonstrated 75% heterogeneity in their susceptibility to vancomycin. Strain 41 (CDSM) was the most heterogeneous of all ribotype 027 strains tested (Figure 3.4).

Strains 98 & 102 were homogenous and susceptible to metronidazole. Strain 95 was homogenous but had reduced susceptibility to metronidazole MIC =4mg/L (Figure 3.3).

Heterogeneous CDRM strains 97, 98 and 103 had some susceptible colonies and others with reduced susceptibility to metronidazole (Figure 3.3). All strains both CDRM and CDSM showed susceptibility to vancomycin with MICs ≤2mg/L. The heterogeneity observed, showed distribution of most of the colonies were at 0.5mg/L or a dilution higher with the exception of strain 99 that had 11 colonies with MICs at 2mg/L (Figure 3.4). Strain 96 is equally susceptible to metronidazole and vancomycin. CDSM strains 41 and 37 had similar response in heterogeneity and susceptibility to both antibiotics with just one dilution difference.

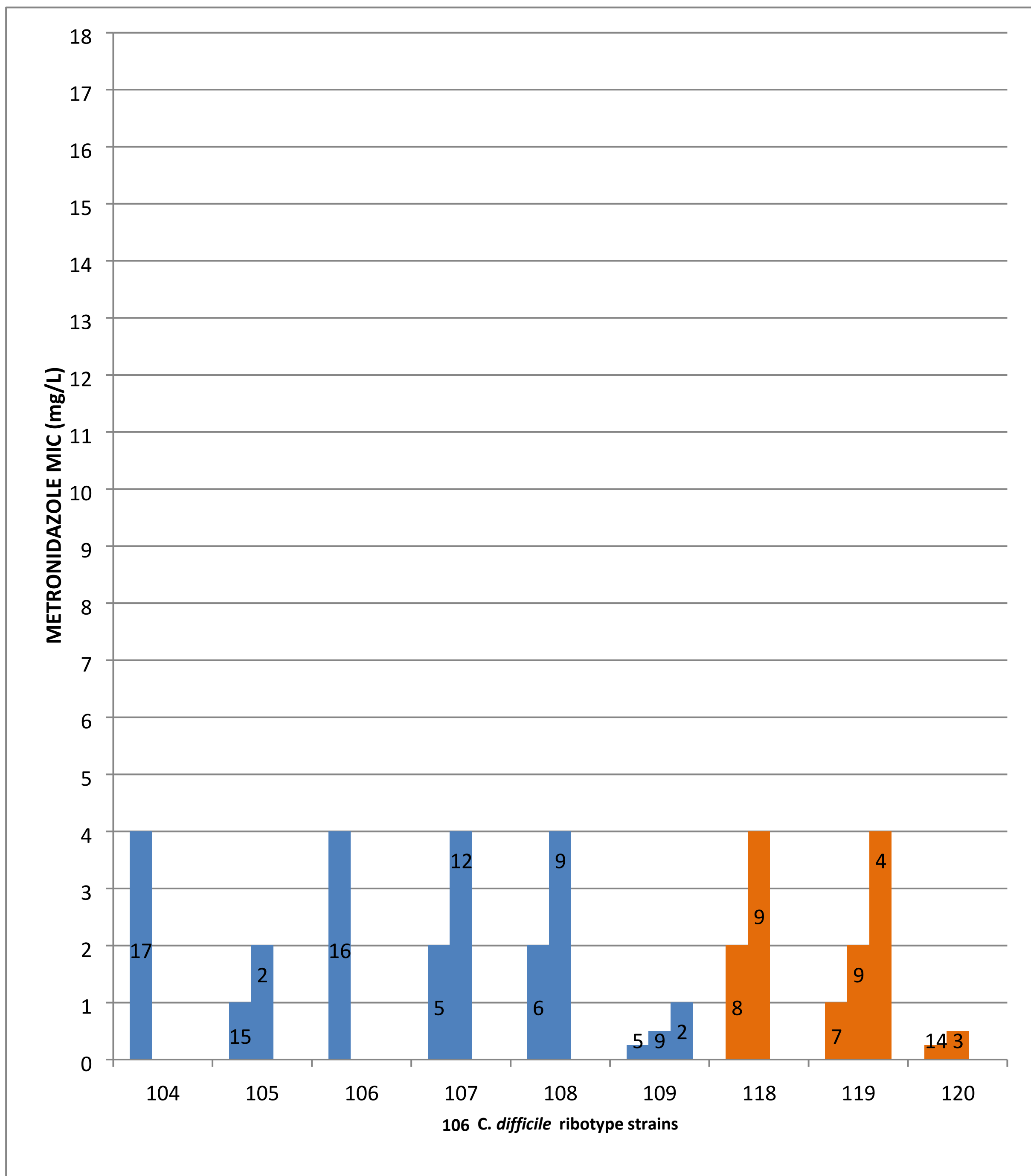

Figure 3. 5 Metronidazole MICs (mg/L) of ribotype 106 showing heterogeneity. Metronidazole reduced susceptible *C. difficile* strains (CDRM) (blue bars) and metronidazole susceptible *C. difficile* strains (CDSM) (orange bars) of individual clones/colony forming units determined using an agar incorporation MIC method. The numbers in the bars show the number of colonies having that MIC.

Sixty seven percent of the CDRM strains of *C. difficile* ribotype 106 showed heterogeneity to metronidazole compared with 100% heterogeneity observed in CDSM strains. *C. difficile* strain 109 (CDRM) and 119 (CDSM) were most heterogeneous in MICs to metronidazole.

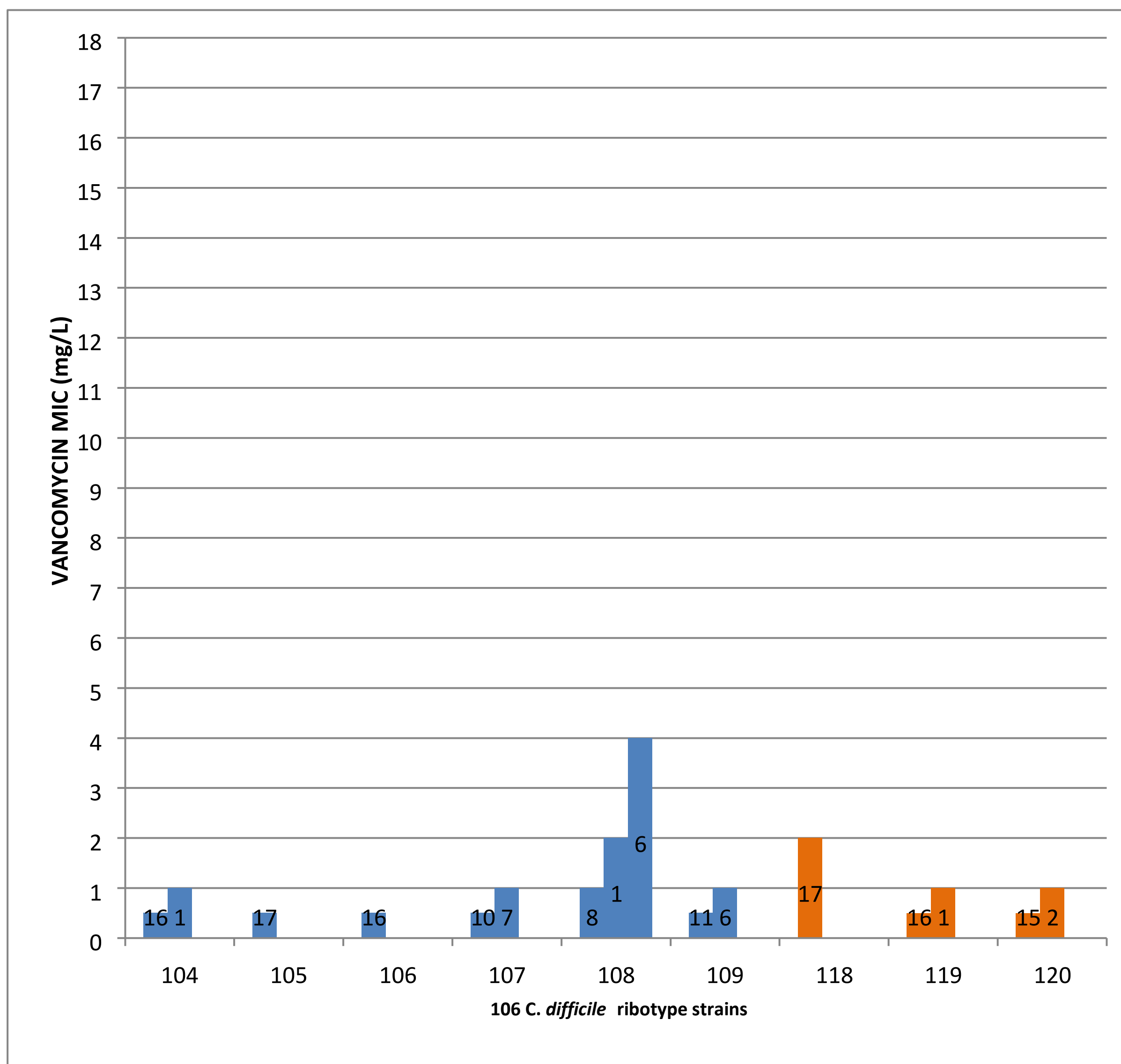

Figure 3. 6 Vancomycin MICs (mg/L) of ribotype 106 showing heterogeneity. Metronidazole reduced susceptible *C. difficile* strains (CDRM) (blue bars) and metronidazole susceptible *C. difficile* strains (CDSM) (orange bars) of individual clones/colony forming units determined using an agar incorporation MIC method. The numbers in the bars show the number of colonies having that MIC

Sixty seven percent of both CDRM and CDSM strains of ribotype 106 showed heterogeneity to vancomycin. *C. difficile* strain 108 (CDRM) was most heterogeneous to vancomycin and demonstrated three MIC values.

C. difficile ribotype 106 strains all demonstrated MICs ≤4mg/L to metronidazole. Two strains of purported CDRM ribotype 106 appeared as CDSM, with MICs of 0.5 mg/L (strains 105 & 109) showed susceptibility to metronidazole (Figure 3.5). Strain 118 & 119 of purported CDSM strains demonstrated colonies that showed reduced susceptibility to metronidazole. Strains 109 and 119 were the most heterogeneous to metronidazole of *C. difficile* ribotype 106 (Figure 3.5), with three MICs evident in these experiments.

All strains of ribotype 106 were susceptible to vancomycin with MICs ≤2mg/L, except strain 108, which demonstrated some of its colonies were with apparent reduced susceptibility to vancomycin (MICs 4mg/L). Strain 108 was also the most heterogeneous of the ribotype 106 strains to vancomycin.

Ribotype 106 was the most heterogeneous with regards to metronidazole MICs amongst the 3 ribotype groups tested in this study for CDRM strains alone with 67% heterogeneity. On considering the total CDRM and CDSM strains analyzed ribotype 106 was also the most heterogeneous of the 3 ribotypes with 78% heterogeneity.

Table 3.2 Number of *C. difficile* strains (CDRM and CDSM separated) heterogeneous in susceptibility to metronidazole and vancomycin

Ribotype	**Phenotype**	**Number of strains heterogeneous to metronidazole (%)**	**Number of strains heterogeneous to vancomycin (%)**	**Total number of strains analyzed**
001	CDRM	11 (50%)	11 (50%)	22
	CDSM	3 (75%)	2 (50%)	4
027	CDRM	5 (56%)	5 (56%)	9
	CDSM	2 (50%)	3 (75%)	4
106	CDRM	4 (67%)	4 (67%)	6
	CDSM	3 (100%)	2 (75%)	3

Table 3.3 Number of *C. difficile* strains (combination of CDRM and CDSM) heterogeneous in susceptibility to metronidazole and vancomycin.

Ribotypes	Number of strains heterogeneous to metronidazole (%)	Number of strains to Heterogeneous vancomycin (%)	Total number of strains analyzed
001	14(54)	13(50)	26
027	7(54)	8(62)	13
106	7(78)	6(67)	9
Total	28(58)	27(56)	48

The number of heterogeneous *C. difficile* strains to both antibiotics were almost same, 28 and 27 to metronidazole and vancomycin respectively (Table 3.3). Also for both antibiotics the number of heterogeneous strains compared to non-heterogeneous strains was more than 50%. Ribotype 106 has the highest level of heterogeneity with 78% and 67% heterogeneity to metronidazole and vancomycin respectively. It was observed in the hetereogeneity results obtained separately for CDRM only, that for all ribotypes, the same percentage of strains heterogenous to metronidazole was heteregenous to vancomycin (Table 3.2). Ribotype 106 was still the highest in hetereogeneity to metronidazole when CDSM and CDRM were analysed separately (Table 3.2).

Table 3.4 Total number of strains analysed (including CDSM strains) in this research highlighting those with heterogeneity MIC distribution across two MICs or more in their susceptibility to metronidazole and vancomycin.

Ribotypes	**Number of strains heterogeneous to metronidazole (%)**	**Number of strains heterogeneous to vancomycin (%)**	**Total number of strains analysed**
001	3(12)	2 (8)	26
027	2 (15)	1 (8)	13
106	2 (22)	1 (11)	9
Total	7 (15)	4 (8)	48

The percentage number of strains with heterogeneity result of MIC distribution across two MICs or more, was ≤ 15% for both antibiotics, with ribotype 106 demonstrating the highest percentage of heterogeneous isolates, with 22% and 11% of isolates being heterogeneous for metronidazole and vancomycin respectively.

3.4 DISCUSSION

The presence of reduced susceptible clones amongst CDSM strains at MIC≥4mg/L as observed in this hetereogeneity experiment shows the possibility of it reverting into a CDRM strain. In comparism with the susceptibility range obtained in chapter 2, ribotype 027 and 106 reflected that there were more susceptible colonies as observed in the heterogeneity assay within the CDRM strains. But CDRM ribotype 001 showed clones with metronidazole reduced susceptibility higher than the range obtained in chapter 2. Though after individual strain analysis it was observed that the 2 fold increase in range was from 3 CDRM strains not necessarily the bulk of the strains analysed for ribotype 001.

The range of MIC observed per ribotype, due to the heterogeneity of the colonies taken from strains tested to metronidazole and vancomycin was moderate, mostly spanning between two to five MIC's however the widest was observed in ribotype 001 to metronidazole from 0.125 - 32mg/L (CDRM). Amongst the 3 ribotypes CDRM strains tested, *C. difficile* ribotype 106 had the highest heterogeneity to metronidazole and vancomycin. While amongst the CDSM strains ribotype 106 also showed a higher heterogeneity to vancomycin than that observed in ribotypes 027 and 001.

Susceptible strains for *C. difficile* ribotype 106 showed a higher degree of heterogeneity to metronidazole and vancomycin compared to the CDRM strains. There was a similar observation in the *C. difficile* ribotype 001 group to metronidazole. *C. difficile* ribotype 027 CDRM strains showed a higher heterogeneity to metronidazole compared to susceptible strains but the reverse was observed to vancomycin. Therefore, the heterogeneity rates of CDRM versus CDSM was different with different ribotypes and antibiotics.

As was mentioned above, an average of 16 colonies for a total of 48 strains (CDRM and CDSM strains) were tested for heterogeneity in MICs to metronidazole and vancomycin. On analysing the total number of strains in this research irrespective of ribotypes, it was showed that with an almost negligible difference the number of heterogeneous strains were more with metronidazole than vancomycin.

The heterogeneity in metronidazole MICs observed among the CDSM strains, explained earlier was not reflective of the data from an earlier report that reported susceptible *C. difficile* strains were homogeneous to metronidazole (Peleaz *et al.,* 2008). However, the ribotype(s) evaluated in those experiments were not stated, so it could be that different ribotypes were studied (Peláez *et al*., 2008). In the present study, the heterogeneity in MICs of ribotype 001 strains corresponded with the observations of a previously published study, where three strains of

ribotype 001 were analysed with agar incorporation and agar dilution methods, and on all four strains showed heterogeneity in metronidazole MICs and elicited between two to five MICs (Moura *et al*., 2012).

The summary of MIC results for all strains in this study for both antibiotics indicated that the number of heterogeneous strains compared to non-heterogeneous strains was more than 50%. This is concerning and could contribute to uncertainty in the analysis and interpretation of clinical MIC data, if scientists do not select a mixture of colony forming units to test; experimentation involving just one colony would not represent the whole population (Weel *et al.,* 1996). It could also be a concern clinically as a decrease in the efficiency of administered antibiotics may also be due to heterogeneity in the offending microbial population (Moura *et al.,* 2012). Under a metronidazole selection pressure in the human colon, given the low faecal concentrations of metronidazole that may be observed in humans following oral dosing it may be that a proportion of the *C. difficile* population is not inhibited by metronidazole. For example, faecal concentrations of metronidazole have been demonstrated to be on average 9.3 mg/L (Bolton & Culshaw, 1986), therefore some CDRM clones in the present study would have been inhibited, whereas others from the same strain would not have been inhibited; allowing potential clonal expansion while the selection pressure existed and compromising treatment. Given the much higher concentrations of vancomycin observed in faecal contents following oral administration, i.e. 1350 mg/L (Abujamel *et al,* 2013), any heterogeneity in MICs would likely be insignificant in its effect on *C. difficile* infection (CDI) treatment with the drug, since MICs up to the maximum observed in this study would be several hundred times lower than the faecal concentration. Most of the heterogeneity observed in the present study may not be of great impact since the accepted error in agar incorporation MIC methods is one doubling dilution, but the notable heterogeneity observed in this study was ≤ 15% of the total number of strains analysed, as the hetereogeneity was ≥2 doubling dilutions.

Researchers should be aware that heterogeneity in MICs can be observed readily in *C. difficile* with respect to both vancomycin and metronidazole across a range of PCR ribotypes. Several colony forming units must be inoculated into antimicrobial susceptibility tests in order that a full assessment of susceptibilities can be made. Although only vancomycin and metronidazole were evaluated in the present study, it seems probable that for other antimicrobials, whether treatment agents for CDI or antimicrobials that may initiate CDI in susceptible demographic groups, heterogeneity in MICs will be observed which could potentially have implications for treatment or induction of CDI.

Heterogeneity in the MIC of frontline antibiotics for CDI therapy as observed in this research may also pose clinical problems and this research maybe reflection of the ungoing status of CDI in hospitals as these strains are clinical isolates. The presence of heteroresistance in *C. difficile* has been earlier linked to therapy failure (Peleaz *et al,* 2008), which emphasizes the need for continuous surveillance

Further research is warranted to gain a fuller understanding of the response of *C. difficile* to antimicrobial agents both in the absence and presence of an antimicrobial agent selection pressure.

4.0 Population analysis profiling

4.1 INTRODUCTION

Population analysis profiling (PAP) is a method used to determine resistant features of a microbial population (Berger-Bachi *et al*, 1986). PAP has also been used to detect three strains of *S. aureus*, resistant to methicillin (MRSA), determining its level of susceptibility to vancomycin (Hiramatsu *et al,* 1997). Though PAP has been used as a reference method in past experiments particularly in detection of vancomycin resistance in *S. aureus,* it has also been described in literature to be a labour intensive procedure (Satola *et al*, 2010). PAP was previously used to identify vancomycin hetero-resistant *S. aureus* (HVRSA) and vancomycin resistant *S. aureus* (VRSA) amongst 100 methicillin resistant *S. aureus* (MRSA) collected over a period of 16 years, in an effort to determine the prevalence of HVRSA and VRSA in clinical centres in the United Kingdom (Wootton *et al*, 2000). PAP, Hiramatsu's screening method, an agar incorporation method (AIM) and antibiotic gradient plates were methods employed in the study done by Wootton and colleagues (2000). Though all methods had varying results, PAP was reported to accurately identify HVRSA in *S. aureus* strain MU3 and none in the hundred MRSA analysed (Wootton *et al*, 2000). Additionally, it was also previously reported in an experiment to evaluate the specificity and sensitivity of seven methods, that PAP was used as a reference method to determine the effectiveness of these seven methods used to detect *S .aureus* strains with reduced susceptibilities to vancomycin (SRSV) (Walsh *et al,* 2001). Furthermore, there have been reports on anaerobic organisms like *B. fragilis* where PAP was used to determine heterogeneity in resistance to cefoxitin (Soki *et al*, 2011). This method was also used to determine the colony forming units (CFU) of *B. fragilis* and *E. cloacae* in relation to antibiotic MIC's, on exposure to different concentrations of piperacillin-tazobactam, ceftizoime and piperacillin, in an experiment to detect the resistant strains and antibacterial activity of the samples (Stearne *et al,* 2004). However, there are no published reports of PAP use with *C. difficile* in relation to either therapeutic agents used to treat *C. difficile* infection (CDI) or antimicrobials with a propensity to induce CDI.

4.11 Aims

To perform population analysis profiling of *C. difficile* for vancomycin and metronidazole.

4.12 Objective

To use agar incorporation of antimicrobial agents alongside total viable counting of metronidazole reduced susceptible *C. difficile* (CDRM) and metronidazole susceptible *C. difficile* (CDSM) strains to inform on the susceptibility of *C. difficile* to metronidazole and vancomycin at the population level.

4.2 METHOD

4.21 *C. difficile* isolates

Two CDRM strains and one CDSM strain was selected from UK isolates of ribotype 001, 106 and 027 along with a control 110 strain of ribotype 010 for this experiment. The CDRM strains selected had metronidazole MIC in antimicrobial susceptibility test/ heterogeneity test ≥4mg/L. Except strain 95 after the heterogeneity test was homogenously 4mg/L.

Table 4.1 Selected strains based on metronidazole susceptibility profile and their respective MICs detected in chapter 2, to be used for PAP test

RIBOTYPE	**STRAIN**	**MIC (mg/L)**
001 CDRM	80	8
	86	8
001 CDSM	114	0.5
027 CDRM	95	2
	99	4
027 CDSM	12	1
106 CDRM	108	4
	109	4
106 CDSM	120	0.125

4.22 Population analysis profiling (PAP) test

Each of the selected *C. difficile* strains was cultured on Braziers agar (LAB160, Neogen Europe Ltd, Ayr, UK) plate supplemented with 2% (v/v) lysed horse blood (SR0050, Oxoid, Basingstoke, UK) to grow overnight in the anaerobic cabinet (N_2:H_2:CO_2, 80:10:10) (Don Whitley Scientific, UK). A sterile swab was then used to transfer the *C. difficile* colonies from the agar plate to a 7ml Schaedler's anaerobe broth (CM0497, Oxoid, Basingstoke, UK) and anaerobically incubated overnight. The OD_{600} of the overnight cultures was determined and used to create standardised inocula of OD_{600} of 0.1 in Schaedler's anaerobe broth in duplicate. The *C. difficile* cultures were then allowed to grow until reaching the required start optical density (OD) (detected from a preliminary experiment that was done as an effort towards starting all strains at a similar range of CFU count). Then serial 10-fold dilutions of *C. difficile* cultures in peptone water (CM0009, Oxoid, Basingstoke, UK) to 10^{-7} were performed, then each dilution (20µL) was plated in duplicate onto Brazier's agar containing antibiotics (metronidazole and vancomycin (Sigma-Aldrich, UK)) in doubling concentrations from 0.03–8mg/L alongside control plates without antibiotics. Colony counts were recorded after anaerobic incubation (24hrs, 37°C). Total viable counts were determined from the colony counts, where ideally 20-200 colony forming units (cfu) were counted and colony counts were multiplied by the appropriate dilution factor and expressed as log_{10}-cfu/ml, this value was deducted from the control (without antibiotics) log_{10}cfu/mL. The decline obtained was incorporated in a graph, plotted against the respective antibiotic concentrations. This PAP test was designed following experiment by Wootton et al (2000). The area under the curve (AUC) was calculated for *C. difficile* strains in order to assess the population susceptibility to antimicrobials using Graphpad prism software (GraphPad Software, Inc., La Jolla, CA, USA).

4.3 RESULTS

CDRM strains 80 and 86 of ribotype 001 were largely unaffected by all concentrations of metronidazole used in this study and viable counts remained constant up until 8mg/L of drug, despite prior MICs of 8mg/L being observed. As shown in figure 4.1 the population decline of CDRM strains of ribotype 001 when exposed to antibiotics, from the control plate without antibiotic exposure, was negligible. Conversely, CDSM strain 114 viable counts had a rapid decline at 1mg/L of metronidazole (Figure 4.1).

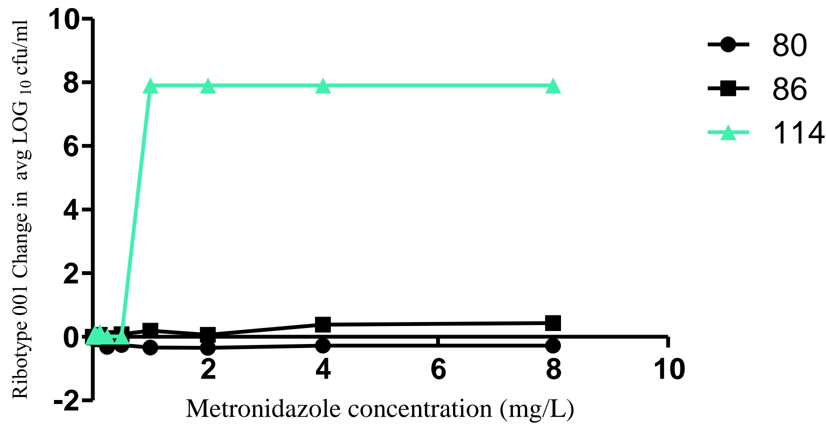

Figure 4. 1 Population analysis profiling (PAP) of two CDRM (80 and 86) and one CDSM (114) ribotype 001 strains using doubling concentrations of metronidazole (mg/L) in Oxoid Wilkin'sChalgren agar.

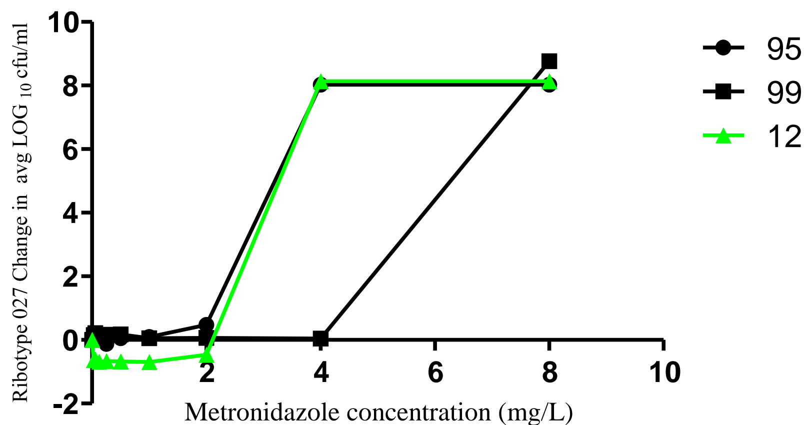

Figure 4. 2 Population analysis profiling (PAP) of two CDRM (95 and 99) and one CDSM (12) ribotype 027 strains using doubling concentrations of metronidazole (mg/L) in Oxoid Wilkin's Chalgren agar.

Ribotype 027 viable counts of CDSM strain 12 and CDRM strain 95 showed a rapid viable population decline only after exposure to 2mg/L. Viable counts of CDRM strain 99 were unchanged until exposure to 4 mg/L metronidazole (Figure 4.2)

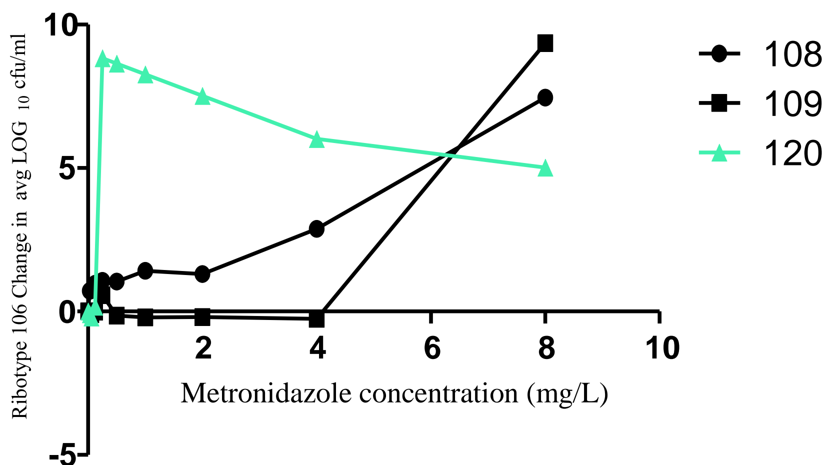

Figure 4. 3 Population analysis profiling (PAP) of two CDRM (108 and 109) and one CDSM (120) ribotype 106 strains using doubling concentrations of metronidazole (mg/L) in Oxoid Wilkin'sChalgren agar.

Rapid decline in viable counts of CDRM strain 108 was not observed until 2mg/L metronidazole CDRM strain 109 was inhibited at one dilution higher of metronidazole. Interestingly, CDSM strain was inhibited by a very low concentration of metronidazole (0.25mg/L), but a pronounced reduction in antimicrobial activity of metronidazole was observed at a higher concentration (Figure 4.3).

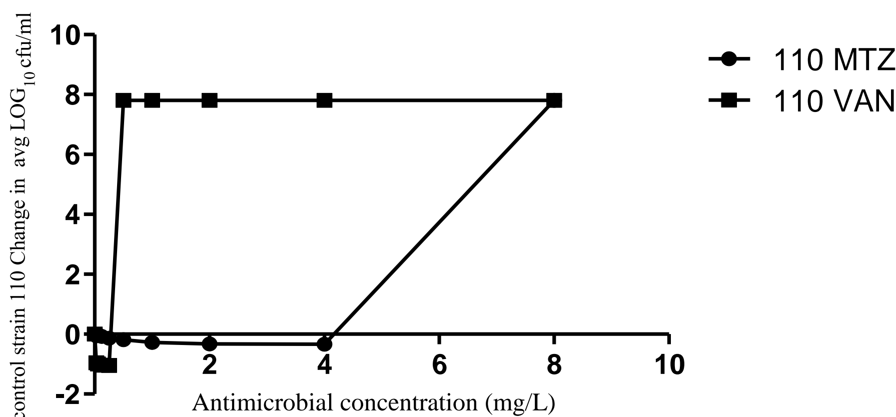

Figure 4. 4 Population analysis profiling (PAP) of *C. difficile* control strain E4 (ribotype 010) strains using doubling concentrations of metronidazole and vancomycin (mg/L) in Oxoid Wilkin's Chalgren agar.

Control *C. difficile* strain E4 coded as 110 (Figure 4.4) was inhibited by 8mg/L metronidazole and viable counts were constant at lower concentrations of the drug. For vancomycin, *C. difficile* strain E4 was inhibited to below the limit of detection by 0.5mg/L. The AUC for metronidazole and vancomycin were calculated to be 16.14 and 59.69 respectively for this *C. difficile* strain.

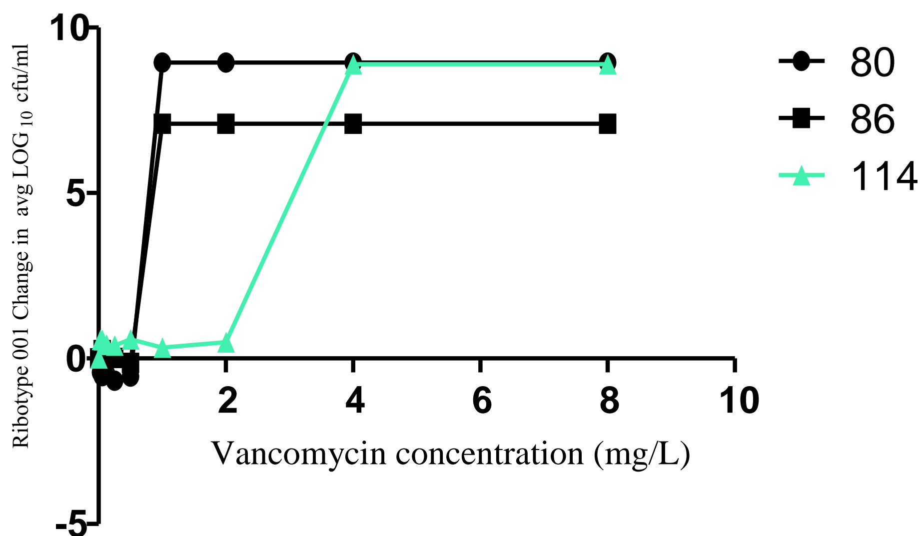

Figure 4. 5 Population analysis profiling (PAP) of two CDRM (80 and 86) and one CDSM (114) ribotype 001 strains using doubling concentrations of vancomycin (mg/L) in Oxoid Wilkin's Chalgren agar.

Ribotype 001 CDRM strains 80 and 86 behaved similarly in this experiment with vancomycin and were both inhibited to below the limit of detection at 1mg/L of drug (Figure 4.5). CDSM strain 114 appeared less susceptible to vancomycin and required exposure to 4mg/L of drug before viable counts declined below the limits of detection.

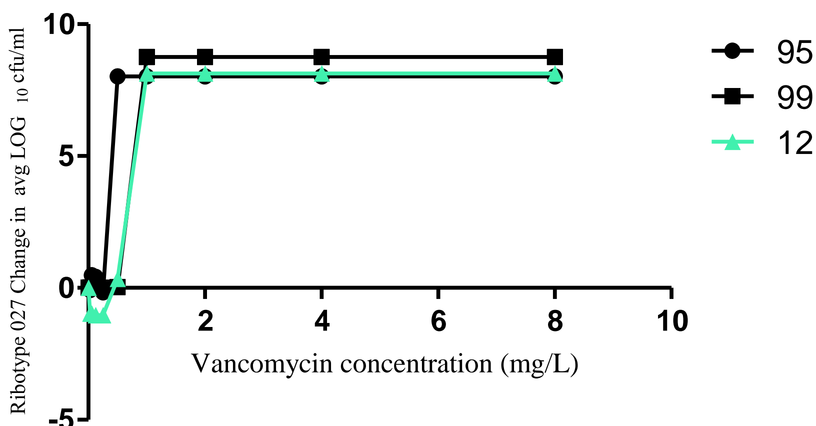

Figure 4. 6 Population analysis profiling (PAP) of two CDRM (95 and 99) and one CDSM (12) ribotype 027 strains using doubling concentrations of vancomycin (mg/L) in Oxoid Wilkin'sChalgren agar.

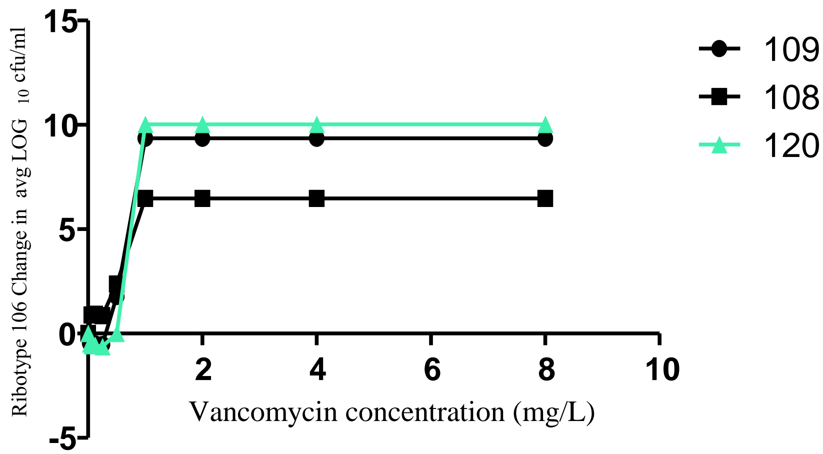

Figure 4. 7 Population analysis profiling (PAP) of two CDRM (108 and 109) and one CDSM (120) ribotype 106 strains using doubling concentrations of vancomycin (mg/L) in Oxoid Wilkin'sChalgren agar.

All the three strains tested of ribotypes 027 and 106 were inhibited by low concentrations of vancomycin and viable counts were below the limit of detection following exposure to 1mg/L vancomycin for all ribotype 027 and 106 strains (Figure 4.6 and Figure 4.7)

Table 4. 2 Area under the curve (AUC) data for the 3 *C. difficile* ribotypes analysed for PAP test, for metronidazole and vancomycin

Ribotypes	**strains**	**Metronidazole**	**Vancomycin**
001	80	2.32	65.04
	86	2.32	51.41
	114 (CDSM)	57.37	45.86
027	95	40.93	61.19
	99	17.93	63.53
	12(CDSM)	41.48	59.37
RT106	108	27.33	68.53
	109	19.06	48.18
	120 (CDSM)	50.50	72.86

The AUC data was directly proportional to susceptibility of the organism thus increased AUC equals increase in susceptibility to the inciting antibiotics (Table 4.2) (Note: this is as a result of the graph used to detect the AUC Fig 4.1-4.7). CDSM strains were observed to have higher AUC compared to CDRM strains when exposed to metronidazole (Table 4.2). All strains had higher AUC on exposure to vancomycin, however ribotype 001 and 027 CDSM strains had lower AUC compared to CDRM strains of the same ribotypes on exposure to vancomycin. The lowest AUC was observed in ribotype 001 CDRM strains (Table 4.2)

4.4 DISCUSSION

Heteroresistance is of unknown significance in CDI in terms of its contribution to the efficacy of treatments and also the induction of CDI, but for *S. aureus,* heteroresistant methicillin resistant *S. aureus* (MRSA) are a complication for treating infections. This study was carried out to examine in greater depth the effects of metronidazole and vancomycin on CDRM and CDSM of UK origin using a PAP method which has been employed previously as a reference method in detecting antimicrobial heteroresistance in bacteria. Due to the laborious nature of PAP methods, only a selection of *C. difficile* strains were analysed, i.e two CDRM and one CDSM per ribotype, in addition to a control strain (E4, CDRM coded as 110).

It is important to note that the viable counts of the cultures used in the present studies were more variable than those that were used in standard MIC testing experiments (see 2.22). This is due to the nature of PAP and the fact that mid-log phase cultures are preferable for these experiments in comparison to the overnight cultures used in standard MIC testing which are likely to be late-log phase or early stationary phase. Also, there is substantial variability between the viable counts that result from different *C. difficile* cultures of different strains with the same apparent OD^{600} (data not shown). Despite these experimental challenges, the present study results are interesting and are additive to the data generated in previous chapters. *C. difficile* strain E4 which was used as the control in the present study (ribotype 010) was inhibited in the present study at 8mg/L metronidazole, which reflected the expected MIC range of 4-16 mg/L for this strain. Furthermore, the data generated following exposure to vancomycin also correlated with the expected MIC to this drug of 0.5mg/L.

All ribotypes with the exception of strain 114 of ribotype 001 showed a higher susceptibility to vancomycin than metronidazole in this PAP study. Strain 114 amongst all the susceptible strains showed reduced susceptibility to vancomycin in this PAP study. Though the susceptibility test in chapter 1 showed an MIC of 2mg/L, the reduced susceptible phenotype observed in this PAP test has earlier been detected in the hetereogeneity test, where 11 clones had a vancomycin MIC of 4mg/L of the 17 clones, of strain 114 analysed. The mechanism of action of vancomycin targets the peptidoglycan precursors during cell wall synthesis and previous research of *Staphylococcus* strains resistant to vancomycin identified that the reason could be the hardening of the cell wall (Hiramatsu *et al*., 1997). There are no published explanations for reduced susceptibility to vancomycin in *C. difficile*, and this phenomenon has been reported in previous studies (Freeman *et al.,* 2015). This PAP test showed that ribotype 001 CDRM strains were reduced susceptible to metronidazole and increases in metronidazole concentrations up to 8mg/L had little or no effect on the viable counts of the CDRM strains tested. The maximum concentration of 8mg/L was selected based on the previous MIC data

generated for the strains in this PAP study from Chapter 2. All ribotype 027 strains showed reduced susceptibility to metronidazole including the CDSM which was expected to be susceptible to metronidazole. The reason for this CDSM strain persisting in the presence of metronidazole at concentrations higher than the apparent MIC is interesting and may be explained by a slightly higher inoculum used in the present study, or perhaps it may be evidence that persistence/tolerance may be possible for *C. difficile* strains. The CDRM strains of 106 like the other 2 ribotypes were not susceptible to metronidazole however decline in CFU for strain 109 and 108 started on exposure to a metronidazole concentration of more than 2mg/L and 4mg/L respectively. Though it has been mentioned repeatedly in literature that PAP is one of the most reliable of the susceptibility test available, there has been no mention of PAP with relation to *C. difficile* in earlier research.

Ribotype 106, strain 109 that had reduced susceptibility in antimicrobial test and was observed to be susceptible in heterogeneity test was however confirmed to be reduced susceptible to metronidazole in PAP. While strain 108 of ribotype 106 strain showed reduced susceptibility to metronidazole in the three methods however the heterogeneity test revealed that its colonies were a mix of susceptible colonies and colonies with reduced susceptibility to metronidazole such cases could have adverse therapeutic implications.

The reliability of PAP as seen in this research confirmed earlier works which also observed susceptibility to vancomycin amongst 330 *C. difficile* strains (Pituch *et al.,* 2011). The susceptibility in 001 ribotype to vancomycin has also been observed in earlier research using agar incorporation and Etest (Baines *et al.,* 2008).

The AUC data reflected the results obtained in the figures in more detail. The lowest AUC was observed in ribotype 001 CDRM strains which depicted a high level of reduced susceptibility in comparison with the other ribotypes this was observed in chapter 2 where 001 ribotype was observed to have the highest level of reduced susceptibility. The AUC also observed the susceptibility of CDSM strains to metronidazole in comparison with CDRM strains. As well as the susceptibility of all strains to vancomycin in a higher level compared to metronidazole (except CDSM strain 114 as previously described) thus the population analysis profile confirms susceptibility results obtained in chapter 2 and 3.
The reliability of the method used was also observed in ribotype 001 and 106 with the reduced susceptibility seen in strain 12 showing susceptibility at 4mg/L and rebounce of strain 120, both strains earlier observed to be susceptible to metronidazole. The rebounce of strain 120 could be as a result of presence of subpopulations with reduced susceptibility to metronidazole

which was selected and able to undergo clonal expansion due to the clearance of majority of strain 120 populations susceptible to metronidazole (Baines *et al,* 2008).

Conclusion and future work

This PAP test confirmed most of the susceptibility profile observed in chapter 2 and 3. The detection of the AUC of a standard *C. difficile* heterogeneous strain, would have aided more information, to be obtained with respect to heterogeneity. This could be a further work as the PAP test would not just confirm susceptibility levels but also reflect heterogeneity levels.

5.0 Stability and mutability of *C. difficile* with reduced susceptibility to metronidazole

5.1 INTRODUCTION

In the clinical environment, bacteria exposed to antimicrobial agents are exerted to a selection pressure, which depending on the antimicrobial agent, its concentration and mechanism of action, may facilitate the development of single nucleotide polymorphisms (SNPs) within the genome of the bacterium.

Serial passage experiments in the presence and absence of antimicrobial agent selective pressures are employed mainly in research settings to assess the stability and mutability of microorganisms that are of wild-type, or mutated genotypes/phenotypes. Cell expansion is required to generate sufficient results from laboratory experiments such as antibiotics susceptibility and resistance, growth rates studies etc, hence the practice of serial passage (Stothard, 1998). Though not all diagnosis require cell expansion, these information generated from laboratory experiments might be applied clinically which could be affected by lack of monitoring the serial passage procedure.

Serial passage studies involve the sequential repetition of cell expansion without constant contact with the original cells and the cells grown in each step are a source of inoculum for the next step (Leeds *et al.,* 2013). Serial passage experiments with antimicrobial agents can be carried out with elevated drug concentration (Leeds *et al.,* 2013).

Though a useful technique serial passage has been observed in several cases to result to or select for mutant cell strains (Gilbert *et al.,* 2001) irrespective of cell type. However, the development of mutants is strain dependent and depends on the condition of growth (Gilbert *et al.,* 2001).

5.1.2 Development of resistant mutant microorganisms on continuous exposure to antibiotics.

Serial passage experiments have been used to study the likelihood and stability of developing antimicrobial resistance in a range of pathogens. Gilbert and colleagues (2001) studied 123 microbes including *Serratia marcescens*, *Pseudomonas aeruginosa*, methicillin susceptible and methicillin resistant strains *Staphylococcus aureus*, *Enterobacter cloacae* and *Escherichia coli* were exposed to three fluoroquinolones and serially passaged 10 times. Before the serial passage, the minimum inhibitory concentration (MIC) that inhibited 50% of the *C. difficile* strains studied (MIC_{50}) for all microbes analysed and for all fluoroquinolones was within the range 0.004 – 0.5µg/ml, after serial passaging the MIC_{50} was within the range 0.25-256 µg/ml. Though there was variation in the onset of resistant bacteria with respect to the bacteria and

the drug used, the research deduced that the use of serial passage could select for bacteria resistant to fluoroquinolone (Gilbert *et al.,* 2001).
Furthermore, Drago *et al.* (2005) carried out a research working with nosocomial isolates of *Acinetobacter spp* and *Pseudomonas aeruginosa* exposed to 2 fluoroquinolones individually as well as in combination with amikacin and four β-lactams. Five serial passages were performed individually with the seven antimicrobials used and then five serial passages with a combination of antimicrobials (Drago *et al.,* 2005). It was observed that after the first passage, all strains of *P. aeruginosa* had significantly elevated (minimum inhibitory concentrations) MICs, when passaged with individual antibiotics. All strains that were initially susceptible to imipenem and amikacin became resistant after the first passage, and most *P. aeruginosa* strains had become resistant after the fifth passage (Drago *et al.,* 2005).

After the first serial passage involving the addition of amikacin or β lactams to fluoroquinolones, there was a fourfold increase in MIC for all except one antibiotic combination in at least one strain. Also onset of resistant *P. aeruginosa* strains were greatly reduced even after the fifth passage compared to the single antibiotics administration, where ≥ 3 strains developed resistance for each antibiotic tested, except for imipenem that had no resistant strain (Drago *et al.,* 2005).
Acinetobacter was more susceptible to the single antibiotics than *P. aeruginosa* however resistant strain was observed after the final passage to all antibiotics except imipenem. Significant elevated MIC and resistant strains was also observed after last serial passage when serial passage was done with combinations of antibiotics but at a reduced rate compared to single antibiotics (Drago *et al.,* 2005).

Interestingly serial passage has also been observed to affect metabolic activity in bacteria, as reported in a six week serial passage study of *Staphylococcus aureus*. Production of the citric acid cycle enzyme aconitase, which has previously been observed to affect the production of virulence factors in *S. aureus,* was assessed. A decrease in aconitase activity was observed after the first week of serial passage experiments compared to parent strain and a 38% ($P<0.001$) decrease in aconitase activity was then observed after the sixth week. Conversely, a 12% increase in growth yield was observed in *S. aureus* derived from the sixth week of serial passage experiments (Somerville *et al.,* 2001). Therefore, these data suggest that it is important to consider the physiological changes that may occur in bacteria during serial passage experiments, independent of exposure to sub-inhibitory concentrations of antimicrobial agents,

and that these changes may indirectly affect a microorganism to influence growth and/or antimicrobial susceptibilities.

5.1.3 Development of resistant anaerobes on exposure to antibiotics

Many strains of anaerobic bacteria have been assessed for their propensity to mutate following exposure to sub-inhibitory concentrations of metronidazole. Larson and Fiehn (1997) studied metronidazole resistance development in strains of: *Actinobacillus, Fusobacteroium, Peptostreptococcus, Porphyromonas,* and *Prevotella* and these researchers detected resistance development to metronidazole (Larsen and Fiehn, 1997). MICs of most strains for metronidazole were ≤ 0.5mg/L, whereas serial passage experiments in the presence of sub-inhibitory concentrations of metronidazole increased metronidazole MICs 1-32 fold (Larsen and Fiehn, 1997). Serial passage in the absence of antimicrobial agents, and therefore in the absence of a selection pressure, may result in MICs declining and a reversion of a wildtype genotype/phenotype. Gal and Brazier (2004) reported that a decrease in MIC was observed in several metronidazole-resistant *Bacteriodes fragilis* strains, serially passaged seven times in the absence of metronidazole (Gal and Brazier 2004). Conversely, in a study of a fluoroquinolone-resistant *Bacteriodes fragilis* strain, the strain mutated following exposure to the fluoroquinolone sparfloxacin. Serial passage for 10 days and monitoring of MICs using broth microdilution, demonstrated stability in the resistant phenotype (Peterson *et al.,* 1999). Therefore, the likelihood of resistance development following serial passage experiments and the stability of the resistance phenotype/genotype may be species and/or antimicrobial agent dependent.

5.1. 4 Serial passage experiments with *Clostridium difficile*

Serial passage studies with *Clostridium difficile* have been performed previously, and have predominantly examined novel or existing antimicrobial agents for the treatment of CDI. Leeds and colleagues (2013) performed ten serial passages on four *C. difficile* strains, strains (ATCC43255, and 3 other clinical isolates REA type AA, REA type J, and an untyped isolate) using three antimicrobial agents to provide a selective pressure, i.e. vancomycin, LFF571 (a novel therapeutic in development), and fidaxomicin. Two of the strains showed ≥ 8-fold decline in susceptibility to vancomycin and fidaxomicin at different points during the serial passage experimental time series. REA type AA demonstrated less than a 2-fold change in MIC to fidaxomicin and vancomycin, whereas REA type J susceptibility to fidaxomicin after 10

passages yielded a 4-fold shift. These susceptibility changes were however not observed to the novel antimicrobial agent LFF571 after employing serial passage technique. Whole genome sequencing was performed and mutations in strains with reduced susceptible phenotypes were analysed. Change in susceptibilities was linked with many pathways including mutations in the proteins MarR (fidaxomicin-exposed *C. difficile*) and MurG (vancomycin), which are implicated in the activation of antibiotic resistance/oxidative stress responses and peptidoglycan precursor transfer during cell wall synthesis in bacteria. The authors of this study concluded that continuous serial passage overtime predisposes to genetic mutation (Leeds *et al.,* 2013).

Surotomycin is an orally administered bactericidal cyclic lipopeptide antimicrobial that is in Phase III clinical trials for the treatment of CDI (Lee *et al.,* 2016; Biox *et al,* 2017) *C. difficile, Enterococcus faecalis and Enterococcus faecum* were exposed to an oral lipopeptide antibiotic CB 183,315 also known as surotomycin (Mascio *et al.,* 2014). Serial passage experiment was done under selective pressure to detect multistep resistance and with the exception of one strain, no change was observed by the 15th day (Mascio *et al.,* 2014). Also SMT19969, one of the novel agents used for therapy in *C. difficile* infection (CDI) cases was observed for stability in susceptibility as *C. difficile* strains were serially passaged for more than 14 days within subinhibitory concentrations of the drug. Amongst all the isolates tested 31 were of 027, 001 and 106 ribotype. No change in MIC of SMT19969 was observed (Vickers *et al.,* 2011).

C. difficile strains have also previously been exposed to cadazolid in serial passage experiments; this antimicrobial is a novel oxazolidinone-fluoroquinolone hybrid antimicrobial currently in phase III clinical trials for treating CDI, acts by preventing protein synthesis and is, at an higher concentration, involved in DNA synthesis inhibition (Locher *et al.,* 2014). The experiment involved 13 passages which resulted in MIC increases after about 3 steps on exposure to linezolid and cadazolid (Locher *et al.,* 2014). However, the increase cadazolid MICs observed were reported to be insignificant, whereas 16-fold MIC increases were observed against moxifloxacin (Locher *et al.,* 2014).

Development of resistance to antibiotic can occur *in vivo* following exposure to antimicrobial agents in the clinical setting. In the laboratory, scientists can mimic the exposure of microorganisms to antimicrobial agents using serial passaging experiments with sub-MIC of antimicrobials, in order to assess the propensity of antimicrobial agents to select for resistant clones. Additionally, the stability of a resistant (or reduced susceptible) genotype/phenotype can be studied *in vitro* to assess the likelihood of reversion of the phenotype/genotype to the wild-type distribution in the absence of the antimicrobial agent selection pressure. Metronidazole is one of the frontline antibiotics for treating CDI, therefore if selection of

resistant mutants occurred at a high mutation frequency then this would impact on the use of this antimicrobial agent in the clinical setting.

5.1.5 Aim

Assessment of the stability of the Metronidazole Reduced susceptible *C. difficile* strains (CDRM) phenotype and the propensity to elevate metronidazole MICs in *C. difficile.*

5.1.6 Objectives

To assess the stability of the CDRM and Metronidazole susceptible *C. difficile* strains (CDSM) phenotype using serial passage experiments in drug-free medium and monitor any changes in metronidazole and vancomycin MICs using an agar incorporation MIC method.

To use serial passage experiments with vancomycin and metronidazole.

To expose CDRM and CDSM strains to sub-inhibitory concentrations of antimicrobials and monitor any changes in MICs using an agar incorporation MIC method.

5.2 METHOD

5.21 *C .difficile* strains

Thirty seven strains of CDRM from three PCR ribotypes: 027, 001, and 106 were evaluated in this study, compared with 11 CDSM strains of the same ribotypes, in addition to two control strains E4 and ATCC700057 (ribotypes 110 and 111 respectively). The susceptibilities to metronidazole and vancomycin were confirmed by determining the minimum inhibitory concentrations (MIC) of these antibiotics using the same method as described previously in 2.22.

5.22 Serial passage without antibiotic exposure in drug free medium

C. difficile strains were grown on Columbia blood agar (PB0122, Oxoid, Basingstoke, UK), then sterile pre-reduced Schaedler's anaerobe broth (CM0497, Oxoid, Basingstoke, UK) was inoculated and incubated anaerobically overnight at 37ºC MIC was determined using agar incorporation method on Wilkins Chalgren agar as described in section 2.21.

The strains were then retrieved from individual blood agar plates and re-cultured on fresh blood agar, then Schaedler's anaerobe broth was inoculated and MICs were then determined. The transfer of strains to fresh blood agar from the previous step was repeated continuously until the 6th fresh blood agar plate was inoculated and MIC was determined at every step.

MIC determination was perfomed for metronidazole and vancomycin.

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5.23 Serial passage with sub inhibitory concentration of metronidazole

5.23.1 *C .difficile* strains

Twenty eight strains of *C. difficile* were selected from previous MIC testing and heterogeneity in MIC experiments carried out in this research reported in section 2 and 3, the selected strains were those with colonies showing MIC of ≥2mg/L. Thirteen strains belonged to *C. difficile* ribotype 001, eight were from ribotype 027, and seven were from ribotype 106. In addition to the CDRM strains evaluated, two CDSM strains for each ribotype were also assessed in this experiment, along with the *C. difficile* E4 control (CDRM) strain.

5.23.2 Serial passage

C .difficile strains were cultured anaerobically from spore stocks at 37ºC on Columbia blood agar, then were transferred into sterile pre-reduced Schaedler's anaerobe broth for overnight incubation. MICs were determined using agar incorporation method on Wilkins Chalgren agar as described in section 2.22.

Where possible, the *C. difficile* strains were then retrieved from the Wilkins Chalgren agar containing subinhibitory concentrations of metronidazole which was mostly 2 dilutions below the MIC, except the colonies on that dilution was low then that strain will be retrieved from 3 dilutions less than the MIC. The retrieved strains were resuspended in 150µl of saline. Then 110µl of saline with respective *C .difficile strains* were transferred into the multipoint inoculator block, anaerobically. The strains were then inoculated on Wilkins Chalgen agar containing doubling concentrations of metronidazole from 0.03mg/L – 128 mg/L using a 36-pin multipoint inoculator. The inoculated Wilkins Chalgren agar plates were incubated in the anaerobic cabinet for 48 hours. Then MIC was determined and the strains were re-cultured from the new MIC plates 2 dilutions below the MIC, this process was done 6 times and the MICs were determined at every step.

5.3 RESULTS

The results obtained from serial passage experiment without antibiotics, differed amongst strains with respect to the antibiotic tested.

5.3.1. Serial passage without antibiotic exposure in drug-free medium

CDRM strains serially were passaged 5 times and MIC of metronidazole were detected at each passage. MIC decline was observed at the final passage compared to the initial MIC detected. This observation was same for all ribotypes analysed, as shown in table 1, ribotype 001 shifted from an initial MIC range of 0.5-16mg/L with an MIC_{50} of 4mg/L to 0.1252mg/L with an MIC_{50} of 0.5mg/L. All except one CDRM strain of ribotype 001 had MIC of
≥2mg/L at the first detection of MIC but after last passage all had MIC ≤2mg/L. Of the 22 CDRM strains of ribotype 001 tested only 3 strains had an MIC of 2mg/L after the last passage.

Table 5.1. MICs of metronidazole and vancomycin against clinical *C. difficile* from ribotypes 027, 106, and 001 before serial passage and after five serial passages in drug-free Oxoid Schaedler's anaerobe broth.

		Passage					
RT (N)	**Antibiotic**	**0***	**1st**	**2nd**	**3rd**	**4th**	**5th**
001 CDRM	**MTZ range**	0.5 -16	0.25-16	0.25-8	0.125-8	0.25-2	0.125-2
	MIC_{50}	4	4	4	2	2	0.5
	VAN range	0.5-4	1-4	0.5-4	0.5-4	0.5-4	0.06-4
	MIC_{50}	1	2	1	1	1	1
001 CDSM	**MTZ range**	0.5-2	0.25-0.5	0.25-0.5	0.25-0.5	0.25-1	0.25-0.5
	MIC_{50}	1	0.5	0.5	0.5	0.5	0.5
	VAN range	1-4	1-4	1-4	1-2	1-2	1-4
	MIC_{50}	1	1	1	1	1	1
027 CDRM	**MTZ range**	2-8	2-4	1-2	2	1-2	0.5-1
	MIC_{50}	2	2	2	2	2	1
	VAN range	1-2	2-4	1-2	1-2	1-2	1-2
	MIC_{50}	1	2	2	2	2	1
027 CDSM	**MTZ range**	4	2-4	1-2	1-2	2	1-2
	MIC_{50}	4	2	1	2	2	1
	VAN range	2-4	1-2	1	1	1	1
	MIC_{50}	2	1	1	1	1	1
106 CDRM	**MTZ range**	1-4	2-8	1-4	1-4	1-2	0.5-1
	MIC_{50}	2	4	4	2	2	1
	VAN range	0.5-1	2-4	0.5-2	1-2	1-4	0.5-2
	MIC_{50}	1	2	1	2	1	1
106 CDSM	**MTZ range**	0.5-4	0.25-2	0.25-1	0.25-1	0.25-2	0.25-1
	MIC_{50}	0.5	0.25	0.25	0.25	0.25	0.25
	Van range	1-2	1-2	1-2	1	1-2	1-2
	MIC_{50}	1	1	1	1	1	1

Most ribotype 027 CDRM strains, showed stability with the usual strain variability of ±1 dilution. Except strain 95 & 98 that showed a decrease of ≥2 dilutions in Metronidazole MIC. The CDSM ribotype 027 strains initial metronidazole MICs (passage 0) were higher than expected (MIC_{50} 4 mg/L), but a gradual decline in MICs was observed as the number of serial passages increased, finally with MIC_{50} of 1mg/L after 5 passages (Table 5.1). Most of ribotype

106 CDRM strains increased in MIC on the first serial passage (1 doubling dilution) and then a general decrease in metronidazole MICs was observed but CDSM strains did not demonstrate any notable change in metronidazole MICs. In contrast to metronidazole MICs, vancomycin MICs of all CDRM and CDSM ribotypes were stable table 5.1.

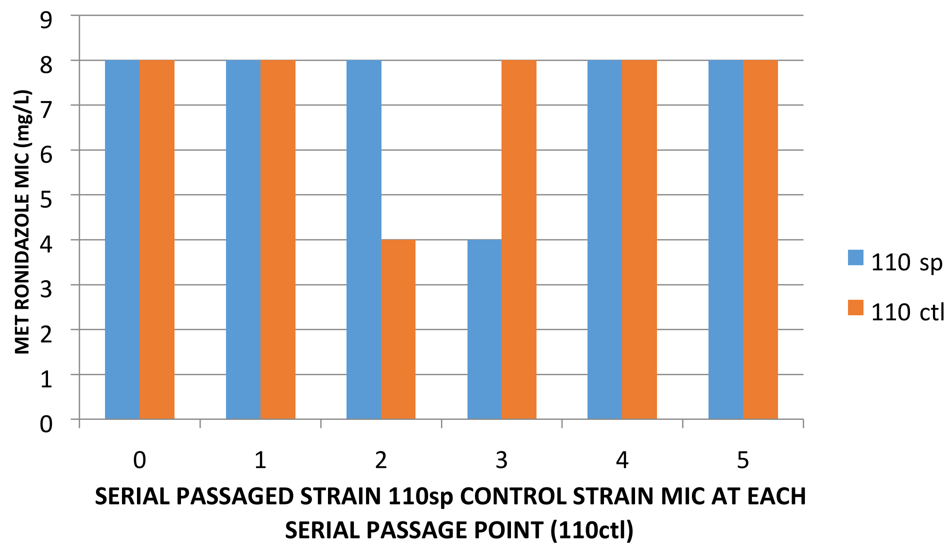

Figure 5. 1. MICs of metronidazole against control *C. difficile* strain E4 (ribotype 010 coded as 110) before and after five serial passages (110sp) & 110CTL, MIC determined from spore stocks without serial passaging

The metronidazole and vancomycin MICs observed from the serially passaged *C. difficile* E4 (Figure 5.1, 5.2) (110 sp) and the control E4 strain were very similar all through the experiment with very little variation. There was a 2-fold reduction in MICs by the end of the experiment, but this is within the normal variability for MICs determined by this method.

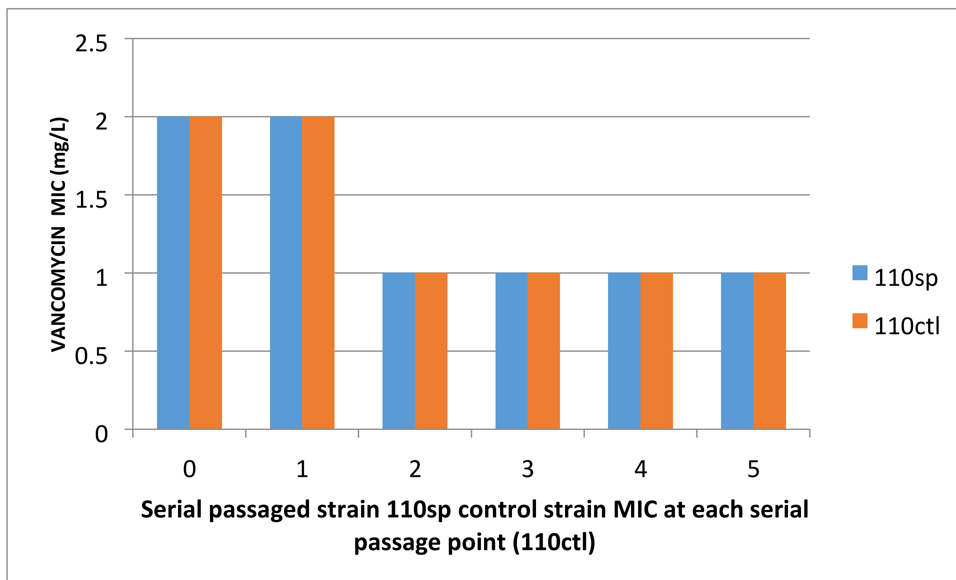

Figure 5. 2 MICs of vancomycin against control *C. difficile* strain E4 (ribotype 010 coded as 110) before and after five serial passages in drug-free medium and control (CTL) 110, MIC determined from spore stocks without serial passaging.

5.3.2 Serial passage with sub-inhibitory concentrations of metronidazole

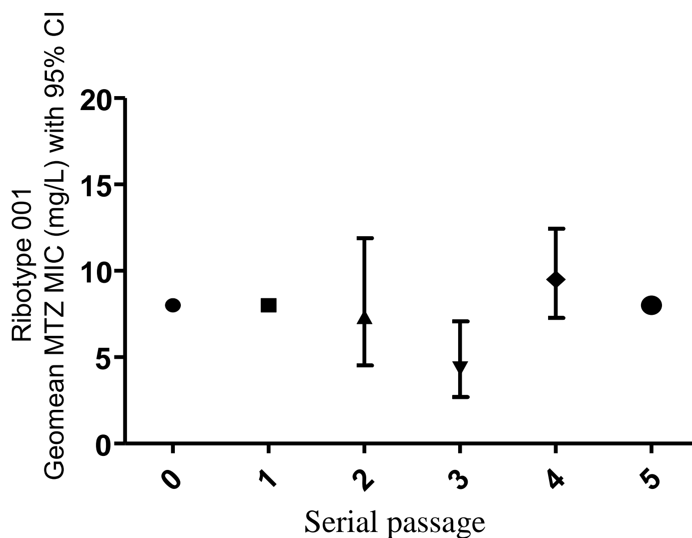

Figure 5. 3 Geometric mean MICs of CDRM ribotype 001 strains exposed to sub-inhibitory MICs of metronidazole during five serial passages

Statistical analysis of the figure above was done using one way Analysis of variance (ANOVA), demonstrated that there was statistical significance between MIC values (P=0.0087) as P < 0.05. Post hoc testing was done using Bonferroni's Multiple Comparison Test and significant difference was only found between serial passage 3 and 4.

CDRM strains of ribotype 027 unfortunately died on subculture, therefore this data is lacking from this report on this group

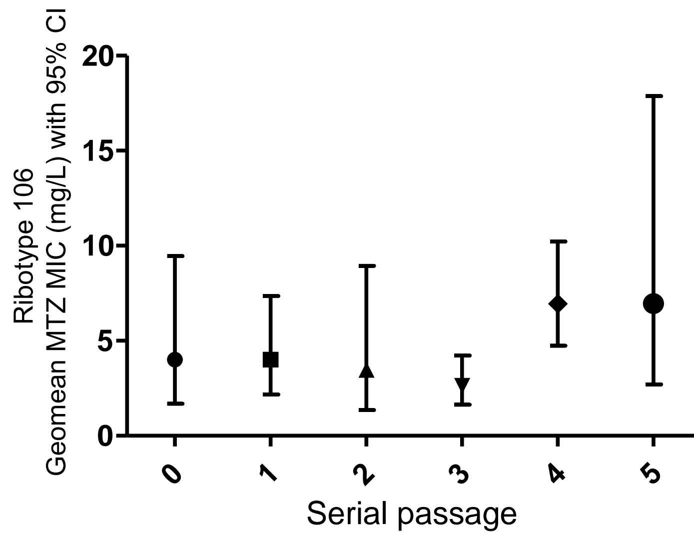

Figure 5. 4 Geometric mean MICs of CDRM ribotype 106 strains exposed to sub-inhibitory MICs of metronidazole during five serial passages

Statistical analysis of the Figure 5.4 above was done using Kruskal – Wallis test and P value =0.1084 thus non-significant. Post hoc testing was done using Dunn's Multiple Comparison Test and no significant difference was observed.

5.4 DISCUSSION

The effects of serial passage without antibiotic exposure were observed to result in declines in metronidazole MICs of CDRM strains. As observed in table 5.1. Reduced susceptible strains of ribotype 001 had MIC_{50} shift from an initial metronidazole MIC_{50} of 4mg/L to 0.5 mg/L at the fifth passage. This decline was however not as much in other two ribotype groups of CDRM strains. Conversely, vancomycin MICs were predominantly stable irrespective of *C. difficile* ribotype or the susceptibility phenotype of the strain. Therefore, serial passage was observed to affect the MICs of metronidazole but had no substantial effect on vancomycin MICs and it had a higher effect on CDRM phenotype strains of ribotype 001 than other ribotypes. Though Leeds *et al,* 2013 have reported a decline in vancomycin MIC in 2 *C. difficile* strains as a result of serial passage but it is useful to note that the experiment reported, entailed a selection pressure (Leeds *et al,* 2013).

Serial passage without selective pressure had little or no effect on the *C. difficile* control strains E4 for both vancomycin and metronidazole. The CDRM control strain E4 did not lose its reduced susceptible phenotype despite continuous exposure to a non-antibiotic pressured environment as was observed in an earlier research that reported a metronidazole resistant strain in a patient who had not had metronidazole therapy (Schapiro *et al.,* 2004).

With exposure to subinhibitory antibiotic concentrations, the effect of serial passage was minimal irrespective of ribotype. It was however noted that most 027 strains could not be recovered on the first passage after the initial MIC was obtained, this was also observed for the 027 CDSM strains after the first passage. The other 2 ribotypes CDRM strains showed MIC stability between passages when strains were exposed to sub inhibitory antibiotic concentration.

It was also observed that the CDSM strains did not stably select for resistant phenotype at the end of passage for all ribotypes. Thus this test has shown that serial passage effect on metronidazole MIC is more when the passage environment has no antibiotic pressure. Thus the CDRM strains tend not to retain the reduced susceptible phenotype if passaged in the absence of antibiotics. However, this phenotype is stable if experimental conditions of serial passage is performed under an antibiotic selection pressure. This finding was not in line with a previous research on serial passage experiments involving strains of peptostreptococcus, an increase in metronidazole MIC from 1-32 fold was observed (Larsen and Fiehn, 1997) though the disparity could be as a result of differences in microbes analysed.

As was earlier mentioned both condition have been observed in previous literature with the serial passaging practice. The MIC stability observed under selective antibiotic pressure in this

research was also reported in a serial passage experiment with surotomycin and SMT19969 on *C. difficile* (Mascio et al., 2014; Vickers et al., 2011)

Conclusion and future work

In conclusion, serial passage is a technique that enable the accumulation of mutations through several generations (Leeds *et al* 2013). The data derived in this experiment suggests that serial passaging of UK *C. difficile* under selective antibiotic pressure does not affect its susceptibility phenotype to metronidazole. Thus this technique under selective antibiotic pressure may not contribute to the development of a resistance phenotype to metronidazole but could give false susceptibility profile if practiced without selective pressure. Thus depicting the CDRM phenotype may be an unstable phenotype. These passaging could occur invivo not only in laboratory practice, this research has shown that it could rarely lead to a resistant phenotype. The instability of the CDRM phenotype could pose a problem in detecting underlying mechanisms that could be responsible for the CDRM phenotype. Thus the practice of this serial passage technique has to be limited both in research and clinically. Future work could be done involving more passages as well as carrying out one step passage experiments using super-inhibitory concentrations of metronidazole as opposed to multiple step passage with sub inhibitory concentrations done in this research to compare outcomes.

6.0 Nitroreductase assay

6.1 INTRODUCTION

Nitroreductase are enzymes that has been observed to reduce nitrocompounds to nonmutagenic compounds and to activate prodrugs (Bryant *et al*, 1981). Nitroreductases have been applied to detect chemicals in the environment as a biosensor for pollutants as well as: in the identification of vapours from explosives (Gwenin et al. nd). Nitroreductases could be involved in bioremediation e.g *pnrA* is a gene that encodes a nitroreductase observed to increase phytoremediation after it was introduced into a transgenic aspen used for phytoremediation of 2, 4, 6 trinitrotoluene an environmental pollutant (Van Dillewijn *et al*, 2008).

Nitrocompounds have been observed to be mutagenic to bacteria, as was confirmed in a research study involving 2,4 dinitrotoluene and 2,6 dinitrotoluene, which were shown to be mutagenic to *Salmonella typhimurium* (Padda *et al*, 2003). However, incidence of mutagenic stimulation may be lower than expected because of the conversion of nitrocompounds into their metabolites which are less harmful (Rafii *et al*, 1991). One bacterial genus observed to play a significant role in the conversions of nitro-compounds is *Clostridium* (Rafii *et al*, 1991). It was confirmed in an earlier research study which exposed faecal microbial flora of human origin to 1-nitropyrene, that organisms with clear zones around areas of microbial growth were isolated and identified. Though the experiment was limited in that the faecal material was from one human source, it served as platform for more research (Raffii *et al,* 1991). The organisms that metabolized 1-nitropyrene as identified by the clear zones were *Clostridium clostridioforme, Clostridium paraputrificum*, *Clostridium leptum, Clostridium sp* and *Eubacterium sp.*

The presence of nitroreductase enzymes in microrganisms has been useful clinically in activation of prodrugs:, e.g. 5-(aziridin-1-yl)-2, 4-dinitrobenzamide (CB1954) is a therapeutic agent against tumour cells has been observed to be reduced by NfsA and NfsB, which are *E. coli* nitroreductase enzymes (Knox *et al*, 1988, Anlezark *et al*, 1992, Vass *et al,* 2009). Additionally, benznidazole is a prodrug that becomes active against *Trypanosomal cruzi* following its reduction initiated by a nitroreductase (Hall and Wilkinson, 2012).

6.11 Nitroreductase in anaerobic organisms

Some nitroreductase genes have been isolated whose activation or inactivation has been associated with resistance to metronidazole. Metronidazole is a nitroimmidazole and a prodrug that requires the action of nitroreductases for its activation. Anaerobic bacteria such as *Helicobacter pylori* the cause of peptic ulcer, *Bacteroides fragilis* and *Clostridium* species which are normal colonic flora, that also could be pathogens, are sometimes being treated by antimicrobials requiring the activation by nitroreductase enzymes. As a result the activation

and inactivation of the genes encoding these nitroreductase enzymes, could have an effect on efficacy of antimicrobial therapy.

Nitroreductase activity is naturally occurring within microorganisms, thus it does not require an external influence for its activity (Raffii *et al*, 1998) this is because it is involved in the normal cellular processes, e.g glucose metabolism. Nitroreductase involvement in normal metabolic processes was also reported in previous research by Britz and Wilkinson (1979), where the products of glucose metabolism in metronidazole resistant *B. fragilis* were compared with a metronidazole susceptible parent strain. It was observed that the increase in resistance to metronidazole was associated with a concurrent decrease in pyruvate dehydrogenase activity. The difference in production of other metabolic end products in the resistant *B .fragilis* strain relative to the wild type correlated to the decrease in pyruvate dehydrogenase activity (Britz and Wilkinson 1979).

The resistant mutants were of two levels of resistance at 25µg/ml MIC & highly resistant *B .fragilis* at 100µg/ml MIC (Britz and Wilkinson 1979). A quarter of the normal dehydrogenase level was produced in the lower level resistant strain; and pyruvate accumulation and increases in lactate concentrations were also observed. There was almost no dehydrogenase activity observed for the highly resistant *B. fragilis* strain and increased levels of CO_2 lactate and ethanol were produced. The results observed in the Britz and Wilkinson (1979) study suggested that resistant *Bacteroides fragilis* strains show lower energy yields obtained from glucose. When compared with normal *B. fragilis*, a decline in growth rates were observed in some resistant *B. fragilis*. The accumulation of pyruvate in the moderately resistant *B. fragilis* showed that it was unable to metabolize pyruvate via other pathways unlike the highly resistant *B. fragilis*.

However, conflicting data were reported by Tabeqchali *et al* (1983), where pyruvate dehydrogenase activity was observed to be active in metronidazole reduced susceptible *B. fragilis,* (MICs of 2-8mg/L) and also in highly resistant *B. fragilis* strain (MIC of 64mg/L) as well as metronidazole – susceptible *B. fragilis* strains with MIC 0.5mg/L). These observations question the indication that pyruvate dehydrogenase activity could be associated with reduction in susceptibility to metronidazole (Tabeqchali *et al*, 1983).

Nitroreductase activity is probably not the single cause of resistance to nitroimidazole, papers have also identified decreased uptake of antibiotics (Lacey *et al*, 1993). During therapy against *Bacteriodes fragilis* using metronidazole, *Enterococcus faecalis* has been observed to inhibit

the susceptibility of *Bacteriodes fragilis* to metronidazole by inactivating metronidazole. Thus the resistance of *Bacteriodes fragilis* to metronidazole could also be influenced by other microbes in the environment. (Nagy and Foldes, 1991). Metronidazole resistance in *Bacteriodes fragilis* has also been observed to be conveyed by *nim* genes (Gal and Brazier, 2004).

As was stated earlier the bacteria frequently reported for metabolising nitrocompounds that could be detrimental to the human health was isolated and identified to be of *Clostridium* genus. *Clostridia* were identified to metabolise -1- nitropyrene and associated compounds to amine derivatives (Rafii *et al,* 1991). This conversion was carried out by a nitroreductase which was confirmed using a nitroreductase assay. The nitroreductase enzyme was further characterised to be extracellular, requiring anaerobiosis for its activity as well as an optimal pH of 8 (Rafii *et al,* 1991). The isolates comprising of *Eubacterium sp, Clostridium leptum, Clostridium clostridioforme, Clostridium paraputrificum and Clostridium sp* all showed nitroreductase activity without the addition of FAD except *Clostridium sp.* One distinct nitroreductase was identified per isolate (Rafii *et al,* 1991). The redox potential necessary for the reduction of metronidazole is -450mV (Edwards, 1980). The reduction of metronidazole not only activates the bactericidal effect of the drug but also increases its concentration intracellularly (Edwards., 1980)

Sisson *et al*., 2002 determined the specificity of a range of nitroreductase enzymes e.g Pyruvate oxidoreductase (POR), RdxA, FrxA in activating of certain nitrocompounds:
Metronidazole nitrofurans and nitazoxanide. It was elucidated that POR reduced nitrofurans but not to the same extent as FrxA, all three nitroreductases reduced nitazoxanide. The only nitroreductase that reduced metronidazole in *Helicobacter pylori* (*H. pylori)* was observed to be RdxA (Sisson *et al.,* 2002). This paper not only identified the nitroreductase specific for metronidazole but also showed that some nitroreductase enzymes can activate multiple substrates. The *rdxA* gene encodes an oxygen insensitive NADH nitroreductase activity and was also identified as a potent contributor to metronidazole resistance as a functional rdxA gene from *H. pylori* was transformed into normally metronidazole resistant *E. coli* and *H. pylori* and both strains became susceptible to metronidazole (Goodwin *et al*, 1998). Goodwin *et al*, (1998) paper also proposed that mutations in *rdxA* causing resistance could be as a result of continuous use of the drug and this supports Jenks *et al*, (1999) who worked on mice and found *rdxA* to be stable when not exposed to metronidazole. Resistance to metronidazole was linked to mutation in *rdxA* following exposure to metronidazole (Jenks *et al.,* 1999). However, the resistant phenotype was observed to be stable despite consecutive subcultures in the absence of metronidazole (Jenks *et al.,* 1999). Not all resistant *H. pylori* had a mutation in the *rdxA*

gene, thus showing the involvement of other factors in conferring resistance in *H. pylori* (Jenks *et al*., 1999).

Another nitroreductase that has been implicated in metronidazole resistance is *frxA,* which encodes an oxygen insensitive NAD(P)H - flavin nitroreductase (Sisson *et al.,* 2002). Comparison of the gene sequence of 9 pairs of *rdxA* and *frxA* susceptible and resistant in *H. pylori*, identified mutations linked to either of these genes in 8 of the pairs however one pair had no obvious mutation in *rdxA, frxA* and *recA* a gene for DNA repair in bacteria (Marais *et al,* 2003). RecA of *H. pylori* was identified to also contribute to metronidazole sensitivity by identifying the effects of mutant *recA* in *H. pylori* this as well affected DNA repair and transformations (Marais *et al,* 2003).

On frxA it was identified that *frxA* without mutation on *rdxA* gene can confer resistance to metronidazole in *H .pylori* as one of the 8 metronidazole resistant strains had mutation only in *frxA* gene not in *rdxA*. The role of *frxA* was further emphasized in a transformation experiment using *frxA* from a *H. pylori* susceptible to metronidazole, which was inserted into a metronidazole resistant *E. coli* which became susceptible. Thus it was suggested that *frxA* by itself could affect susceptibility metronidazole (Marais *et al*, 2003).

A similar experiment was performed by Kwon *et al*, (2000) transferring *frxA* from a metronidazole resistant *H. pylori* cloned gene into metronidazole sensitive *H. pylori* (minimum inhibitory concentrations (MIC) = 0.5-1 mg/L) yielded moderate level resistance to metronidazole (MIC =32mg/L). Metronidazole sensitive *H. pylori* strains had increased level of MIC (32mg/L) when their *rdxA* genes were inactivated (Kwon *et al,* 2000) also one strain was reported to have Metronidazole MIC increase to 128mg/L, thus showing the possibility of the inactivation of *rdxA* being involved in metronidazole resistance (Kwon *et al,* 2000).

Nitroreductase assay was carried out on *Clostridium* species resistant to nitrofurantoin. The cell free supernatant of the isolates mutants or wild type also metabolised nitrofurantoin which was detected by the disappearance of the nitrofurantoin peak using high performance liquid chromatography (HPLC). The use of cell free supernatant further indicates that nitroreductases are extracellular, and confirmation of nitroreductase presence was detected by the conversion 4- nitrobenzoic acid to 4 aminobenzoic acid (a known role of nitroreductases (Rafii *et al*., 1991). Using a Nitrofurantoin susceptible bacillus a bioassay was used to detect that nitrofurantoin was converted to a non-bactericidal substance by 2 of the mutants. This research confirms that both susceptible and resistant *Clostridium* species could produce nitroreductase but would convert substrate to different metabolites (Rafii and Hansen, 1998). This same

characteristics was observed in *Enterococcus casseliflavus* and *Enterococcus gallinarum* (Rafii *et al*, 2003).

Nitroreductase activity can be assayed for by measuring the conversion of 4- nitrobenzoic acid (PNBA) to 4-amino benzoic acid (PABA) which is an action carried out by the enzyme (Rafii *et al.,* 1991) the concentration of PABA is quantified by the production of a purple azo dye that occurs after a series of reaction that ends with addition of N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) to produce the azo dye that is measured using spectrophotometer.

6. 1.2 Aim

To analyse metronidazole reduced susceptible *C. difficile* strains (CDRM) from each ribotypes 001, 106 and 027 were selected along with metronidazole susceptible *C. difficile* strains (CDSM) strains and for nitroreductase activity.

6.1.3. Objectives

Use of a spectrophotometry-based nitroreductase assay for the conversion of 4-nitrobenzoic acid (PNBA) to 4-aminobenzoic acid (PABA) in Metronidazole reduced susceptible *C. difficile* strains (CDRM) and Metronidazole susceptible *C. difficile* strains (CDSM)

6.2 METHOD

6.21 *Clostridium* strains

CDRM strains from ribotypes 001 (n=4), 106 (n=2), and 027 (n=5) were analysed in this experiment alongside CDSM strains 001 (n=2), 027 (n=1). Control strains 110 (E4) and 111(ATCC 70057) were incorporated into these experiments alongside *Clostridium prefringens* (Rafil *et al*, 1991) which was previously reported as a nitroreductase positive control (Rafil *et al,* 1991).

6.22 Nitoreductase assay

Overnight culture of *Clostridium difficile* strains in BHI broth (CM1135, Oxoid, Basingstoke, UK) were standardized to OD_{600} = 0.1 in sterile BHI broth. Then 500µg/ml of 4-nitrobenzoic acid (PNBA) (101553376 Sigma Aldrich, Poole UK) was added in a 1:10 dilution to new culture anaerobically and allowed to incubate for two hours. The culture mixture was then centrifuged for 30 mins at 4000rpm. Subsequently, the 4-aminobenzoic acid (PABA) (Sigma Aldrich, Poole UK) concentration in supernatant was detected by adding the following chemicals at 1 in 10 dilution as was described in Rafii *et al.,* 1991.

Trichloroacetic acid (0.21% w/v) (11964921 Fisher Scientific, Loughborough, UK) thereafter, sodium nitrite (0.007%w/v) (563218, Sigma Aldrich, Poole, UK) was added and incubated at room temperature for 20 minutes. Next ammonium sulfamate (0.04%w/v) (51512, Sigma Aldrich, Poole, UK) was added to neutralize the sodium nitrite following a 3min incubation period at room temperature. After NEDD N-(1- Naphthyl) ethylenediamine dichloride (NEDD 0.35%w/v) (1001844308, Sigma Aldrich Poole, UK) was added to a mixture to yield a purple dye. The PABA concentration was then determined using a Spectrophotometer (Cecil instruments, Cambridge, England) set to an absorbance of 540nm (Rafil *et al,* 1991)., one unit of enzyme is one microgram of PABA.

6.3 RESULTS

Table 6. 1 Nitroreductase (Nit) activity demonstrated by *Clostridium difficile* strains from ribotypes 001, 027, 106 using a spectrophotometry based PNBA reduction assay at 540nm.

***C. difficile* ribotype**	**Number of CDRM strains nitroreductase positive (%)**	**Number of CDSM strains nitroreductase positive (%)**
001	1(25)	0(0)
027	0(0)	0(0)
106	2(100)	No strains analysed

The strains with positive enzyme activity were all approximately one enzyme unit which was very low. Variability was also observed in this assay.

C. perfringens ATCC 3626 demonstrated nitroreductase activity in the assay but the two internal *C. difficile* control strains did not. Only a low proportion of CDRM strains demonstrated nitoreductase activity. None of the CDSM strains analysed showed detectable nitroreductase activity.

6.4 DISCUSSION

This research has shown that majority of *C. difficile* strains were nitroreductase negative with the exception of three strains using the method of detection described by Rafii *et al.,* 1991 where *Clostridium* species actually showed positive nitroreductase activity. Although the nitroreductase assay used in this experiment yielded positive nitroreductase data for the *C. perfringens* strain used as a positive control, there was inconsistency in the data generated from *C. difficile* strains, both clinical CDRM isolates and CDSM isolates and in the internal control strains used.

Rafii *et al.,* 1991 stated that the nitroreductase activity was extracellular and constitutive however metronidazole activation has been reported to occur intracellularly (Edwards and Mathison 1970). It has also been stated previously that nitroreductase enzymes are substrate specific (Sisson *et al*, 2002) thus there is a possibility that the nitroreductase enzyme responsible for conversion of PNBA to PABA is extracellular and that of metronidazole is intracellular. The method used in the present study was following the method reported for the detection of nitroreductase activity in wild type bacteria and nitrofurantoin resistant mutants (Raffii *et al,* 1998). Nitrofurantoin is a nitrofuran antimicrobial agent and this group of nitro compounds has been identified to have redox potentials ranging from -250mV to -270mV, nitazoxanide has a redox potential of -360mV while that of metronidazole is -485mV (Sisson *et al*., 2002). The substrate specificity was observed to be largely linked to the redox potential requirement of each substrate. Therefore, in *Helicobacter pylori,* POR was more active to reduce nitazoxanide, RdxA reduced metronidazole and FrxA reduced nitrofurans, while both RdxA and FrxA were also observed to reduce nitazoxanide (Sisson *et al.,* 2002). Furthermore, Raffii *et al.,* (2003), has observed nitroreductase activity in metronidazole resistant *Enterococcus* species, which metabolised metronidazole to a non-bactericidal metabolite and the nitroreductase activity was detected by measuring PABA production. It was also elucidated that PNBA conversion was not affected by the presence of metronidazole thus there was no substrate competition which should not be the case when PNBA was converted and metronidazole metabolised (Raffii *et al.,* 2003), this further emphasizes the likelihood of different reductase enzymes involved in the nitroreduction in bacteria. Thus indicating that perhaps the conversion of PNBA to PABA might be performed by specific nitroreductases, distinct from those responsible for the activation of metronidazole, or perhaps this conversion could occur by a wide range of nitroreductases. This present research and previously published literature have shown that due to the substrate specificity of different nitroreductase enzymes,

any experiment with the aim of detecting nitroreductase activity has to be modified with respect to the substrate and potentially the microorganism under study.

Conclusion and future work

In conclusion the method employed was not efficient to detect the nitroreductase enzyme reduction of metronidazole, since the method detects extracellular nitroreductase. Devising a method using radio labelled metronidazole (Tally *et al.*, 1978) and monitoring the decline in the radiolabel signal, which is proportional to the level of nitroreduction, may be a more useful method in correlating nitroreductase activity to the CDRM phenotype. This approach may also provide an insight into the level and rate of activity of reductases. Thus would likely be more sensitive in the detection of nitroreductase activity.

There is yet to be a nitroreductase enzyme associated with *Clostridium difficile* with respect to metronidazole activation but as a frontline therapy for CDI, this warrants further investigations. Future work on the identification of nitroreductases, both intracellularly and extracellularly expressed are warranted in *Clostridium difficile* in order to more fully understand the process of nitroreduction in *Clostridium difficile*. Whole genome sequencing in *C. difficile* would identify putative nitroreductases, potential mutations (SNPs or deletions), and mutagenesis experiments and gene knockouts (Jeong *et al,* 2000, Jeong *et al,* 2001) targeted at the nitroreductases would yield valuable data on the role of these enzymes in *C. difficile* physiology and in the activation of metronidazole.

7.0 *Nim* Genes: A Potential Mechanism of Reduced Susceptibility to Metronidazole in *C. difficile*

7.1 INTRODUCTION

Nitroimmidazole resistance genes (*nim* genes) encode for nitroreductases that aid the reduction of metronidazole to non-antimicrobial compounds (Schapiro *et al,* 2004). The contribution of *nim* proteins to reduced susceptibility to nitroimmidazoles has been reported following a comparative study of *nim* positive and *nim* negative *Bacteroides fragilis* strains. Both strains were exposed to a dimetronidazole, which is a 5-nitroimidazole compound. The *nim* negative strain reduced dimetronidazole to a nitroso radical anion then to 5, 5' –azobis-(1,2-dimethylimidazole) but the *nim* positive strain reduced it to 5 amino-1, 2, dimethylimidazole which was nontoxic to *Bacteroides fragilis* (Carlier *et al,* 1997). Thus elucidating that the *nim* positive strains code for an enzyme that reduces metronidazole to a less harmful derivative compared to the *nim* negative strains (Carlier *et al,* 1997). Land and Johnson (1999) correlated the features associated with metronidazole decreased activity, in varying organisms including anaerobic bacteria, parasitic anaerobic protists as well as microaerophilic bacteria. It was concluded that metronidazole resistance was multifactorial, but a common link was observed to be due to metronidazole activation which is metronidazole inactivation or lack of activation (Land and Johnson, 1999). The above findings from Carlier *et al*, 1997 showing the roles of *nim* genes have supported these statements.

Mostly, *nim* genes have been observed in *Bacteroides fragilis* but there has been some controversy in their presence causing reduced susceptibility to metronidazole. Gal and Brazier (2004) analysed the susceptibilities of the *Bacteriodes* spp. sent to the Anaerobe Reference Laboratory (ARL, Cardiff, UK) and correlated susceptibility with the presence of specific *nim* genes. The total *Bacteriodes* species analysed were 206 isolates of human origin of which 142 were *Bacteriodes fragilis*. Fifty of these isolates were observed to be *nim* positive out of which *nimA* was more frequently observed (Gal and Brazier, 2004). The high occurrence of the *nimA* gene in *nim* positive strains analysed was also observed in a 2005 research, where of 30 *nim* positive *Bacteroides fragilis* strains the most common was *nimA* gene which was more than half of the population (Lofmark *et al*, 2005).

Another study by Schaumann *et al* (2005) examined metronidazole reduced susceptible, resistant and susceptible *Bacteroides fragilis* strains for the presence of *nim* genes. Some of these strains were reference strains of known *nim* activity. Those with reduced susceptibility were *nim* positive and those with susceptibility were *nim* negative. However, after serial passaging *B. fragilis* with the sub-inhibitory concentrations of metronidazole, all strains

demonstrate increased MICs up to >256mg/L irrespective of the *nim* status (positive or negative).This was therefore suggestive of the need for further research in elucidating metronidazole reduced susceptibility mechanisms in *B. fragilis* without ruling out the association of *nim* genes. The decreased metronidazole susceptibility is likely to be a multifactorial process (Schaumann *et al*, 2005). Consequently, it could be that increase in metronidazole concentrations observed in patients following antimicrobial therapy has a way of switching on/off genes linked either to the activation, uptake, extrusion or inactivation of metronidazole (Schaumann *et al,* 2005).

The frequency of *Bacteroides fragilis* with decreased susceptibility to metronidazole was relatively low when the published literature in this area were analysed (Lofmark *et al*, 2005). Further to this, the frequency of *nim* positive strains has also been shown to be low, as observed in Lofmark *et al,* (2005) which showed only 30 strains out of 1502 *Bacteroides fragilis* strains analysed, were *nim* positive (2%) (Lofmark *et al,* 2005). It was also observed that *nim* positive strains had a higher propensity to show further decreases in their susceptibility when exposed to metronidazole *in vitro* compared to strains without *nim* genes. The stability of the mechanism(s) responsible for these further elevations in metronidazole MICs was questionable, given that less than 20% of the strains that showed elevated MIC where irreversibly induced. The controversy on the role of nim genes in metronidazole reduced susceptibility/resistance was questioned in a study where 21 *nim* positive anaerobe strains were observed to be susceptible to metronidazole (Theron *et al,* 2004).

7.1.1 Presence of *Nim* Genes in Other Microorganisms

Nim genes have also been identified in anaerobic Gram positive cocci, mostly of *Peptostreptococcus* species, most of which were *nimB* positive. However, metronidazole susceptibility was also observed in these *nimB* positive strains (Theron *et al,* 2004) except for two strains of *F. magna* which were resistant to metronidazole with a MIC of >128mg/L.

A South African study identified the presence of *nimA* in a strain of *Clostridium bifermentans* following the analysis of 64 anaerobes, and reported the presence of *nim* genes in 35 anaerobes, including some strains of *Bacteriodes fragilis* and *Prevotella spp* and *Actinomyces species* (Lubbe *et al,* 1999)

The *nim* gene primers were designed by Trinh and Resset in their 1996 research article. They designed two polymerase chain reaction (PCR) primers (*nim3* and *nim5*) which were observed to lead to the amplification of a product consisting of 458bp irrespective of gene location (plasmid or chromosome). These primers were also identified to be specific for *nim* genes due to the absence of cross reactivity with other regions of bacterial genomes. However, the

sensitivity of the PCR assay was dependent on specificity and copy number of the *nim* target (Trinh and Reysset, 1996). The common *nim* genes identified in previous published research are *nimA* - *nimF*, however other *nim* genes have also been discovered including; *nimG, nimH, nimJ*, and *nimI.* NimI genes have been isolated in *Prevotella* species and not to be associated with previously identified *nim*-linked IS elements and were located on the chromosome (Alauzet *et al.,* 2010). NimG genes were observed in *Bacteriodes spp* (Gal and Brazier, 2004) and *nimJ* was identified in *Bacteriodes fragilis* strains with a different sequence that could not be amplified from previously designed primers. Due to SNPs in the nucleotides encoding *nimJ*, the PCR was unsuccessful, however the amino acid sequences encoding nimJ were identical (Husain *et al.,* 2013). Nagy and foldes (1991) screened *Enterococcus faecalis* known for the inactivation of metronidazole for the presence of *nim* genes but these facultative anaerobes were found to be lacking *nim* (Trinh and Reysset, 1997). It may have been that enterococcal mediated metronidazole inactivation was not due to presence of *nim* genes, or the universal *nim* primers cannot amplify the sequences of the nim genes of *Enterococcus faecalis.*

As a result of the numerous associations of *nim* genes with resistance to metronidazole in *Bacteroides fragilis*, researchers have evaluated their presence in metronidazole resistant *Clostridium difficile*. However, there is yet to be a research study that has positively identified *nim* genes in *Clostridium difficile* with a resistant or reduced susceptible phenotype to metronidazole though some efforts have been made at genomic level. The first *Clostridium difficile* strain identified in UK to show reduced susceptibility to metronidazole was reported to show a negative result to assay for *nim* gene (Brazier *et al.,* 2001). This *C. difficile* strain (*C. difficile* E4, ribotype 010) was isolated in a care home in Leeds and the strain possessed a metronidazole MIC of 16mg/L when susceptibility to metronidazole was carried out with use of E-test (Brazier *et al.,* 2001). *NimB* is the only *nim* gene that has been associated with *C. difficile* in a proteomic assay (Chong *et al.,* 2014).

7.1.2 Aim

To evaluate CDRM strains for the presence of nim genes. *NimA –E* was screened for in 3 ribotypes, ribotype 001, 027 and 106. Susceptible and reduced susceptible strains will be compared.

7.1.3 Objectives

Polymerase chain reaction experiments will be performed to analyse CDRM strains from ribotypes 001, 027 and 106 for the presence of *nimA – nimE* as the universal primers for these genes were used.

7.2 METHOD

7.21 *Clostridium difficile* strains

Twenty eight *Clostridium difficile* strains were selected from previous MIC and heterogeneity experiments carried out earlier in this research (see Figure 3.1, 3.2 and 3.6) the selected strains were those demonstrating metronidazole MICs of ≥2mg/L. Thirteen, eight and seven strains were selected of ribotypes 001, 027 and 106. These strains were inclusive of two *C. difficile* strains known to have being susceptible to metronidazole (CDSM strains) for each ribotype. Two control strains where also involved E4 and ATCC 700057 of ribotype 010 and 038 respectively, as well as the positive control for *nimB* which was *Bacteroides fragilis* strain BF8 (Soki *et al.,* 2006).

7.22 *Nim* gene detection

A suspension of *C .difficile* colonies for each strain was made in sterile water (100µl) (Eitel *et al.,* 2013). The suspension was incubated for 15mins in 95ºC then it was centrifuged at 14000 rpm for 2mins to obtain the DNA template. This was performed for all sample strains as well as the positive control for *nimB* which was BF8 (Soki *et al.,* 2006). PCR mixture contained 1µl of DNA template, 1µl of primers (Primers NIM3 (5′-ATG TTC AGA GAA ATG CGG CGT AAG CG-3′) NIM5 (5′-GCT TCC TTG CCT GTC ATG TGC TC-3′)), 12.5µl of Amplitaq Gold™ PCR mastermix (10289234, Applied Biosystems™, Thermo Fisher Scientific, Loughborough UK) and 9.5-10µl of sterile distilled water. The primers used were the universal primers NIM-3 and NIM-5 known to be used for the amplification of *nimA nimE.* The *nim* genes PCR cycling conditions used were 35 cycles of: 94ºC for 15seconds, 62ºC for 30secs, and 72ºC for 30 seconds. The cycle was done (Eitel *et al*, 2013) in a thermal cycler (Biocompare, sanFrancisco, California, US). Then each PCR product was mixed with 2.5µl of loading dye and loaded in a tris boris EDTA gel, for gel electrophoresis alongside a 100bp ladder (1002389655, Sigma Aldrich, Poole, UK). Gel red (Biotium, Hayward, California, US) was incorporated in the agarose gel to enable detection of PCR products following exposure to UV light.

7.3 RESULTS

The *nim* gene PCR amplification was only observed in the *nim* positive control strain *BF8* (about 400 bp). All samples tested including ribotype 001 (appendix1) 027 and 106 CDSM and CDRM strains showed no *nim* PCR product (Figure 7.1)

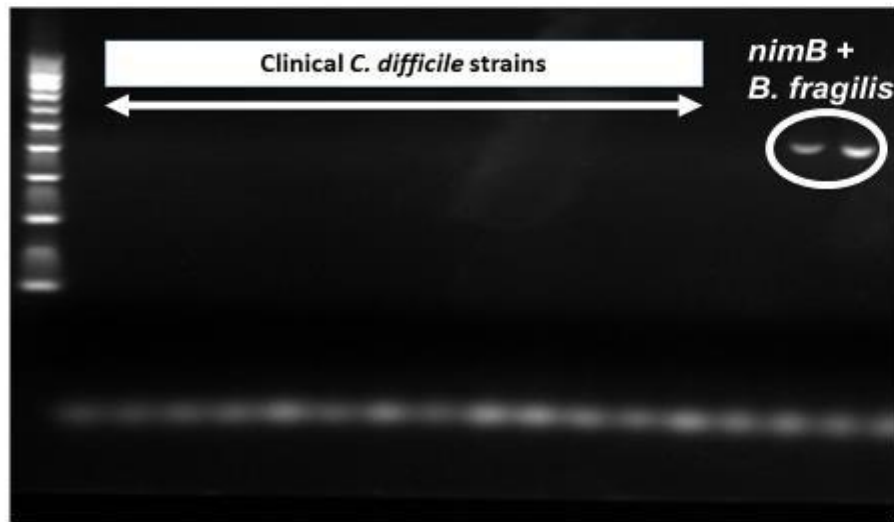

Figure 7. 1 PCR amplification of *C. difficile* strains and *nimB* positive control *BF8*.The first well contained 100 base pair ladder, other wells contained *C. difficile* strains except the last 2 wells that contained the positive control *BF8*

7.4 DISCUSSION

Nim genes, as earlier mentioned, have been implicated in previous reports as being involved in contributing to decreased susceptibility to metronidazole in anaerobic organism such as *Bacteroides fragilis*. In the present study *C. difficile* PCR ribotypes 001, 027 and 106 were screened for the presence of *nimA-E* genes. Most of the strains screened were strains observed to be CDRM and the study also included CDSM for each of the ribotypes analysed. This research confirmed the absence of *nim* genes in *C. difficile* UK ribotypes irrespective of metronidazole susceptibility patterns. As shown in Figure 7.1, no *nim* genes were amplified in any of the ribotypes irrespective of susceptibility of the strains analysed. PCR amplification of putative *nim* was only observed in the *nim* positive control strain of *Bacteriodes fragilis* (*BF8*). *Bacteriodes fragilis* (*BF8*) as was earlier mentioned is *nimB* positive, positive control strains for the remaining *nim* genes could not be sourced as part of this study however, given that these cycling conditions have successfully amplified other nim genes previously (Eitel *et al,* 2013), had these *nim* genes been present then the PCR process would have successfully amplified them, as the primers used were universal primers for *nimA-E*. It is interesting that *nimB* was absent in the *C. difficile* strains evaluated, since research by Chong and colleagues (2014) suggested that *nimB* was present in both metronidazole susceptible and resistant ribotype 027 variants of *C. difficile.*

As earlier stated there has been some controversy correlating *nim* to decrease in metronidazole activity. This controversy is further confirmed in an earlier research where 2% of *Bacteriodes* spp. from Hungary where reported to have reduced susceptibility to metronidazole. However just 5% of these decreased susceptibility strains were *nim* positive with the *nimA* and *nimB* genes (Urban *et al,* 2002). The present study is also in line with a previous research that involved observing twenty four isolates of *Clostridium difficile* for the presence of *nim* genes. Fourteen out of these isolates were identified to be metronidazole resistant, however *nim* genes were not found in the isolates (Pelaez *et al*, 2008). Whole genome sequencing was used also to analyse three *C. difficile* strains of ribotype 010 originally from Spain, one of the strains was reduced susceptible to metronidazole, the other metronidazole resistant, as well as susceptible strain. *Nim* genes were not observed in the genomic analysis of these *C. difficile* isolates (Moura *et al,* 2014).

Conversely, a proteomic assay was carried out on three 027 ribotype strains which were metronidazole susceptible, reduced susceptible and resistant to metronidazole. *NimB* was detected and *NimB* expression was observed to be 3-fold higher in the reduced susceptible and metronidazole resistant strain compared to the susceptible strain (Chong *et al*., 2014). However,

these same strains had been earlier sequenced using next generation sequencing and *nim* genes were absent (Lynch *et al,* 2013). The possibility of *nimB* gene is further emphasized in the reclassification of *C. difficile* into the family of *Peptostreptococcacaee* that led to its renaming as *Peptoclostridium difficile* (Yutin & Galperin, 2013). The *Peptostreptococci* have previously been reported to possess *nimB* genes (Theron *et al,* 2004).

The present study has confirmed earlier findings showing the absence of *nim* in *C. difficile*, thus indicating that metronidazole reduced susceptibility may be due to factors other than the conversion of metronidazole into a less harmful derivative as facilitated by *nim* genes. It also, along with observations in published literature depicts the potential inability of the *nim* universal primers to identify *nim* sequences in all bacterial species. This issue was previously encountered in *Bacteriodes fragilis,* where the universal primers could not amplify *nimJ* because it had a different nucleotide sequence even though the proteins where same with the universal primers. Therefore, this would have given a false *nim* negative result if the research had been limited to PCR amplification using the universal primers (Hussain *et al,* 2013).

Conclusion and Future work

In conclusion this research has shown inability to amplify nim gene using the universal primers. Though, the present study did not assess at the proteomic level for the presence of Nim as in the study of Chong *et al* (2014), therefore the presence of Nim in the *C. difficile* proteome of strains in the present study cannot be ruled out. The proteomic assay which demonstrated *nimB* expression that was not identified at genomic level (Lynch et al., 2013 and Chong *et al* 2014) indicates a different gene sequence that could have similar proteins as *nimB*. Thus this could be an aspect for future research as genomic assays is yet to identify *nim* presence in *Clostridium difficile*.

The submission of the *C. difficile* Nim protein sequence to Phyre Protein Fold recognition server could also aid in the comparison with other *nim* protein structures using Dali server (Hussain *et al,* 2013). As well as converting the *C. difficile* Nim protein sequence it to its gene sequence, which would aid identification of genetic differences with other *nimB* sequences in published literature.

8.0 Metronidazole Uptake as a factor contributing to reduced susceptibility

8.1 INTRODUCTION

The rate or magnitude of accumulation of an antimicrobial agent in a microbe can affect the efficacy of the antimicrobial and ultimately the susceptibility levels of the microorganisms, as has been researched and reported previously for the protozoal pathogen, *Trichomonads vaginalis* (Muller and Gorell, 1983)

Metronidazole uptake in anaerobic bacteria has been observed to be dependent on the low redox potential present within the anaerobes, which enables metronidazole reduction to its active form that causes DNA degradation and then the resultant antimicrobial effect. The reduction of metronidazole to its active form has been observed to aid its uptake, as the intracellular metronidazole concentrations decline due to activation, thus enabling further extracellular metronidazole to diffuse passively into cell due to the concentration gradient (Edwards *et al,* 1993). However, it is notable to emphasize that antimicrobial susceptibility related to drug uptake could be influenced by oxygen in the environment thus reducing the redox potential, as well as other metronidazole uptaking microorganisms such as *E. coli* and *E. faecalis* in the environment that could lead to decrease in concentrations below therapeutic levels (Edward, 1980).

Activity of metronidazole is not solely governed by the rate or magnitude of metronidazole uptake or accumulation within microbes Tally *et al.,* 1978 studied ^{14}C-metronidazole uptake by *E.coli* and *Bacteroides* spp drug uptake was successful in both susceptible and resistant samples bacteria, however the differences in metronidazole susceptibility was due to the ability to metabolise metronidazole (Tally *et al.*, 1978). Metronidazole uptake in *Helicobacter pylori* has also been previously studied (Lacey *et al.,* 1993), where *H. pylori* cultures were exposed to metronidazole (10mg/L) and uptake was assessed indirectly using a HPLC assay to determine residual metronidazole left in the broth cultures. The authors cross referenced metronidazole uptake to the total viable count of *H. pylori*. It was observed that with decrease in metronidazole concentration in broth there was a parallel increase in Total viable counts (Lacey *et al.*, 1993).

8.1.1 Aim

To study metronidazole uptake in *C. difficile* as a possible mechanism of developing reduced susceptibility to the antibiotic.

8.1.2 Objectives

To expose metronidazole reduced susceptible *C. difficile* strains (CDRM) and metronidazole susceptible *C. difficile* strains (CDSM) to a fixed concentration of metronidazole and assess directly and indirectly the uptake of metronidazole.

8.2 METHOD

8.2.1 *C .difficile* strains

CDRM strains from three PCR ribotypes: 027 (n=6), 001 (n=9), and 106 (n= 3) were evaluated in this study, and compared with CDSM strains of ribotypes 027 (n=2), 001 (n=1), in addition to two control *C. difficile* strains E4 coded as 110 and ATCC 700057 coded as 111 (ribotype 010 and 038 respectively).

8.2.2 Sample preparation

C. difficile overnight broth cultures were used to inoculate fresh Brain heart infusion (BHI) broth (CM1135, Oxoid, Basingstoke, UK) standardised to $0.1OD_{600}$, and cultures were monitored until they reached mid –log phase of approximately $0.55OD_{600.}$ Metronidazole (M3671, Sigma- Aldrich, Poole, UK) was then added to *C. difficile* cultures in duplicate at a final concentration of 10mg/L, alongside control cultures were anaerobically incubated overnight at 37°C. OD readings were obtained after overnight exposure to metronidazole and total viable counts were determined. The procedure for determining total viable counts involved serial 10-fold dilution of cultures in pre-reduced peptone water (CM0009, Oxoid, Basingstoke, UK) to 10^{-7}. Next twenty microliters of cultures were inoculated in duplicate onto Brazier's agar under anaerobic conditions and plates were incubated anaerobically at 37°C. Between 20-200 colony forming units were counted and on each replicate and total viable counts were determined after calculation using the dilution factor and sample volume inoculated onto the agar plates, with total counts expressed as $\log_{10}$ cfu/ml. Calibrators were prepared to create a standard curve to detect metronidazole concentration.

8.2.3 Uptake assay

A microbiological bioassay was carried out using the susceptible control *C. difficile* ATCC 700057 strain as the indicator organism, to detect the presence of residual metronidazole in the cell free broth. An overnight broth culture of this susceptible strain in BHI broth was incorporated in molten Wilkins Chalgren agar before pouring into square (100mm) Petri dishes (11349273, Sterilin^{TM}, Fisher Scientific, Loughborough, UK). On drying, wells (10mm) were cut into the agar with a sterilised cork borer and 20µL of samples, controls, as well as calibrators

were assigned wells. The plates were then incubated anaerobically overnight, the zone of inhibition (mm) around calibrator and cell – free cultures of *C. difficile* were determined. The standard with known metronidazole concentration were used to plot a line graph from which the metronidazole concentrations of the samples solution were extrapolated.

8.2.4 Determination of Intracellular concentration of metronidazole

C. difficile exposed to metronidazole and controls were centrifuged to pellet cells, this cells were washed with sterile water to remove broth that contained metronidazole. The cells were then resuspended in sterile water and added to ready to use 2ml lysing matrix tubes containing specialised beads (Mpbiomedicals LLC, UK) which was spun to shear cells in a FastPrep - 24sample preparation system (Mpbiomedicals, California, USA) an effort to release metronidazole uptaken into the cells which was then used to carry out a bioassay as reported in section 8.2.3

8.3 RESULTS

Spectrophotometric readings were obtained as well as total viable count, after cultures were exposed to 10mg/L metronidazole in duplicates. The spectrophotometric readings showed as observed in the Figure 8.1 below, a decrease in OD_{600} compared to the control that was not exposed to metronidazole for all strains analysed except for strain 82 that had higher OD_{600} higher with test samples than control samples.

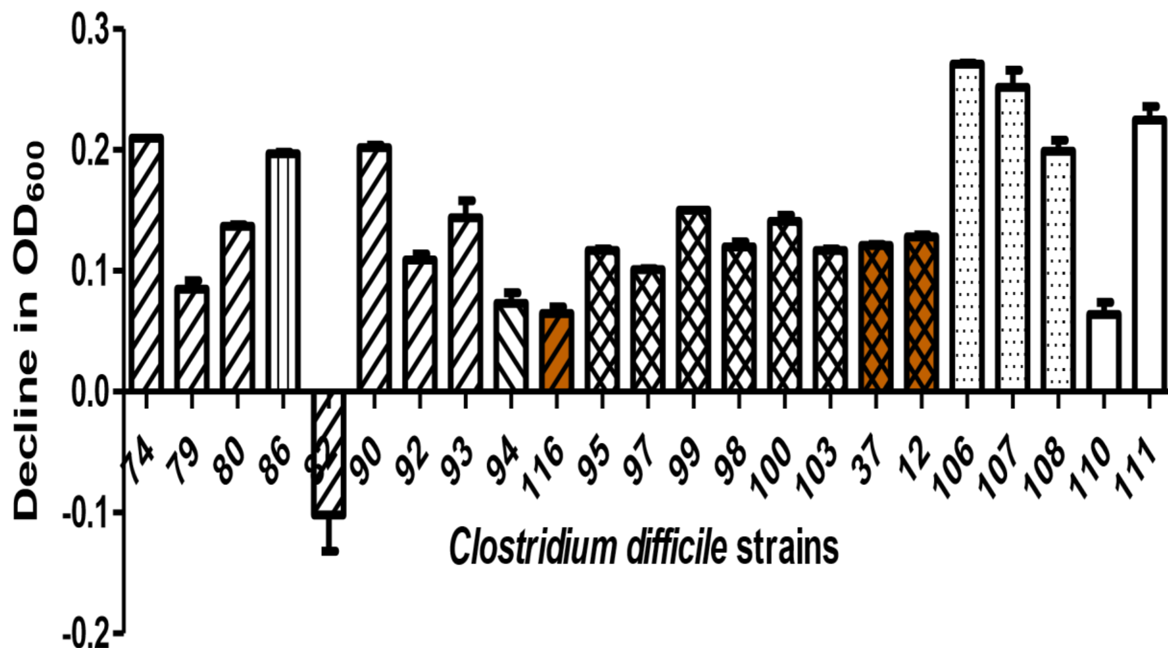

Figure 8. 1 Decline in OD600 of *C. difficile* strains exposed to metronidazole (10mg/L) in an uptake assay. Showing ribotype 001 (striped bars) 027 ribotype (crossed bars), Ribotype 106 (dotted bars). Clear bars are control strain E4 (110) (CDRM) and ATCC 700057 (111) (CDSM). CDSM strains were coloured brown

Decline in OD_{600} was observed to be ≤0.3 for all strains analysed, metronidazole susceptible *C. difficile* strains (CDSM) control strain ATCC700057 (111) had a higher OD decline compared to metronidazole Reduced susceptible *C. difficile* strains (CDRM) control strain E4 (110).

This difference was not reflected in the samples analysed between CDSM and CDRM strains. Ribotype 027 CDSM strains had similar values to the CDRM strains. While 001 CDSM strain 116 had the lowest decline of all tested 001 strains

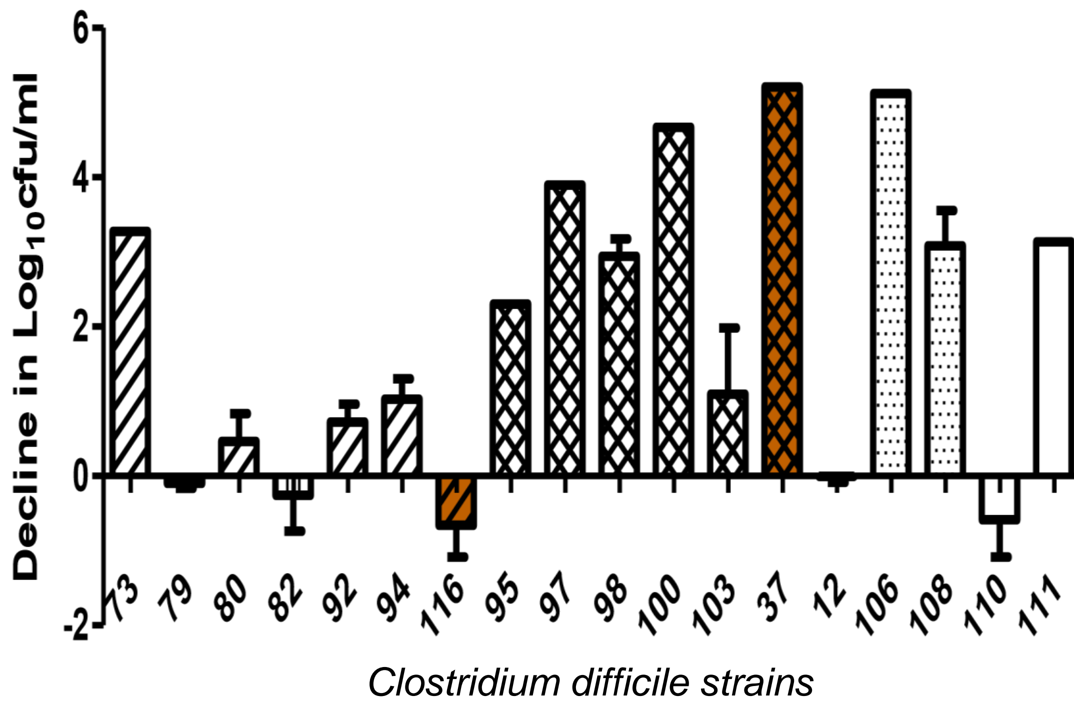

Figure 8. 2 Decline in total viable counts (log10-cfu/mL) of *C. difficile* strains exposed to metronidazole (10mg/L) in an uptake assay. Showing ribotype 001 (striped bars) 027 ribotype (crossed bars), Ribotype 106 (dotted bars).Clear bars are control strain E4 (110) (CDRM) and ATCC 70057 (CDSM), CDSM strains were coloured brown. CDSM strain 12 bar too minute to be coloured.

Decline in TVC was varied with strains and was not dependent on susceptibility phenotype as observed in Figure 8.2 above.

Table 8. 1 Mean metronidazole (MTZ) concentrations detected in cell-free cultures of *C. difficile* strains exposed to MTZ (10 mg/L); using a microbiological bioassay. *only one replicate demonstrated detectable MTZ

Ribotype 001 strain code	MTZ (mg/L)	**Ribotype 027 strain code**	MTZ (mg/L)	**Ribotype 106 strain code**	MTZ (mg/L)
73	0	**95**	0	**106**	3.05*
79	0	**97**	2.09*	**107**	0
80	0	**98**	0	**108**	0
86	1.36*	**99**	3.75		
82	0	**100**	0		
92	0	**103**	0		
94	0	**37(CDSM)**	8.4		
110	0	**12(CDSM)**	6.4		

8.4 DISCUSSION

Metronidazole has been reported to demonstrate bactericidal activity against *C. perfringens* and *B. fragilis in vitro* (Ralph & Kirby, 1975) and is generally considered a bactericidal antimicrobial (Nemeth, Oesch, & Kuster, 2014); which results in a 1000- fold decline in viable counts of bacteria. The results from the present study indicated that exposure of *C. difficile* (CDRM and CDSM) to 10mg/L metronidazole resulted in bactericidal activity in 7/19 *C. difficile* strains. Metronidazole susceptibility status did not correlate strongly with whether bactericidal activity was observed. The activity of antimicrobial agents against bacteria is known to vary with the medium composition, antimicrobial concentration, and also whether a solid medium or liquid medium is employed for MIC testing (O'Connor *et al.,* 2008). Therefore, the limited activity of metronidazole against some *C. difficile* strains in the present study may reflect the fact that in this assay a liquid medium was used to engage *C. difficile* vegetative cells and metronidazole, which is a different interaction than used in previous experiments (See Chapters 2 and 3) where a liquid culture was inoculated onto a solid medium containing metronidazole. It is possible that, although the *C. difficile* cultures were standardised to log-phase (approx. 0.55 OD_{600}), some *C. difficile* strains might have been early-log phase/late-log phase depending on their growth rate and maximum biomass yield which were not analysed in this study and might have affected the antimicrobial effect of metronidazole. The concentration of metronidazole might also affect the Pharmacodynamics of metronidazole, and 10 mg/L was selected in order to reflect the study of Lacey and colleagues (1993), but this is also a concentration of metronidazole that is reflective of the magnitude of drug seen in the faeces of patients and volunteers following oral therapy (Bolton & Culshaw, 1986). Regardless of these experimental variables, the study of Lacey and colleagues (1983) clearly demonstrated in *B. fragilis,* exposure of bacterial cultures to 10mg/L of metronidazole resulted in loss of free drug from cell-free culture supernatants, and the data observed from the present study reflected these findings regardless of metronidazole susceptibility status in the *C. difficile* strains evaluated. With respect to metronidazole susceptibility in anaerobes, it remains a possibility that reduced uptake of drug might contribute to metronidazole reduced susceptibility or resistance. When considering the CDRM and CDSM strains evaluated in the present study, there is little evidence that metronidazole uptake was attenuated in CDRM strains. In most *C. difficile* culture supernatants there was no recoverable metronidazole detected. Only six *C. difficile* cultures yielded recoverable metronidazole, one ribotype 001(CDRM), four ribotype 027 (2 for CDRM, 2 for CDSM), and one ribotype 106 (CDRM)

The growth changes in strains exposed to metronidazole compared with their control counterparts having no metronidazole did not correlate with their susceptibility profile. As was

observed using the OD readings the difference in growth as a result of exposure to metronidazole varied between strains and ribotypes. *C. difficile* strain E4 (Brazier *et al*., 2001) was less affected by 10mg/L in comparison to ATCC 700057 which was substantially adversely affected, the results correlated with their MIC of 16mg/L and 0.5mg/L respectively.

Interestingly, Tally *et al*, (1978) also reported that metronidazole uptake was observed both in susceptible and resistant bacteria which confirms the bioassay data in this research however in their research only the susceptible anaerobic bacteria metabolised the drug which was not in line with observation made in the present research.

All *C. difficile* strains exposed to metronidazole and their unexposed counterparts were also lysed in order to measure the intracellular concentrations of metronidazole using the bioassay technique but no metronidazole was observed in any samples. The data from this present experiment, suggest that the intracellular activated metronidazole concentrations does not play a role in CDRM phenotype. Metronidazole reportedly enters bacterial cells by passive diffusion, influenced in a positive loop by the intracellular reduction of metronidazole (Edwards *et al*, 1980). Thus the undetectable intracellular metronidazole observed in the present research indicates its reduction which facilitated the maximal uptake from the broth and correlated with an earlier report that the toxic radical formed from metronidazole is very short-lived and it disintegrates further into tiny nontoxic particles rapidly (Edwards *et al*, 1980).

Conclusion and future work

This study had shown that uptake of metronidazole is not a factor that is likely to contribute to metronidazole phenotype. In consideration of the above data discussed, future work in the determination of mechanism of development of reduced susceptibility in *C. difficile*, should be directed more towards the downstream processes involved in the mechanism of action of metronidazole. This might encompass the targets of the redox radical as suggested by Muller and Gorrell, 1983. It also useful to note that this research has observed that metronidazole reduction and uptake is taking place irrespective of the level of susceptibility. As activation of metronidazole increases the pressure diffusions of metronidazole into the cell. However, there is a possibility that a different mechanisms of metronidazole reduction take place for the CDRM strains compared to CDSM strains. This present research cannot also rule the possibility of metronidazole being reduced to its non-toxic amino radical (Carlier *et al,* 1997).

9.0 Effect of Hemin on The Susceptibility Level of *C. difficile* Strains to Metronidazole.

9.1 INTRODUCTION

Heme has been described as the split portion in haemoglobin that contains iron (Das, 2012) it is also referred to as the iron protoporphyrin section of haemoglobin that is insoluble (Saunders, 2003) and hemin is the chloride form of heme. In anaerobic bacterial growth, heme synthesis, which produces the cytochrome system is needed for nitrate reduction. Jacobs and colleagues demonstrated the importance of adding exogenous hemin to growth media containing nitrate, in order to stimulate growth under anaerobic conditions when studying six strains of *Staphylococcus* spp. (Jacobs *et al,* 1964). Microorganisms like all other living organisms require nutrient to grow and multiply, as well as transition elements like iron to aid metabolic processes (Awad *et al*, 2016). Iron levels in living organisms have been stated to be of importance as lowered levels decrease bacteria growth and excess iron cause chronic oxidative stress. Published data also suggest that directly or indirectly iron or iron related metabolites are associated to decrease in metronidazole susceptibility (Veeranagouda *et al,* 2014).

Metronidazole resistant mutants of *Bacteroides fragilis* (number of strains =32) were observed to have a mutated FeoAB gene (Veeranagouda *et al,* 2014). The minimum inhibitory concentration (MIC) reverted back to that of the parent strain when the growth medium was supplemented with iron. FeoAB is a protein linked to iron uptake in bacteria, thus reducing iron uptake may be linked to reduced susceptibility to metronidazole (Veeranagouda *et al*, 2014). It is possible that the metronidazole activation process linked to Pyruvate ferredoxin oxidoreductase (PFOR) is iron dependent. The importance of FeoB in growth and expression of virulence factors was reported in a *C. perfringens* strain with a mutated FeoB strain which demonstrated growth defects, reduced manganese uptake, reduced gas production, and reduced toxin production that were later resolved by adding iron supplements to the growth medium (Awad *et al,* 2016). The research noted FeoB a subunit of FeoAB was more prevalent of the 13 iron uptake systems identified using bioinformatics tools on *C. perfringens* (Awad *et al,* 2016). Another iron related protein linked to metronidazole susceptibility in bacteria is the ferric uptake regulator (Fur). Analysis of Fur, a bacterial regulator of iron homeostasis in bacteria, demonstrated increased Fur expression in metronidazole reduced susceptible *C. difficile* strains (CDRM) and fully-resistant ribotype 027 strains of *C. difficile* compared to a susceptible phenotype (Chong *et al.,* 2014). This result indicates Fur expression, and correspondingly iron bioavailability in bacteria, is associated with the decline in metronidazole susceptibility in *C. difficile* (Chong *et al,* 2014). Consequently, the present study was undertaken to evaluate if iron availability might be associated with the CDRM phenotype.

9.2 METHOD

Agar incorporation MICs were performed as described previously (see 2.22) but with the following alterations. Overnight Schaedler's broth cultures of *Clostridium difficile* were exposed to doubling dilutions of metronidazole (0.03- 64mg/L) on Wilkins-Chalgren agar, Wilkins-Chalgren agar supplemented with 5mg/L hemin (51280, Sigma Aldrich, Poole, UK), Brucella agar and Brucella agar supplemented with 5mg/L hemin. The CDRM strains studied were of three ribotypes 001 (N=7), 027 (N=5), and 106 (N=5), along with CDSM strains of ribotypes 001 (N=2), 027 (N=2). The ATCC 700057 metronidazole susceptible strain was used as an experiment control. Brucella agar was made from Brucella medium base (CM0169, Oxoid Basingstoke, UK) by adding 1.5% Bacteriological agar (LP0011, Oxoid Basingstoke, UK) supplemented with 10µL/L Vitamin K_1 (V3501, Sigma Aldrich, Poole, UK) and 5% (v/v) defibrinated horse blood (SR0050, Oxoid, Basingstoke, UK). The inoculated MIC plates were then incubated anaerobically at 37°C for 48 hours and MIC endpoints determined as previously described (see 2.22).

Statistical analysis was performed using Graphpad Prism 7.0, geometric mean MICs for antimicrobial agents' activity against the individual ribotype groups were calculated along with 95% confidence intervals.

A Mann Whitney test was used to test for statistical significance of MIC differences between groups and a P-value of >0.05 was considered statistically significant.

9.3 RESULT

On Wilkins Chalgren agar, MICs were consistent with or without supplementation of additional hemin (Figure 9.1) with exception of four Metronidazole reduced susceptible *C. difficile* strains (CDRM) strains (106 ribotype N=3, and 001 ribotype N=1) which had +/- 1 dilution difference in MIC. MIC was between 4-8mg/L (Figure 9.1) for CDRM strains. Metronidazole susceptible *C. difficile* strains (CDSM) 027 ribotype strains were observed to be 2mg/L MIC.

On Brucella Agar, MIC with Hemin ranged between 2-8mg/L for CDRM strains and MIC without hemin ranged between 2-4mg/L (Figure 9.2). Ribotype 027 CDSM strains MICs were observed to be 1mg/L without hemin and 2mg/L with hemin. MIC increase with hemin was observed in 56% of the total strains and approximately 63% of the CDRM strains when tested in Brucella agar.

Tests for significance demonstrated that there were no significant differences in metronidazole MICs in the absence of additional hemin in Wilkins Chalgren agar for all ribotypes (P>0.05)

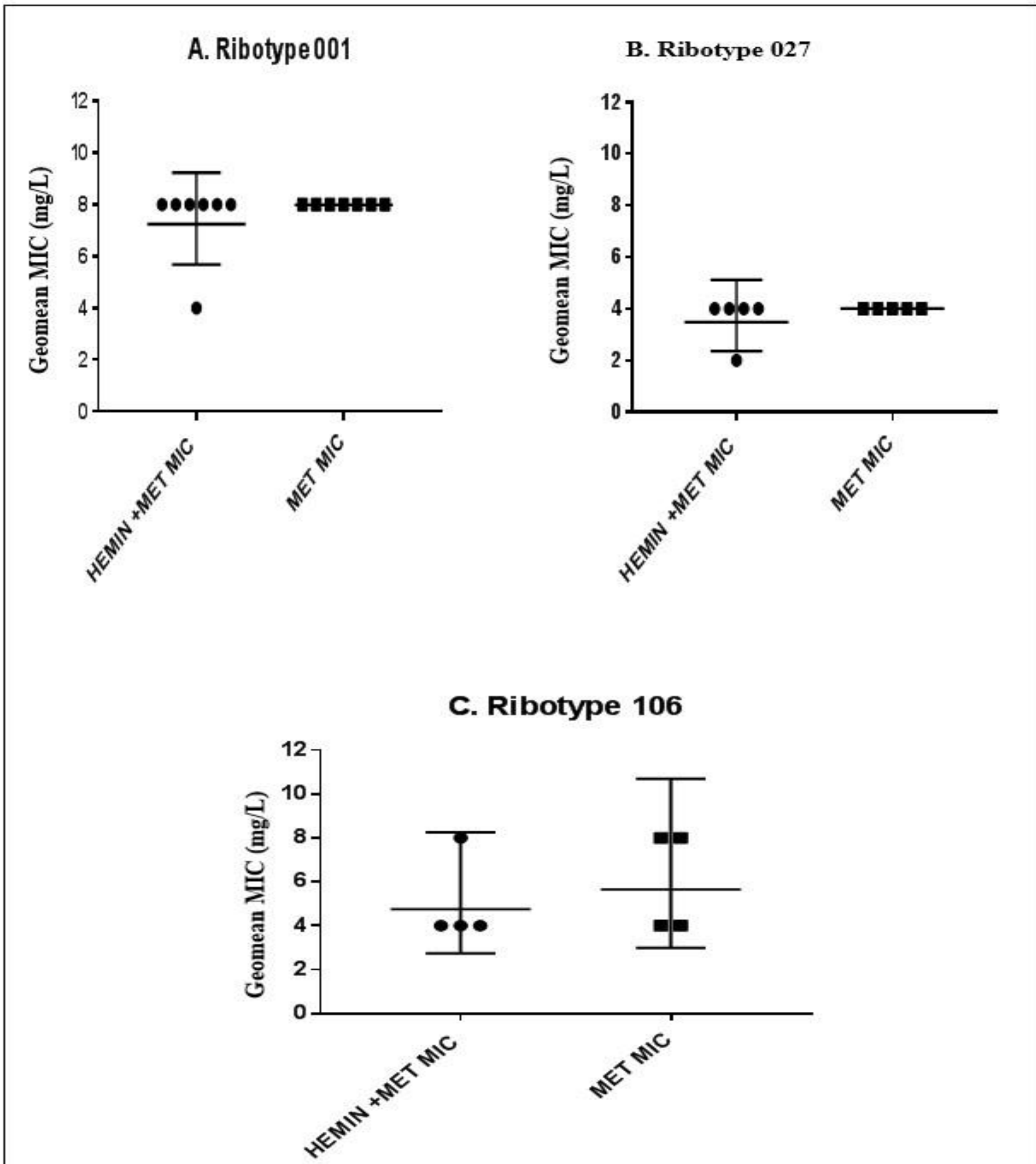

Figure 9. 1 Geometric mean metronidazole (MET) MICs (mg/L, ±SE) against *Clostridium difficile* with reduced susceptibility to metronidazole from PCR ribotypes 001 (N=7), 027 (N=5), and 106 (N=4) on Oxoid Wilkin's-Chalgren agar ±5 mg/L additional hemin.

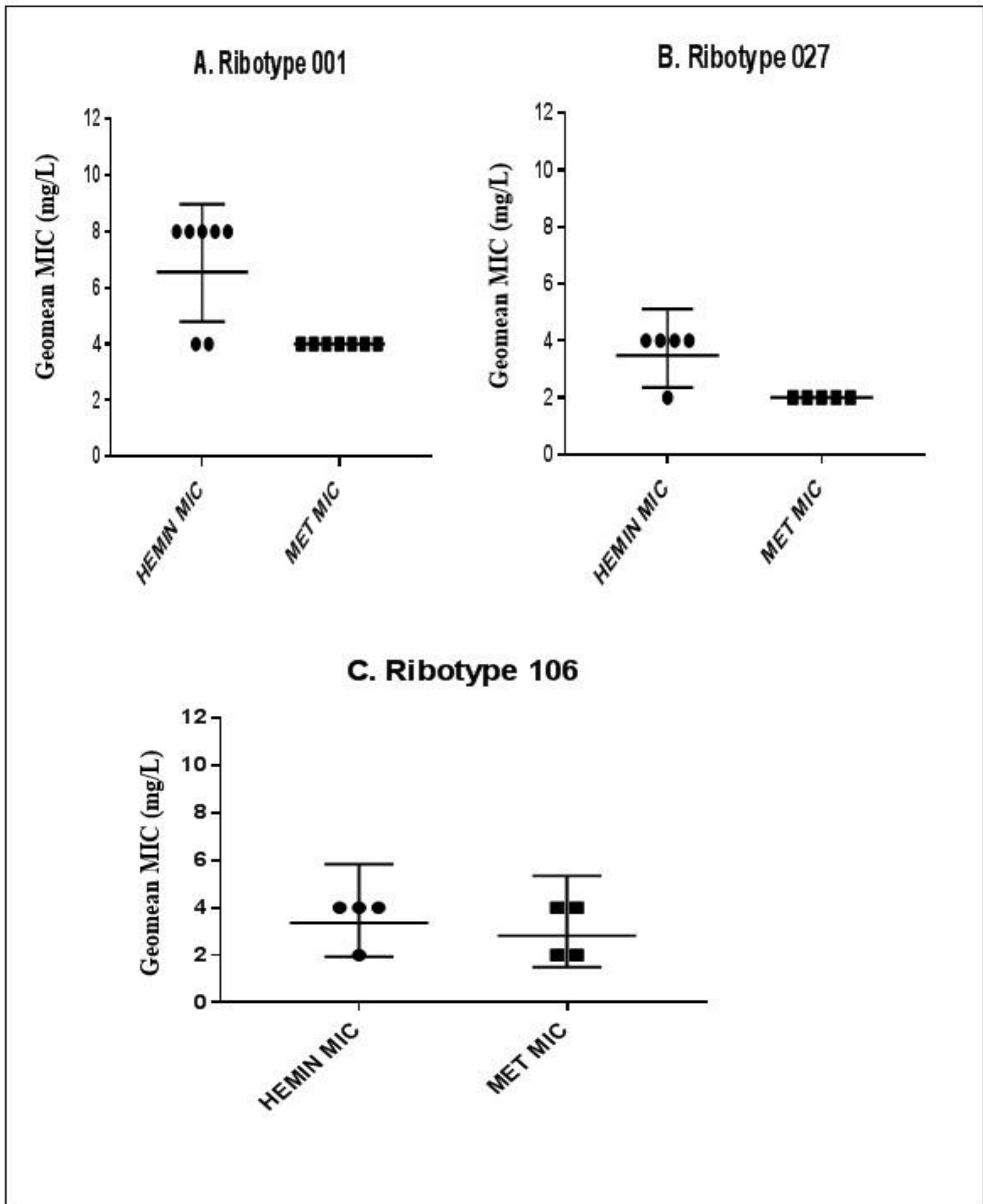

Figure 9. 2 Geometric mean metronidazole (MET) MICs (mg/L, ±SE) against *Clostridium difficile* with reduced susceptibility to metronidazole from PCR ribotypes 001 (N=7), 027 (N=5), and 106 (N=4) on Brucella blood agar ±5 mg/L additional hemin.

For CDRM strains on Brucella blood agar, tests for statistical significance demonstrated for ribotype 001, that addition of hemin significantly increased MICs (P=0.021, Figure 9.2) as was also the case for ribotype 027 (P = 0.048); however, for ribotypes 106 addition of hemin did not significantly affect metronidazole MICs.

9.4 DISCUSSION

C. difficile strains with reduced susceptibility to metronidazole have been reported previously and the mechanism and clinical significance of this reduced susceptibility remains to be determined (Baines *et al*., 2008). Baines and colleagues (2008) demonstrated that the CDRM phenotype was method dependent and Wilkin's-Chalgren agar was able to detect CDRM, whereas the Clinical & laboratory standards institute (CLSI) recommended method for the susceptibility testing of anaerobes using Brucella blood agar (no hemin) was not. In the present study of CDRM these elevated minimum inhibitory concentrations (MICs) on Wilkin's Chalgren agar were again demonstrated, although supplementation of Wilkins Chalgren agar with additional hemin did not further increase the MICs. Interestingly, the effect of hemin supplementation using Brucella blood agar clearly demonstrated significantly elevated MICs for CDRM from ribotypes 001 and 027, but not for ribotype 106. This raises the possibility that there may be a differential mechanism in metronidazole reduced susceptibility in *C. difficile* ribotypes. One possibility also is that the addition of hemin to Brucella agar increased the growth of the *C. difficile* inoculum on the agar, therefore an elevated inoculm might have produced an 'inoculum effect' and increased the MIC as has been observed in *H. pylori* for metronidazole (Hartzen *et al.,* 1997). Iron, a secondary product of hemin though has been observed to affect growth and metronidazole susceptibility in *C. perfringens* deficient of iron (Awad *et al,* 2016), hemin effect on growth was also observed in six staphylococci strains that were facultative anaerobes as well as in *Bacillus subtilis* an obligate aerobe (Jacobs *et al*, 1964). However, data from Awad *et al,* 2016 earlier mentioned, conflict with the observations of Wu and Hurdle (2015), where iron containing molecules (10 µM $FeCl_3$, $FeSO_4$) were not observed to affect metronidazole MICs. Wu and Hurdle (2015a) reported the effect of hemin supplementation on unstable metronidazole reduced susceptibility in *C. difficile.* Also, this present study observed that in Brucella agar 100% decline in MIC was observed without hemin for CDRM strains of ribotype 027, thus confirming the results reported on the effect of hemin on ribotype 027 strains (Wu and Hurdle, 2015a). Interestingly, Wu and Hurdle (2015a) showed that CDRM or resistant isolates metronidazole MICs declined to within the susceptible range (0.25-1.0 mg/L) in the absence of hemin and that Fe^{2+} and the heme catabolites biliverdin and bilirubin had no effect on MICs. It was also noted by the authors that the effect of hemin on metronidazole MICs was not consistent across all of the ribotypes studied, again reflecting observations from the present study. The decline in MICs observed in the present study in the absence of hemin-supplemented media was less than observed in previous studies, approximately a 2-fold reduction in geometric mean MIC compared to a 4 -10-fold reduction seen by Wu and Hurdle (2015a). Again, this difference along with the reported stability

differences in MICs may be suggestive of differing mechanism of reduced susceptibility to metronidazole. Also of note was the moderate difference in the concentration of hemin supplemented into Brucella agar; in the present study 5mg/L (7.7 µM) was used, whereas Wu and Hurdle (2015a) used 10µM (6.5 mg/L), and this may have potentially affected the results observed.

Notable to point that though the absence of hemin resulted in metronidazole MIC decline, it was observed that all reduced susceptible strains still retained the reduced susceptible phenotype. Hemin supplementation resulted in MIC increase from 1mg/L to 2mg/L for CDSM strains of ribotype 027 (Appendix). As these strains were originally selected as CDSM strains their MICs were not involved in the statistical analysis thus there MIC fluctuation was only mentioned in result section but it had no effect on the statistical result presented.

Also as earlier stated the observed hemin effect on metronidazole susceptibility could be associated with the effect of iron related factors on gene expression/hypermutability in *C. difficile*. Some *C. difficile* strains passaged in the presence of metronidazole were cultured at passage 10, 13 and 15 with and without hemin. At passage 15, hemin addition resulted in an increase of metronidazole MIC much more than at passages 10 and 13 and the authors demonstrated mutations in the iron sulfur cluster regulator IscR (Wu and Hurdle, 2015b). IscR is a global gene regulator confirmed by its role in *P. aeruginosa* one of its roles is intracellular iron homeostasis modulation (Romsang *et al,* 2014). Though one of the known roles of IscR is heme metabolism, more research is required to detect the role of IscR mutations in *C. difficile*.

In the study of Chong *et al* (2014), two genes related to iron were observed to have been linked to reduce susceptibility to metronidazole and resistance in *C. difficile*. FeoB, a ferrous iron transporter protein B, and an iron compound ABC transporter substrate protein decreased in expression in the metronidazole-resistant *C. difficile* strain analysed after addition of metronidazole.

While the ferric uptake regulator (Fur), an iron homeostasis central regulator, was observed in the absence of metronidazole to be more highly expressed in the resistant and reduced susceptible *C. difficile* compared to the susceptible phenotype tested, addition of metronidazole did not elicit much changes in Fur expression (Chong *et al,* 2014).

Conclusion and future work

Further research analyzing of the genome sequence of reduced susceptible phenotype would provide more in-depth information on factors contributing to metronidazole reduced susceptibility. The results from the present study clearly demonstrate that hemin affects the susceptibility of CDRM to metronidazole, and offers an intriguing possibility that heme

containing substances (e.g. occult blood) might contribute to reduced efficacy of metronidazole in CDI patients where bleeding occurs.

10.0 Whole genome sequencing (WGS) of *Clostridium difficile*

10.1 INTRODUCTION

Whole genome sequencing (WGS) can be employed to elucidate possible genetic variations in bacterial genomes including single nucleotide polymorphisms (SNPs) which could be a possible cause of phenotype traits including resistance to antimicrobials, and can also be used as a molecular typing approach which has a similar resolution to highly discriminatory techniques such as MLVA.

Dominguez *et al* (2015) typed *C .difficile* strains using next generation sequencing to analyse 27 *C .difficile* isolates from children during a clinical outbreak (Dominguez *et al,* 2015). Whole genome sequencing was also used to analyse samples of *C .difficile* obtained from adult patients showing *C. difficile* infection symptoms, in the hospital and community settings in the UK. Single nucleotide variants (SNV) were detected and *C. difficile* isolates in 3 years were observed to be different genetically from earlier isolates (Eyre *et al.,* 2013). WGS has also been used in the Netherlands to detect the similarity of the ribotype 078 isolates isolated from animals and farmers, and the researchers demonstrated no SNP differences in the genome sequences of *C. difficile* isolates, thus removing the possibility of an intermediate host (Knetsch *et al.,* 2014). Additionally, the same insertion of tetracycline and streptomycin resistance determinants were reported in the ribotype in both animals and human. The authors postulated that transmission between human and animals might be possible based on these data, through the faecal oral route especially from pigs to humans. However it was not ruled out that it was possible that both populations could have been infected from the same source since both are exposed to similar environment (Knetsch *et al.,* 2014).

Analysis of short gene sequences have been used to replace susceptibility testing of the phenotype as it tends to be more rapid and practicable in clinical sampling, although genotypic variations do not always confer phenotypic resistance to antimicrobials. Whole genome sequence for susceptibility testing, though not yet in practice, could be advantageous in obtaining a variety of antimicrobial susceptibility profile rapidly (Feuerriegel *et al*, 2014). This was demonstrated in an earlier research by Koser *et al*., (2013) where Illumina MiSeq was used for rapid whole genome sequencing and 39 genes known to confer resistance in *Mycobacterium tuberculosis* were interrogated. Other than the susceptibility profile, a mutation in a gene responsible for the activation of one of the drugs- paraaminosalicyclic acid was identified. The use of WGS for susceptibility testing is more advantageous when certain mutations have already been linked to resistance to specific antimicrobial agent. Thus, the use of WGS may

not be fully aimed at replacing phenotypic testing but only to get a rapid antimicrobial susceptibility profile of a clinical sample (Koser *et al.,* 2013), which is important as not all mutations confer resistance. The maximal analysis of a sample sequence is advised to prevent false positives or false negatives that could be as a result of silent genetic variations in the genome (Koser *et al.,* 2013). An earlier study mapped out 18 genes linked to resistance to ten antimicrobial agent in *Mycobacterium spp.* Seventy one bacterial strains were analysed and 121 polymorphisms were found; 58 of which were markers linked phylogenetic properties and these data was suggested to aid future genotypic assays design (Feuregelle *et al*, 2013).

WGS was also used to identify resistance genes in *Bacteroides thetaiotamicron* to metronidazole as well as other antibiotics such as tetracycline and the results showed a number of resistance genes including *tetX, tetQ,* and *ermF.* Additionally, a plasmid-located *nimD* was identified along with a second *nim*-like sequence (Sadarangani *et al*, 2014)

In a 2012 study, a novel method of detecting resistance genes was developed using ResFinder, which was developed to screen published genomes for resistance genes. The study indicated that though Polymerase chain reaction (PCR) has been in use but this can only detect specific genes targeted by PCR primers, thus excluding other resistance genes that could be present (Zankari *et al*., 2012). Analysing the whole genome gives this benefit and new genes can be found. The use ResFinder gives the advantage to high weight data as is generated by whole genome sequence. Resfinder was used to analyse some NCBI stored whole genome sequences of 30 isolates, and the genes were identified accurately (Zankari *et al*., 2012).

A novel organism *Bacteroides genomospecies* was identified in United States using WGS; the organism was earlier wrongly identified as *Bacteroides fragilis* using known accurate techniques MALDI-TOF mass spectrometry and 16S RNA sequencing. Thus, the inclusion of WGS in bacterial identification might be of clinical importance as it could aid accurate detailed specification of the offending pathogen (Salipante *et al.,* 2015). Genome sequencing was used along with experimental evolution and functional analysis was used to map out *E. coli* antimicrobial cross resistance and detected the underlying cause (Lazar *et al,* 2014)

Whole genome sequencing has been used to characterize the metronidazole susceptibility profile of hypervirulent *C .difficile* ribotype 027 (Lynch *et al.,* 2013). Identifying genetic variations that could have led to phenotypic changes for the resistant strain (Lynch *et al.,* 2013). Moura *et al,* 2014 also characterised the resistance to metronidazole phenotype in *C .difficile* non toxigenic ribotype by sequencing and analysing the genome.

The present study was performed in order to assess the whole genome sequences of two *Clostridium difficile* strains with reduced susceptibility to metronidazole in order to gain an insight into the potential molecular mechanisms which may contribute to metronidazole reduced susceptibility when genomes were analysed comparatively with metronidazole susceptible strains.

10.1.1 Aim

To analyse the genome of two *C. difficile* isolates with the reduced susceptibility to metronidazole phenotype, with the aim of detecting mutations that can be linked to reduced susceptibility to metronidazole.

10.1.2 Objectives

To extract, purify, and quantify genomic DNA of *Clostridium difficile* strains 80 (ribotype 001) and E4 (ribotype 010 coded as 110) such that it is suitable for WGS.

To use PacBio sequencing and genome assembly and annotation of *Clostridium difficile* strains 80 (ribotype 001) and E4 (ribotype 010) and bioinformatics analysis to interrogate the genomes for potential genetic variations that might contribute to the metronidazole reduced susceptible *C. difficile* strains (CDRM) phenotype.

10.2 METHOD

10.2.1 DNA extraction

Overnight brain heart infusion broth (CM1135, Oxoid, Basingstoke, UK) cultures of individual CDRM strains of ribotype 001 and ribotype 010, strains 80 and 110 respectively, were centrifuged at 16000 rpm for 2mins to pellet the cells. To the cells was added: 120µL of 20 mg/ml lysozyme (L6876, Sigma-Aldrich, Poole, Dorset, UK), 3µL of 10µg/µL RNase A and 89 µL of TE was added to lyse cells. The mixture was incubated overnight at 37°C and vortexed after the addition of 150µl of Promega Wizard™ lysis solution and 10µL of proteinase K (A1120, Promega UK, Southampton, UK).

The lysed cell mixture was then incubated at 70°C for 15 minutes on a heating block (Grant Dry Block, Grant Instruments, Royston, UK), vortexing every 5 minutes. Then the cell extracts were chilled on ice for 5 minutes and 175µL of protein precipitation solution from the Promega Wizard™ extraction kit was added and extracts were vortexed for 10 seconds, and centrifuged at 4°C at 16000rpm for 15 minutes. The supernatant was then transferred to a new tube containing 3µL of Rnase A, mixed gently, and then incubated at 37°C for 30 minutes. To the supernatant 500µL of isopropanol (I9516, Sigma Aldrich, Poole, UK) was added and the tubes were inverted several times. Tubes were then centrifuged at 4°C at a speed of 16000 rpm to pellet DNA. The supernatant was then carefully decanted to avoid removing the pellet and the pellet was washed with 500µL 70% ethanol (652261, Sigma Aldrich, Poole, Dorset, UK). Residual ethanol was removed following centrifugation at 16000rpm for 2 minutes and the DNA pellet was the air-dried for 5 minutes at room temperature and resuspended in 35-50µL of Rnase-free water.

The purity and quantity of DNA was detected using Nano drop (1000 3.8.1, Thermo Scientific, UK) and qubit fluorimeter (3, Invitrogen life technologies, UK). The DNA was then sent for sequencing, using a Pacific Biosciences RSII sequencer along with subsequent genome assembly and annotation at the Earlham Institute (Norwich Science Park, University of East Anglia, Norwich UK)

10.2.2 Bioinformatics analysis

Nucleic acid sequence data obtained from the Earlham Institute was viewed using File Viewer Plus (Sharpened productions, US) and Snap gene software (GSL Biotech Kenwood Avenue, Chicago). These were used to search for 33 genes of interest (Table 10.1) that have previously been associated with metronidazole resistance (Lynch *et al.,* 2013; Chong *et al*., 2014). Identified genes were aligned with reference genome *Clostridium difficile* strain 630 known to

be susceptible to metronidazole (MIC = 0.25 mg/L). *C. difficile* strain 110 (E4) sequences were also compared with the previously published genome of a metronidazole resistant ribotype 010, strain 7032989 (MIC = 32 mg/L), which was a non-toxigenic *C. difficile* strain isolated from Spain (Moura *et al.,* 2014). Nucleotide sequences were aligned using National center for biotechnology information (NCBI) basic local alignment search tool (BLAST).

To determine the effect of the identified SNPs on amino acid sequence, further analysis was carried out on the genes detected to possess SNPs. The amino acid sequence of these genes of were aligned with that of CD 630 using pair wise alignment tool on Geneious software (R10 Biomatters, Auckland, New Zealand)

Table 10. 1 Gene targets assessed using bioinformatics tools for *C. difficile* PCR ribotype 001 (strain 80) and ribotype 010 (strain 110, E4) with reduced susceptibility to metronidazole.

GENE DESCRIPTION	**GENE**
Gyrase A	*gyrA*
Anti-sigma factor protein	*rsbW*
DNA directed RNA polymerase	*rpoA*
Glycerol-3-phosphate dehydrogenase	*glyC*
UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase	*murD*
V-type ATP synthase subunit C	*ntpC*
Homoserine dehydrogenase	*Hom1*
Stage 0 sporulation protein A	*Spo0A*
DNA topoisomerase 1	*topA*
Ferric uptake regulation protein	*fur*
Thiamine biosynthesis protein	*thiH*
Putative germination specific protease	*cspC*
Endonuclease IV	*nfo*
Toxin A	*tcdA*
Rubrerythrin	*rbr*
Endonuclease subunit C	*sbcC*
Anaerobic nitric oxide reductase flavorubredoxin	*norV*
exodeoxyribonuclease	*exoA*
5-nitroimidazole reductase	*nimB*
Ferrous iron transport protein A	*feoA1*
Ferrous iron transport protein B	*feoB1*
thioredoxin	*trxA1*
Thioredoxin reductase	*trxB1*
Thiol peroxidase	*bcp*
Thioredoxin reductase	*trxB2*
Thioredoxin	*trxA2*
Thioredoxin reductase	*trxB3*
Electron transfer protein	*prdC*
Excinuclease ABC subunit B	*uvrB*
Excinuclease ABC subunit C	*uvrC*

GENE DESCRIPTION	GENE
Excinuclease ABC subunit A	*uvrA*
Pyruvate flavodoxin oxidoreductase	*nifJ*
Oxygen independent coproporphyrinogen III oxidase	*hemN*

10.3 RESULTS

Table 10. 2 DNA yield and purity for DNA preparations from *C .difficile* PCR ribotypes 001 and 010, strains 80 and 110.

Strains	Ribotype	DNA(ng/ µl)	Approx. volume (µl)	total	260/280	260 /230
110	010	296	200	59200	1.83	2.35
80	001	272	100	27200	1.85	1.90

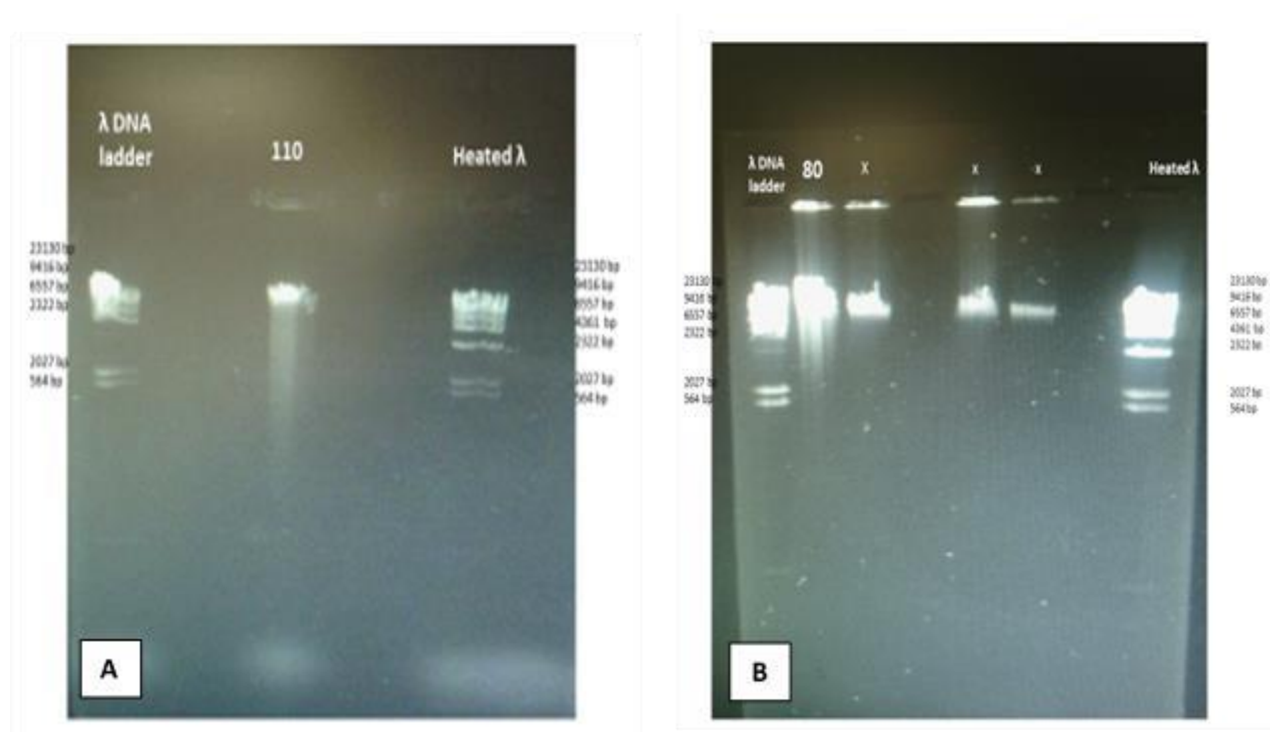

Figure 10. 1 Purified high molecular weight DNA from *C. difficile* strains 110 (A, ribotype 010) and 80 (B, ribotype 001) visualised on an agarose gel (1% w/V) alongside a lambda DNA high molecular ladder (11873943, Fisher Scientific, Loughborough, UK) the X lane are DNA in tubes not sent for sequencing.

10.3.1 Whole Genome Sequence (WGS) Analysis

Strain 110 of ribotype 010, had 16 genes aligned successfully, alignment with metronidazole susceptible reference strain CD630, yielded 10 genes with SNPs, the other genes had 100% identity. While alignment with metronidazole resistant strain of ribotype 010 CD7032989 showed 13 genes had 100% identity and 4 genes had variants as observed in table 2 below.

Table 10. 3 Gene analysis for sample 110 with reference genome, identifying percentage similarity and identifying SNPs

Protein	**GENE**	**Nucleotide identity (%) to CD630**	**SNP location**	**Nucleotide identity (%) to CD7032989**	**SNP location**
gyrase A	*gyrA*	2425/2427 (99%)	2106 (C-T) 245 (T-C)	99%	245 (T-C)
Glycerol-3phosphate dehydrogenase	*glyC*	1018/1020 (99%)	495(A-G), 351 (T-A)	100%	
V-type ATP synthase subunit C	*ntpC*	100%		100%	
Stage 0 sporulation protein A	*Spo0A*	100%		100%	
DNA topoisomerase 1	*topA*	2084/2088 (99%)	572 (C-T) 651 (g-A) 1141 (A-G) 1743(G-T)		1016 (a- gap)
Ferric uptake regulation protein	*fur*	100%		100%	
Endonuclease IV	*nfo*	835/837(99%)	765(G-A), 797 (A-G)	99%	539 (gap-T)

Protein	**GENE**	**Nucleotide identity (%) to CD630**	**SNP location**	**Nucleotide identity (%) to CD7032989**	**SNP location**
Anaerobic nitric oxide reductase flavorubredoxin	*norV*	1192/1194 (99%)	45(T-A), 861 (T-C)	100%	
exodeoxyribonuclease	exoA	752/753 (99%)	747 (G-A),	100%	
5-nitroimidazole reductase	*nimB*	467/468 (99%)	389 (C-A)	100%	
Thiol peroxidase	*bcp*	465/465 (100%)		465/466 (99%)	408 (gap-A)
Electron transfer protein	*prdC*	100%		100%	
Excinuclease ABC subunit B	*uvrB*	1966/1971 (99%)	1374 (T-C)	100%	
Excinuclease ABC subunit C	*uvrC*	1818/1818 (100%)		100%	
Pyruvate flavodoxin oxidoreductase	*nifJ*	3532/3540 (99%)	813 (C-T) 2796 (A-G) 2946 (T-C) 2607(T-C) 3223 (C-T) 3290(T-C) 3462(G-A) 3174 (T-C)	100%	

Protein	**GENE**	**Nucleotide identity (%) to CD630**	**SNP location**	**Nucleotide identity (%) to CD7032989**	**SNP location**
Oxygen independent coproporphyrinogen III oxidase	*Hem N*	1172/1176 (99%)	510(C-T) 615(T-G) 891 (C-T) 949 (a-G)	1176/1176 (100%)	

10.3.2 Amino acid sequence analysis.

The genes with observed SNPs were selected to undergo amino acid sequence analysis, silent mutations and point mutations were identified as shown below.

Mutations in genes with SNPs of strain 110

Figure 10. 2 Alignment of Oxygen independent coproporphyrinogen III oxidase gene of sample 110 with same gene in CD630 detecting point mutations

Figure 10. 3 Alignment of Exodeoxyribonuclease gene of sample 110 with same gene in CD630 detecting point mutations

Figure 10. 4 Alignment of Gyrase A gene of sample 110 with same gene in CD630 detecting point mutations

Figure 10. 5 Alignment of Endonuclease IV gene of sample 110 with same gene in CD630 detecting point mutations

Figure 10. 6 Alignment of 5-nitroimidazole reductase gene of sample 110 with same gene in CD630 detecting point mutations

Figure 10. 7 Alignment of Gyrase A gene of sample 110 with same gene in CD7032989 detecting point mutations

10.3.4 Sample 80

Ten genes from *C. difficile* strain 80 (ribotype 001) were aligned to those of comparator *C. difficile* genes, others were either not present or had multi subunits. Six genes were observed to have SNPs while the remaining genes demonstrated 100% identity with the same gene in CD630 as observed in table 10.3 below.

Table 10.3: Gene analysis for sample 80 with reference genome, identifying percentage similarity and identifying SNPs

Protein	**GENE**	**Nucleotide identity (%) CD630**	**Position /snp(q-s)**
Stage 0 sporulation protein A	*Spo0A*	824/825(99%)	802 (T-C)
Ferric uptake regulation protein	*fur*	459/459(100%)	
Endonuclease IV	*nfo*	837/837(100%)	
Toxin A	*tcdA*	8130/8133(99%)	7713(G-A) 4331(C-T)
Anaerobic nitric oxide reductase flavorubredoxin	*norV*	1193/1194(99%)	861(T-C)
exodeoxyribonuclease	*exoA*	752/753(99%)	603 (G-A)
5-nitroimidazole reductase	*nimB*	468/468(100%)	
Thiol peroxidase	*bcp*	463/465(99%)	84(C-T), 171 (A-T)
Electron transfer protein	*prdC*	1314/1314(100%)	
pyruvate flavodoxin oxidoreductase	*nifJ*	3523/3540(99%)	1587 (G-A), 1674 (T-C) 1794 (C-T), 2358 (G-A), 2372 (G-T)2387 (T-C), 2390(C-T)

10.3.5 Gene of sample 80 with same gene in CD630 detecting point mutations

700 710
Consensus Cys Ser Phe Ile Cys Pro His Xaa Cys Ile Arg Pro Val Leu Val Thr
Identity
1. CD 630 PFOR Cys Ser Phe Ile Cys Pro His Ala Cys Ile Arg Pro Val Leu Val Thr
2. PROKKA_001... Cys Ser Phe Ile Cys Pro His Ser Cys Ile Arg Pro Val Leu Val Thr

730 740
Consensus Gly Leu Glu Gly Leu Lys Tyr Arg Xaa Gln Xaa Ser Pro Leu Asp Cys Thr Gly
Identity
1. CD 630 PFOR Gly Leu Glu Gly Leu Lys Tyr Arg Ile Gln Val Ser Pro Leu Asp Cys Thr Gly
2. PROKKA_001... Gly Leu Glu Gly Leu Lys Tyr Arg Met Gln Leu Ser Pro Leu Asp Cys Thr Gly

790 800
Consensus Ala Pro Lys Xaa Asp Val Met Pro Xaa Asn Thr Val Lys Gly
Identity
1. CD 630 PFOR Ala Pro Lys Val Asp Val Met Pro Ala Asn Thr Val Lys Gly
2. PROKKA_001... Ala Pro Lys Gly Asp Val Met Pro Val Asn Thr Val Lys Gly

900 910
Consensus Glu Leu Val Ser Met Xaa Ile Cys Glu Asp Ala Lys
Identity
1. CD 630 PFOR Glu Leu Val Ser Met Asp Ile Cys Glu Asp Ala Lys
2. PROKKA_001... Glu Leu Val Ser Met Gly Ile Cys Glu Asp Ala Lys

1,060 1,070
Consensus y Ala Asp Lys Asn Gln Phe Ile Lys Ala Val Xle Glu Ala Glu Lys His Asp
Identity
1. CD 630 PFOR y Ala Asp Lys Asn Gln Phe Ile Lys Ala Val Leu Glu Ala Glu Lys His Asp
2. PROKKA_001... y Ala Asp Lys Asn Gln Phe Ile Lys Ala Val Ile Glu Ala Glu Lys His Asp

Figure 10. 8 Alignment of Pyruvate flavodoxin oxidoreductase showing mutations gene of sample 80 with same gene in CD630 detecting point mutations

Figure 10. 9 Alignment of Toxin A first point mutation gene of sample 80 with same gene in CD630 detecting point mutations

Figure 10. 10 Alignment of Toxin A second point mutation gene of sample 80 with same gene in CD630 detecting point mutations

Silent mutations in genes with SNPs of strain 110 (E4) on alignment with CD630 was observed in excinuclease ABC subunit B gene, Glycerol-3-phosphate dehydrogenase, Anaerobic nitric oxide reductase flavorubredoxin, DNA topoisomerase 1, Pyruvate flavodoxin oxidoreductase. Silent mutations in genes with SNPs of strain 80 on alignment with CD630 was observed in anaerobic nitric oxide reductase flavorubredoxin, Thiol peroxidase, exodeoxyribonuclease and SpoA gene.

Both strains 110 and 80 had SNPs observed in anaerobic nitric oxide reductase flavorubredoxin (*norV*), exodeoxyribonuclease and pyruvate flavodoxin oxidoreductase gene relative to CD630. In addition, strain 80 had SNPs in Stage 0 sporulation protein A (Spo0A) gene, Toxin A tcdA, thiol peroxidase (bcp).

While for strain 110, the genes SNPs relative to strain CD630 were gyrase A (*gyrA),* Glycerol-3-phosphate dehydrogenase (*glyC),* Homoserine dehydrogenase (*Hom1*), Thiamine biosynthesis protein (*thiH*), Endonuclease IV (*nfo*), exodeoxyribonuclease(*exoA*), 5nitroimidazole reductase (*nimB*), Ferrous iron transport protein A (*feoA1*), Ferrous iron transport protein B(*feoB1*), Thioredoxin reductase (*trxB3*), Excinuclease ABC subunit B (*uvrB*), Excinuclease ABC subunit A (uvrA), Excinuclease ABC subunit C (*uvrC*).

10. 4 DISCUSSION

Metronidazole reduced susceptibility and resistance presents a clinical issue for the treatment of CDI, as metronidazole is a cost-effective first-line therapy. Since metronidazole achieves only low luminal concentrations in the colon of CDI patients following oral dosing, even small MIC increases may be clinically significant. Resistance to metronidazole in *C. difficile* has been reported previously (Pelaez, *et al.,* 2002, Pelaez*, et al.,* 2008, Brazier *et al.,* 2001) but the mechanisms of resistance were not determined, other than ruling out the involvement of *nim* genes. Several recent reports have suggested potential mechanisms of resistance in *C. difficile* following whole genome sequencing bioinformatics analysis (Lynch *et al.,* 2013, Chong, *et al.,* 2014, Moura *et al.,* 2014).

Lynch *et al*, (2013), characterized a stable metronidazole resistant *C. difficile* strain of ribotype 027 phenotypically and at the genomic level. Some phenotypic features were linked to gene mutations, one of which was the aberrant growth *in vitro.* The high level metronidazole resistant *C. difficile* strain had low cell density and an extended lag phase compared to the reduced susceptible and susceptible strain, thus depicting a nutrient limitation. At the genetic level, a mutation in thiamine biosynthesis protein (*thiH)* and Oxygen independent coproporphyrinogen III oxidase *(hemN)* genes were observed (Lynch *et al.,* 2013), in line with Lynch *et al*, (2013), mutations were also observed in the present research in *thiH* and *hemN* gene in strain 110 (E4). Lynch *et al.,* (2013), also showed a point mutation in *Spo0A* that was present in the resistant phenotype (CD26A54_R) but absent in the reduced susceptible phenotype tested which was indicated to be a possible cause of lack of sporulation up to 48hours in CD26A54_R. The absence of mutation in the reduced susceptible phenotype on the *Spo0A* gene was in line with our research as no mutation was found for strain 110 (E4) *Spo0A* gene. Contrastingly, the clinical ribotype 001 isolate analysed did possess a SNP in *spo0A,* but the phenotypic impact of this mutation remains to be determined given that sporulation capacity was not evaluated in the present study.

Also genome analysis of a metronidazole resistant ribotype 027 has previously led to the identifications of mutations in Pyruvate flavodoxin oxidoreductase (*nifJ*) and glycerol-3phosphate dehydrogenase *(glyC*), these genes has also been observed to be linked to the electron transport (Lynch *et al*, 2013). Mutations in these genes as was observed in the present experiment could predispose to alteration of the electron transport which has been reported to affect redox potential within the cell and production of energy thus could affect the activation and diffusion of metronidazole into the cell (Lynch *et al*, 2013).

Mutations in DNA repair proteins such as excinuclease and endonuclease IV were observed in strain 110 which were also observed previously in the metronidazole resistant and reduced susceptible 027 ribotype, thus increased levels of DNA repair can predispose to reduced susceptibility in metronidazole (Lynch *et al*, 2013). Chong *et al*, (2014) also reported increase in gene expression in DNA repair proteins such as excinnuclease ABC, endonuclease IV, exodeoxyribonuclease after carrying out a proteomic assay to detect changes that could affect susceptibility to metronidazole in *C. difficile*. Lynch *et al*, (2013) also observed point mutation in the ferric uptake regulator gene (*fur)* as well as increase in expression of the gene in a *C. difficile* proteomic assay as a contributor to metronidazole resistance (Chong *et al,* 2014). However, in the present study 100% alignment with the *fur* gene in CD630 was observed in both strains 80 and 100. Thus at genomic level mutations in the *fur* gene have not contributed to metronidazole reduced susceptibility which is in line with the findings of Moura *et al,* (2014), where the genome of *C. difficile* strains of the ribotype 010 were analysed and no genetic mutations were observed in the *fur* gene.

Point mutations were further observed in anaerobic nitric oxide reductase flavorubredoxin (*norV*) and thiol peroxidase that codes for redox active proteins which was suggested to protect against nitric oxide (Emerson *et al,* 2008) and has been earlier reported to increase in expression, in reduced susceptible and metronidazole resistant *C. difficile* (Chong *et al.,* 2014).

NimB was observed by Chong *et al,* (2014) to increase in expression by 3 fold more in reduced susceptible and metronidazole resistant *C. difficile* compared to the susceptible strain. These expression increases were observed without pre-exposure to metronidazole (Chong *et al.,* 2014). *Nim* genes have been observed to convert nitro-imidazole to a nontoxic derivative, therefore contributing directly to metronidazole resistance in *Bacteroides fragilis* and other anaerobes (Gal and Brazier, 2004, Theron *et al.,* 2004). The mutation in this gene reported in the present study in strain E4 (110) could have activated the gene as this SNP exist in the resistant reference strain CD7032989 thus 100% alignment was observed with the strain E4 (110) sequence. Therefore, metronidazole may be up-taken and converted into an amino radical by *nimB* and not a nitroradical which would affect the toxicity of the antibiotic (Carlier *et al*, 1997). Further studies involving knocking out *nimB* and complementing *nimB* back into the CDRM strain E4, would be required in order to determine if this is indeed the case.

Silent mutations and four single nucleotide polymorphisms were as well observed in the present research in the *hemN* gene in strain E4 (110). The *hemN* gene codes for a protein that is part of heme biosynthesis, coproporphyroinogen 111 oxidase. Mutations in this gene could account

for slow growth, decrease in hemin biosynthesis and affect electron transport which could decrease the amount of metronidazole activated (Roggenkamp *et al.,* 2004, Chong *et al.,* 2014).

Conclusion and future work

In summary, the action of metronidazole as reported by Edward *et al*, (1980, 1993) was dependent on the DNA of the cell and activation of the drug which influences the uptake, and subsequent toxicity of the nitro-radical formed. The activation is dependent on the electron transfer occurring within the cell. In this chapter we have identified single nucleotide polymorphisms in some of the genes linked to electron transfer, activation, oxidative stress and DNA repair. Thus indicating that the multifactorial feature of metronidazole resistance earlier observed in 027 ribotypes (Lynch *et al*, 2013) and in Spanish strains of 010 ribotype (Moura *et al*, 2014) is also present in metronidazole reduced susceptible UK isolates of 010 and 001 ribotype as observed in this current research.

Future work, could entail analysis of non-protein coding regions for example the enhancer, regulatory DNA and promoter regions to identify mutations linked to metronidazole reduced susceptbility. Future work may also involve WGS of additional clinical *C. difficile* strains in order to look for consistency in the postulated genomic components that might be involved in CDRM as well as others. Structural analysis identifying the potential impacts of SNPs on protein structures of the relevant proteins evaluated in this study would enlighten as to the domains in the proteins/enzymes where mutations occurred and therefore predictions might be made about their functionality. Unpicking the factors that contribute most strongly to CDRM would be a complex process and is beyond the scope of this current study, but is certainly warranted to enhance the knowledge of the *C. difficile* community with relation to the activity of metronidazole

CONCLUSION

This research has studied the phenotypic and genotypic features of metronidazole reduced susceptible *Clostridium difficile* (CDRM) UK isolates. Metronidazole is a cost-effective firstline therapy for CDI, so metronidazole reduced susceptibility and resistance presents a clinical issue for the treatment of CDI, thus indicating the importance of characterising the metronidazole reduced susceptible phenotype of *Clostridium difficile*.

In this present study it was observed that the CDRM phenotype is heterogeneous and the phenotype is unstable in the absence of metronidazole, except for the control CDRM strain 110 (E4) which was stable. The MIC heterogeneity assay gave an insight to the MIC distribution of CDRM and CDSM strains of varying ribotypes, thus depicting the variability of the susceptibility phenotype in distinct clones of the same *C. difficile* isolate. The serial passage experiments evaluating CDRM and CDSM clones in the presence and absence of sub-inhibitory concentrations of metronidazole led to a consistent decrease in MIC in the absence of a selection pressure, thereby confirming the unstable nature of the reduced susceptible phenotype of UK *C. difficile* isolates. The AUC data obtained from the population analysis profile has further characterised the metronidazole reduced susceptibility phenotype of the colony forming units in the ribotypes analysed, indicating that the greatest AUCs were observed for ribotype 001 CDRM strains, which was the first ribotype identified in which CDRM emerged in 2008 (Baines *et al*., 2008). Confirming the susceptibility test that ribotype 001 had a higher reduced susceptible level compared to the other ribotypes analysed.

The serial passage experiments highlighted the instability of the reduced susceptible phenotype to be only in the absence of metronidazole, where the highest level of instability was also observed in *C. difficile* ribotype 001, which showed a decline in metronidazole MIC_{50} from 4mg/L – 0.5mg/L after passage experiment without sub-inhibitory concentrations of metronidazole. Although not much literature is available on *Clostridium difficile* serial passage experiments with a metronidazole selection pressure, the MIC stability observed under selective antibiotic pressure in the present study was also reported in a serial passage experiments with surotomycin and SMT19969 against *C. difficile* (Mascio *et al.,* 2014; Vickers *et al.,* 2011). However this study has done 5 serial passages, future work might increase the number of passages to detect the effect on the CDRM phenotype.

Metronidazole is a prodrug that requires activation following entry into the cell in order to demonstrate antimicrobial activity, therefore, a nitroreductase assay was performed in the

present study to assess the activation of metronidazole in CDRM. Nitroreductase activity was not consistently detected in *C. difficile* using method of Rafil *et al.,* (1991), this may be attributed to the substrate specificity in nitroreduction processes described by Sisson *et al.*, (2002). The cell death observed in the PAP test confirmed that metronidazole is activated in the UK *C. difficile* isolates tested. This is further confirmed by the metronidazole uptake assay, as more than 50% of strain analysed were observed to uptake metronidazole such that residual metronidazole in the cell-free medium was below the limits of experimental detection. Consequently, there were no deficiencies in metronidazole uptake in most CDRM strains evaluated in the present study and although nitroreduction was studied, there needs to be refinement in the nitroreduction assay in order to gain a more definitive assessment of the efficacy of nitroreduction in CDRM strains. All strains were able to reduce metronidazole, since metronidazole was active at the MIC, however, there may potentially be deficiencies in the efficacy of nitroreduction which could not be definitively evaluated in the present research.

The metronidazole uptake assay also clarified that the CDRM phenotype was not related to deficiencies in drug reduction and uptake. It does not however eliminate possibility of metronidazole being reduced to its non-toxic amino radical as earlier observed by Carlier *et al.*, (1997). This function was observed to be carried out by *nim* genes, and although no *nim* genes were observed by using PCR amplification of universal primers against *C. difficile*, which also reflected the *nim* PCR results observed in the present study. *NimB* has been observed in earlier proteomic studies of *C. difficile* ribotype 027 (Chong *et al.,* 2014). Furthermore, in the present research using WGS, a *nimB* gene was identified in the CDRM strain E4 (coded as 110 of ribotype 010) which possessed a SNP when aligned with CD630 metronidazole susceptible strain *nimB*. Contrastingly, 100% alignment was observed when CDRM strain E4 *nimB* was aligned with a metronidazole-resistant *C. difficile* strain in this current study. This does not just confirm the presence of the *nim* gene in *C. difficile* but also highlights the possibility that the SNP detected could activate the conversion of metronidazole to its non-toxic amino radical, as *nimB g*ene has been observed both in susceptible and resistant *C. difficile* but with SNP.

Additionally, mutations in the *hemN* gene that encodes coproporphyroinogen 111 oxidase which is involved in heme biosynthesis, were detected in this study in CDRM strain E4 (110), mutation in this gene has earlier been observed by Lynch *et al.,* (2013). It was also observed in this study that supplementation of hemin predisposed to increase in MIC of the CDRM strains of ribotype 027 and 001 UK isolates, which is in line with Chong *et al.,* (2014) where addition of hemin was observed to aid the growth of resistant 027 *C. difficile* ribotype.

The WGS study also identified SNPs in other genes in the CDRM strains analysed which may be linked to metronidazole mechanism of action, which were in pyruvate flavodoxin oxidoreductase (*nifJ*) and glycerol-3-phosphate dehydrogenase *(glyC*). Mutations in these genes observed in the present experiment, could predispose to alterations of electron transport which has been reported to affect redox potential within the cell and production of energy thus could affect the activation and diffusion of metronidazole into the cell (Lynch *et al*, 2013). Mutations in DNA repair proteins such as excinuclease and endonuclease IV were also identified in strain E4 (110). Metronidazole is converted to its nitro radical that damages the DNA, so increased DNA repair can predispose to reduced susceptibility in metronidazole (Lynch *et al*, 2013).

Future work, will be needed to detect the expression of these genes as well as other genes such as *fur* gene where no SNP was observed in this study, as change in the expression of these genes could as well affect the susceptibility to metronidazole (Chong *et al,* 2014). Also protein structure of these genes would be a useful addition to detect the actual locations of the SNPs which would provide more knowledge on the impact of the SNPs detected.

This is the first known study to identify the *nimB* gene in a genomic assay of CDRM *C. difficile* UK isolates, as well as the SNP that could be responsible for its activation into a nontoxic variant. Further studies would be required in the analysis of the genome and amino acid sequence, to design its primers as it has been confirmed in this study, as well as in previous study that the universal *nim* primers that have been observed to amplify other *nimB* genes, does not amplify these *nimB* in *C. difficile.* Also a *nimB* gene knockout experiments would identify the actual functionality of this gene alongside complementing back into a CDRM strain a wild-type *nimB* to see if the metronidazole susceptible phenotype is restored. A similar approach could also be undertaken for the other genome components linked to the activity of metronidazole.

In summary, this study aimed to characterize reduced metronidazole susceptibility in UK *C. difficile* isolates, which has been achieved by characterizing phenotypic and genotypic features of these isolates detecting its level of heterogeneity and instability in the absence of metronidazole, as well as hemin nutrient dependence and the presence of SNPs in genes linked to the metronidazole mechanism of action which could predispose to reduced susceptibility to metronidazole in *C. difficile*.

Reflective feed back

The aim of this work is to characterize the metronidazole reduced susceptibility phenotype of *C. difficile* isolates. A reflection of my study on completion, has shown that if I was to do the work again, I would change the order in which I carried out my research. I would carry out the whole genome experiment after doing the susceptibility test but before detecting factors that could predispose to the metronidazole reduced susceptibility phenotype, as that would have changed the dynamics of the work.

The whole genome sequence (WGS) analysis has highlighted genes linked to the metronidazole reduced susceptibility phenotype for example snps were observed in PFOR. Thus instead of just doing a nitroreductase assay, perhaps a research detecting PFOR activity would have been done.

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Appendices

APPENDIX 1.0

Table A.1.1: MIC results for CDRM strains tested using agar incorporation method

Ribotype	Strain	Metronidazole (mg/L)	Vancomycin (mg/L)
001	73	4	2
	74	8	0.5
	75	4	0.5
	76	4	4
	77	4	0.5
	78	4	0.25
	79	4	0.5
	80	8	0.5
	81	4	0.5
	82	4	0.5
	83	4	0.5
	84	0.125	0.5
	85	4	0.5
	86	8	1
	87	4	0.5
	88	4	0.5
	89	2	1
	90	4	0.5
	91	4	0.5
	92	4	0.25
	93	4	0.25
	94	8	0.5

Ribotype	Strain	Metronidazole (mg/L)	Vancomycin (mg/L)
027	95	2	0.5
	96	2	0.5
	97	4	0.5
	98	4	0.5
	99	4	0.5
	100	4	0.5
	101	2	0.5
	102	2	0.5
	103	2	0.5
106	104	4	0.5
	105	2	0.5
	106	2	0.5
	107	4	0.5
	108	4	0.5
	109	4	0.5
	110	8	1
	111	0.125	0.5

Table A.1.2. MIC results for CDSM strains tested using agar incorporation method

Ribotype	Strains	Metronidazole (mg/L)	Vancomycin (mg/L)
001	114	0.5	2
	115	0.25	0.5
	116	0.5	2
	117	0.5	0.5
106	118	2	1
	119	1	0.5
	120	0.125	0.5
027	35	0.5	0.5
	37	0.5	0.5
	41	0.5	0.5
	12	1	0.5

APPENDIX 2.0

POPULATION ANALYSIS PROFILE

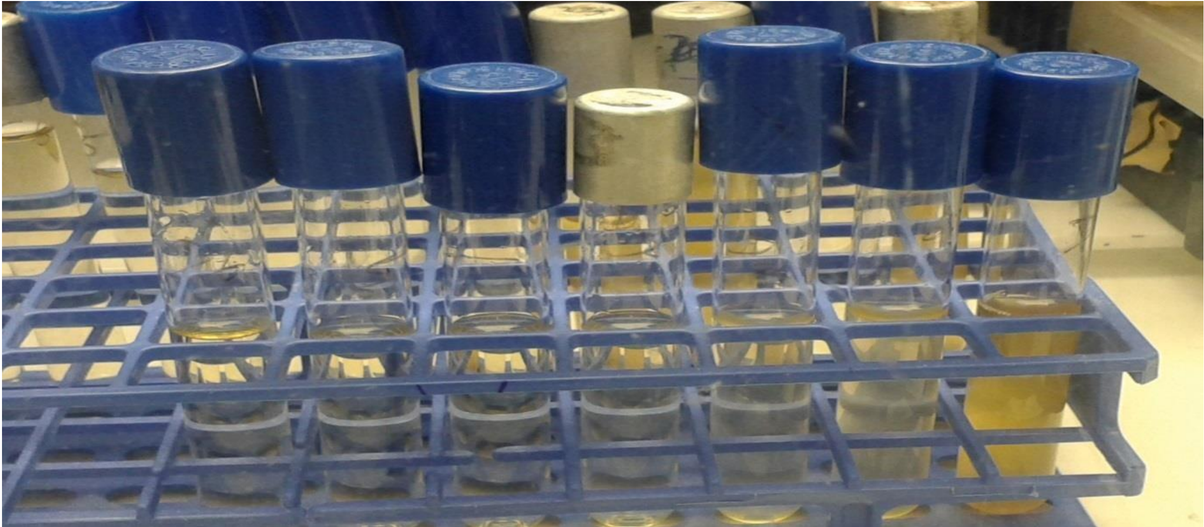

Figure A.2.1: Serial dilution of neat culture from the right to left for PAP test.

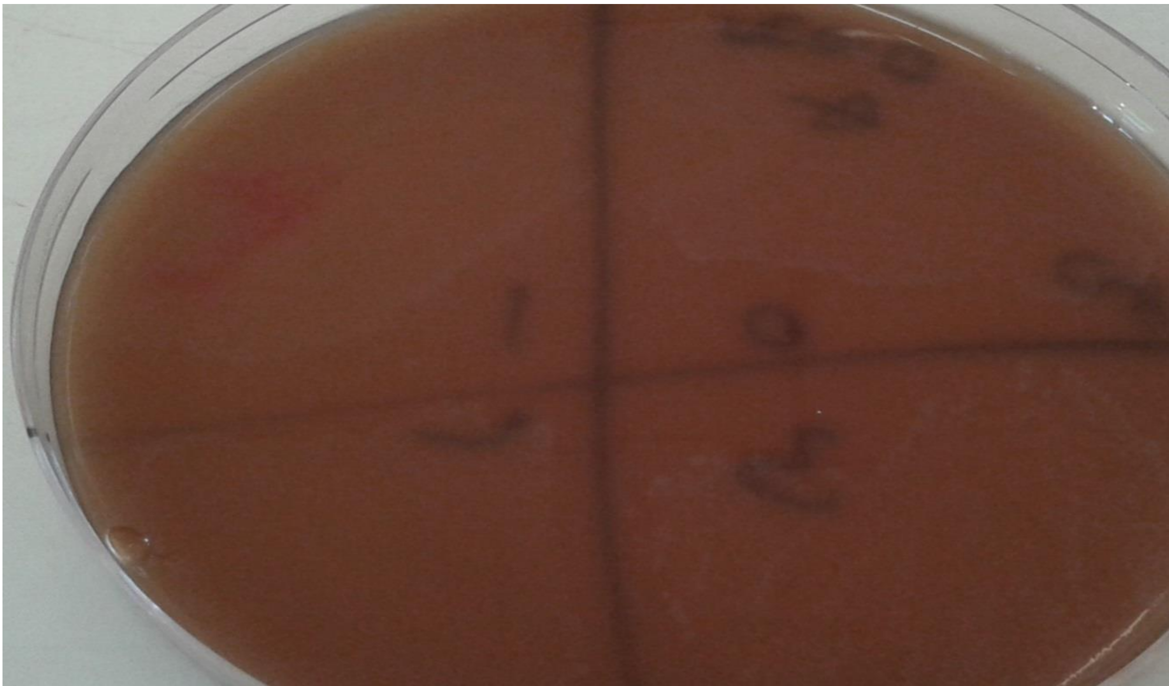

Figure A.2.2: Braziers agar inoculated with *C. difficile* diluted from neat culture to 10^3

Figure A.2.3: Braziers agar inoculated with *C. difficile* showing dilutions from 10^4 to 10^7

Population analysis profile graph flipped to show cfu decline

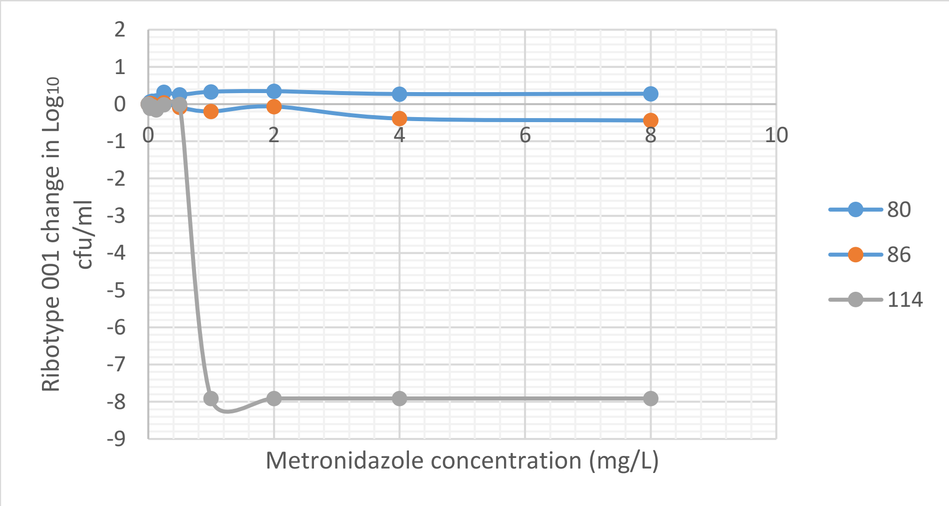

Fig A.2.4. Population analysis profile in ribotype 001 CDRM (blue) and CDSM (GREY) strains, exposed to doubling concentrations of metronidazole (mg/L) in Oxoid Wilkin's Chalgren Agar

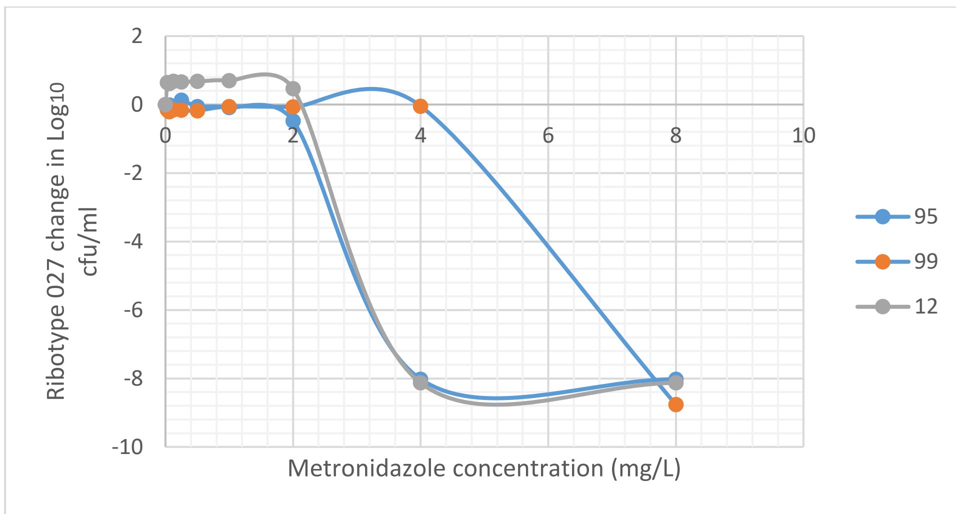

Fig A.2.5 Population analysis profile in ribotype 027 CDRM (blue) and CDSM (GREY) strains, exposed to doubling concentrations of metronidazole (mg/L) in Oxoid Wilkin's Chalgren Agar

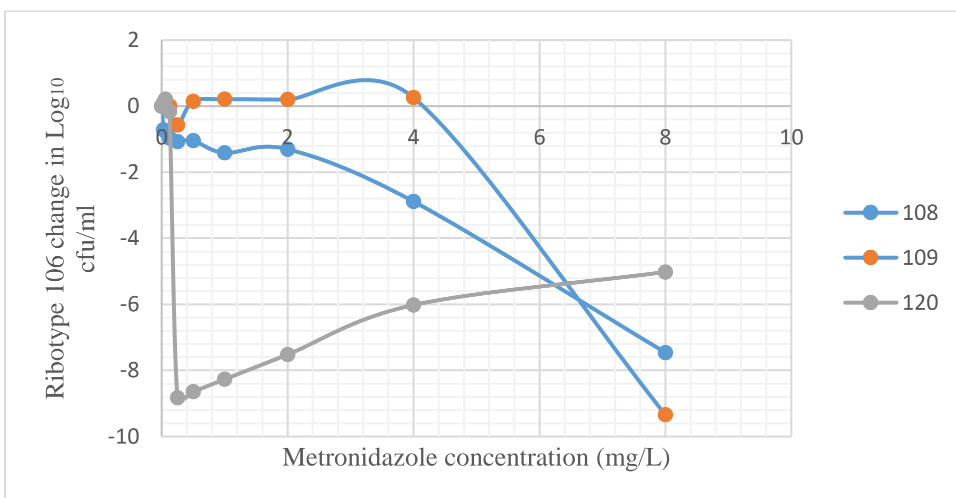

Fig A.2.6 Population analysis profile in ribotype 106 CDRM (blue) and CDSM (GREY) strains, exposed to doubling concentrations of metronidazole (mg/L) in Oxoid Wilkin's Chalgren Agar

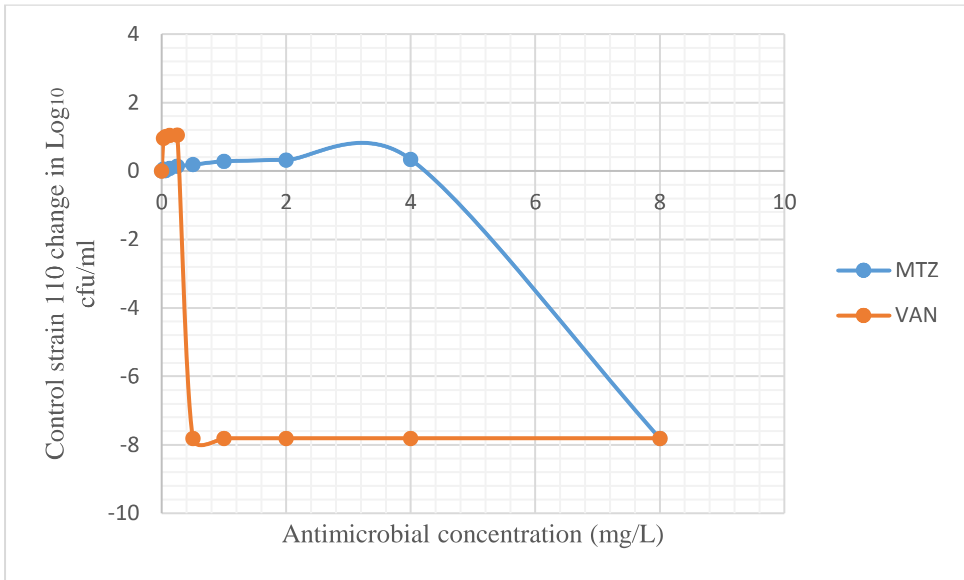

Fig A.2.7. Population analysis profile in control strain 110 CDRM (blue) and CDSM (GREY) strains, exposed to doubling concentrations of Antibiotics (mg/L) in Oxoid Wilkin's Chalgren Agar

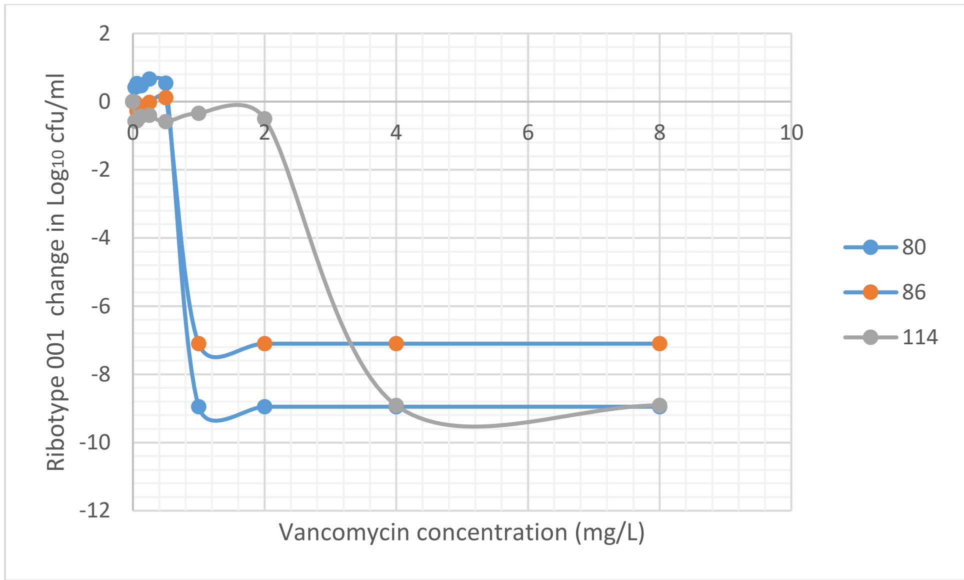

Fig A.2.8. Population analysis profile in Ribotype 001 CDRM (blue) and CDSM (GREY) strains, exposed to doubling concentrations of vancomycin (mg/L) in Oxoid Wilkin's Chalgren Agar

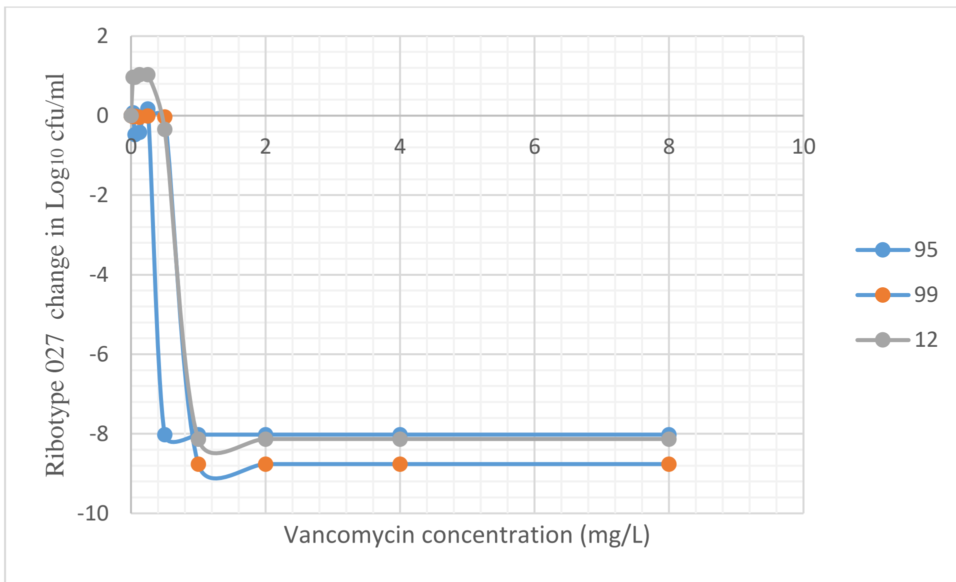

Fig A.2.9 Population analysis profile in Ribotype 027 CDRM (blue) and CDSM (GREY) strains, exposed to doubling concentrations of vancomycin (mg/L) in Oxoid Wilkin's Chalgren Agar

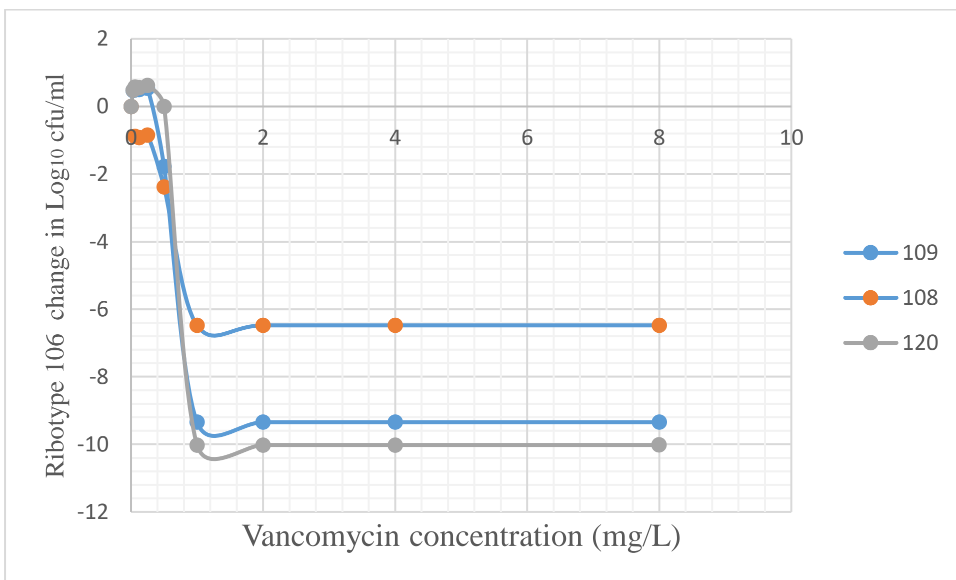

Fig A.2.10 Population analysis profile in Ribotype 106 CDRM (blue) and CDSM (GREY) strains, exposed to doubling concentrations of vancomycin (mg/L) in Oxoid Wilkin's Chalgren Agar

APPENDIX 3.0

Serial passage results for all strains in the *C. difficile* ribotypes analysed in exposure to metronidazole. Fig 3.1-3.6 has some strains with yellow bars these indicate CDSM strains.

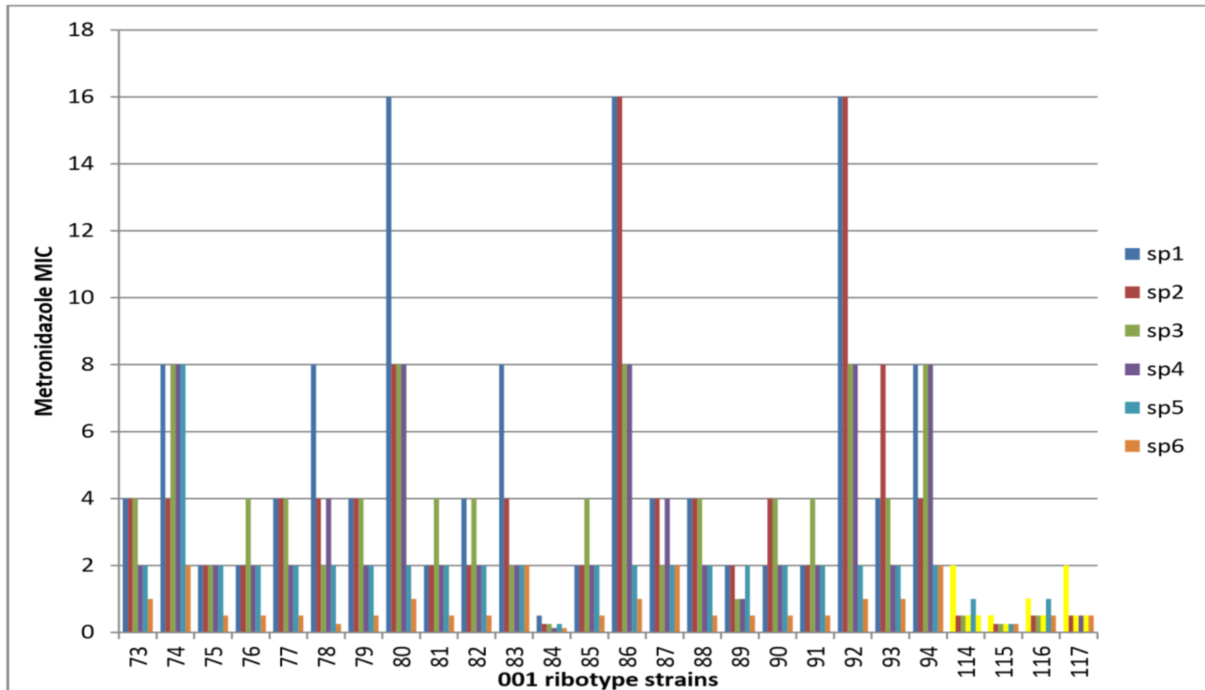

Figure A.3.1: serial passage of 001 ribotype strains without subinhibitory concentrations of metronidazole. CDSM strains were the set bars with yellow colour strain 114, 115, 116, 117

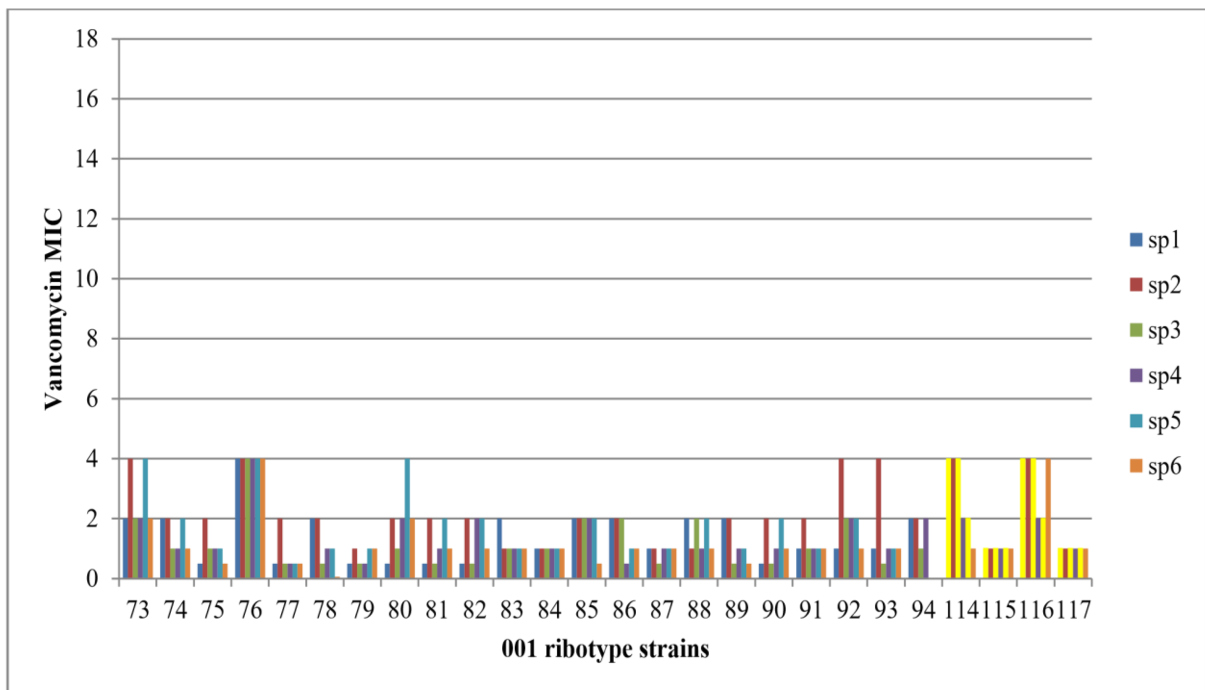

Figure A.3.2: serial passage of 001 ribotype strains without subinhibitory concentrations of vancomycin. CDSM strains were the set bars with yellow colour strain 114, 115, 116, 117

.

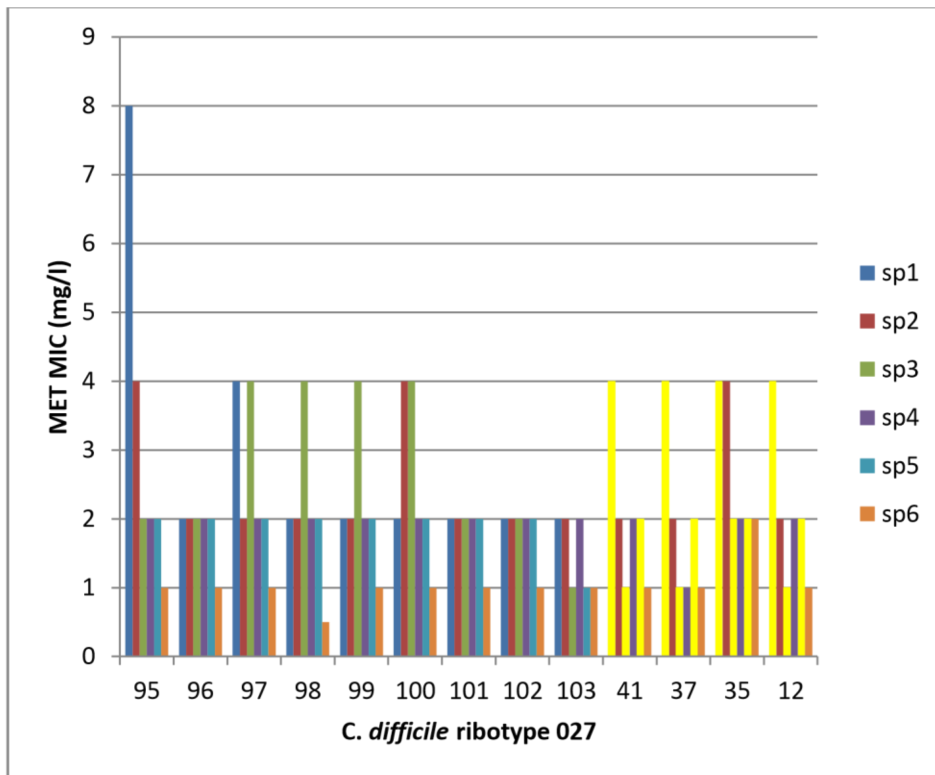

Figure A.3.3: serial passage of 027 ribotype strains without subinhibitory concentrations of metronidazole. CDSM strains were the set bars with yellow colour strain 41, 37, 35, 12

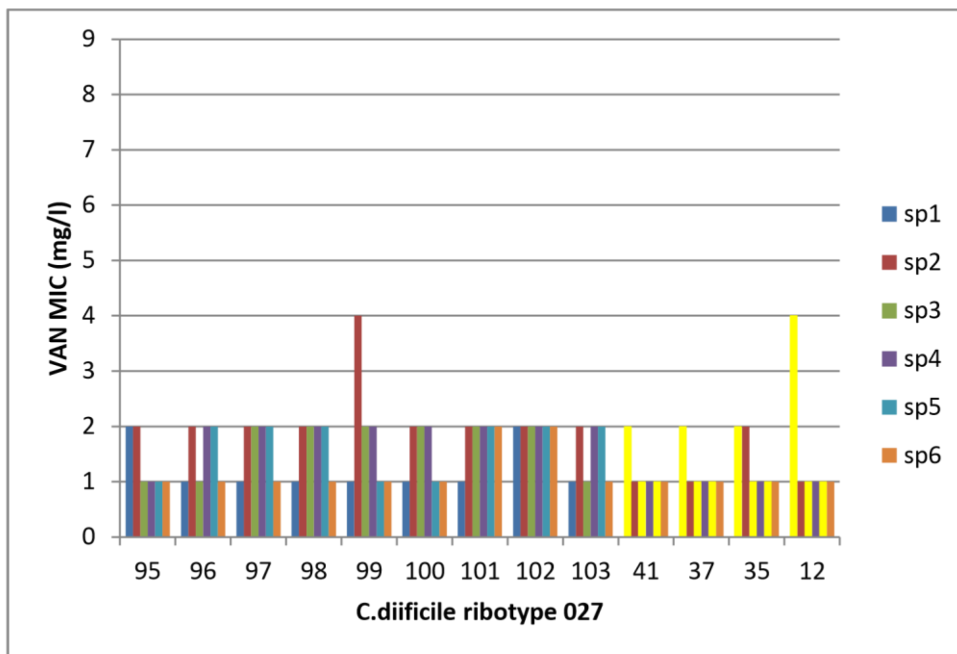

Figure A.3.4: serial passage of 027 ribotype strains without subinhibitory concentrations of Vancomycin. CDSM strains were the set bars with yellow colour strain 41, 37, 35, 12

.

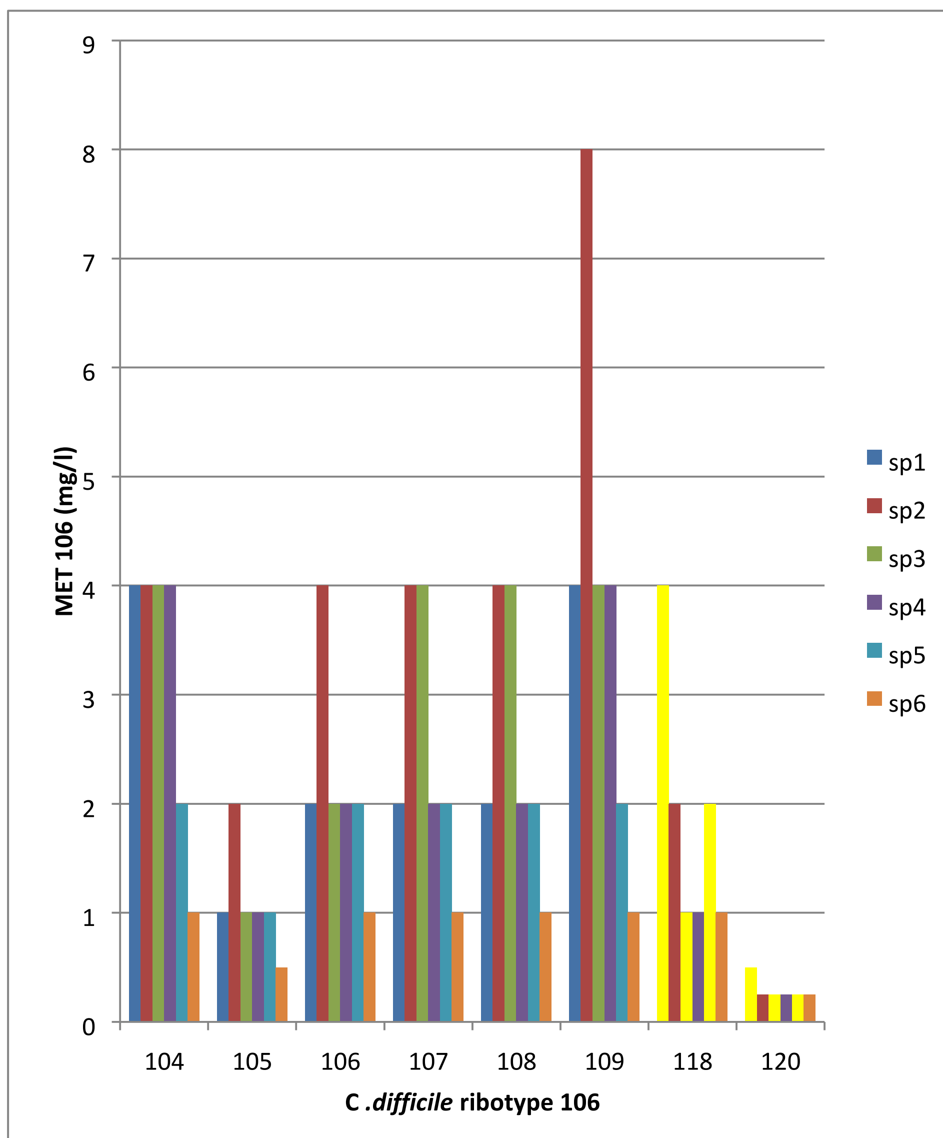

Figure A.3.5: serial passage of 106 ribotype strains without subinhibitory concentrations of metronidazole. CDSM strains were the set bars with yellow colour strain 118, 120

.

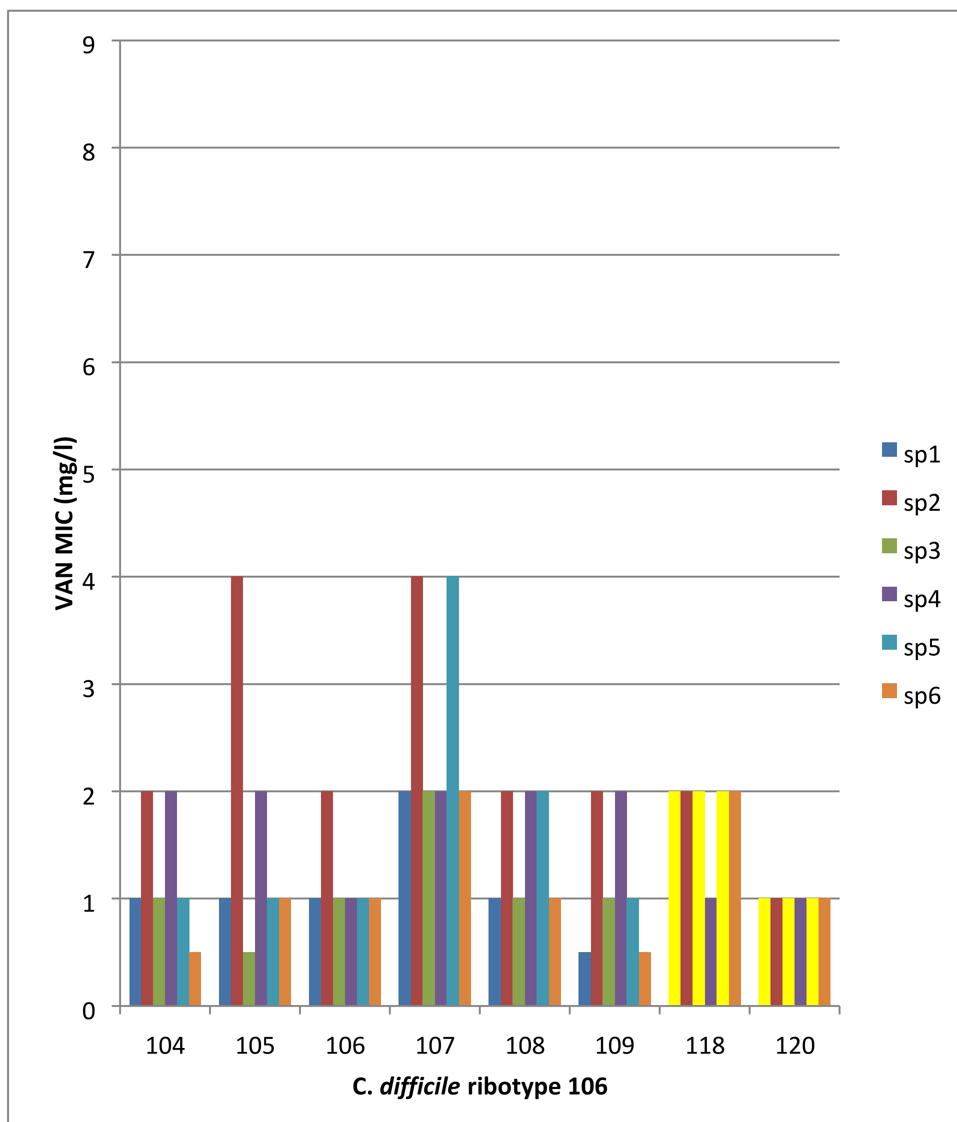

Figure A.3.6: serial passage of 027 ribotype strains without subinhibitory concentrations of vancomycin. CDSM strains were the set bars with yellow colour strain 118, 120

APPENDIX 4

Hemin effect on *Clostridium difficile* susceptibility to metronidazole

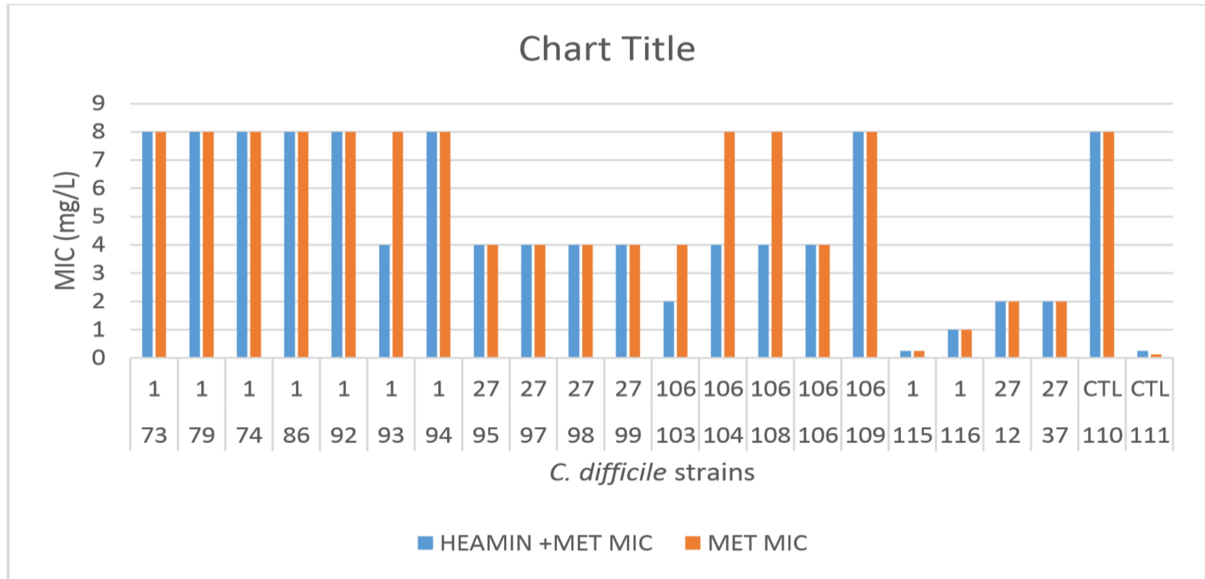

Figure A.4.1: Hemin effect on *Clostridium difficile* susceptibility to metronidazole incorporated in Wilkin Chalgren agar.

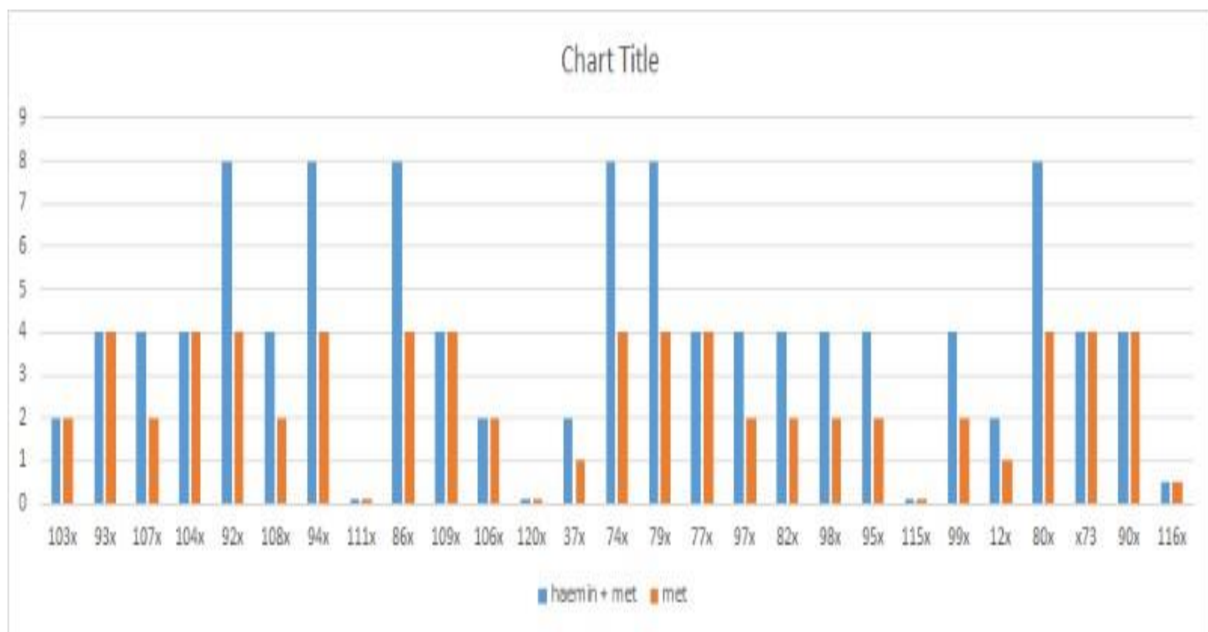

Figure A.4.2: Hemin effect on *Clostridium difficile* susceptibility to metronidazole incorporated in Brucella agar.

APPENDIX 5.0

UPTAKE ASSAY

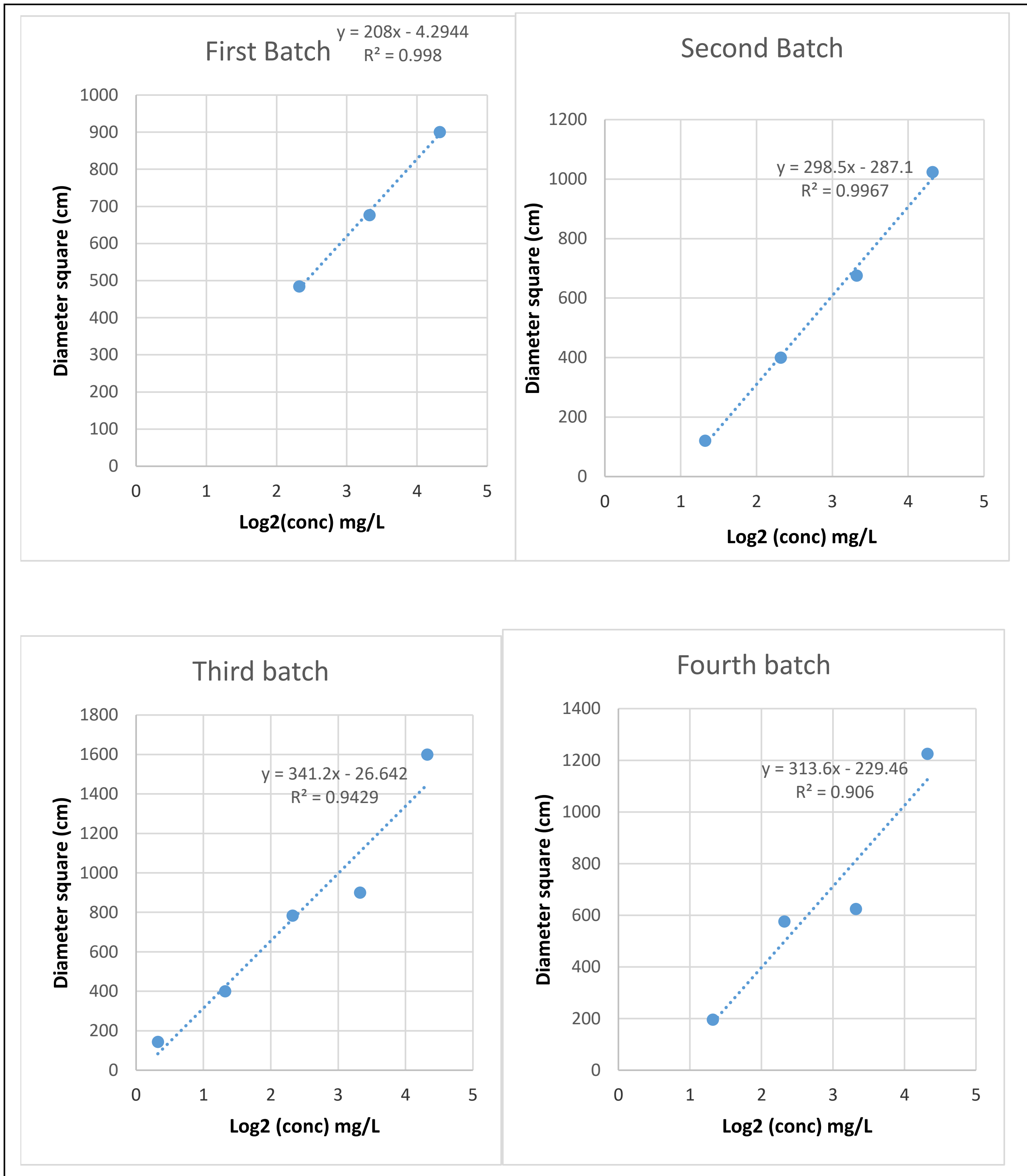

Fig A.5.1 Calibration graph for uptake assay using standards with known metronidazole concentrations