BIOLOGICAL EVALUATION OF MEMBRANE-ACTIVE COMPOUNDS AS TREATMENT FOR *Clostridium difficile* INFECTION

By

MD. ZAHIDUL ALAM

Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT ARLINGTON

December 2015

Acknowledgements

First of all, I would like to express my honest gratitude to my Ph.D. supervisor, Dr. Julian G. Hurdle, for his continuous support in my Ph.D. program. I believe that his guidance has helped me to make myself motivated in the field of drug-development research. Moreover, I really appreciate his time and effort that he gave me while writing this dissertation.

Besides my Ph.D. supervisor, I would like to thank my committee member: Dr. Laura Mydlarz, Dr. Michael Roner, Dr. Woo-Suk Chang, and Dr. Shauna McGillivray, for their valuable comments and encouragements in my Ph.D. research. I also believe that their comments helped me to improve my writing proficiencies as well as to progress in various aspects of my academic career.

In addition, I also like to thank my lab mates who helped me not only in the laboratory work but also to involve myself in the scientific discussions and seminars. Especially, I would like to thank Dr. Xiaoqian Wu, the postdoc in my lab, whose insightful comments and judgments really helped me to understand and to ask research questions.

Last of all, I would like to thank my parents for their continuous supports in the whole period of my Ph.D. study.

November 12, 2015

Abstract

BIOLOGICAL EVALUATION OF MEMBRANE-ACTIVE COMPOUNDS

AS TREATMENT FOR *Clostridium difficile* INFECTION

MD. ZAHIDUL ALAM, Ph.D.

The University of Texas at Arlington, 2015

Supervising Professor: Julian G. Hurdle

Clostridium difficile infection (CDI), which is caused by a spore-forming, gram-positive anaerobe, named *C. difficile*, is the leading cause of the hospital-acquired antibiotic-associated diarrhea. Hospitalized elderly patients, who receive broad-spectrum antibiotics, are mostly affected. However, recent clinical observations demonstrate that the incidence of CDI is also increasing in the community and occurs without previously established risk factors. With the recent emergence of epidemic strains of *C. difficile*, such as BI/NAP1/027, the severity of the CDI has increased in the North America, as well as in other parts of the world. In 2011, *C. difficile* caused 29,000 deaths in the United States alone. Despite the increase of incidence and the severity of the disease, the treatment options for CDI are limited. Metronidazole and vancomycin have been the first line treatment for the last three decades, and recently, in 2011, the FDA approved fidaxomicin for the treatment of CDI. The pathogenesis of *C. difficile* relies on the production of toxins (A and B), and sporulation that primarily occurs in late logarithmic and stationary-phase cells, which are characteristically resistant to antibiotics. We hypothesize that the lower efficacy of vancomycin in treating CDI is due to its less effectiveness against the slow growing cells of *C. difficile*. Furthermore, the effect of metronidazole is hampered by its poor oral pharmacokinetics. Conversely, compounds targeting the bacterial membrane may have superior efficacy by retaining potent activity against growing and non-growing cells. This dissertation, therefore, examined the properties of the membrane-active compound, surotomycin, in *C. difficile*.

Surotomycin, also known as CB-183,315, is an orally active lipopeptide and in phase 3 clinical trials for the treatment of CDI. Although, *in vivo*, this compound demonstrated better clinical efficacy in CDI, compared to other commonly used drugs, and caused membrane potential disruption in *S. aureus*, its action is not well characterized in *C. difficile*. Therefore, this study, using *in vitro* assays, provide validation of surotomycin's mode of action and effects on virulence. It was observed that surotomycin demonstrates concentration-dependent killing of both logarithmic and stationary-phase cells of *C. difficile*. The exposure of *C. difficile*'s cultures to surotomycin at their minimum bactericidal concentration resulted in a significant reduction in viability with simultaneous reduction in toxin production and spore numbers; and these effects are due to the disruption of Clostridial membrane potential, and not due to membrane pore formation. In addition, as a part of a collaborative team, this study evaluated the gastrointestinal pharmacokinetics and the *in vivo* efficacies of novel hybrid compounds, metronidazole-tetramic acid (MTZ-TA), using hamster model of CDI (Chapter V). This *in vivo* study observed better efficacy of the hybrid compounds compared to metronidazole alone, which is due to the lower absorption across the intestinal epithelium, as supported by the plasma and the fecal concentrations of these compounds in hamster model of CDI.

Table of Contents

Acknowledgements iii

Abstract iv

List of Figures x

List of Tables xii

Abbreviations and glossaries xiii

Chapter I: Overview of *Clostridium difficile* infection and hypotheses 1

1.1 History and overview of *Clostridium difficile* infection 1

1.2 Epidemiology 2

1.3 Pathogenesis of *C. difficile* infection 6

1.4 Community-acquired *C. difficile* Infection 12

1.5 Hyper-virulent strains of *C. difficile* 13

1.6 The Role of the Normal Gut Flora in Preventing the Gastrointestinal Infections 14

1.7 Recurrence of *C. difficile* Infection 17

1.8 Current treatment options for the CDI and their limitations 18

1.8.1 Antibiotic treatment 19

1.8.2 Non-antibiotic treatment 22

1.9 Membrane targeting compounds in controlling the recurrent *C. difficile* 24

1.10 Hypotheses 26

1.11 Aims 26

Chapter II: Evaluation of the mode of action of surotomycin in *C. difficile*.....27

2.1 Introduction.....27

2.2 Materials and methods……28

2.2.1 Compounds, bacterial strains, and growth media……...28

2.2.2 Determination of MICs) and MBCs……...29

2.2.3 Time-kill kinetics…….....29

2.2.4 Macromolecular biosynthesis inhibition assay……...30

2.2.5 Determination of the membrane potential and permeability using FACS……..30

2.2.6 ATP-leakage assay……..32

2.2.7 Determination of growth inhibitory concentration…….33

2.2.8 Quantitative Reverse Transcriptase PCR (qRT-PCR) assay……...33

2.2.9 Statistical analysis……...34

2.3 Results…….35

2.3.1 Surotomycin is bactericidal against *C. difficile*……...35

2.3.2 Surotomycin kills *C. difficile* in a concentration-dependent manner……..36

2.3.3 Surotomycin dissipates membrane potential without pore formation…….38

2.3.4 Surotomycin inhibits multiple macromolecular biosynthetic processes…….42

2.3.5 Surotomycin caused increased expression of certain membrane-active genes …..44

2.4 Discussion……...47

Chapter III: Role of surotomycin in reducing the virulence factors from *C. difficile*……51

3.1 Introduction……51

3.2 Materials and methods……...53

3.2.1 Compounds, bacterial strains, and growth media……..53

3.2.2 Detection of toxin A and toxin B……...53

3.2.3 Sporulation assay……...54

3.2.4 Bioassay of compounds……55

3.2.5 Statistical analysis……..55

3.3 Results……....56

3.3.1 Surotomycin significantly reduced toxin production……56

3.3.2 Surotomycin significantly reduced spore numbers……....62

3.3.3 Surotomycin's activity decreases with prolong incubation……...68

3.4 Discussion……..69

Chapter IV: *In vivo* evaluation of metronidazole-tetramic acid (MTZ-TA) hybrids…….....73

4.1 Introduction……....73

4.2 Materials and methods……...75

4.2.1 Compounds and bacterial strain……75

4.2.2 Hamster model of CDI……...76

4.2.3 Pharmacokinetic studies……76

4.2.4 Statistical analysis……..77

4.3 Results……....77

4.3.1 MTZ-TA hybrids demonstrate better efficacy compared to metronidazole……..77

4.3.2 MTZ-TA hybrids achieved low concentration in plasma ……81

4.4 Discussion……..83

Chapter V: Conclusions, limitation of the studies, future directions and prospects of membrane-active agents for the treatment of *C. difficile* infection.....85

5.1 Conclusions.....85

5.2 Limitation of the *in vitro* assays.....86

5.3 Lack of the LCMS data.....86

5.4 Sensitivity of the ELISA kit.....87

5.5 Limitation of the hamster model of CDI.....87

5.6 Future direction.....88

Elucidation of biological target……..88

5.7 Prospects of membrane-active agents.....89

Appendix: Published papers……..…….91

References.....93

Biography.....111

List of Figures

Figure 1-1: Incidence of hospital-acquired *C. difficile* infection (CDI).....3

Figure 1-2: Spread of epidemic fluoroquinolone resistance strain of *C. difficile* 5

Figure 1-3: Stages of *C. difficile* colonization during the pathogenesis of CDI. 8

Figure 1-4: Genomic location of pathogenicity locus (PaLoc) in *C. difficile*.. 9

Figure 1-5: Pathogenesis of *C. difficile* infection.. 10

Figure 1-6: Effect of glycosylation of G-protein by toxin A and toxin B.....11

Figure 1-7: Role of normal gut flora in preventing the gastrointestinal infections.....16

Figure 1-8: Chemical structure of the commonly used antibiotics for CDI treatment.....19

Figure 2-1: Time-kill assay against BAA 1875 (PCR ribotype 078).. 36

Figure 2-2: Time-kill assay against R 20291 (PCR ribotype 027).....37

Figure 2-3: Effect of surotomycin on *C. difficile*'s (BAA-1875) membrane. 39

Figure 2-4: Effect of surotomycin on *C. difficile*'s (R20291) membrane. 40

Figure 2-5: Effect of surotomycin on the leakage of ATP from cells.....41

Figure 2-6: Effect of surotomycin on *C. difficile*'s (BAA-1875) macromolecular biosynthesis....42

Figure 2-7: Effect of surotomycin on *C. difficile*'s (R20291) macromolecular biosynthesis.....43

Figure 2-8: Growth inhibitory concentration (GIC) of various compounds against R20291. 45

Figure 2-9: Expression of selected genes (*kdpA*, *kdpB*, *cls*1, *cls*2, *murA*, and *fabK*) 46

Figure 3-1: Effect of surotomycin on *C. difficile*'s (BAA-1875**)** toxin production.....57

Figure 3-2: Effect of surotomycin on C. difficile's (R20291) toxin production 58

Figure 3-3: Effect of surotomycin on BAA-1875 in reducing toxin production (Quantitative) 60

Figure 3-4: Effect of surotomycin on R20291 in reducing toxin production (Quantitative) 61

Figure 3-5: Effect of surotomycin on sporulation against R20291, in condition 1.....63

Figure 3-6: Effect of surotomycin on sporulation against R20291 in condition 2.....64

Figure 3-7: Effect of surotomycin on sporulation against BAA-1875 in condition 1.....65

Figure 3-8: Effect of surotomycin on sporulation against BAA-1875 in condition 2..... 66

Figure 3-9: Ratio of spore numbers to total viable cells (TVC) in BAA-1875 strain..... 67

Figure 3-10: Ratio of spore numbers to total viable cells (TVC) in R20291 strain..... 68

Figure 4-1: Hybridization of MTZ to tetramic acid motif of reutericyclin……78

Figure 4-2: *In vivo* efficacy of MTZ-TA hybrid compounds……..80

Figure 4-3: Pharmacokinetic properties of MTZ-TA and MTZ after oral dosing……..81

Figure 4-4: Fecal-concentration of hybrid compounds after single oral dosing……82

List of Tables

Table 2-1: *In vitro* activities of surotomycin against various strains of *C. difficile*. 35

Table 2-2: List of genes for RT-PCR assay. 44

Table 3-1: Bioassay (μg/mL) of surotomycin and other corresponding drugs. 69

Table 4-1: *In vitro* activities and structures of MTZ-TA hybrids……...79

Abbreviations and glossaries

ATP	Adenosine triphosphate
BAA-1875	A strain of *C. difficile* belongs to PCR ribotype 078
BI/NAP1/027	Hyper-virulent strain of *C. difficile*
BHI	Brain Heart Infusion
CA-CDI	Community-acquired *C. difficile* Infection
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CDI	*C. difficile* Infection
CDAD	*Clostridium difficile*-associated diarrhea
CDC	Centers for Disease Control and Prevention
Cdc42	A protein belongs to the Rho GTPase family
CFU	Colony Forming Unit
Cwp66	Cell wall protein 66
Cwp84	Cell wall protein 84
$DiBAC_4(3)$	Bis-(1,3-dibutylbarbituric acid) trimethine oxonol
$DiOC_2(3)$	Diethyloxacarbocyanine iodide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence-activated Cell Sorting
FDA	Food and Drug Administration
FliC	Flagellin C
FliD	Flagellar cap protein
FMT	Fecal Microbiota Transplant
FlowJo X 10.0.7	Software used to analyze the flow cytometric data

GIC	Growth Inhibitory Concentration
GTPase	An enzyme that hydrolyzes guanosine triphosphate
IVIG	Intravenous Immunoglobulin
Dalton, symbol Da	Standard unit used to indicate molecular mass
LLOQ	Lower limit of quantification
MTZ	Metronidazole
MTZ-TA	Metronidazole-tetramic acid
MIC	Minimum inhibitory Concentration
MBC	Minimum Bactericidal Concentration
Nosocomial infection	Hospital-acquired infection
OD_{600nm}	Optical density at the wavelength of 600 nm
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PLoc	Pathogenicity locus
PMF	Proton Motive Force
PMNs	Polymorphonuclear cells
Pseudomembranous colitis	Severe inflammation of the colon due to CDI
qRT-PCR	Quantitative Reverse Transcriptase PCR
Rac	Signaling G proteins, belongs to Rho family of GTPases
Rho GTPase	Any member of the Rho family of GTPases
RNA	Ribonucleic acid
VRE	Vancomycin Resistant Enterococci

Chapter I

Overview of *Clostridium difficile* infection and hypotheses

1.1 History and overview of *Clostridium difficile* infection

Clostridium difficile is a gram-positive, obligate anaerobic, spore-forming bacterium (1). After the first isolation, in 1935, from the feces of neonates, it was coined *Bacillus difficile* by Hall and O'Toole, because of the difficulty in isolation and growing of this pathogen in the laboratory (2). However, due to the widespread use of the broad-spectrum antibiotics, it is now problematic to control the spread of *C. difficile*, which is the frequent agent of antibiotic-associated colitis in the hospital settings. Previously, the disease was termed *C. difficile* associated diarrhea (CDAD); though, this term is now largely obsolete and replaced by *C. difficile* infection (CDI). CDI is largely considered as a nosocomial infection that mostly affects elderly patients who receive broad-spectrum antibiotics for the treatment of other infections (3, 4). It is widely believed that broad-spectrum antibiotics disrupt the normal protective gut flora, thereby allows pathogenic *C. difficile* to flourish. Antibiotics most frequently associated with CDI are cephalosporin, clindamycin, amoxicillin and fluoroquinolones (5). In the milieu of the disrupted gut flora, vegetative cells of *C. difficile* produce toxins that cause the inflammation and necrosis of the gut epithelium. In severe infection, CDI can cause pseudomembranous colitis and toxic megacolon, which are the life-threatening complications (5, 6).

Interestingly, not all patients colonized with *C. difficile* develop the colitis. Two factors play central roles in the pathogenesis of colitis. These are the host immune response and the virulence of the bacteria (1). Since 2001, due to the emergence of epidemic strains of *C. difficile*, there is an increase in the severity and the pathogenicity of CDI, mainly, in the hospitals of North America (7). These strains show a propensity to cause severe CDI. The CDC identified the strains as the BI/NAP1/027, which was later characterized to

exhibit fluoroquinolone resistance and an increase in the ability to produce toxins. After the infection with the epidemic strains, there is an increase in the recurrence of CDI as well as in the mortality rate, which is three times higher, compared to the less-virulent strains of *C. difficile* (5, 7).

1.2 Epidemiology

CDI is a major health problem, mainly as a nosocomial infection, in the developed countries (7, 8). It is estimated that *C. difficile* causes 75 % of all the cases of the antibiotic- associated colitis, and 90 to 100 % of all the cases of the pseudomembranous colitis (9). Unfortunately, the incidence and the severity of CDI have been increasing in North America, Europe, Australia, and in many countries of Asia (7, 10). According to the report of the US CDC, each year, at least 250,000 people are infected, and of them, 14,000 died, due to CDI, only in the Unites States (11). In addition to high mortality, the economic burden is high, which cost about 4,000 USD, per case of CDI or more than 1.5 billion USD, per year (9).

Since the beginning of the new century, a rapid rise in the incidence and the severity of CDI has been observed in many parts of the world. In the USA, before 2001, in the mid or late 1990s, the number of CDI cases in the hospitals was 30 per 100,000 of patients (2, 7). However, this number rose to 50 in 2001, and in 2005, the incidence rate increased to 84 (per 100,000 of hospitalized patients), which is almost 3-fold higher than the incidence rate of the 1990s (2, 7, 12). Furthermore, other recent data obtained from 28 hospitals of the southern part of USA, indicate that CDI is now higher than the incidence of MRSA infections, which is the most common cause of the nosocomial infection in the USA (13). Figure 1-1 shows the incidence of CDI cases among different age groups in the USA.

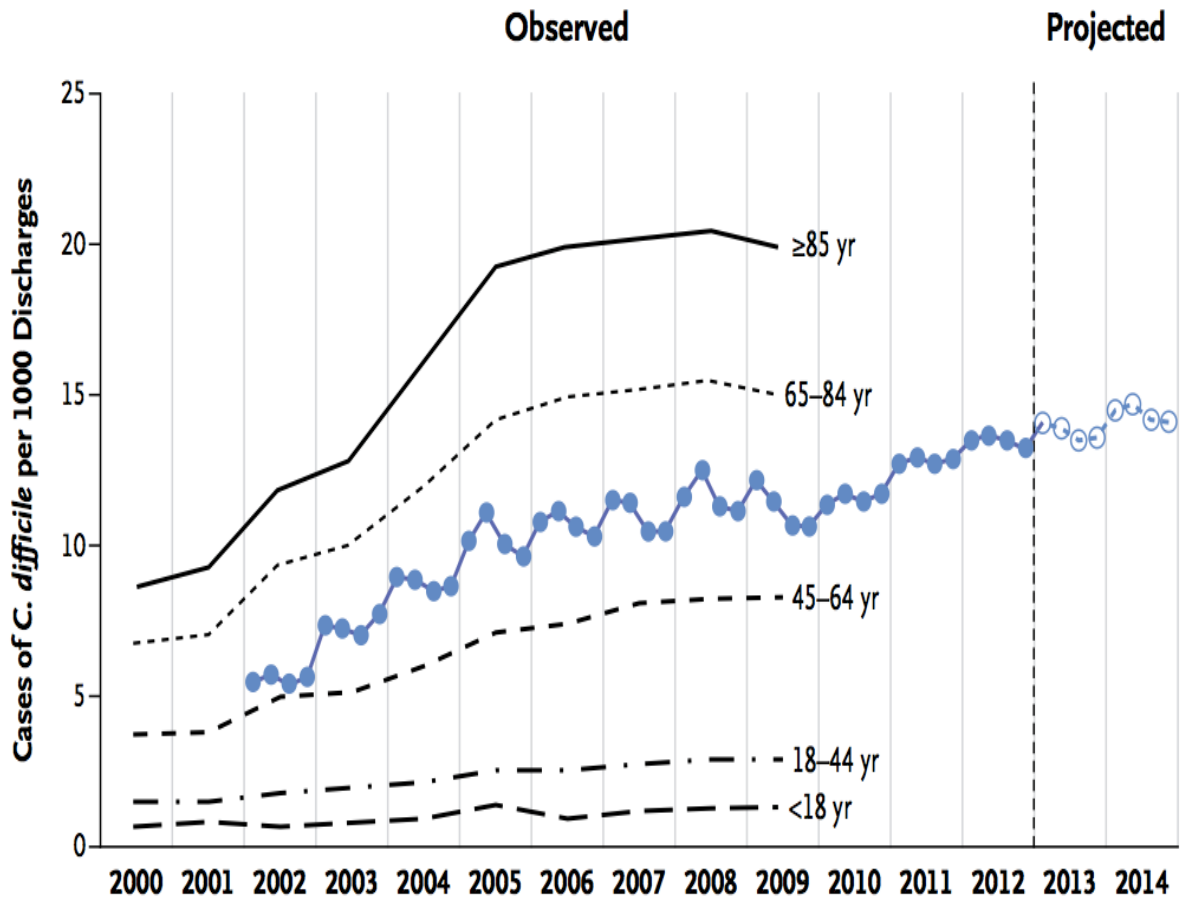


Figure 1-1: Incidence of hospital-acquired *C. difficile* infection (CDI) in patients across various age groups. There is a gradual rise of CDI incidence among all the age groups, especially, in high age group (>65 year), where the rise of incidence is very rapid (seven patients per 1000 hospital discharge at the year 2000 to more than 20 patients per 1000 hospital discharge at 2010). This figure also shows the projected incidence of CDI (blue closed circles). This graph is reused after obtaining permission from the *New England Journal of Medicine*. (Daniel et al. 2015).

In Canada, the incidence and the severity of CDI are also alarming. Through a retrospective study in Quebec, it was observed that the incidence rate of CDI per 100,000 of people increases by 4.5-fold, from 1991 to 2003 (7, 8, 14). In the same period, there is also a rise in the rate of complicated infection, from 7.1% to 18.2%, and the mortality rate (30- day mortality), from 4.7% to 13.8% (7, 12, 14). Clinical studies performed later in many other sites (Montreal and Sherbrook) of Canada, also demonstrated high incidence rate, which was about 22.5 cases of CDI per 1000 of hospital admissions (7). In addition, the mortality rate was found high, which was 6.9% (7, 12).

In Europe, the continuous increase of CDI is associated with hospital outbreaks. Ribotype 027, the hyper-virulent strain was found to be responsible for the outbreaks in 18 different countries of Europe (7, 8). First hospital outbreaks were observed in the UK, from 2003 to 2005, where the mortality rate was 11-12% (7, 8, 13). After the UK, there were hospital outbreaks in Netherland, Belgium, Finland, France, Luxemburg, Switzerland, and in Ireland (7).

In Germany, the incidence of CDI increased by 5-fold from 2002 to 2006, per 100,000 of people (15). Moreover, hospital discharge of elderly patients (age >65 year) diagnosed with CDI has also increased, for example, it has risen by 3-fold in Spain, from 1997 to 2005 (7, 15).

The rapid rise in the incidence and the severity of CDI in North America and in Europe is largely due to the emergence of the epidemic strain of *C. difficile*. The prevalent epidemic strain in the North America is named BI/NAP1/027 strain, while the prevalent strain in the Europe is BI/NAP1/078 (7). Both of the strains can cause severe infections. Figure 1-2 shows the spread of the epidemic strain in USA, and various parts of the world. This ribotype (027) produces more toxins, compared to other strains of *C. difficile* (SE 20, SE 21 and SE 30) (16) and causes more severe infection. Furthermore, due to its resistance to fluoroquinolones, a widely used drug in the hospitals, BI/NAP1/027 strains can survive and transmit from patient to patient in the presence of the selection pressure of quinolones, and cause the epidemics of CDI in hospitals (8, 12).

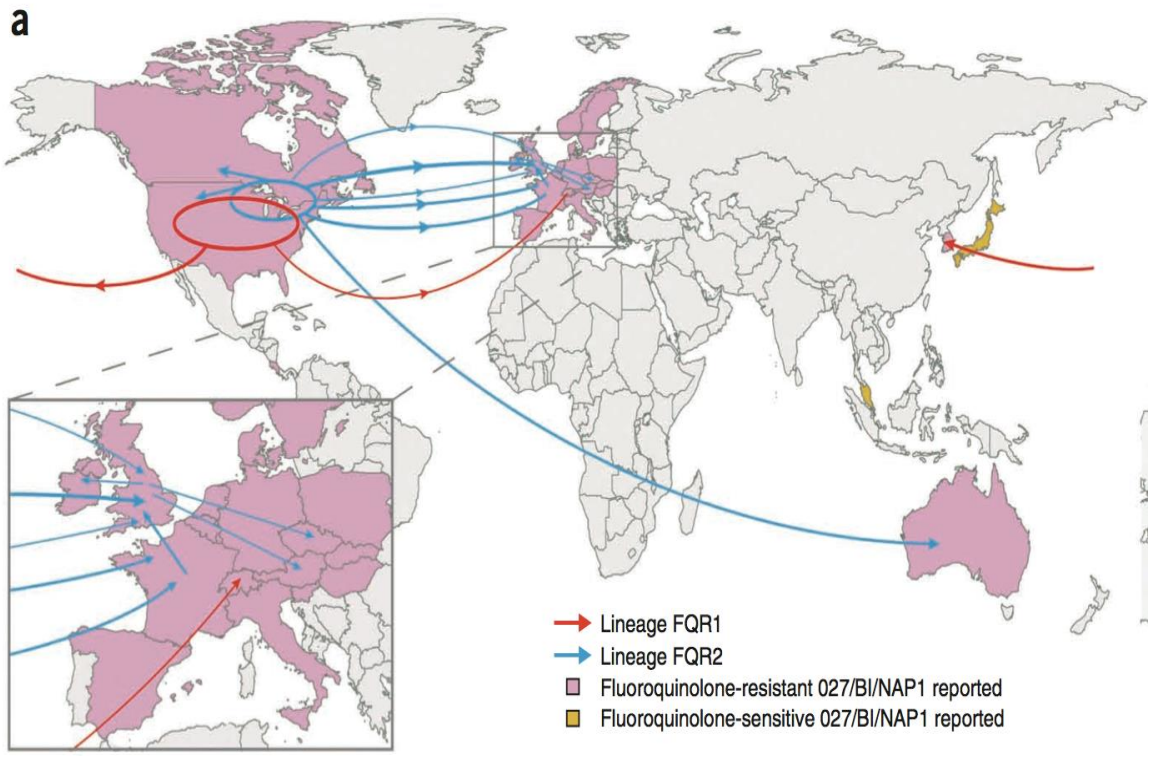


Figure 1-2: Spread of epidemic fluoroquinolone resistance strain of *C. difficile* in the USA and the other parts of the globe. (Reprinted from Miao et al. 2013. Emergence and global spread of epidemic healthcare-associated *C. difficile*. *Nature Genetics*. With permission from Macmillan Publishers Ltd.).

There are incidences of CDI in many countries of Asia, mostly, in Korea, Japan, Taiwan, Singapore, Hong Kong, Indonesia, Malaysia, India, and in Bangladesh (7, 10). Interestingly, the ribotypes isolated from the Asian region, were detected as 017, 018, 014, and 002, in contrast to ribotypes 027 and 078, which are prevalent in North America and in Europe (7, 10). The 017 ribotype is toxin B positive but toxin A negative (A^-B^+), and can produce the same signs and symptoms like epidemic strains (027). However, there are three

unique factors found to be related with 017 ribotype infection. These are- exposure to the chemotherapeutic agents, use of naso-gastric tubes, and stay in particular wards (7, 10).

The epidemiology of CDI in Australia is different from the other countries. Although, the incidence was high in western part of Australia during the 1980s (2.09 cases/1000 discharge), it decreased significantly (0.89 cases/1000 discharge) in 1998-1999 (7). This decrease in incidence is thought to be due to the discontinuation of the use of cephalosporin drugs in the hospitals (7). However, a recent study by Foster et al. (17), performed in two tertiary level hospital of Western Australia, has observed the increase in the incidence of CDI. The incidence of CDI varied from 3.9 to 16.9 cases per 10,000 of admitted patients in one hospital, and 5.2 to 8.1 cases per 10,000 of hospitalized patients in another hospital. Moreover, the rate of severe CDI was found to be high, 40%, among diagnosed patients (17).

The epidemiology and the severity of CDI have changed recently. Although CDI was first recognized in Western Europe and North America, it has now become a global health-related threat.

1.3 Pathogenesis of *C. difficile* infection

CDI is an opportunistic infection of the human gut (1, 4). It is widely believed that when the normal gut flora is disrupted, mainly by the broad-spectrum antibiotics, *C. difficile* can overgrow, produce toxins, and cause the signs and symptoms of CDI (1, 3). *C. difficile* produces toxin A and toxin B. However, some strains, in addition of toxin A and B, produce another toxin, named binary toxin (1, 18). Toxin A (enterotoxin) and toxin B (cytotoxin) primarily affect the large intestine, and cause inflammation of the epithelial cells (1, 3, 4). Due to this inflammation, patchy necrotic lesions are formed over the surface of the large intestine, which looks like a membrane- the characteristic feature of the pseudomembranous colitis (1, 19).

Not all the patients infected with *C. difficile* develop pseudomembranous colitis (1). The signs and symptoms of CDI vary that ranges from the asymptomatic colonization to mild and severe infections (2, 20). In mild to moderate infection, patients develop diarrhea (with or without blood), abdominal pain (mild), and fever; while in severe infection, patients develop bloody diarrhea, leukocytosis and high-grade fever with severe abdominal pain, which could be the clinical manifestations of the pseudomembranous colitis and toxic mega colon (2, 9, 20).

After the disruption of the normal gut flora, usually by broad-spectrum antibiotics (for example, cephalosporin, ampicillin, clindamycin, fluoroquinolones, etc.), exogenous or endogenous spores of *C. difficile* germinates in the colon to give raise the vegetative cells (4, 21). The presence of bile salts in the intestine contributes in the germination of *C. difficile* spores (21). Vegetative cells multiply and pass through the mucus layer by the help of various hydrolytic enzymes secreted from the cells as well as by the flagellar activity, to bind with the epithelial cells of the gut (1, 3, 4). Figure 1-3 shows the stages of colonization of *C. difficile*.

Several adhesion proteins have been identified that help *C. difficile* to bind to the gut epithelium (Figure 1-4). The most well-known are the surface layer protein A (SlpA), Cwp84, Cwp66, FliD (flagellar cap protein), FliC (Flagellin), and the fibronectin binding protein (1, 3).

After colonization, *C. difficile* secretes toxin A and toxin B. Some strains also produce binary toxin, in addition of Toxin A and Toxin B (1). Toxin A and Toxin B, which are large proteins and have the molecular masses of 308 KDa and 269 KDa, respectively, are the main virulent factors in causing the symptoms of CDI (19). The genes for the toxins (*tcdA* and *tcdB*) are located in the pathogenicity locus (PLoc) of the *C. difficile* genome along with other three genes (Figure 1-4), which regulate the expression and the secretion of the toxins (1, 3, 4). The other three genes are *tcdR*, cause positive regulation of toxin genes expression;

tcdC, cause the negative regulation of toxin genes expression and; *tcdE*, cause a holin-pore formation and help to secrete the toxins outside of the cell (1).

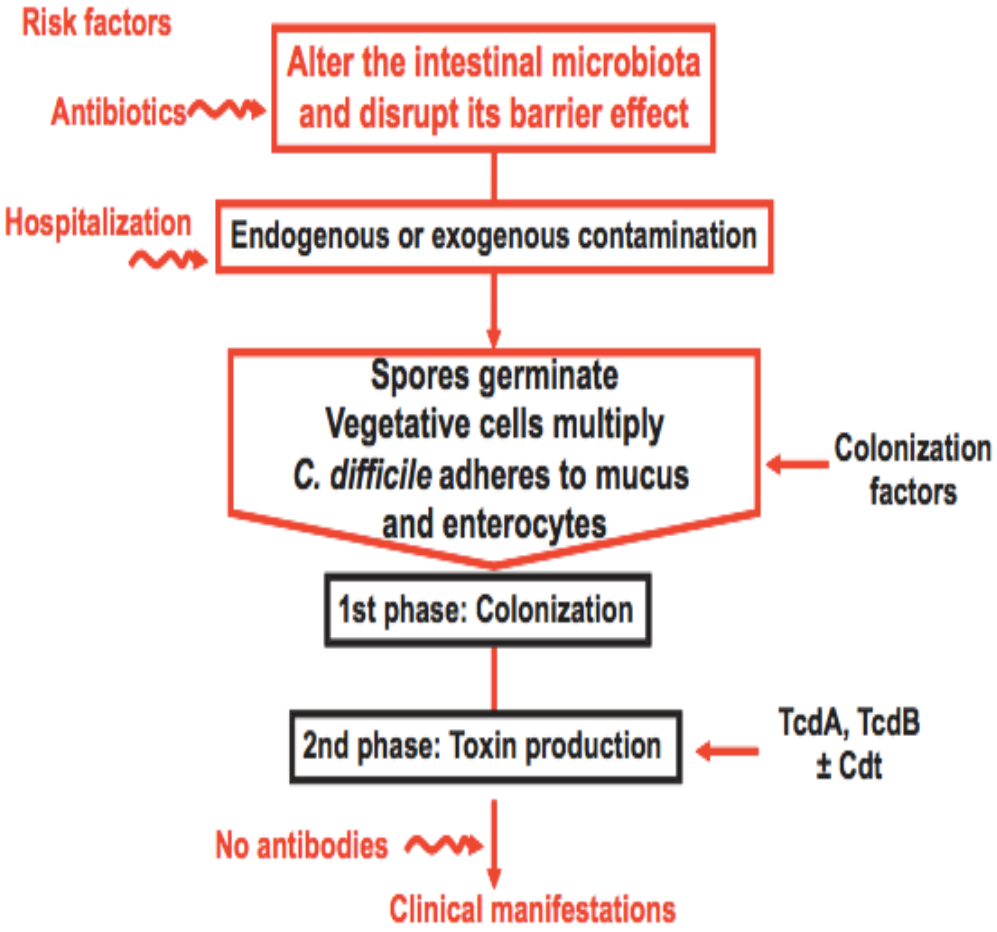


Figure 1-3: Stages of *C. difficile* colonization during the pathogenesis of CDI. (Reprinted from Deneve et al. 2009. New trends in *Clostridium difficile* virulence and pathogenesis. *International Journal of Antimicrobial Agents*, with permission from Elsevier Publishers Ltd.).

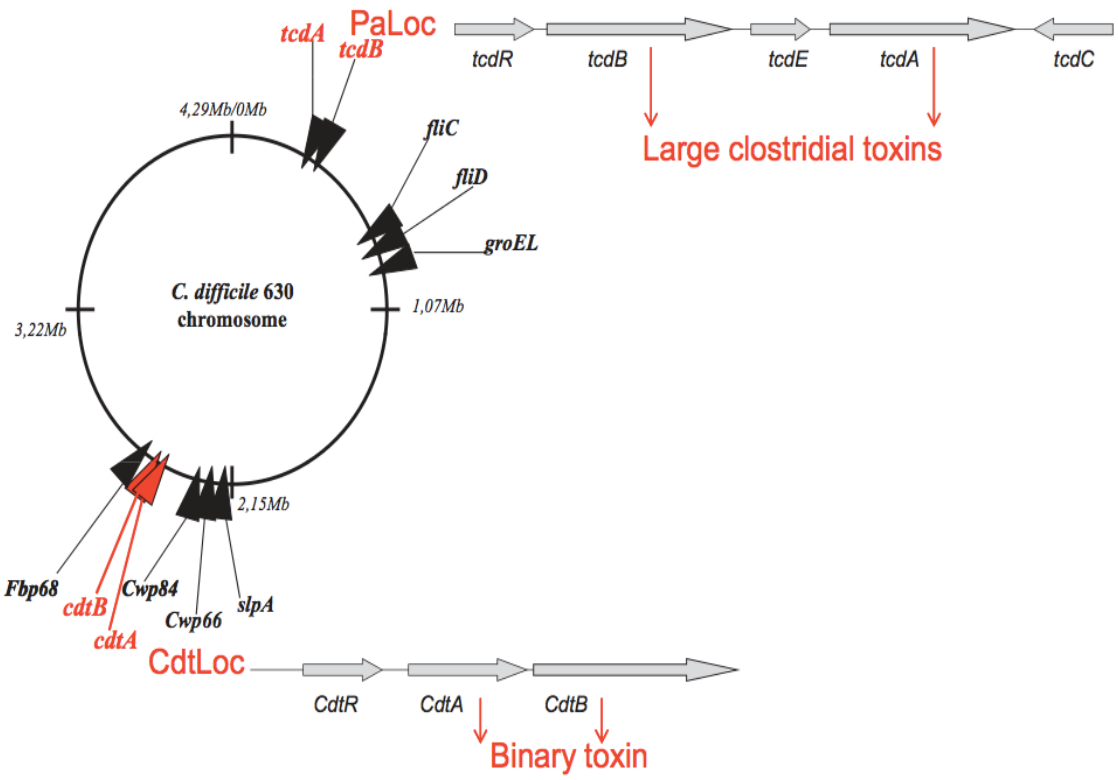


Figure 1-4: Genomic location of surface proteins *C. difficile*. Pathogenicity locus (PaLoc) is also depicted, which shows the relative locations of the toxin genes (Toxin A and Toxin B), in relation to other regulatory genes of toxin production (*tcdR*, *tcdC*, and *tcdE*) (Reprinted from Deneve et al. 2009. New trends in *C. difficile* virulence and pathogenesis. *International Journal of Antimicrobial Agents*, with permission from Elsevier Publishers Ltd.).

Figure below (Figure 1-5) shows how the toxin A and toxin B cause inflammation and give raise tissue damage, inflammation and symptoms of CDI. Toxin A stimulates the intestinal mucosal cells to produce cytokines, and other pro-inflammatory factors, which attract polymorphonuclear (PMNs) cells to the site of infection (1, 19).

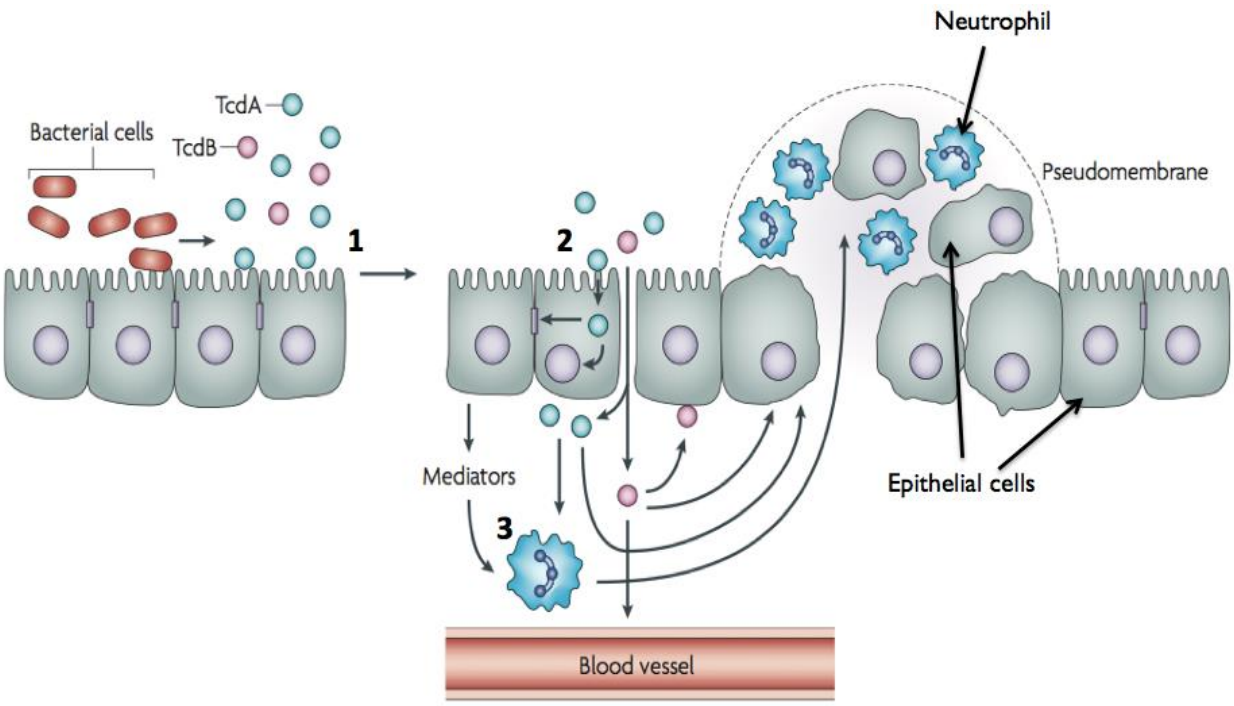


Figure 1-5: Pathogenesis of *C. difficile* infection. Toxigenic strain of *C. difficile* produces toxin A and toxin B. (1) Toxin A binds to the apical surface of the intestinal epithelial cells, and after internalization, causes cytoskeletal changes, and disruption of the tight epithelial junctions. (2) Disruption of tight junctions causes the release of fluid in the gut lumen and helps toxins to enter into the sub-mucosa layer for further tissue damage. (3) Both toxins are cytotoxic and induce the release of immunomodulatory mediators from the epithelial cells resulting in increased migration of polymorphonuclear cells (e.g., neutrophils) to the site of infection. (Reprinted from Rupnik et al. 2009. *C. difficile* infection: New developments in epidemiology and pathogenesis. *Nature Reviews Microbiology*, with permission from Macmillan Publishers Ltd.).

After reaching the site of infection, polymorphonuclear cells, mostly, the neutrophils, cause the damage of the mucosa, and break the tight junctions between the epithelial cells of the gut (19). This effect has two consequences; firstly, it causes leakage of fluid from the tissues to the lumen, and secondly, it helps the

toxin B to cross the mucosal membrane, leading to sub mucosal damage (19). Furthermore, toxin A stimulates the enteric nervous systems and affects the gastrointestinal motility, which plays a role in causing diarrhea (1, 19).

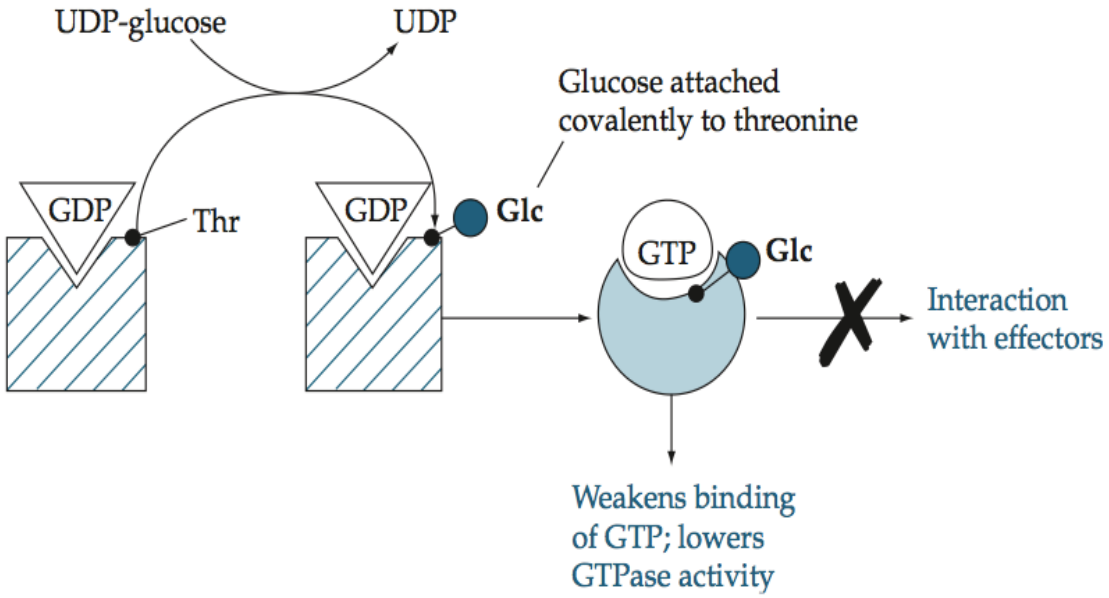


Figure 1-6: Effect of glycosylation of G-protein by toxin A and toxin B. (Ref: Wilson et al. *Bacterial Pathogenesis: A Molecular Approach*. Third Edition. Page 429).

Both the toxin A and toxin B have molecular targets inside the cell. Primarily, these toxins cause the glycosylation of the G-protein targets (Figure 1-6), which maintain several important functions inside mammalian cells (1, 19). Toxin A and toxin B cause the transfer of glucose from the UDP-glucose, to the target molecule, for example to Cdc42, Rac, and Rho, G-proteins situated inside the cell (1, 19). These proteins perform many crucial functions, and loss of these functions affects the normal physiology of the eukaryotic cells. Among many functions, these proteins control the polymerization and the de-polymerization of the actin proteins, and inhibition of this function results in loss of the shape (cell

rounding) of the cells, apoptosis and tissue necrosis (1, 3, 19). The dead mucosal cells, in addition of the inflammatory cells, produce a membrane on the surface of the epithelium, which look like a pseudo-membrane, the characteristic feature of pseudomembranous colitis (1, 3, 19).

1.4 Community-acquired *C. difficile* Infection

Although, it was previously known that *C. difficile* infection is primarily a nosocomial infection, and mainly affects the hospitalized elderly patients receiving broad-spectrum antibiotics for other infections, recent observations suggest that CDI is now common in the community (22).

According to the clinical practice guidelines of CDI (2010), as set by the Society for Health Care Epidemiology of America (SHEA) and the Infectious Disease Society of America (IDSA), the CDI is considered to be community-acquired (CA-CDI), if the signs and symptoms of CDI occurs in persons with no previous stay in hospitals or any other health care facility centers, at least for 12 weeks before the onset of the symptoms (7, 23).

Data from the North America and Europe suggest that the incidence of CDI is increasingly recognized in the community. A statewide surveillance, performed in Connecticut, in 2006, by the Centers of disease control and prevention, found 60% rate of CA-CDI, which were diagnosed by the positive toxin assay and by the gastrointestinal symptoms (24). Another study by Khanna et al., performed among the residents of Olmsted County, Minnesota, from 1991 to 2005, also observed high incidence of CA-CDI, which was 41% of 385 definite cases of CDI (25). In these studies, younger patient groups (Median age 50), without any history of prior antibiotic exposure, were found to be affected (7, 25). Studies from the other parts of USA also demonstrate a high occurrence of CA-CDI (7, 8).

The incidence in Europe is also on rise. In the UK, a study performed by Wilcox et al., over the year of 2000, observed high incidence of CA-CDI, which was 60%, in the urban areas (26). In addition, they did not observe any prior exposure of the risk factors (use of antibiotic, use of proton pump inhibitors, etc.). In 1995, a nationwide survey in Sweden, reported that the CA-CDI rate as high as 28%, of all the diagnosed cases of CDI (27). The incidence is also high in Netherlands. In a prospective study, which was performed in three independent labs in Netherlands, from 2007 to 2008, showed that CA-CDI affects all the age groups and many of them had no previous risk factors (7, 28).

Interestingly, a vast majority of the data suggests that CA-CDI occurs in younger people, without having any risk factors, like antibiotic exposure, use of acid suppressant drugs, and the history of hospitalization. These findings pose a new challenge in identifying the risk factors for the CA-CDI. In a study performed by collecting the environmental dust and other household samples, for example, the shoe bottoms, bathroom surfaces, house floor dust, etc., from 30 houses in the Houston area, showed that the presence of *C. difficile* is ubiquitous in the community house-holds of the urban area (29). In addition, while recognizing the possible source of infection of CA-CDI, zoonotic transmission of *C. difficile* spore, is considered to be important. Many recent studies show the presence of *C. difficile* spore in the meat products, for examples, in chicken, beef, pork, etc. (30, 31) that could be the potential sources of CA-CDI. Community-acquired CDI is usually considered as a mild infection; however, it can cause severe infection with complications (32).

1.5 Hyper-virulent strains of *C. difficile*

Since 2001, due to the emergence of the epidemic strains of *C. difficile*, there was an increase in the epidemic of CDI in the US hospitals (1, 33). The epidemic strain is determined as BI/NAP1/027 (33). Molecular analysis revealed the identity of the epidemic hyper-virulent strain responsible for the outbreaks. By the use of restriction endonuclease analysis (REA), the strain is characterized as BI; by the pulse field gel-electrophoresis (PFGE), as NAP1; and by the PCR-ribotyping, as ribotype 027 (1, 2).

There are two main factors, which make this ribotype more virulent and provide advantages to cause the epidemics, compared to the other ribotypes of *C. difficile* (1, 2). Firstly, BI/NAP1/027 carries deletion mutation in the *tcdC* gene, which product causes the negative regulation in the production of the toxins (Toxin A and Toxin B). It is observed that BI/NAP1/027 strain produces 16-fold and 23-fold higher level of toxin A and toxin B, respectively, compared to the non-virulent strains (33). Secondly, BI/NAP1/027 is resistant to fluoroquinolones, which are the most commonly used antibiotics in the hospitals (2, 33, 34). Due to this resistance, BI/NAP1/027 can spread from patient to patient; in spite of the selection pressure produced by fluoroquinolones, and can cause the epidemics of CDI (1, 33, 34). Furthermore, this hyper-virulent epidemic strain produces binary toxins (CDT), which role in CDI pathogenesis has recently been explored by Aktories et al. 2011. Their study showed that CDT causes ADP-ribosylation of actin protein (at the position of arginine-177) resulting in complete depolymerization of the intracellular cytoskeletal system (35).

Based on the immune status, patients infected with BI/NAP1/027 develop severe CDI, as evident by the high white blood cell count and severe diarrhea (2, 33, 34). Elderly debilitated people are mostly affected and develop severe infection as because they have lower immunity and produce low antibody titers against *C. difficile* (36). For example, elderly people produce low level of IgA, which is a local antibody that protects the gut from CDI. In addition, when people are affected with the BI/NAP1/027 ribotypes, there is an increase chance of developing pseudomembranous colitis and toxic mega-colon, which suggests the strong association of the epidemic strain, BI/NAP1/027, with the severe complicated CDI (1, 2).

1.6 The Role of the Normal Gut Flora in Preventing the Gastrointestinal Infections

The human body is colonized with a great number of bacteria, viruses, archaea, and other unicellular microorganisms (37-39). These microorganisms, known as the normal microbiota, reside in the human body without causing any harm to the host (37). It is estimated that the human body contains 10^{14} bacteria in their

normal flora, a number, which is ten times higher than the number of total cells that make the human body (37). As the normal commensal, the most of the bacteria that comprise more than 70 % of the bacteria of the total microbiota, are present in the human gut (37, 40).

The human gut flora consists of different types of bacteria; however, most of the bacteria belong to two phyla– Bacteroidetes and Firmicutes (37, 39). In addition, there are bacteria from the Actinobacteria and Proteobacteria phylum that comprise a small portion of the microbiota (37).

The normal flora performs various important functions including digestion, metabolism, nutrition, and maturation of the gut immune system (37, 39, 41, 42). Apart from these, a major function of the gut microbiota is to prevent the infections caused by endogenous or the exogenous pathogenic microorganisms (37-39, 41, 42).

The Normal commensal performs this function by various ways. The gut flora protects the host from pathogens by acting as a physical barrier (37, 42). Normal flora occupies the surface of the intestinal epithelium, and prevents the attachment of the pathogens, which acts as the deterrent for the colonization of the disease causing bacteria (37, 42). In addition, the normal commensal competes for the nutritional sources with the pathogenic bacteria and plays a role in preventing the infection (37). Figure 1-7 shows the mechanisms, by which normal commensals prevent the infections caused by *C. difficile*, and other pathogenic bacteria.

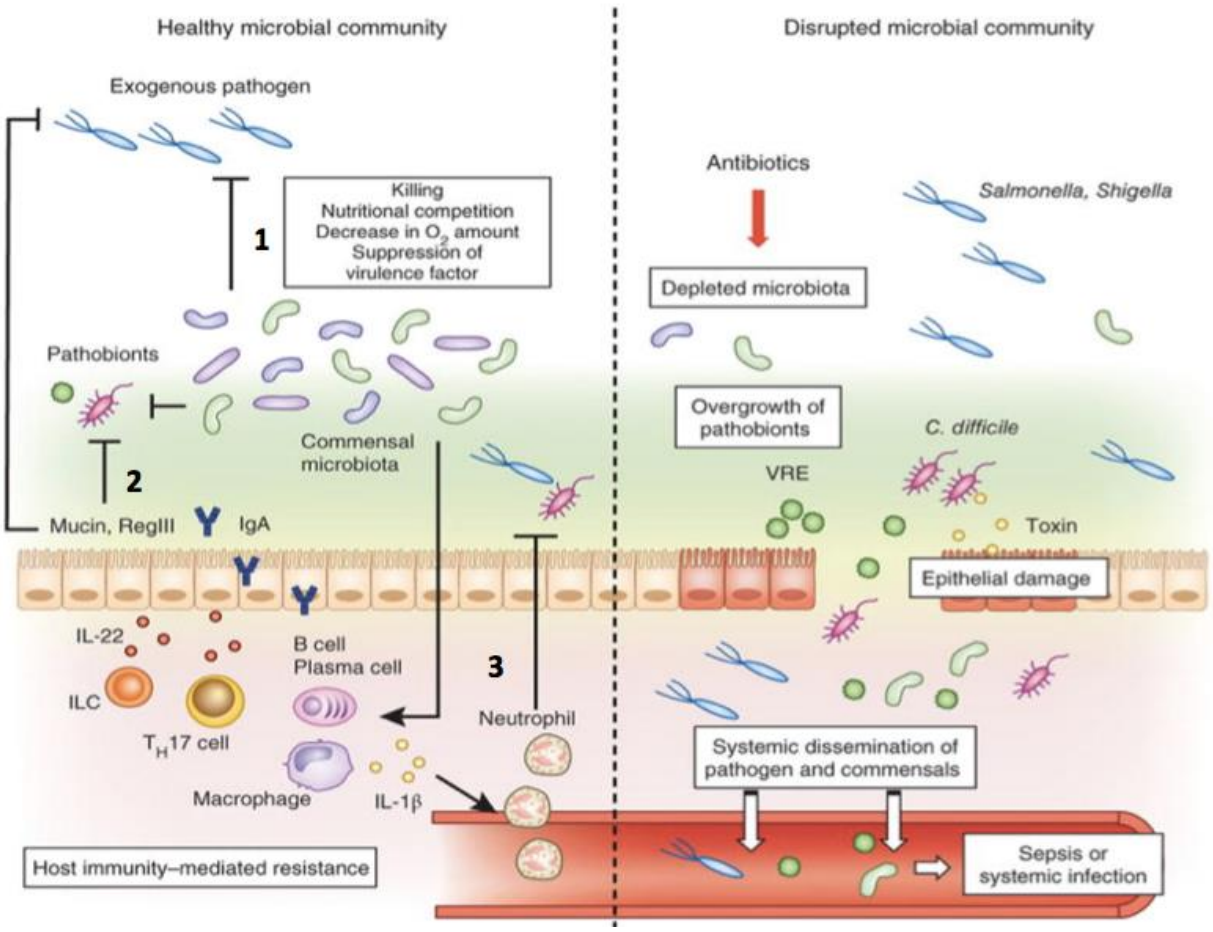


Figure 1-7: In a healthy gut environment (Left side of the figure), the commensal microbiota prevent the colonization of exogenous pathogenic bacteria by various mechanisms. (1) By consuming residual oxygen and metabolites, commensal bacteria modify the expression of virulence factors in pathogens. (2) Commensal microbiota maintain the normal host barrier function by upregulating the secretion of mucous, antimicrobial peptides (e.g., RegIII), and IgA. (3) Commensal bacteria also cause increased migration of the polymorphonuclear cells to the site of infection by priming the intestinal macrophages to secrete more pro-IL-1β. Antibiotic treatment (right side of the figure) disrupts the normal gut flora, which results in increased colonization of pathogenic bacteria (e.g., *C. difficile*). ILC: Innate Lymphoid Cells; T_H17: Helper T cell; IL: Interleukin; VRE: Vancomycin Resistant Enterococci. RegIII: A protein produced from the paneth cell. (Reprinted from Kamada et al. 2013. Control of pathogens and pathobionts by the gut microbiota. *Nature Immunology*, with permission from Macmillan Publishers Ltd.).

Furthermore, the gut flora stimulates the secretion of numerous antimicrobial agents from the intestinal cells (37, 42-44). The metabolic products of the microbiota stimulate the paneth cells, found at the base of the intestinal crypts, to secrete various kinds of antimicrobial peptides (AMP), for instance, defensins, C-type lectins, cathelicidins, etc. (37, 42-44). Short-chain fatty acids (SCFA) and lithocholic acids, which are the metabolic byproducts of several bacteria of the normal commensal, also stimulate the expression of LL-37 cathelicidin, a strong antimicrobial peptide with immunomodulatory effect (37). In addition, many bacteria of the normal flora help to activate the secreted AMP. For example, paneth cell secrete pro-defensin, which needs to be proteolytically cleaved to form the defensin, the active form of the peptide (37). In a study in mice, it was observed that *Bacteroides thetaiotaomicron* possess the enzyme matrilysin that breaks the pro-defensin and cause the release of the defensins (37). Moreover, certain species of *Lactobacillus* produces lactic acids and tetramic acid like substances, which inhibit the growth of a wide range of pathogenic bacteria (both gram-positive and gram-negative) and play a role in the innate immunity of the gut epithelium (37).

1.7 Recurrence of *C. difficile* Infection

Recurrences of CDI after the treatment of the primary infection commonly occur. It is estimated that 20% to 30% of the patients, who are treated with the traditional drugs (metronidazole or vancomycin), go on to the recurrence within eight weeks of treatment (45-47). The recurrence occurs due to the re-colonization and growth of the vegetative cells produced by spores that were not eradicated by the primary treatment, or by a new strain from the environment (45, 46, 48, 49). Recurrence of CDI is a big challenge. In addition of morbidity and mortality, the financial burden of recurrent CDI is high, which costs about 13,000 USD for the treatment of each case of recurrent CDI (45).

There are certain risk factors of recurrent CDI; which are, increased age of the patient (>65 years), prolonged hospitalization, severe primary infection, history of the recurrence, and continued use of the broad-spectrum antibiotics for other diseases (45, 46). Furthermore, host immunity is considered an

important factor to determine the outcome of the recurrence of CDI. It was observed that patients who develop recurrent CDI, has a higher chance of the second recurrence (40%), and interestingly, patient with the second recurrence, has more chance (more than 60%) to develop three or more recurrences (2).

Patients with recurrent CDI have more disruption of the normal gut flora, compared to the patient with the first episode of the disease (46). In addition of reduced number of the *Bacteroidetes* spp, the protective anaerobic bacteria, patients with the recurrent attack, also have less diversity of the gut microbiota, compared to the healthy controls (45, 46). This observation is further supported by the fecal bacteriotherapy, where patients' normal gut microbiota are restored by healthy donors' fecal materials (45, 46, 50).

Treatment of the recurrent CDI is a big challenge, and there are no clear-cut treatment options for it. However, there are some widely accepted approaches (2, 20). For the first recurrence, treatment is like the primary treatment, with oral metronidazole or vancomycin for 14 days (2, 20). For the second recurrence, oral vancomycin with tapering doses for a long period (Eight weeks); and for the treatment of third or the subsequent episodes, oral vancomycin with the adjuvant, rifaximin for 14 days (2, 20). If the patient is refractory to these treatments, then the last resort of treatment is the fecal bacteriotherapy (2, 20).

1.8 Current treatment options for the CDI and their limitations

There are limited numbers of antibiotics currently in use for the treatment of CDI. Moreover, those antibiotics often suffer from several shortcomings in treating CDI. Figure 1-8 shows the chemical structure of the commonly used antibiotics for CDI. In addition to antibiotics, including those in clinical trials, there are other treatment options, for example: fecal microbiota transplant, monoclonal antibodies, vaccines, and probiotics for the treatment and the prevention of CDI. Below are the details of the individual treatment options for the CDI, with their shortcomings.

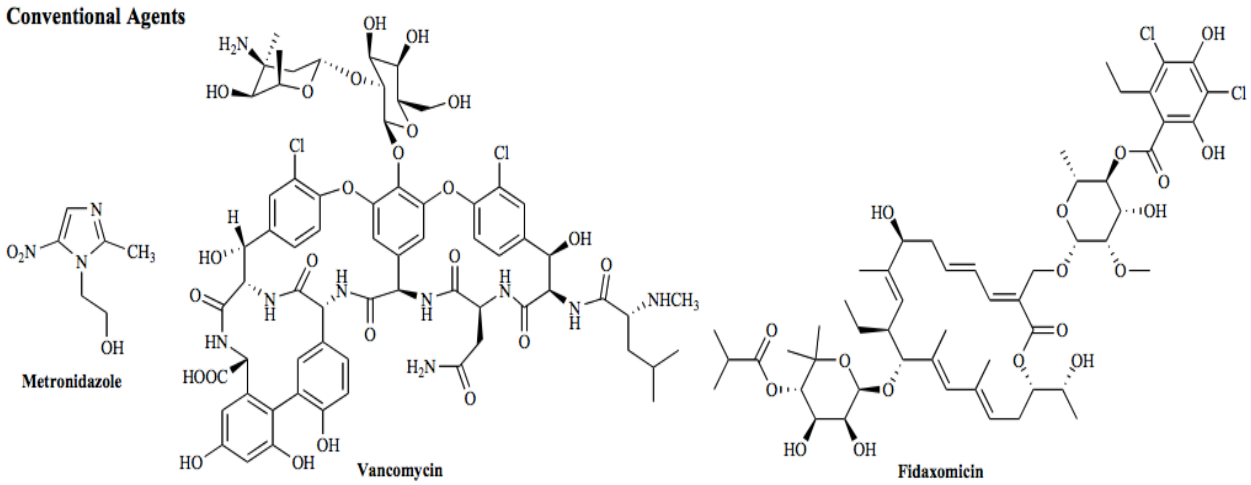


Figure 1-8: Chemical structure of the commonly used antibiotics for CDI treatment. (Reprinted from Tsutsumi et al. 2014. Progress in the Discovery of Treatments for *C. difficile* Infection: A Clinical and Medicinal Chemistry Review. *Current Topic in Medicinal Chemistry*, with permission from Bentham Science Publishers).

1.8.1 Antibiotic treatment

1.8.1.1 Metronidazole

Metronidazole (MTZ) is the treatment of choice for the mild to moderate CDI (20, 51). It is an orally active cost-effective drug that has been used for the last three decades (2, 9). MTZ, a nitroimidazole, is a pro-drug, which needs to be biochemically modified to its active form before it exhibits antibacterial activities (51-53). It undergoes reduction to produce the reactive species. Due to its chemical properties, and small molecular weight, metronidazole can diffuse easily through the bacterial membrane and enters the cell cytoplasm, where the reduction of the 5-nitro groups occurs (51, 52, 54). The active reduced form then binds to the DNA, and causes bacterial DNA breakage, as well as destabilization of the DNA helix (52,

55). MTZ is bactericidal *in vitro* and is effective against a wide range of anaerobic bacteria and protozoa. However, there are some potential problems using metronidazole in treating CDI (9, 51). Firstly, owing to its high absorption rate across the intestinal wall, metronidazole may not always achieve the bactericidal concentration in the colon to kill the vegetative cells of *C. difficile* (56-59). It only reaches 0.5 μg/g to 10.4 μg/g in the stool (59). The reduced therapeutic effectiveness of MTZ against epidemic NAP1 strains, many believe, is due to its poor pharmacokinetics while treating gut infections (52, 60, 61). Secondly, MTZ is a broad-spectrum antibiotic and kills other anaerobic bacteria, which are the part of the normal gut commensal, for example, *Bacteroidetes* spp. This property might contribute to the recurrence of CDI after the treatment of primary infection (62, 63). The action of MTZ on gut flora may hinder successful rebounding of organisms. Thirdly, though it was believed previously that the use of vancomycin can give rise to the Vancomycin-Resistant Enterococci (VRE), a recent study shows that use of MTZ can also accelerate the emergence of VRE, which cause serious nosocomial infections and are refractory to most of the antibiotics (63). Lastly, though the resistance is unstable, there are emergences of *C. difficile* strains in hospitals that show high MICs against MTZ (56, 60, 64).

1.8.1.2 Vancomycin

Vancomycin (VAN), which is a lipoglycopeptide, is indicated for the treatment of moderate to severe CDI (20, 65). VAN is only effective against gram-positive bacteria and inhibits bacterial peptidoglycan biosynthesis by preventing the binding of D-Ala-D-Ala to the growing peptidoglycan layer (65, 66). There are certain properties that make VAN superior to other antibiotics while treating CDI. After oral administration, VAN has low absorption rate from the gut, in contrast to MTZ, and can achieve high concentration to kill the vegetative cells of *C. difficile* (56, 67, 68). This property also minimizes the systemic side effects of VAN (68). Moreover, no clinical strain of *C. difficile*, resistant to vancomycin, has been detected yet, except in *in vitro* study (69). However, the problems, similar to MTZ remain, which are reduced success in preventing recurrence of the disease due to its activities against *Bacteroides* spp and the risk of causing the emergence of the VRE strains (62, 63). Furthermore, VAN primarily works on growing

cells and has poor activity on slow-growers (70, 71). This observation led us to speculate that VAN efficacy can be affected in treating CDI.

1.8.1.3 Fidaxomicin

In 2011, fidaxomicin (FDX), a nature derived macrocyclic antibiotic, was approved by the FDA, for the treatment of CDI (51, 72). It is the RNA polymerase inhibitor, and prevents the initial steps (inhibits sigma subunit and the mobile-clamp domain beta of RNA polymerase) in the synthesis of mRNA(73), which is different than the other classical RNA polymerase inhibitors, for example, streptolydigin, rifaximin, etc. (51, 74-77). FDX is a narrow-spectrum antibiotic, and has no inhibitory effect on the growth of the other major commensals (74, 78). For instance, it is inactive against *Bacteroidetes* spp, a common genus of anaerobes that helps to protect the gut epithelium from the pathogens. In clinical studies, FDX was found to be more effective in preventing the relapse of the disease, compared to vancomycin, after the primary infection. This reduction in recurrence is believed due to its narrow-spectrum of activities on the gut microbiota (74, 78). FDX can be given orally, and after oral administration, it can attain a good concentration in the gut lumen to kill the *C. difficile*. However, due to its specific, simple mechanism of action, bacteria can become resistant to FDX easily, and resistant strains of *C. difficile* have already been emerged in hospitals (69, 76, 79). Furthermore, in several clinical studies it was observed that FDX was not superior to VAN while treating the CDI caused by the epidemic strains (NAP1) of *C. difficile* (80-82).

1.8.1.4 Rifaximin

Rifaximin (RFX), another macrocyclic antibiotic, has been used in combination with VAN, for the treatment of recurrent CDI (2, 20, 51, 83). RFX inhibits the synthesis of bacterial RNA by binding with the DNA-dependent RNA-polymerase enzyme (83, 84). Although RFX is mainly used for the treatment of travelers' diarrhea caused by *E. coli*, due to its potent anti-difficile activity and low gastrointestinal absorption after oral administration, this drug is also indicated in combination with VAN for the treatment

of multiple recurrences (usually third recurrence) of CDI (2). The third recurrence is treated by VAN (125 mg 4 times daily) for 14 days followed by oral RFX (400 mg twice daily) for another 14 days. However, the main problem with this antibiotic is the development of drug-resistance, which occurs by point mutations in bacterial DNA-dependent RNA-polymerase enzyme (77, 85, 86). This mutation readily arises and is very common in the hospital settings. Moreover, while treating the recurrence, RFX possesses limitations against the epidemic strains of *C. difficile*. In a retrospective study performed by Matilla et al., it was observed that RFX demonstrated higher MIC values against the NAP1 strains (0.46 μg/mL), compared to the non-epidemic strains (<0.002 μg/mL) of *C. difficile* (83).

1.8.2 Non-antibiotic treatment

1.8.2.1 Intravenous immunoglobulin (IVIG) and human monoclonal antibodies

IVIG is used as a passive immunization for the treatment of CDI (9, 51). It was observed that patients with severe CDI, develop low levels of antibodies against the toxin A and toxin B. Therefore, the rationale behind using the antibodies (IVIG), which are believed to reduce the severity and the duration of CDI, is to neutralize the toxins produced during the infection (87). Nevertheless, there is the lack of studies regarding the recommended doses of IVIG in active infection (87). In addition of IVIG, human monoclonal antibodies are also in clinical trials (51). Patients receiving monoclonal antibodies with MTZ or VAN were observed to have reduced recurrence of CDI (51, 88). Recently, Merck Sharp & Dohme Corporation initiated the phase 3 clinical trials to study the efficacy of monoclonal antibodies (MK-6072, MK-3415, and MK-3415A), which are designed against the toxin A and toxin B, in preventing the relapse of CDI (89).

1.8.2.2 Vaccines

Vaccines have entered in clinical trials for the prevention of CDI (9, 51). Asymptomatic carriers of *C. difficile* develop high serum titer of antibodies (IgG) against the *C. diffcile*'s toxins. Moreover, there are evidences that antibodies against the toxin A provide protection against CDI (51, 90). Most of the studies

related to *C. difficile*'s vaccine are animal-based. However, Sanofi Pasteur, a company entirely devoted to vaccine development, has developed vaccines from the inactivated toxoid (A and B) of *C. difficile*, and successfully completed phase 1 and phase 2 clinical trials (51, 90, 91).

1.8.2.3 Probiotics

Probiotics are another treatment option for the prevention of CDI (9, 51). Probiotics are live organisms, mostly bacteria, which help to reestablish the normal gut microbiota that are disrupted by the broad-spectrum antibiotics (92). There is no clear evidence about the efficacy of probiotics, though some small and moderate size clinical trials showed that certain microorganisms present in probiotics, for instance, *Lactobacillus*, *Bifidobacterium*, etc. are effective in preventing CDI (92, 93). More controlled studies are needed, before establishing probiotics as effective for the prevention of CDI (51).

1.8.2.4 Fecal Microbiota Transplant (FMT)

Fecal Microbiota Transplant (FMT), an old method and first reported by Eiseman et al. (94) in the year of 1958, has recently gained more attention in treating recurrent CDI. FTM is now used as the last choice of treatment for refractory CDI (9, 20, 51). FTM involves the replenishing of patients disrupted gut flora by the feces of screened healthy donors, preferably, from the same family (95, 96). After the preparation of the donors' feces, it is given by anal (rectal enema) or oral (nasogastric) route. Several clinical studies are ongoing to examine the effectiveness and the safety of FTM, and so far, this procedure is found to be very effective (>90 % effective) in treating recurrent CDI (95, 97). However, there are certain drawbacks of the FMT, for example, there are chances of transmission of infectious diseases through the fecal materials (9, 95, 96). In addition, this procedure needs special equipment (e.g., colonoscope, endoscope, etc.) and is sometimes embarrassing for the patients (51, 95, 96).

Although, the antibiotics that are currently in use for the treatment of CDI can control the mild to moderate infections, it is hard to treat infections caused by epidemic NAP1 strain, which produces severe and complicated disease. In addition, these antibiotics cannot prevent the relapse of the disease, another big challenge while treating CDI (82, 98).

Therefore, the search for antibiotics, which can cure CDI, is really necessary. In this endeavor, compounds that can demonstrate narrow-spectrum of activity (do not disrupt gut microbiota) and possess good gastrointestinal pharmacokinetics with the novel mode of action, will be the promising drugs.

1.9 Membrane targeting compounds in controlling the recurrent *C. difficile*

Maintaining the proper physiology and the integrity of the cell membrane is essential regardless of the metabolic status of the cell. Even at the dormant stage, the cell membrane remains dynamic and performs crucial jobs necessary to maintain the homeostasis of the cell. The cell membrane contains one-third of proteins of the cell that provide active transport of ions and metabolites, help in the production of the ATP for the intracellular functions, cause the active removal of the metabolic waste products, and help in maintaining the proper proton motive force (99). In addition, it plays an important role in the formation of biofilms by establishing the cell-cell communication (99).

In the perspective of the recalcitrant and biofilm infections, where most of the cells remain metabolically dormant, membrane-targeting compounds may be the promising armamentarium to combat those problems. However, the problem remains in the non-specificity of the bacterial membrane with the mammalian cell membrane (100).

Fortunately, there are subtle differences in the composition of the lipid bilayer of mammals and bacteria that guides in finding new targets for the antibacterial drugs. Lipopeptides (e.g., daptomycin) and lipoglycopeptides (e.g., oritavancin) are good examples of membrane targeting agents, which are already

in the market for the treatment of biofilm and recalcitrant infections caused by the gram-positive pathogens (101). However, due to the presence of outer membrane and efflux pumps, these membrane-active drugs are not effective against the gram-negative pathogens. In addition to potent efficacy against gram-positive bacteria, these groups of drugs demonstrate low potential for the development of resistance (102, 103). These drugs preferentially bind with the bacterial cell membrane due to the dominance of the negatively charge phospholipids (e.g., phosphatidylglycerol, cardiolipin, and phosphatidylethanolamine), compared to the mammalian cell membrane (102, 104). For example, in the presence of calcium ions, daptomycin binds with the negatively charged phosphatidylglycerol. These interactions cause the formation of micelle-like structures that results in leakage of essential ions (especially potassium ion), and the disruption of the membrane potential (104). In a similar way, the glycolipopeptides, for example, oritavancin, which carries net positive charge, destabilizes the bacterial membrane containing a large amount of phosphatidylglycerol and cardiolipin (99). Owing to the novel mode of action, potent therapeutic efficacy, and low frequency in developing the resistance, membrane-targeting compounds can be the main focus of treatment for the dormant infection.

Surotomycin, a lipopeptide compound and believed to possess a similar mechanism of action like the daptomycin, is still in the clinical phases for the treatment of CDI. In addition, it is the first membrane-active drug in clinical trials for CDI. However, its proper mode of action, its effect on *C. difficile*'s virulence factors, as well as in preventing the relapse of CDI, still needs to be investigated. In this dissertation, these are intended to be explored.

1.10 Hypotheses

Based on the preliminary results, the entire study revolves around the following two hypotheses.

(1) This study hypothesizes that surotomycin by primarily disrupting *C. difficile*'s membrane potential, exerts its bactericidal effect against the stationary-phase cells resulting in the effective reduction in toxin production and sporulation.

(2) This study also hypothesizes that hybridization of tetramic acid, a minimally absorbable membrane-targeting molecule, to metronidazole increases the lipophilicity of the hybrid compounds (MTZ-TA) and helps to retain the hybrids in the gut to kill the stationary-phase cells of *C. difficile*.

1.11 Aims

The hypotheses as mentioned above are addressed by following two aims

Aim 1: Determine the mode of action (Chapter II) and the efficacies of surotomycin in reducing toxin production and sporulation (Chapter III) against toxigenic strains of *C. difficile* using *in vitro* assays.

Aim 2: Determine the *in vivo* efficacy and the pharmacokinetic properties of MTZ-TA hybrid compounds using hamster model of CDI (Chapter IV).

Chapter II

Evaluation of the mode of action of surotomycin in *C. difficile*

2.1 Introduction

The Gram-positive, spore-forming anaerobic bacterium *Clostridium difficile* is the leading cause of hospital-acquired diarrhea in North America and Europe. Elderly hospitalized patients on broad-spectrum antibiotics are the main target populations, but recent observations indicate there is an increase in the incidence of *C. difficile* infection (CDI) in the community without known risk factors (22, 105). In the United States in 2011 there were an estimated 500,000 cases of CDI resulting in 29,300 deaths (106), reflecting the devastating impact of CDI since the turn of the last century. Furthermore, the number of cases of severe CDI has escalated, coinciding with the emergence of epidemic ribotypes such as BI/NAP1/027 (2, 7). BI/NAP1/027 is now responsible for a significant number of cases of hospital-acquired CDI in North America (2, 106).

For more than 30 years vancomycin and metronidazole have been the first-line treatment choices for CDI (9). Metronidazole is prescribed for mild to moderate CDI, while vancomycin is recommended for severe CDI (2, 20). However, rates of recurrence of 20-25% or higher in severe CDI are common, following treatment with metronidazole or vancomycin (2, 48, 50). The mode of action of vancomycin is well established, involving inhibition of the latter stages of peptidoglycan biosynthesis, which primarily kills rapidly growing *C. difficile* (71). Metronidazole undergoes biochemical reduction to form reactive species that target DNA and is potent in vitro, but only low concentrations reside in the gastrointestinal tract (54, 56-58, 107). Fidaxomicin, which targets the bacterial RNA polymerase inhibitor, has a narrower spectrum of activity than metronidazole and vancomycin and is superior in the prevention of CDI recurrence (80, 108). However, additional novel therapeutics are required to effectively treat CDI and reduce the rates of recurrence following initial therapy.

Surotomycin is a minimally absorbed narrow-spectrum cyclic lipopeptide antibiotic, which is in phase 3 clinical trials as a novel treatment for CDI. It is chemically and structurally related to the antibiotic daptomycin that targets the bacterial membrane thereby exhibiting bactericidal effects (109-111). Daptomycin has been shown to display activities against stationary phase *Staphylococcus aureus* (111), which is a property that would seem amenable to the action of surotomycin in mitigating the pathogenesis of *C. difficile*. This organism produces spores and toxin (TcdA and TcdB), primarily in the late logarithmic and stationary phases of growth (112). However, it is unreported whether the bactericidal activity of surotomycin encompasses the non-growing stationary phase *C. difficile*. Killing of stationary phase cells by membrane-active antibiotics has been shown to lower toxin and spore numbers in vitro, which in principle could contribute to lowering disease severity and rates of endogenous recurrence (71). The basis for surotomycin's potent activity against *C. difficile* is thought to arise from dissipation of the bacterial membrane as shown in *S. aureus* (109). However, direct studies determining if surotomycin dissipates the membrane potential of *C. difficile* have not been reported. Herein, we characterized the mode of action of surotomycin against *C. difficile*, examining its bactericidal effects on logarithmic and stationary phase cells and associated cellular effects linked to dissipation of the membrane potential in *C. difficile*. This study was presented in part as a poster presentation at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC).

2.2 Materials and methods

2.2.1 Compounds, bacterial strains, and growth media

Surotomycin and daptomycin were provided by Merck and Co., Inc. All other antimicrobials were obtained from Sigma-Aldrich (vancomycin, metronidazole, CCCP, ampicillin, fusidic acid, rifaximin and nisin) or Enzo Life Sciences (gatifloxacin). The *C. difficile* strain BAA-1875 (ribotype 078) was from the American Type Culture Collection (ATCC). Strain R20291 (ribotype 027) was kindly provided by Dr. A. L. Sonenshein, Tuft University, Boston USA. Strain IT0843 (ribotype 001) was kindly provided by Dr. Paola

Mastrantonio (Istituto Superiore di Sanità, Rome, Italy). Brain Heart Infusion (BHI; from Oxoid) was used for all the experiments and was supplemented with calcium to a final concentration of 50 mg/L for all experiments with surotomycin and daptomycin. To supplement BHI to a final calcium concentration of 50 mg/L, calcium levels in manufactured lots of BHI were determined by the Laboratory Specialists, Inc., OH. All strains were routinely grown in pre-reduced BHI media under anaerobic conditions in a Whitley A35 anaerobic workstation at 37°C.

2.2.2 Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

Minimum inhibitory and minimum bactericidal concentrations of compounds against *C. difficile* were determined as described by Wu et al. (71, 113). MICs were performed using ~10^6 cfu/mL inoculum of *C. difficile* in 24-well microtiter plates containing 2-fold serial dilutions of compounds in a total volume of 1 mL. MICs were defined as the lowest concentrations of compounds inhibiting visible growth after 24 h of incubation. MBCs were performed against both the logarithmic-phase (MBC_{Log}) and stationary-phase (MBC_{Sta}) cells using logarithmic (OD_{600nm}≈0.3) and 24 h old cultures, respectively. Briefly, in 24-well microtiter plates, cultures were added to 2-fold diluted compounds in a total volume of 1 mL. After 24 h of incubation, the number of viable cells was determined by plating aliquots onto pre-reduced BHI agar containing activated charcoal (10 % w/v). MBCs were defined as the lowest concentrations of compounds causing ≥3 log reduction in viable cells compared to the starting inocula. The MICs and MBCs were determined from two independent starting cultures.

2.2.3 Time-kill kinetics

Time-kill kinetics was evaluated against both the logarithmic and stationary-phase cultures as described by Wu et al. (71, 113). Logarithmic (OD_{600nm} ≈0.3) and stationary-phase cultures were exposed to 1, 4 and 16× the MIC of compounds. Samples (100 µL) were taken at time 0, 1, 2, 4, 6 and 24 h after the addition of

compounds and viable cell counts were determined on BHI agar plates containing activated charcoal (10 % w/v). Bacterial counts were enumerated after 24 h of incubation. This assay were determined from two independent starting cultures.

2.2.4 Macromolecular biosynthesis inhibition assay

Logarithmic cultures of R20291 and BAA-1875 were grown to early logarithmic-phase (OD_{600nm} ≈0.3) under anaerobic conditions and aliquoted for subsequent analysis. To analyze the DNA, RNA, protein and cell wall synthesis inhibition; ^{3}H-Thymidine (2 μCi/mL), ^{3}H-Uridine (2 μCi/mL), ^{3}H-Threonine (2 μCi/mL) and ^{3}H- N-acetyl-Glucosamine (2 μCi/mL) were used respectively. Radiolabelled precursors were added 5 min before the addition of compounds at either inhibitory (1×MIC) and bactericidal (16×MIC) concentrations. Gatifloxacin, rifaximin, fusidic acid and ampicillin were used as controls for DNA, RNA, Protein and cell wall synthesis inhibition, respectively. Against BAA-1875 strain, gatifloxacin, rifaximin, fusidic acid, and ampicillin concentrations at 16×MIC, were 64 μg/mL, 0.96 μg/mL, 2 μg/mL, and 8 μg/mL, respectively. Against R20291, the concentrations at 16×MIC were 512 μg/mL, 2 μg/mL, 2 μg/mL, and 16 μg/mL, for the respective control drugs. Samples (500 μL) were taken at specific time points (30, 60 and 120 min), spun down, and the cell pellet collected and incubated on ice with 10% w/v ice-cold trichloroacetic acid (TCA) for 30 min. Samples were then filtered through Whatman GF/C filters, washed twice with 5% w/v TCA and 95% ethanol. Filters were dried and scintillation counting performed.

2.2.5 Determination of the membrane potential and permeability using FACS

To assess the effects of compounds on the membrane potential and permeability of *C. difficile*, we adopted a fluorocytometric method relying on the use of DiBAC4(3) to assess the membrane potential, and the Propidium Iodide (PI) to assess the membrane permeability (114). Interestingly, no published study has been found on determining the membrane potential in *C. difficile* using FACS. Therefore, this method, based on that reported by Nuding et al. (114) for anaerobic bacteria, is an effort to establish a protocol for

the determination of membrane potential and permeability using FACS in *C. difficile*. During the optimization of this assay, various fluorescence dyes (e.g., Rhodamine123, DiOC3, Oxonol IV) and different conditions were used that could detect the disruption of the membrane potential. The conditions that were used are:

Condition 1: Overnight culture was diluted by 100-fold and let it grow until the OD_{600nm}≈0.2. Fluorescence dye DiBAC4(3) was added at a concentration of 5 μM and incubated for 30 minutes. Then compounds were added, and reading was taken at 30 minutes.

Condition 2: Overnight culture was diluted by 100-fold and then let it grow until the OD_{600nm}≈0.2. Compounds were added at first, and after 10 minutes DiBAC4(3) was added at a final concentration of 5 μM. After adding DiBAC4(3), the culture was incubated for 20 minutes and reading was taken at 30 minutes.

Condition 3: Condition 2 plus an addition of selenium, fumarate, sulphate and nitrate at a final concentration of 10 μM. We believed that these molecules can act as the terminal electron acceptors and might help to increase the potential across the Clostridial cell membrane.

From these three conditions, we observed that condition 2 seemed to be promising in detecting the membrane potential disruption. Therefore, this condition was followed for the subsequent FACS analysis. For this analysis, strains R20291 and BAA-1875 were used, and were exposed to different concentrations of compounds as: 1, 4 and 16× the MIC of compounds. Briefly, cultures were grown anaerobically to an OD_{600nm} ≈0.2 and 10 mL aliquots added to 20 mL serum vials. Compounds were subsequently added and the vials crimped sealed with silicone bungs and removed from the anaerobic chamber. After 10 minutes of adding the compounds, DiBAC4(3) was added via a 23G syringe needle to a final concentration of 5 μM. After an overall 30 min of exposing cells to compounds, at room temperature, fluorocytometric

analysis was performed using BD LSR II flow cytometer. DiBAC4(3) was excited using the 488-nm excitation laser and its fluorescence emission detected using FITC filters. As a positive control, carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Sigma-Aldrich), which completely dissipates the membrane potential was used; vancomycin was used as a negative control. A minimum of three independent cultures was evaluated.

Membrane permeability assays were similarly performed using the protocol described above, except that the membrane impermanent dye propidium iodide was added to a final concentration of 5 μM instead of DiBAC4(3). After 30 minutes of incubation, samples were analyzed in the BD LSR II flow cytometer, with excitation at 488-nm and emission at 630-nm collected using the PI-A filters. Nisin was used as a positive control for membrane damage and vancomycin as a negative control. Daptomycin was also included as a control in these experiments. Resazurin (0.001 gm/L) and sodium thioglycollate (0.5 gm/L) were added to the media to act as an indicator of oxygenation and an oxygen scavenger, respectively. Histogram plots of a number of events against fluorescence of the population were comparatively analyzed using FlowJo X 10.0.7.

2.2.6 ATP-leakage assay

Both the intracellular and extracellular ATPs were determined following the protocol described by Wu et al. (71) and the manufacturer instruction (Promega). Briefly, logarithmic-phase culture (OD_{600nm}≈0.2) of BAA-1875 (ribotype 078) was exposed with various concentrations (1×MIC, 4×MIC, 16×MIC, and 80×MIC) of surotomycin. While incubating in the anaerobic chamber, samples were taken out at 30 minute, 60 minute, and at 120 minute, for the determination of the intracellular and extracellular level of ATP. For the determination of intracellular ATP, after centrifugation at 12,000×g for 6 minutes, pallets were collected and then resuspended with fresh pre-reduced BHI media, while supernatant was collected for the determination of extracellular ATP. Luminescence was measured by multi-mode microplate reader

(BioTek; synergy2) using the BacTiter-Glo kit (Promega), and the ATP level over time was expressed as a percent of the control. This assay was performed with three independent cultures.

2.2.7 Determination of growth inhibitory concentration

Growth inhibitory concentrations of the compounds were determined before the qRT-PCR assay, as because we looked at the expression of the selected genes exposing to the GIC. This assay was performed by following the protocol as described by Arunachalam et al. (115). As GIC acts on the larger number of the cells (OD_{600nm}≈0.3) and halt their growth, it can be considered superior to MIC, while determining the expression of genes. Briefly, overnight grown culture of R20291 (PCR Ribotype 027) were diluted by 100-fold on the next day morning in fresh pre-reduced BHI media and then let the culture grow up to logarithmic-phase (OD_{600nm}≈0.3). Compounds were added to the culture with a various range of concentrations (from 1×MIC to 16×MIC) and then incubated in the anaerobic chamber. Samples were taken at specific time intervals (0.5 hour, 1 hour, 2 hour, 4 hour, and at 6 hour) and optical densities were measured at 600 nm wavelengths (OD_{600nm}) using spectrophotometer (Thermo Scientific: GENESYS 10S). GICs were determined as the concentration of the compounds that prevented the further rise of the optical densities from the baseline (OD_{600nm}≈0.3). Viable cell counting was also performed to see the reduction in log numbers compared to untreated samples at the GIC concentration. Daptomycin and vancomycin were included in this assay.

2.2.8 Quantitative Reverse Transcriptase PCR (qRT-PCR) assay

This experiment was performed according to the protocol described by Manish Kumar et al. (116) and the manufacturer's instruction (Promega). Briefly; under anaerobic conditions, logarithmic-phase (OD_{600nm}≈0.3) cultures were exposed to the compounds at the concentration of GICs as follows: surotomycin at 8 μg/mL, daptomycin at 16 μg/mL, and vancomycin at 16 μg/mL. After 30 minutes of anaerobic incubation, RNAprotectTM reagent (QIAGEN, Valencia, CA) was added with the samples and

then left in the room temperature for 20 minutes. After 20 minutes, samples were spanned down (8,000×g for 5 minutes) to collect the cell pellet. By following the manufacturer protocols, the total RNA was isolated from the cell pellets using SV Total RNA Isolation System (Promega). Primers of the selected genes (Table 2) for the qRT-PCR were designed by using the CLC main workbench 6, and the efficiency of the primers were checked before the assay. To make the cDNA from the RNA, M-MLV reverse transcriptase (Promega) was used followed by using random hexamers. Specific primers and Maxima SYBR Green Master Mix (Thermo Scientific) were used to see the expression of the genes using Applied Biosystems 7300 Real Time PCT System (Applied Biosystems, Grand Island, NY). The total reaction volume was 20 µl and the steps followed for the total 45 amplification cycles were as follows: 2 minutes at 95°C (stage 1), 15 seconds at 95°C (stage 2) and 30 seconds at 72° C (stage 3), followed by a dissociation cycle. This assay was performed in triplicates with three independent cultures and the fold of gene expression was calculated by comparative CT ($\Delta\Delta C^T$) method using 16S rRNA while normalizing the mRNA levels. Data is plotted as a fold of changes in gene expression with standard error bars, using graph pad prism software (version 5).

2.2.9 Statistical analysis

While statistical analysis, the one-way ANOVA was performed for the macromolecular biosynthesis inhibition and the ATP-leakage assay. For the macromolecular biosynthesis inhibition assay, compound-treated samples from three biological replicates were compared with the corresponding untreated control samples. Similarly, in ATP-leakage assay, treated samples, from three biological replicates, were compared with the corresponding untreated samples. GraphPad prism software (Version 5) was used during the analysis, and the differences were considered statistically significant when *P* value was found <0.01.

2.3 Results

2.3.1 Surotomycin is bactericidal against both the logarithmic and stationary-phase *C. difficile*.

As shown in Table 2-1, surotomycin MICs against test strains ranged from 0.125 to 1 µg/mL. The concentration of surotomycin required for bactericidal activities against logarithmic and stationary phase cultures were similar and was 8-128 fold above the MICs (Table 1), which corresponds to 2 to 16 µg/mL. As expected the control metronidazole was also bactericidal, killing both culture types at concentrations between 2 to 16 µg/mL, whereas vancomycin was bacteriostatic and was completely inactive against stationary-phase *C. difficile*.

Table 2-1: Minimum inhibitory concentration (MIC in µg/mL) and minimum bactericidal concentration (MBC in µg/mL) of surotomycin and other conventional drugs against various strains of *C. difficile*.

	***In vitro* Activity of Compounds (µg/mL)**								
	BAA-1875 (078)			**R20291 (027)**			**IT0843 (001)**		
	MIC	MBC_{LOG}	MBC_{STA}	MIC	MBC_{LOG}	MBC_{STA}	MIC	MBC_{LOG}	MBC_{STA}
Surotomycin	0.25	4	2	1	8	16	0.125	8	16
Metronidazole	0.5	2	8	0.5	8	16	0.25	1	16
Vancomycin	0.5	>128	>128	2	>128	>128	2	>128	>128

LOG: Logarithmic-phase cells, STA: Stationary-phase cells

2.3.2 Surotomycin kills *C. difficile* in a concentration-dependent manner.

To determine the effectiveness of surotomycin as the treatment for CDI, time-kill kinetics was performed using several concentrations of surotomycin against logarithmic and stationary-phase culture of *C. difficile*. Toxigenic strain, BAA-1875 and R20291 were tested to assure that results would be largely applicable. The activity of surotomycin was compared to metronidazole, which is also effective against the logarithmic and stationary phase cells, and with vancomycin.

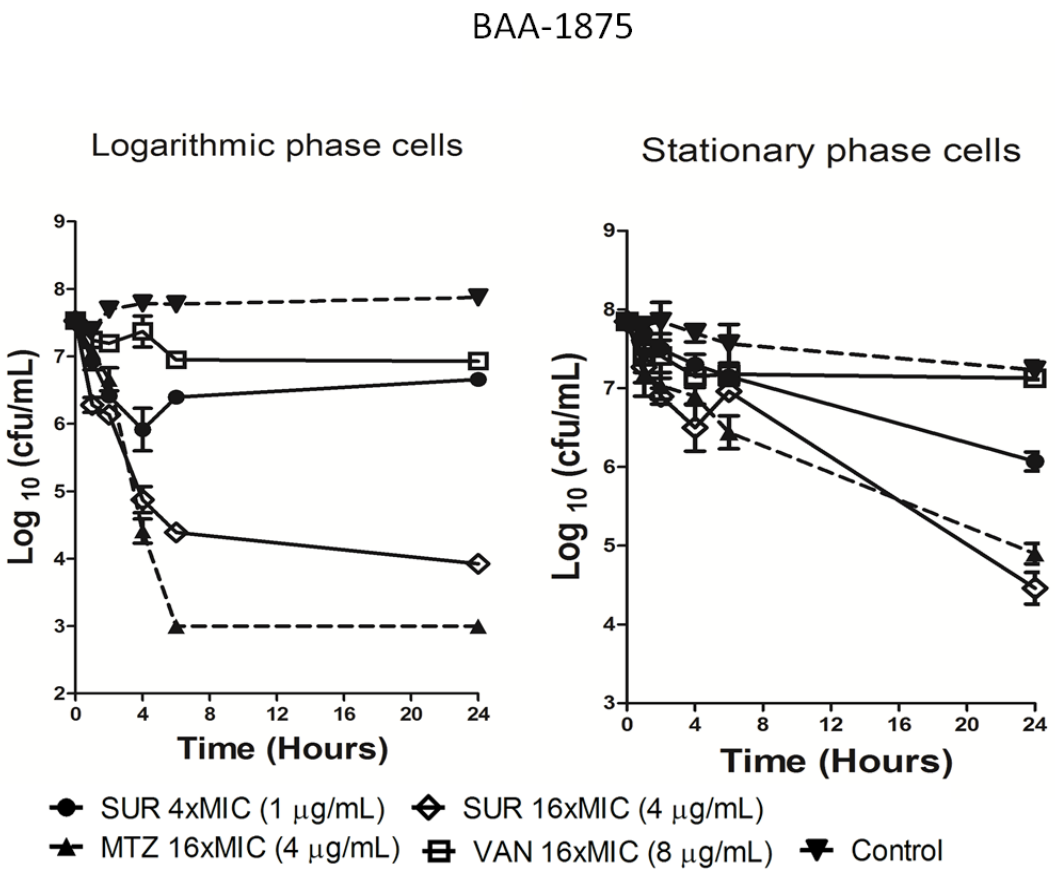


Figure 2-1: Time-kill assay against Logarithmic phase (left) and stationary phase (right) BAA 1875 (PCR ribotype 078). Surotomycin is compared with metronidazole (MTZ) and vancomycin (VAN) at various concentrations shown in the legend. Time-kill assay was performed in duplicates (using two independent cultures) and the data were plotted as mean (± standard errors).

Time-kill assay revealed that surotomycin exhibited a concentration dependent mode of killing against both the logarithmic- and stationary phase cultures of *C. difficile*. Against logarithmic BAA-1875, at 16×MIC (4 µg/mL), surotomycin killed more than 99% of cells in 6 h, whereas 24 h was required to achieve a similar reduction in culture viability, against stationary-phase cells (Figure 2-1, above).

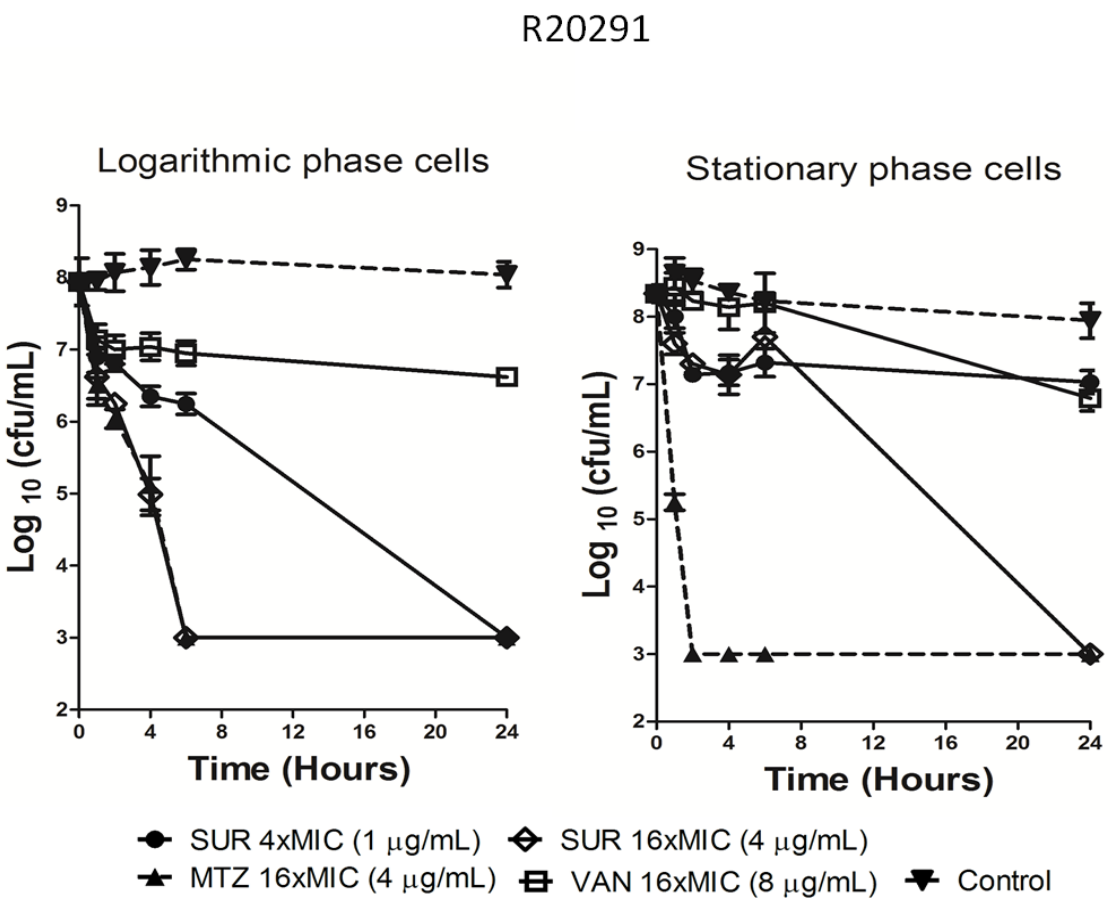


Figure 2-2: Time-kill assay against Logarithmic phase (left) and stationary phase (right) cells of R 20291 (PCR ribotype 027). Surotomycin is compared with metronidazole (MTZ) and vancomycin (VAN) at various concentrations shown in the legend. Time-kill assay was performed in duplicates (using two independent cultures) and the data were plotted as mean (± standard errors).

A similar pattern of killing was observed against R20291, though; this strain seemed more sensitive to surotomycin. At 4×MIC, surotomycin caused a 99% reduction in viable numbers at 24 hour of incubation

against the logarithmic-phase cultures (Figure 2-2, above). This suggests that against R20291, in larger culture volume (10 mL), the MBC is 4 μg/mL, which differs by 4-fold that obtained in 1 mL volumes for the MBC determinations.

Metronidazole was also found to display concentration-dependent killing (against both logarithmic and stationary-phase culture), causing a ≥99% reduction of viable cells at 16×MIC after 24 hours, whereas vancomycin was bacteriostatic and was inactive against the stationary-phase cultures. These observations broadly support the findings of the MBCs data.

2.3.3 Surotomycin dissipates membrane potential without pore formation

Surotomycin causes membrane potential disruption in *S. aureus* (109). However, the effect of surotomycin on *C. difficile*'s membrane potential is not properly known yet. In order to examine whether surotomycin dissipates the membrane potential in *C. difficile*, we adopted the fluorescent probe DiBAC4(3) (114). The fluorescence of DiBAC4(3) changes with the membrane potential status of cells, with depolarized cells demonstrating enhanced florescence, due to DiBAC4(3) entering depolarized membranes and binding to lipid-rich intracellular components, thereby exhibiting increased green fluorescence (FITC-A). When *C. difficile* cultures of R20291 and BAA-1875 were treated with surotomycin, an increase in fluorescence was observed at only 16×MIC, compared to the untreated cultures. Figure 2-3 and figure 2-4 shows the FACS-histograms of Clostridial membrane potential against the BAA-1875 and R20291, respectively, at the concentration of 16×MIC. No changes in the fluorescence of cells were observed at lower concentrations of 1× and 4×MIC. As expected, the negative control vancomycin that inhibits peptidoglycan biosynthesis did not alter the membrane potential, while CCCP that acts as a proton ionophore and disrupts the bacterial membrane potential was shown to increase the fluorescence against R20291 and BAA-1875.

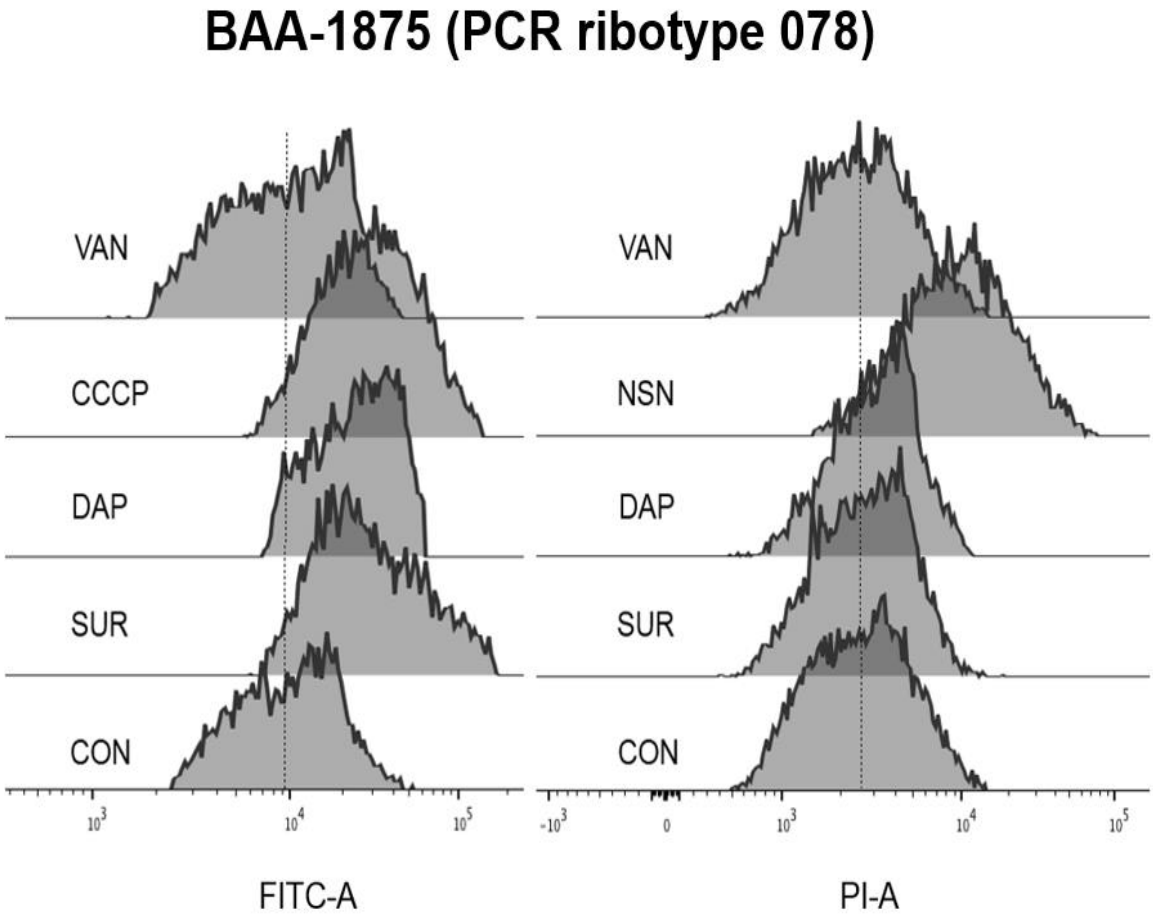


Figure 2-3: Dissipation of *C. difficile*'s (BAA-1875: PCR ribotype 078) membrane potential by surotomycin without an increase in membrane permeability, as detected by the PI uptake assay (right side of the graph). CCCP (Carbonyl cyanide m-chlorophenyl hydrazine) was used as a control for the dissipation of membrane potential, and Nisin, which cause membrane pore formation, was used as a control for the PI uptake assay.

Dissipation of membrane potential by surotomycin at 16×MIC was independent of the membrane pore formation, as cells did not show an increase in red fluorescence (PI-A) compared to the untreated control and vancomycin-treated cultures. In contrast, the pore-forming agent nisin caused membrane pore

formation, which was evident by an increase in the red fluorescence (PI-A) of cells. The non-pore forming effect of surotomycin at the bactericidal concentration is further supported by the ATP-leakage assay (Figure 2-5).

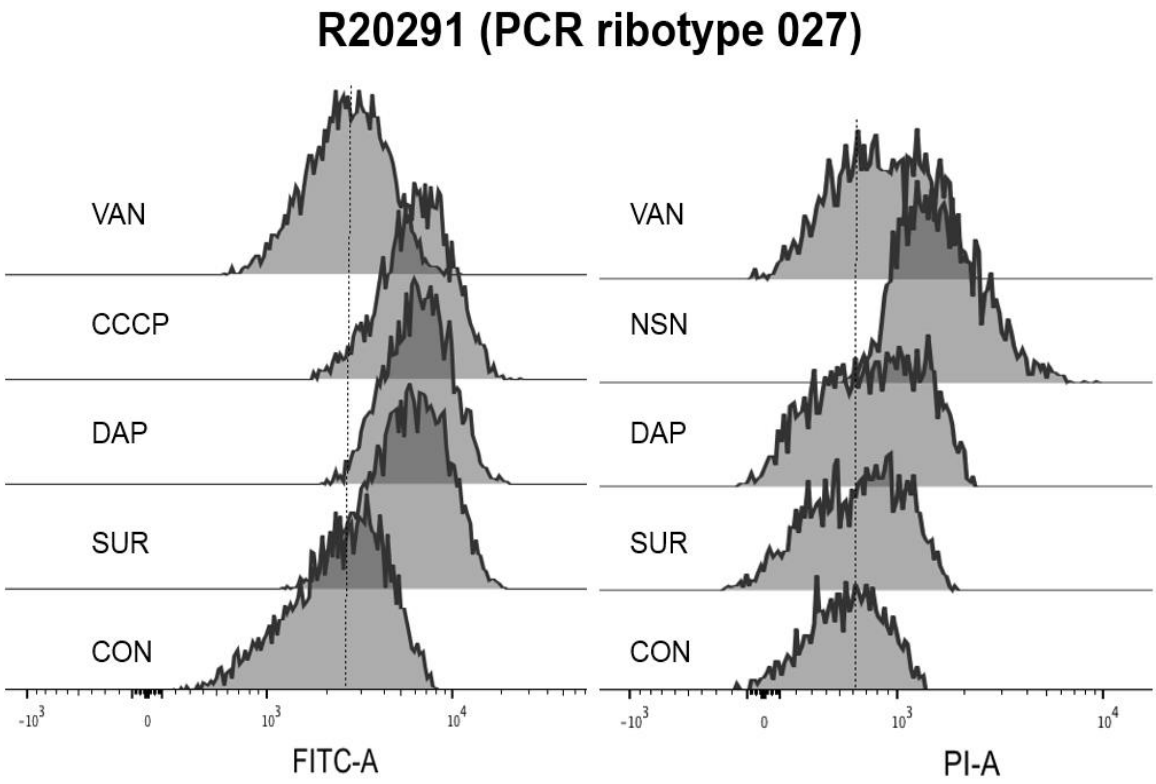


Figure 2-4: The effect of surotomycin at the bactericidal concentration, in disrupting the membrane potential of *C. difficile* ribotype 027, R20291. Like the BAA-1875, similar kinds of effects were observed. CCCP was used as a control for the dissipation of membrane potential, and Nisin was used as a control for the PI uptake assay.

As the above observations are based on 30 min incubation periods, we extended out incubation times. Incubation of cultures with 1× and 4×MIC for up to 2 hour did not lead to an observable difference in the membrane potential status of cells compared to untreated controls (data not shown). Similarly, continued exposure to 16×MIC did not produce further, measurable increases in the dissipation of the membrane

potential. In addition, in the propidium uptake assay, extended incubation times at 1×, 4×, and 16×MIC did not lead to increases in the permeability of cultures to propidium iodide (Figure 2-5).

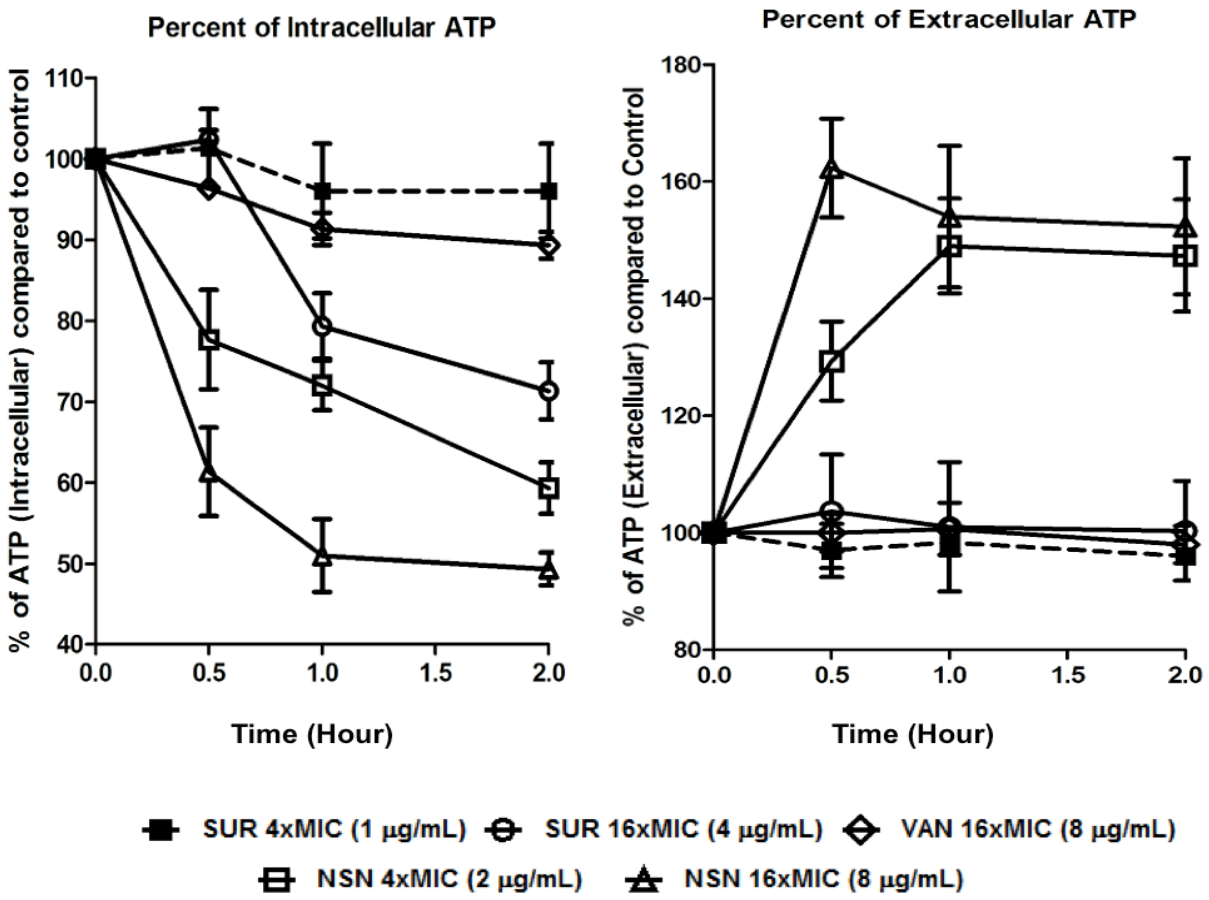


Figure 2-5: Effect (Mean ±SE) of surotomycin in the leakage of ATP from the cell. At 30 minutes, surotomycin at the bactericidal concentration (4 µg/mL), did not cause any leakage of ATP from the BAA-1875 (PCR ribotype 078) strain of *C. difficile*, while nisin caused significant leakage ($P<0.01$) of cellular ATP, as observed by both the intracellular and extracellular level of ATP. Vancomycin did not cause any ATP-leakage up to 2 hour of incubation, even at the 16×MIC (8 µg/mL). Both the intracellular (left side) and extracellular (right side) ATP were measured and plotted in the graph as a percent of untreated samples, obtained from three independent cultures (n = 3).

2.3.4 Surotomycin inhibits multiple macromolecular biosynthetic processes.

Exposure of BAA-1875 and R20291 to inhibitory and bactericidal concentrations of surotomycin resulted in the simultaneous inhibition of DNA, RNA, protein and cell wall. Figure 2-6 and figure 2-7 shows the effect of surotomycin on macromolecular biosynthesis against BAA-1875 and R20291, respectively.

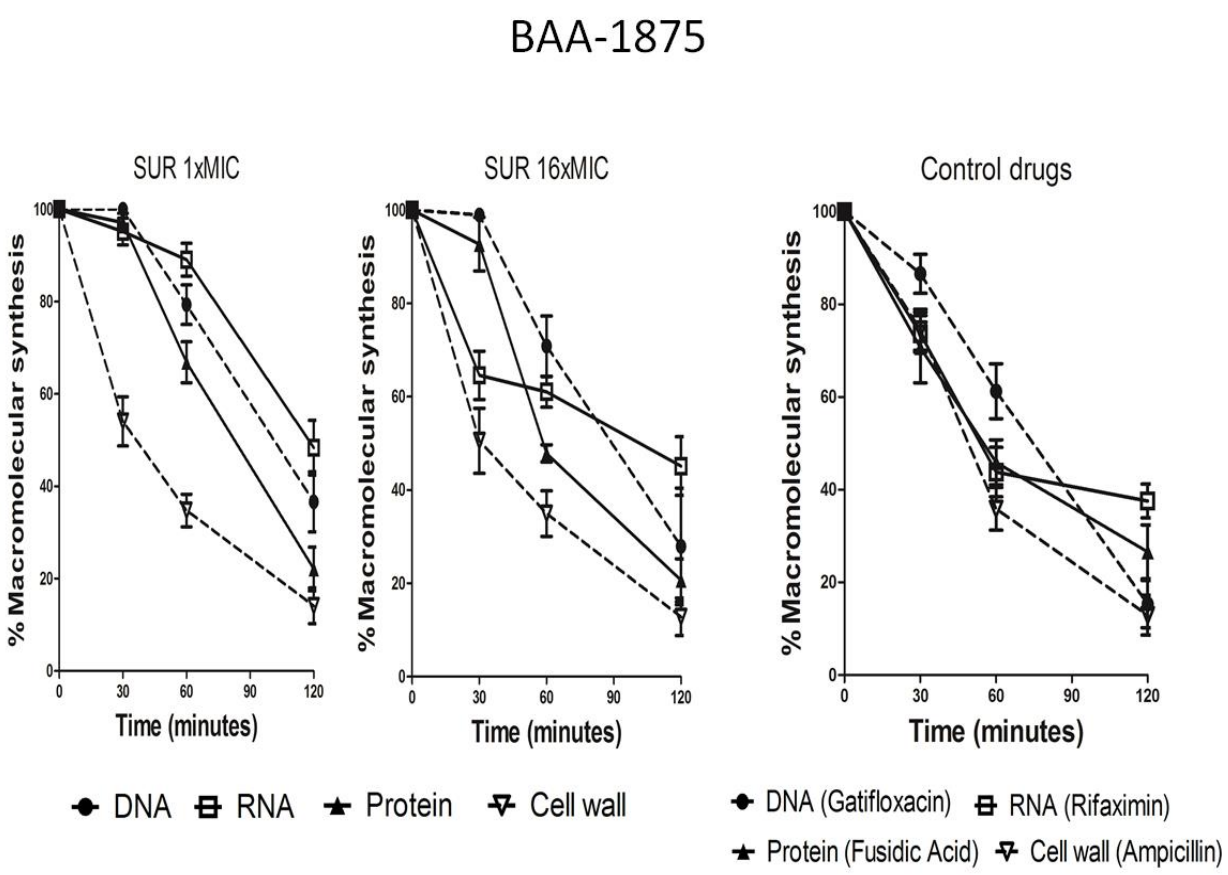


Figure 2-6: Non-specific inhibition of macromolecular biosynthesis by surotomycin at different time intervals (30 min, 60 min and 120 min) against logarithmic-phase *C. difficile* (BAA-1875). At 60 minute and 120 minute, surotomycin inhibits all the macromolecular biosynthesis, which is statistically significant ($P<0.01$). Effect of control drugs is also shown. Statistical significance was determined using ANOVA at $P<0.01$, compared to control samples. Data were plotted as mean (±SE), obtained from three independent cultures (n = 3).

R20291

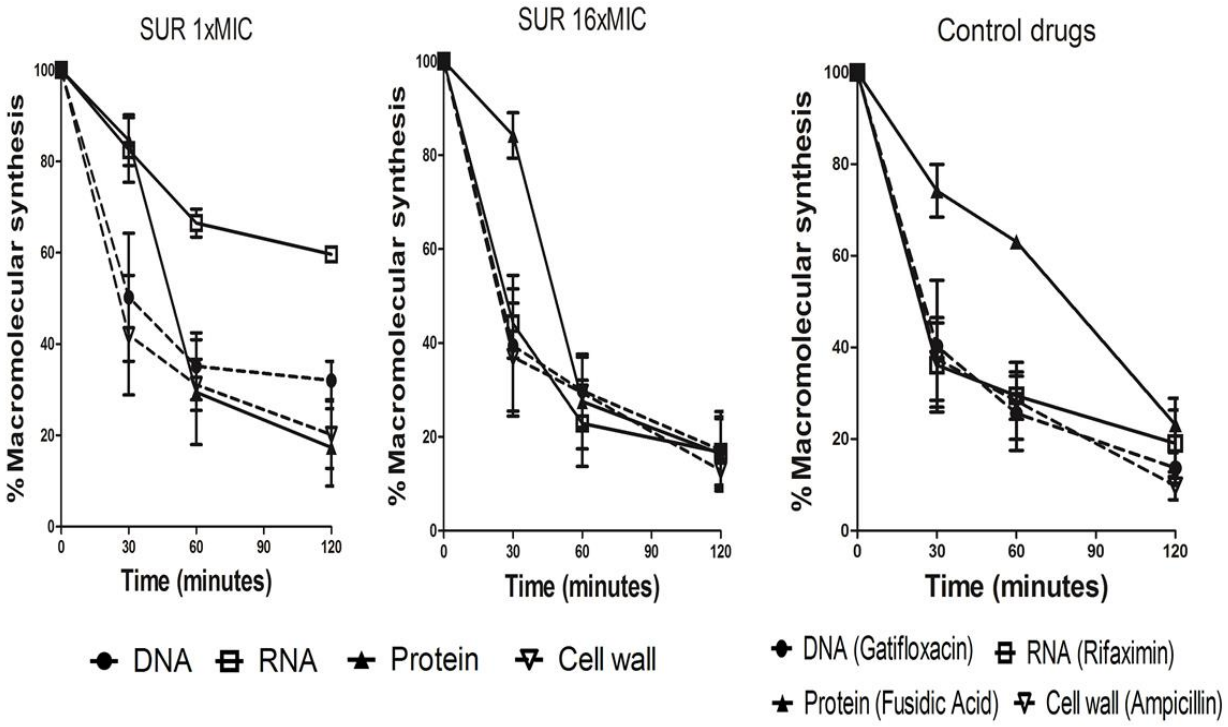


Figure 2-7: Non-specific inhibition of macromolecular biosynthesis by surotomycin at different time intervals (30 min, 60 min and 120 min) against logarithmic-phase R20291. Similar to BAA-1875, at 60 minute and 120 minute, surotomycin inhibits all the macromolecular biosynthesis, which is statistically significant (P<0.01). Effect of control drugs is shown. Statistical significance was determined using ANOVA at P<0.01, compared to control samples. Data were plotted as mean (±SE), obtained from three independent cultures (n = 3).

While it is expected that at the bactericidal concentration, all macromolecular processes would be affected in dying cells, these processes were also affected in cells exposed to inhibitory concentrations. This is consistent with the membrane being the primary target for surotomycin action, thereby imposing multiple cellular effects on processes that require membrane homeostasis.

2.3.5 Surotomycin caused increased expression of genes encoding the potassium transporting ATPase and cardiolipin synthetase protein

The effect of surotomycin and other compounds on the expression of the selected genes (Table 2-2), which can yield further understanding of the mechanism of action of surotomycin, is determined at the concentration of the GIC of the respective compounds (Figure 2-8). These genes were selected based on the daptomycin resistance data in *S. aureus* as well as in *Enterococci* (115, 117).

Table 2-2: Selected genes tested for the gene expression assay (RT-PCR)(115, 118).

Gene	**Gene Product**	**Function**
kdpA	Subunit-A of potassium-transporting ATPase protein	Potassium transport across the cell membrane powered by ATP-hydrolysis
kdpB	Subunit-B of potassium-transporting ATPase protein	Potassium transport across the cell membrane powered by ATP-hydrolysis
cls1	Cardiolipin synthetase	Catalyzes the formation of cardiolipin from the membrane phosphatidylcholine
cls2	Cardiolipin synthetase	Catalyzes the formation of cardiolipin from the membrane phosphatidylcholine
murA	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	Cause the addition of enolpyruvyl to UDP-N-acetylglucosamine, a component in the cell wall biosynthesis
fabK	Trans-2-enoyl-ACP reductase	Regulate the final and rate-limiting step in bacterial fatty acid biosynthesis

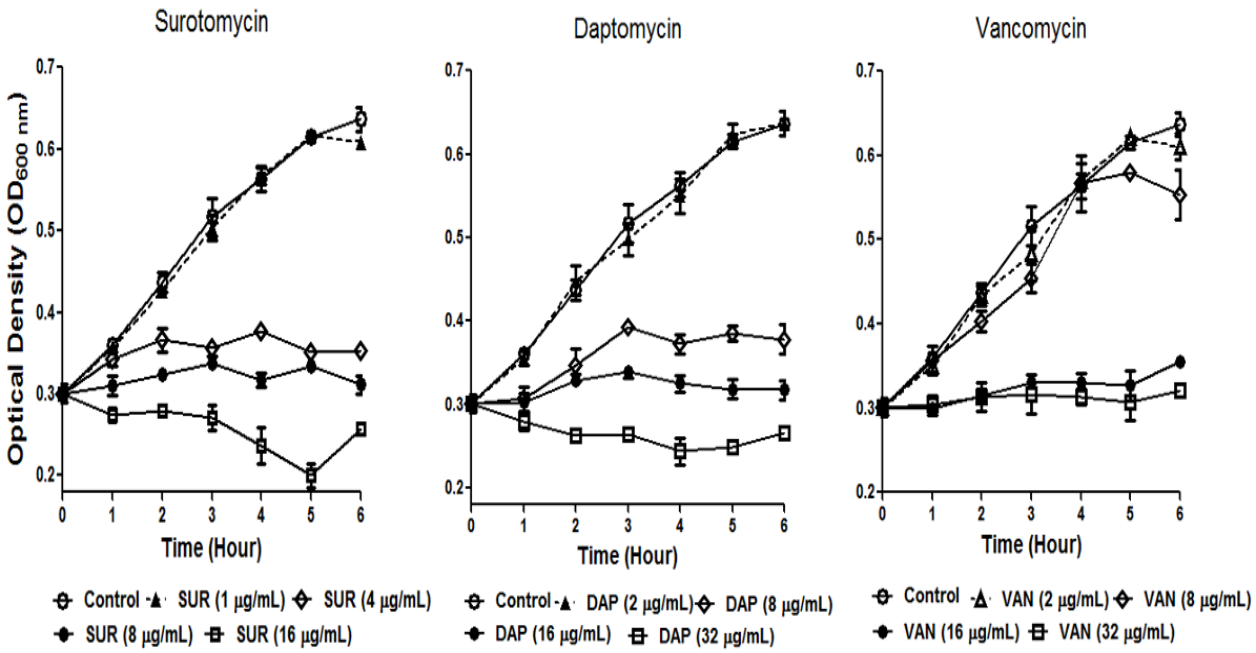


Figure 2-8: Growth inhibitory concentration (GIC) of various compounds against R20291 (PCR ribotype 027). The concentrations used for each compound is shown in the legend at the bottom of the corresponding graph. Optical densities (OD_{600nm}) are plotted in the Y-axis (mean± SE), obtained from three independent cultures (n = 3).

The expression of *kdpA* and *kdpB* genes, which encode the A and B subunit of the potassium transporting system, observed to be significantly expressed (*kdpA* by 3.41±0.95 and *kdpB* by 3.8±0.61) by surotomycin treatment (Figure 2-9), while the *fabK* gene was negatively expressed (0.28±0.18). Daptomycin showed similar effect on the expression of *kdpA* (2.24±0.05) and *kdpB* (2.98±0.26) genes, but no effect on the *fabK* gene. Furthermore, surotomycin caused significant expression of the *cls* 2 (3.49±1.18) and *cls* 1 gene (to a lesser extend; 2.07±0.49). However, interestingly, daptomycin seemed to have no effect on these genes. The expression of *murA* gene was not affected by surotomycin or daptomycin. In contrast, vancomycin did not demonstrate significant effect on any of these genes, except *murA*, which is expressed by 3.4±0.45 fold in all the three cultures tested.

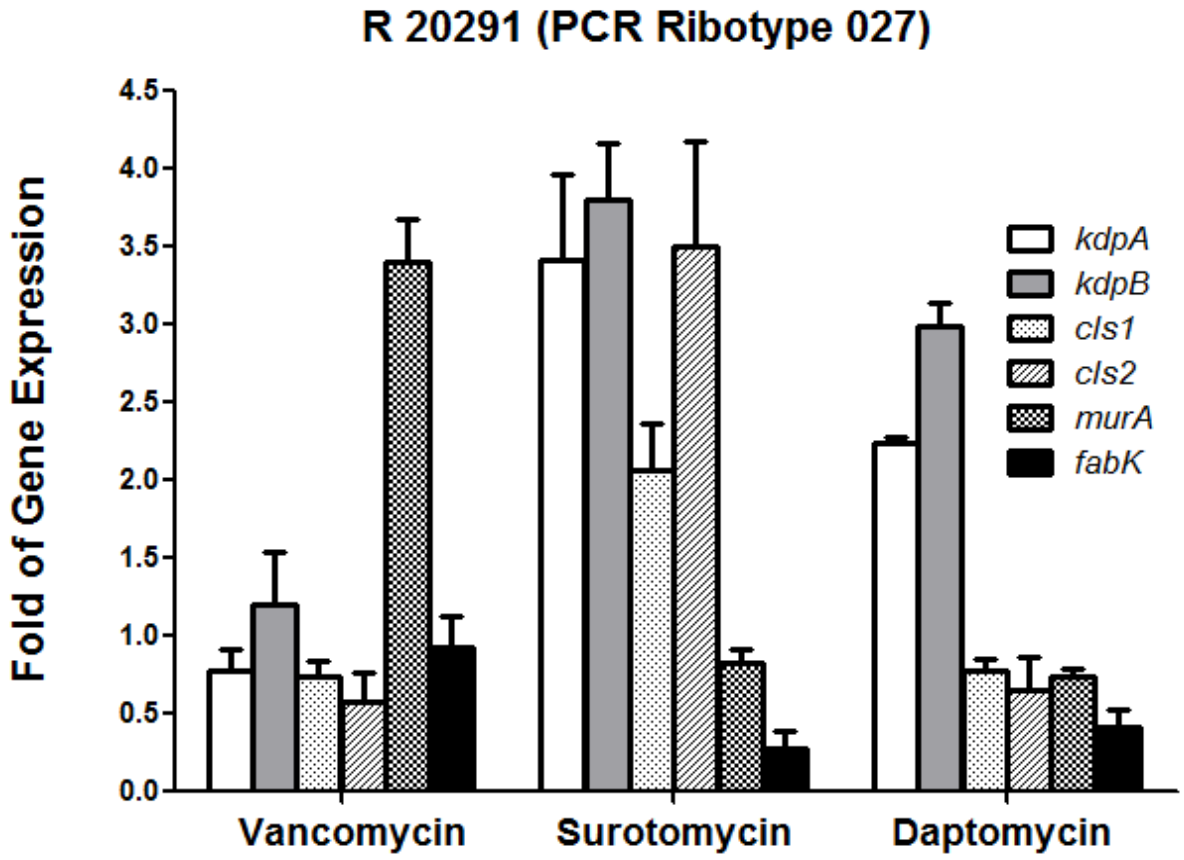


Figure 2-9: Expression of selected genes (*kdpA*, *kdpB*, *cls*1, *cls*2, *murA*, and *fabK*) at 30 minutes, after treating the *C. difficile*, strain R20291 with the GIC concentrations of various compounds (vancomycin 16 μg/mL, surotomycin 8 μg/mL and daptomycin 16 μg/mL. 16S rRNA was used during normalizing the data before calculating the final gene expression (2ΔΔCT). This assay was performed with three independent cultures (n = 3) and the graph is plotted here as mean (±SE).

2.4 Discussion

Maintaining proper membrane potential in necessary for the survival of logarithmic as well as the stationary-phase cells of *C. difficile*, and the agents that cause disruption of membrane potential demonstrate rapid bactericidal activities. In a study by Wu et al. (71), it was observed that membrane-targeting agents, for example, reutericyclin, DCCD, valinomycin, etc. caused bactericidal affect that was associated with the rapid reduction in ATP biosynthesis, against the stationary-phase cells of *C. difficile*. To eradicate CDI, effectiveness of compounds against the stationary-phase cells is important, as because, at this stage *C. difficile* produces toxins and prepare itself for sporulation, which contributes to the recurrence of the disease. Surotomycin, an orally active lipopeptide is a leading example of membrane-targeting agent for treating the CDI. Mascio et al., in 2012, showed that surotomycin disrupts membrane potential in *S. aureus* (109); however, its proper mode of action in *C. difficile* is not properly established. Therefore, this thesis intended to investigate the mode of action of surotomycin against *C. difficile* using fluoroprobe $DiBAC_4(3)$, and validated that surotomycin, at the bactericidal concentration, dissipates *C. difficile*'s membrane potential. In addition, using propidium iodide (PI) uptake assay, this study confirmed that membrane potential disruption is not associated with any membrane-pore formations. This observation is consistent with the study performed by Mascio et al. (109), where they also observed that surotomycin disrupted Staphylococcal membrane potential without causing any membrane pore formations. However, in our study, measurable membrane potential disruption in *C. difficile* occurred only at the bactericidal concentration. This is probably due to the low magnitude of Clostridial membrane potential; and the marginal reduction in membrane potential, at the minimum inhibitory concentration, is not measurable using $DiBAC_4(3)$ dye. Other sophisticated techniques might be required to measure the small changes in *C. difficile*'s membrane potential.

In this study, surotomycin was bactericidal against logarithmic and stationary-phase cells of *C. difficile* and it resulted from the multiple cellular effects, as evident by the extensive non-specific inhibition of

macromolecular biosynthetic processes. In contrast, vancomycin was active against the logarithmic-phase cells but was inactive against the stationary-phase cells. Interestingly, metronidazole was effective in reducing the viability of logarithmic as well as stationary-phase culture of *C. difficile*. Nevertheless, *in vivo*, due to poor oral pharmacokinetics, metronidazole cannot achieve high concentration in the gut to kill the stationary-phase cells (59, 60). In contrary, surotomycin can achieve high concentration in the gut after oral administration as evident by the phase 1 randomized double-blind study, where after treating the CDI patients with oral surotomycin (1000 mg twice daily), the mean fecal concentration was found 6,394 ±3104 μg/g, the concentration that is 200 times higher than the bactericidal concentration (16 μg/mL) of surotomycin against the stationary-phase cells of *C. difficile* (109, 119). Therefore, it is plausible that surotomycin is bactericidal against the stationary-phase cells in the colon, and could be effective in reducing the burden of toxin production and sporulation. In a phase 2 clinical trials, surotomycin caused reduced recurrence of CDI, compared to vancomycin. After treating with surotomycin, at the doses of 125 mg and 250 mg twice daily, the recurrence rate of CDI were 27.9% and 17.2 %, respectively, while after treating with vancomycin at the dose of 125 mg four times daily, the recurrence rate was 35.6% (120). Therefore, in the microbiological perspective, it is tempting to think that due to the bactericidal effect and the narrow spectrum of activities, compared to vancomycin and metronidazole, surotomycin causes the reduction of the virulence factors of *C. difficile*.

To get the further understating in the mode of action of surotomycin, six genes were selected for the quantitative RT-PCR assay. These genes were selected on the basis of literature review, and the predicted guess considering the possible site of action of membrane-targeting compounds. This study found significant expression of the *kdpA* (by 3.41±0.95 fold) and *kdpB* gene (by 3.8±0.61 fold), which encode the A and B subunit of the potassium transporting channel situated in the membrane, in *C. difficile* after treatment with surotomycin and daptomycin. This finding correlates well with the study by Muthaiyam et al. (115), where they observed high expression of the *kdpA* and *kdpB* by daptomycin against *S. aureus*. Our observation supports the role of potassium channel, which were previously reported in *S. aureus* (121, 122)

in the causation of membrane potential disruption in *C. difficile*. However, in our study, in contrast to study by Muthaiyam et al., we did not see any increased expression of the *murA* (0.26±0.075) gene in *C. difficile* after daptomycin or surotomycin treatment. We also observed the increased expression of the *cls*2 (3.49±1.18), and to a lesser extend of the *cls*1 gene that encodes the cardiolipin synthase in *C. difficile*. The role of cardiolipin synthase, in the causation of daptomycin resistance, was demonstrated by Davlieva et al. (117), where they observed mutations in the *cls* gene, as well as alteration of membrane compositions in *Enterococcus*. In addition, in a recent study, in 2015, Kelly Palmer and her colleagues observed mutations in the *cls* gene, after exposing *C. difficile* with surotomycin (118). Interestingly, the *fabK* gene, which product is involved in the final steps of the fatty acid biosynthesis (123), observed to be negatively expressed by surotomycin. These observations suggest that there might be similarities in the mode of action of daptomycin and surotomycin, and their interactions with the cell membrane, as well as in developing the stress response. The increased expression of cardiolipin synthase might be due to the result of the stress response. The compromised cell membrane tried to prevent the further binding of new surotomycin molecules by replacing the phosphatidylglycerol (PG), a possible target of the compound, with cardiolipin (a modified LPG). This phenomenon also may explain the decreased expression of the *fabK* gene, which lower expression might cause decreased production of membrane lipid (phosphatidylglycerol), the probable binding site of surotomycin in presence of calcium ions. The association of the *fabK* gene with surotomycin exposure is further supported by the observation that surotomycin exposed *C. difficile* developed mutation in the *fabK* gene (118). However, we did not see any changes in the expression of the *murA* gene either by surotomycin or daptomycin. In contrast, vancomycin that was used as a control drug, observed to have no effect on the expression of the genes, except the *murA* gene (3.4±0.45), which might be due to the consequence of the cell wall-stress response. Although, this study provides a framework regarding the *in vitro* activities and the mode of action of surotomycin in *C. difficile*, there are certain limitations. Based on the literature review, only selected number of genes is studied in the RT-PCR assay; and this might not be sufficient to get the overall idea regarding the effect of surotomycin on the expression of various genes. To overcome this limitation, expression pattern of the whole *C. difficile* genome need to be explored.

Furthermore, the *in vitro* studies do not provide the explanations how surotomycin is superior, compared to the other commonly used drugs in reducing the recurrence of CDI.

Chapter III

Role of surotomycin in reducing the toxin production and the sporulation from *C. difficile*

3.1 Introduction

Clostridium difficile, the etiological agent in the causation of the *Clostridium difficile* infection (CDI), is the major cause of hospital-acquired diarrhea (2, 15). CDI has caused several hospitals outbreaks in Europe and North America, including the recent outbreaks, in 2011, in many hospitals of Ontario, Canada (7, 124, 125). Toxin production and sporulation by *C. difficile* are considered to be the main virulence factors in the pathogenesis of the primary infection as well as the recurrence of the disease (1).

After colonization in the large intestine, vegetative cells produce toxins (toxin A, toxin B), which are large molecular weight proteins having glucosyltransferase activities (1, 126). In addition to toxin A and toxin B, some strain (epidemic, NAP 027) produces binary toxins, which function is not properly established yet (34). Toxin A and toxin B cause UDP-glucosylation of the GTPase enzymes, such as the Rho proteins resulting in modification of actin cytoskeletal system of the intestinal epithelial cells (126). This enzymatic effect causes the destruction and the apoptosis of the epithelial cells, which produces the symptoms of CDI (1).

C. difficile produces spores, which are resistant not only to the harsh chemical environments but also to all known antibiotics. In addition, some studies suggest that epidemic strains sporulate efficiently and in high levels, which help this strain to disseminate easily in the hospital settings and to cause most of the recurrence of the CDI (18, 34, 127). Relapse of CDI is the most challenging problem while treating this infection. It is estimated that the rate of recurrence is 20 to 30% after the treatment of the primary infection with the standard drugs, and the rate increases with the subsequent attacks (45, 98). Recurrence occurs due to the

persistence of *C. difficile* spores that are not killed previously by the drugs as well as reinfection with a new or the same strains. (127).

The traditional treatment options (vancomycin and metronidazole) are not effective in treating the severe infection caused by the hyper-virulent strains, as well as in preventing the relapse of the disease. The shortcomings of the traditional antibiotics are largely due to their decreased effectiveness against the cells of stationary-phase, the stage where *C. difficile* produces toxins and prepare itself for the sporulation (51, 56). Stationary-phase cells are characteristically resistant to commonly used drugs. Fidaxomicin, the RNA polymerase inhibitor, which is recently approved by the FDA, is shown to inhibit spore formation and prevent the relapse of CDI; however, current clinical data indicate that it is not superior to vancomycin, in preventing the recurrence of CDI caused by the epidemic strains (81, 82). Furthermore, the resistant strain against fidaxomicin has already emerged (79).

Surotomycin, a lipopeptide compound with membrane-active activities, is now in the phase 3 clinical trials for the treatment of CDI (109). In phase 2 clinical trials that include 209 patients of CDI, surotomycin demonstrated better therapeutic potential in the treatment of primary infection, as well as in preventing the recurrence (50% reduction in relapse rate) of the disease, compared to vancomycin (120). However, it is not properly understood regarding the potential reasons why surotomycin possesses better efficacies. Therefore, this study, using *in vitro* assays, intends to look at the effect of surotomycin on the toxin production and sporulation by *C. difficile* that are largely responsible for the pathogenesis and the recurrence of the disease, respectively.

Based on the preliminary results, as presented below, this study hypothesizes that by being active against the stationary-phase cells of *C. difficile* due to its novel mode of action, surotomycin can effectively reduce the burden of toxin production and sporulation.

3.2 Materials and methods

3.2.1 Compounds, bacterial strains, and growth media

Lipopeptides (daptomycin and surotomycin) were provided by Merck and Co., Inc. Metronidazole, vancomycin, fusidic acid, and acridine orange were purchased from Sigma-Aldrich. The *C. difficile* strain BAA-1875 (ribotype 078) was obtained from the American Type Culture Collection (ATCC). Strain R20291 (ribotype 027) was kindly provided by Dr. A. L. Sonenshein, Tuft University, Boston USA. For the sporulation and the toxin inhibition assay, Brain Heart Infusion (BHI; from Oxoid) was the media used which was supplemented with calcium to a final concentration of 50 mg/L. To supplement BHI to a final calcium concentration of 50 mg/L, calcium levels in manufactured lots of BHI were determined by the Laboratory Specialists, Inc., OH. *C. difficile* were grown at 37°C in pre-reduced BHI media under anaerobic conditions using the Whitley A35 anaerobic workstation.

3.2.2 Detection of toxin A and toxin B

Toxin inhibition assay was performed using two toxigenic strain of *C. difficile* (BAA 1875: PCR ribotype 078 and R20291:PCR ribotype 027), as described by Wu et al. (71). Stationary-phase cultures that were grown for 24 hours in BHI media were centrifuged (12,000×g for 5 minutes), and then cell pallets were collected and washed twice with pre-reduced PBS. Supernatants were collected and filtered through 30 K MWCO filter device. Cell pallets were resuspended in the filtered toxin free media. Compounds were added at the concentration of 1×MIC, 4×MIC, 16×MIC and 80×MIC and incubated in the anaerobic chamber for 24 hours. Against BAA-1875, the concentration of surotomycin was 0.25 μg/mL, 1 μg/mL, 4 μg/mL, and 20 μg/mL; while against R20291, the concentration was 1 μg/mL, 4 μg/mL, 16 μg/mL, and 80 μg/mL, respectively. On the next day, samples (1 ml) were taken and spun down to collect the supernatant for the measurement of the extracellular toxin. To measure the total toxin, recovered samples were sonicated for 1 minute to lyse the cells. Then, samples were spun down, and the supernatant was collected to determine the total toxin. Samples were diluted to obtain the proper absorbance reading using microplate reader (BioTeK,

Synergy2) as described by the manufacturer for the *C. difficile* Tox A/B II kit (AlereTMWampoleTM). Toxin inhibition assay was performed in triplicated with three independent cultures. In addition, the qualitative measurement of toxins (Both toxin A and toxin B) was performed using C. diff. toxins A or B quanti (tgcBIOMICS) ELISA kit by following the protocol as described above as well as provided by the manufacturer, to determine the total concentrations (ng/mL) of toxin A and toxin B in stationary-phase cultures.

3.2.3 Sporulation assay

Following the protocol as described by Wu et al. (71, 113), sporulation assay was performed using the same two strains tested for the toxin inhibition assay. However, two different conditions (condition 1 and condition 2) were used while performing this assay. In condition 1, no extra compounds were added during the period of incubation, while in condition 2, extra compounds (same concentrations, as added on day 0) were added at day 2 and at day 4. Briefly, cultures were grown on BHI agar plate containing 0.1 % (w/v) sodium taurocholate (ST). On the next day, broths were prepared after inoculating 2-3 colonies from the plate into fresh pre-reduced BHI media (containing no ST) and were grown up to OD_{600nm}≈0.3. Then cultures were exposed to various concentrations of compounds as tested for the toxin assay, and incubated in the anaerobic chamber (Whitley A35 Anaerobic Chamber) for 5 days. During the period of incubation, samples were taken at day 1, day 3 and at day 5, for the determination of spore numbers by plating the heated samples (60°C for 30 minutes), and total viable cells by plating the non-heated sample on BHI agar plate containing 0.1% (w/v) ST, respectively. Acridine Orange (30 µg/ml) was used as a positive control. All the agar plates were incubated in anaerobic chamber for 24 hour before counting the colonies. This assay was done in triplicates with three independent cultures.

3.2.4 Bioassay of compounds

To check the presence of active compounds over the period of five day's incubation, the bioassay experiment was performed. Briefly, 1 ml of sample was taken from each condition. Samples were centrifuged (12,000×g for 5 minutes) and supernatant was collected to filter through the 0.22 µm filter. Then, this supernatant was used as the starting concentration in the 96-well microtiter plate for the MIC testing. Supernatant was diluted 2-fold in fresh pre-reduced BHI broth, before adding 100 µl of overnight-grown diluted culture. MIC plates were incubated in the anaerobic chamber for 24 hours before taking the reading. *S. aureus* (Newman) was used as an indicator organism during this MIC testing (in BHI media). MICs were performed at day 0, day 3 and also at day 5.

3.2.5 Statistical analysis

GraphPad prism software (version 5) was used for the statistical analysis. While performing the analysis for sporulation assay, compound-treated samples from three biological replicates, were compared with the corresponding untreated control samples. Likewise, in toxin inhibition assay, treated samples, from three biological replicates, were compared with the corresponding untreated samples. One-way analysis of variance (ANOVA) was performed to compare the treated samples with the corresponding controls, for both of the assays. Differences were considered statistically significant when *P* value was found <0.01.

3.3 Results

3.3.1 Surotomycin significantly reduced toxin production as a concentration-dependent manner, from the stationary-phase culture, as determined by qualitative and quantitative analysis

3.3.1.1 Qualitative analysis

Toxin production was inhibited significantly by various concentration of surotomycin, from the stationary-phase *C. difficile*, after 24 hours of anaerobic incubation, as detected by the ELISA assay. Against R20291 (Figure 3-1), surotomycin caused 1.66-fold, 2.37-fold and 3.11-fold decrease in the total toxin, compared to the untreated control at 4×MIC, 16×MIC, and at 80×MIC, respectively. Additionally, extra-cellular toxins were reduced by 1.87-fold, 2.12-fold and 3.16-fold with the above concentrations, respectively, compared to the untreated cells.

Surotomycin, after 24 hours of incubation, against BAA-1875 (Figure 3-2), caused 1.88-fold, 2.26-fold and 4.85-fold decrease in the total toxin amount compared to the untreated samples at 4×MIC, 16×MIC, and at 80×MIC, respectively, which are statistically significant (P<0.0001) as performed by one-way ANOVA (Figure). At 1×MIC, no significant reduction was observed. The concentration-dependent effect of surotomycin against BAA-1875 was also observed while quantifying extracellular toxins, which were inhibited by 2.27-fold, 2.28-fold and 3.57-fold compared to untreated samples, at 4×MIC, 16×MIC, and at 80×MIC, respectively. In contrary, vancomycin, at 16×MIC, caused 1.21-fold decrease in total toxins and 1.19-fold decrease in extracellular toxins, which are statistically non-significant. However, at 80×MIC, only the total toxin (1.54-fold decrease) is significantly inhibited, as evident by the statistical analysis (One-way ANOVA). Fusidic acid, a known protein synthesis inhibitor, observed to cause concentration-dependent inhibition of toxin production from *C. difficile*.

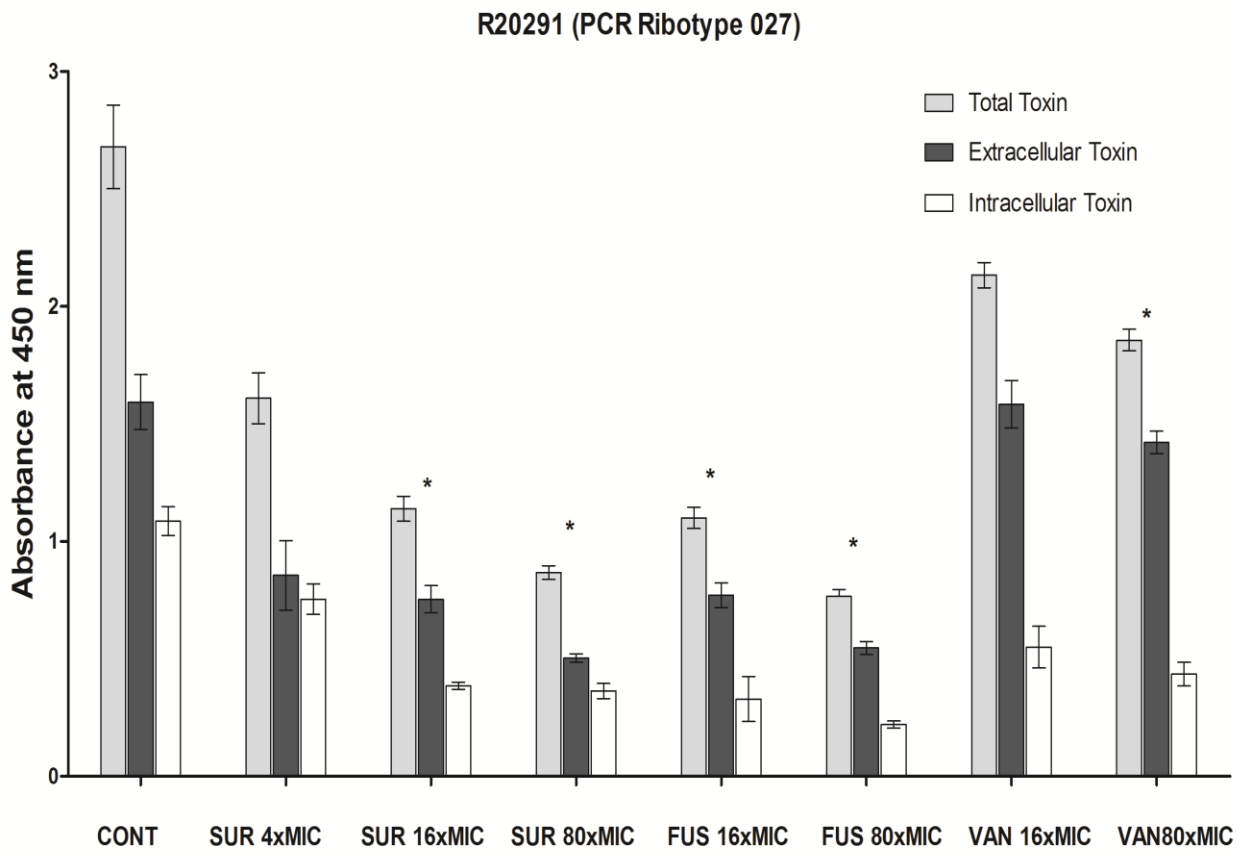


Figure 3-1: Mean (±SE) activity of surotomycin and other comparator compounds in reducing the toxin production in cultures of stationary-phase *C. difficile* (R20291). Surotomycin caused significant inhibition in toxin production at the bactericidal concentration (16×MIC) or more, while vancomycin, only at 80×MIC, caused significant inhibition, compared to control samples. Fusidic acid, used as a positive control, demonstrated similar effect like surotomycin. Inhibition of total toxin, extracellular toxin and intracellular toxin are demonstrated in the same graph. Statistical analysis was performed by one-way ANOVA, and asterisk (*) indicates significant statistical differences, compared to untreated samples at $P<0.01$. Toxin inhibition assay was performed using three independent cultures (n = 3). SUR: Surotomycin, FUS: Fusidic acid, VAN: Vancomycin.

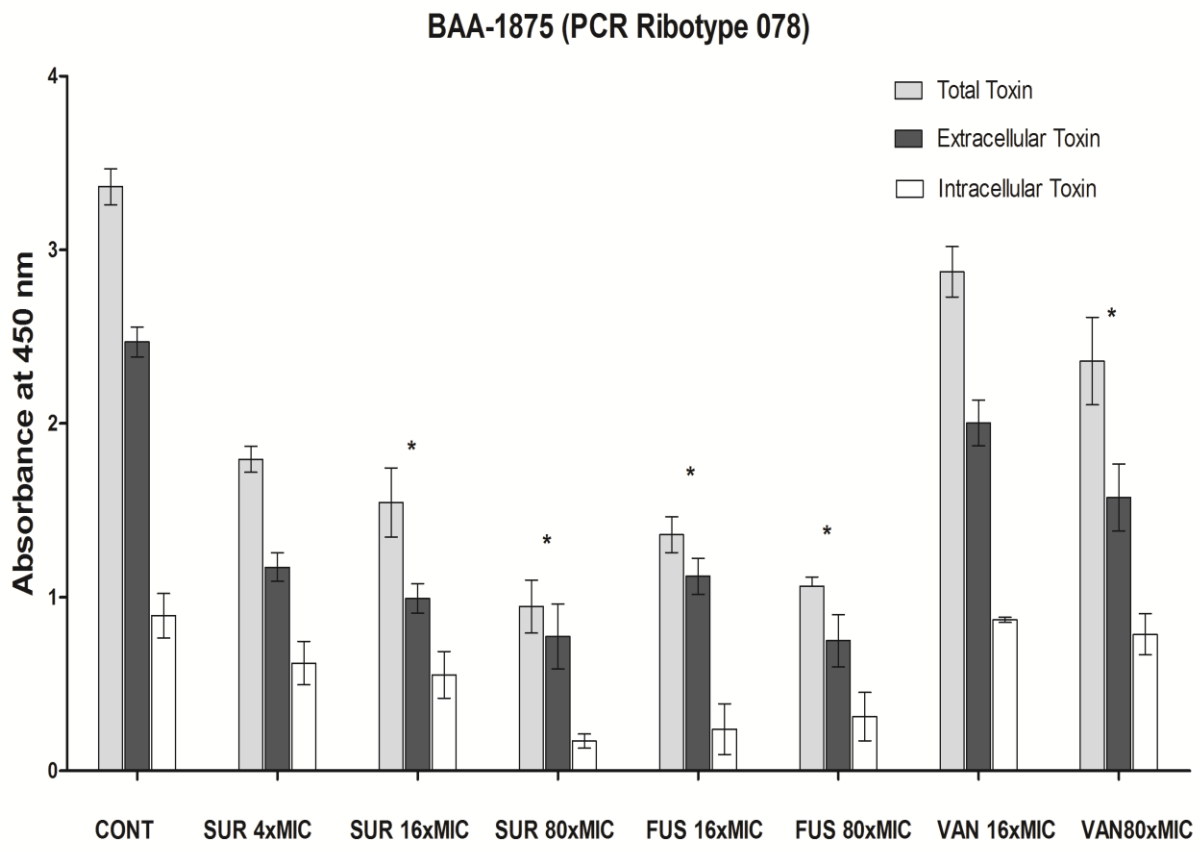


Figure 3-2: Mean (±SE) activity of surotomycin and other compounds, compared to untreated samples, in reducing the toxin production against BAA-1875. Similar to R20291, at the concentration of 16×MIC or more, surotomycin significantly reduced toxin (total, extracellular and intracellular) production. In contrast, vancomycin caused significant reduction in toxin production only at high concentration (80×MIC), compared to the control samples. Inhibition of total toxin, extracellular toxin and intracellular toxin are demonstrated in the same graph. Statistical significance, as shown by asterisk (*) was determined using one-way ANOVA at $P<0.01$. SUR: Surotomycin, FUS: Fusidic acid, VAN: Vancomycin.

3.3.1.2 Quantitative analysis

To validate the data of the ToxA/B ELISA assay (*C. difficile* Tox A/B II kit), which only gives the qualitative estimation of the toxin inhibition, quantitative measurement using (C. diff. toxins A or B quanti; tgcBIOMICS) was performed. The data of the quantitative assay are consistent with the results of the qualitative measurement, and demonstrates similar pattern of inhibition against both the BAA-1875 and the R20291 strain of *C. difficile*.

Against R20291 (Figure 3-3), surotomycin reduced the total level of toxin A by 1.67 ng/mL, 9.3 ng/mL, and 13.97 ng/mL, compared to control samples at 4×MIC, 16×MIC, and at 80×MIC, respectively. Additionally, the toxin B level (total) is reduced by 0.68 ng/mL, 6.67 ng/mL, 11.34 ng/mL with the above concentrations, respectively, compared to the untreated cells.

Against BAA-1875 (Figure 3-4), surotomycin reduced the toxin A (Total level) by 1.4 ng/mL, 11.7 ng/mL, and 14.4 ng/mL, compared to control samples at 4×MIC, 16×MIC, and at 80×MIC, respectively. Also, the toxin B level (total) is decreased by 0.36 ng/mL, 11.94 ng/mL, 14.33 ng/mL with the above concentrations, respectively, compared to the untreated cells.

Against both of the strains tested, at 16×MIC and 80×MIC, surotomycin caused significant inhibition ($P < 0.0001$) of both of the toxin production (Toxin A and B) compared to untreated samples, while vancomycin demonstrated less effect in inhibiting toxin production and caused significant inhibition of toxin production only at high concentration (80×MIC).

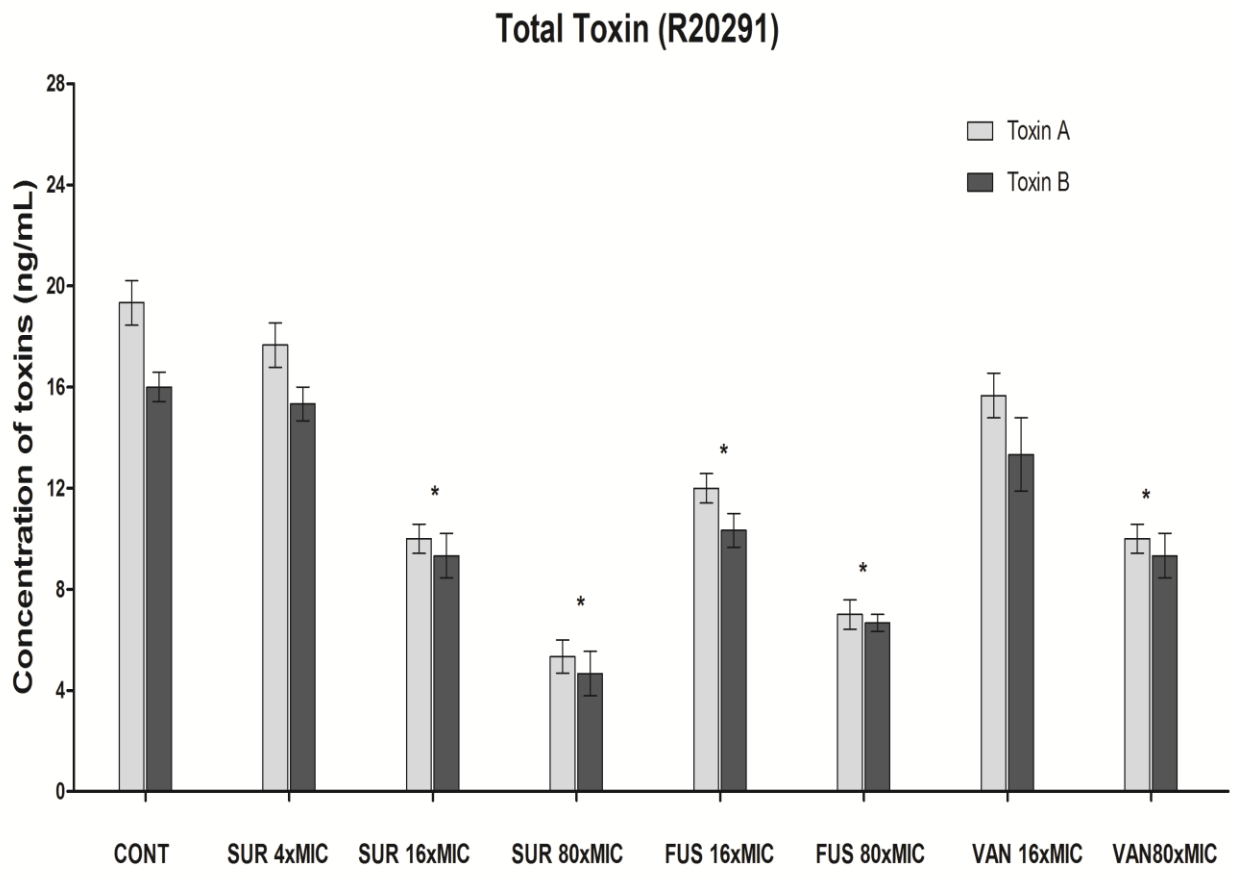


Figure 3-3: Surotomycin's activity in reducing the toxin production against R20291. Similar to qualitative assay, at the concentration of 16×MIC or more, surotomycin significantly reduced total toxin level (both toxin A and toxin B), compared to the control samples. In contrast, vancomycin caused significant reduction only at high concentration (80×MIC). Statistical significance, as shown by asterisk (*), was determined by one-way ANOVA at $P<0.01$. Data were plotted as mean (±SE), obtained from three independent cultures (n = 3). SUR: Surotomycin, FUS: Fusidic acid, VAN: Vancomycin.

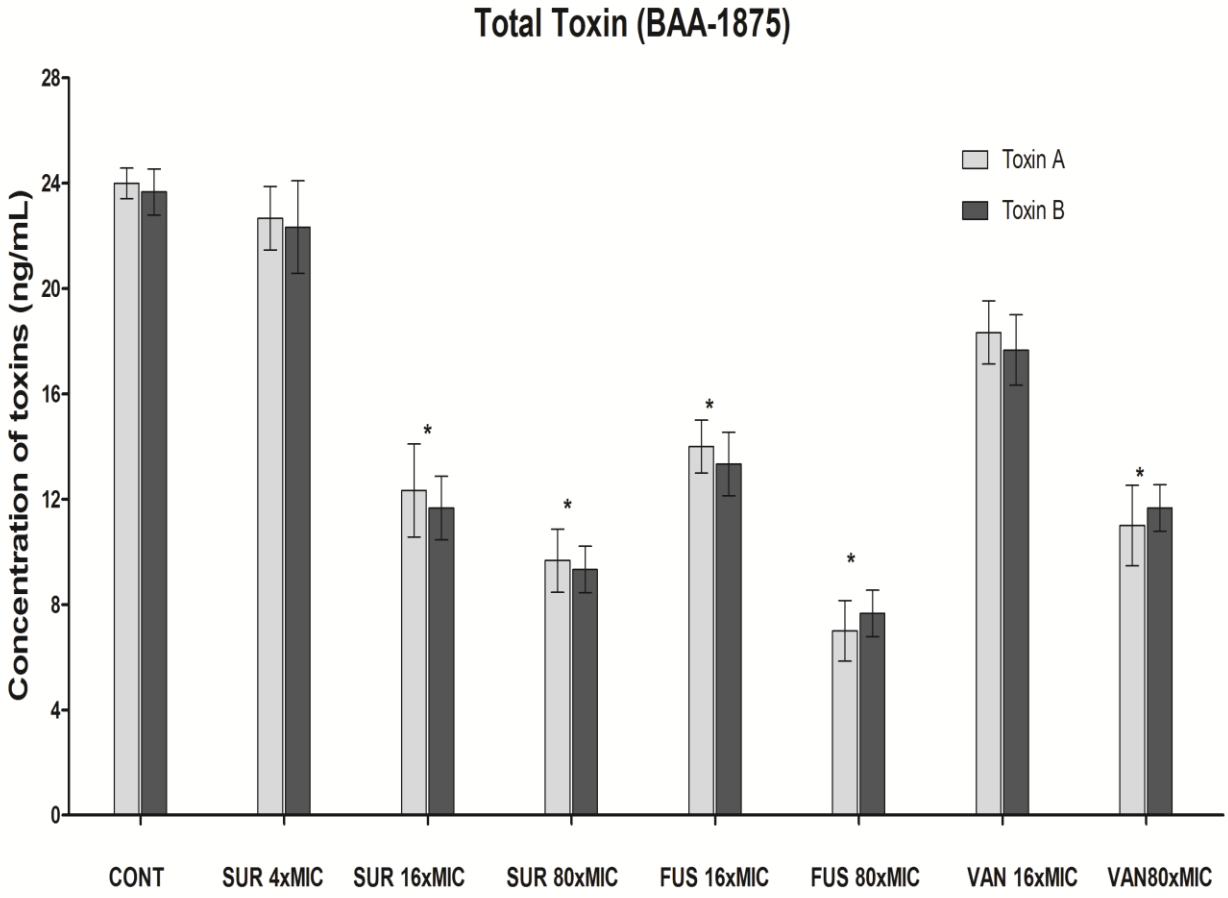


Figure 3-4: Activity (mean ±SE) of surotomycin and other comparator compounds in reducing the toxin production against BAA-1875. Similar to qualitative assay, at the bactericidal concentration (16×MIC) or more, surotomycin significantly reduced total toxin level (both toxin A and toxin B), compared to the control samples. In contrast, vancomycin caused significant reduction only at high concentration (80×MIC). Statistical significance, as shown by asterisk (*), was determined by one-way ANOVA at $P<0.01$. Quantitative assay was performed using three independent cultures (n = 3). SUR: Surotomycin, FUS: Fusidic acid, VAN: Vancomycin.

3.3.2 Surotomycin significantly reduced spore numbers at day 5 in condition 1, and at day 3 and day 5 in condition 2

To evaluate the *in vitro* role of Surotomycin in reducing the spore numbers by *C. difficile*, epidemic strain was tested (ribotype 027). Several studies believe that epidemic strains are more prone to develop spores, which helps *C. difficile* in transmission and in causing recurrence of CDI. Two different conditions (C1 and C2) were used while performing this assay. In C1, no extra compounds were added during the period of incubation, while in C2, extra compounds (same concentrations, as added on day 0) were added at day 2 and at day 4. The main purpose of using these conditions was to observe the stability of surotomycin over the long period of incubation time (five day) at the 37° Celsius. This study observed that surotomycin reduced spore numbers in a concentration-dependent manner at day 3 and at day 5, though, at day 5, the reduction is more significant. At ≥16×MIC, SUR significantly reduced spore numbers in both R20291 and BAA-1875. Against R20291, under C1, (where no extra compound is added), exposure to 16× and 80×MIC of SUR for 3 days reduced spore numbers by 1.5 Logs, compared to the untreated samples (Figure 3-5). However, by day 5, the spore numbers were only 1.2 and 3 Logs less than the untreated control, for the respective concentrations. To evaluate the efficacy (active concentration) of surotomycin over the long period of sporulation assay, studies under condition 2 were performed, where drug was added at days 2 and 4. Under C2, by day 3, similar levels of spore-reductions were observed; however, at day 5, both 16× and 80×MIC reduced spore numbers by >3 Logs compared to untreated controls (Figure 3-6). In contrast, spore reduction with VAN (80×MIC) was inferior to SUR, under condition 1. In condition 2, for VAN (80×MIC),the maximum reduction in spore numbers was 1.8 Logs by day 5, which was inferior to 16× and

80×MIC of SUR. Metronidazole reduced spore numbers significantly (>3 Logs) in both of the conditions at 80×MIC.

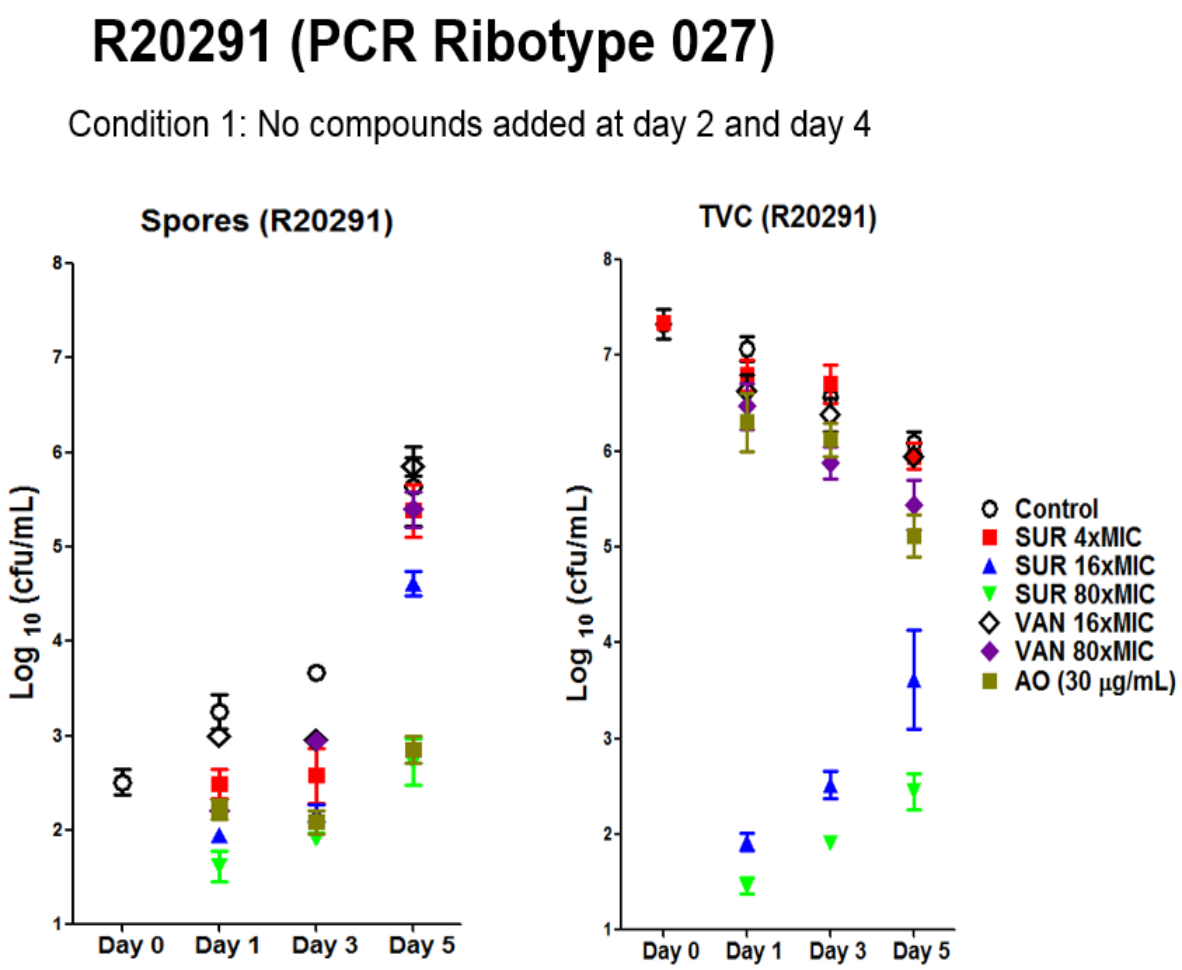


Figure 3-5: Effect of surotomycin and other comparator compounds on sporulation against R20291, in condition 1. Surotomycin significantly reduced spore numbers at day 5, only at the concentration of 80×MIC. In contrast, vancomycin demonstrated no effect in reducing the spore numbers, even at the high concentration (80×MIC). Acridine Orange (30 µg/mL) was used as the positive control in this study. Total viable cell numbers, TVC, (right side of the graph) are also shown with the spore numbers. Statistical significance was determined by one-way ANOVA at $P<0.01$. Data were plotted as mean (±SE).

Data obtained from three independent cultures (n = 3). SUR: Surotomycin, VAN: Vancomycin, AO: Acridine orange.

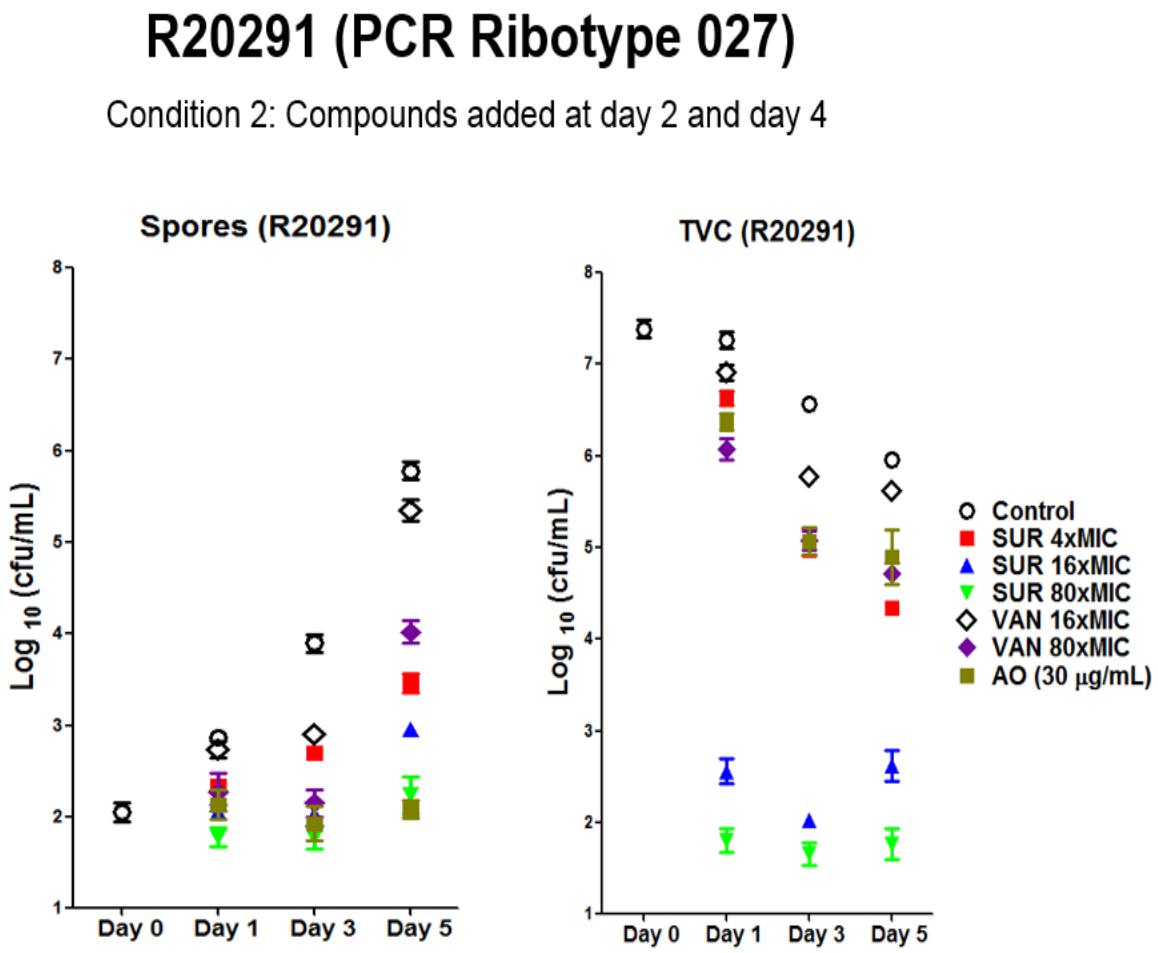


Figure 3-6: Effect of surotomycin on sporulation against R20291 in condition 2, where the same concentrations of compounds were added at day 2 and at day 4. Compared to condition 1, more reduction in the spore numbers was observed. Surotomycin, even at the concentration of 16xMIC, significantly reduced spore numbers at day 3 and day 5. Results of vancomycin are also shown for the comparison. Total viable cell numbers, TVC, (right side of the graph) are also shown with the spore numbers Statistical significance was determined by one-way ANOVA at $P<0.01$. Data were plotted as mean (±SE), obtained from three independent cultures (n = 3). SUR: Surotomycin, VAN: Vancomycin, AO: Acridine orange.

Against BAA-1875, in both conditions (C1 and C2), similar pattern of reduction in spore numbers were observed (Figure 3-7 and figure 3-8).

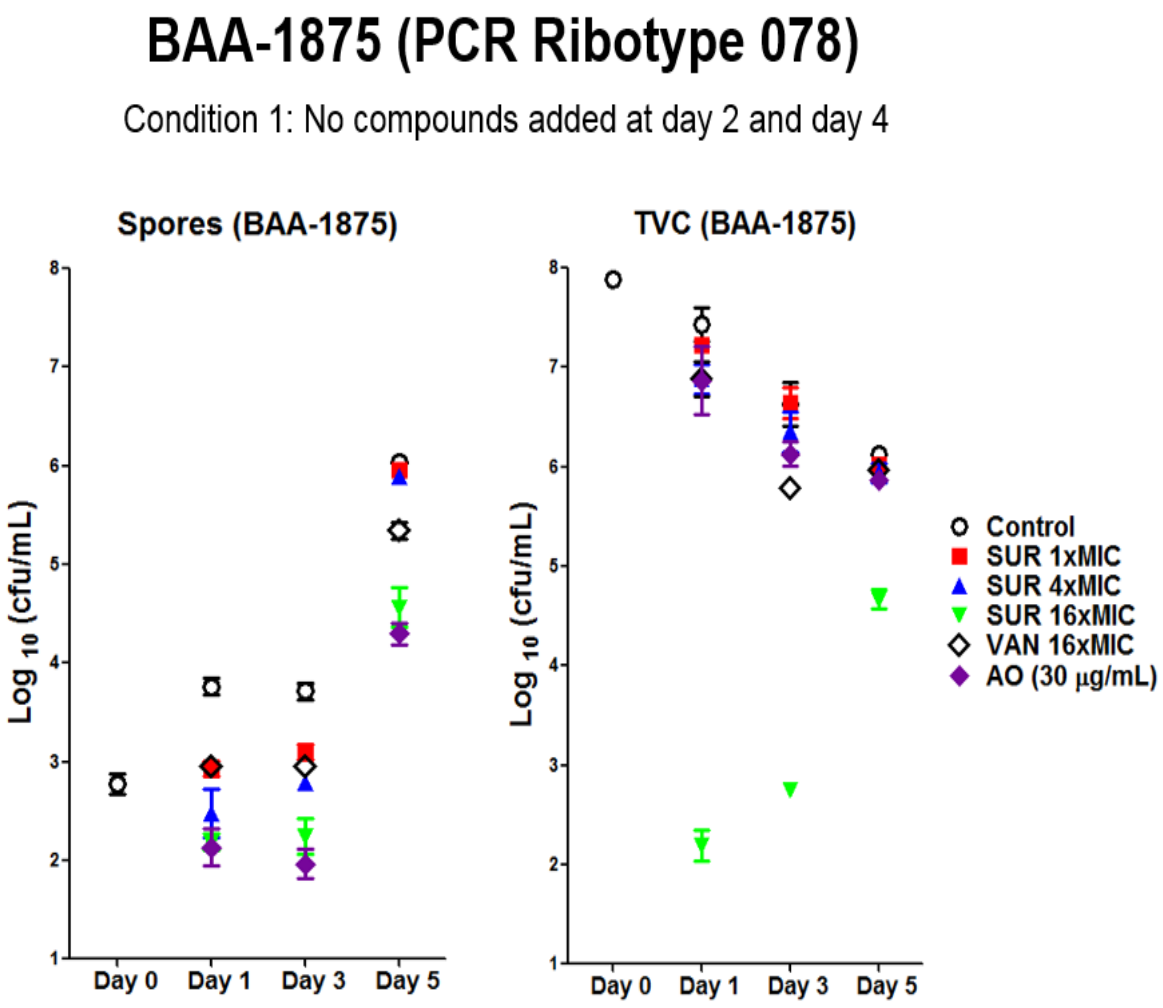


Figure 3-7: Effect of surotomycin on sporulation against BAA-1875 in condition 1, where no extra compounds were added. Concentration-dependent reduction in spore number was observed at day 5, mainly, at the concentration of 16×MIC. Acridine Orange (30 µg/mL) was used as the positive control. Total viable cell numbers, TVC, (right side of the graph) are also shown with the spore numbers. Data were plotted as mean (±SE), obtained from three independent cultures (n = 3), and the statistical analysis was performed by one-way ANOVA. SUR: Surotomycin, VAN: Vancomycin, AO: Acridine orange.

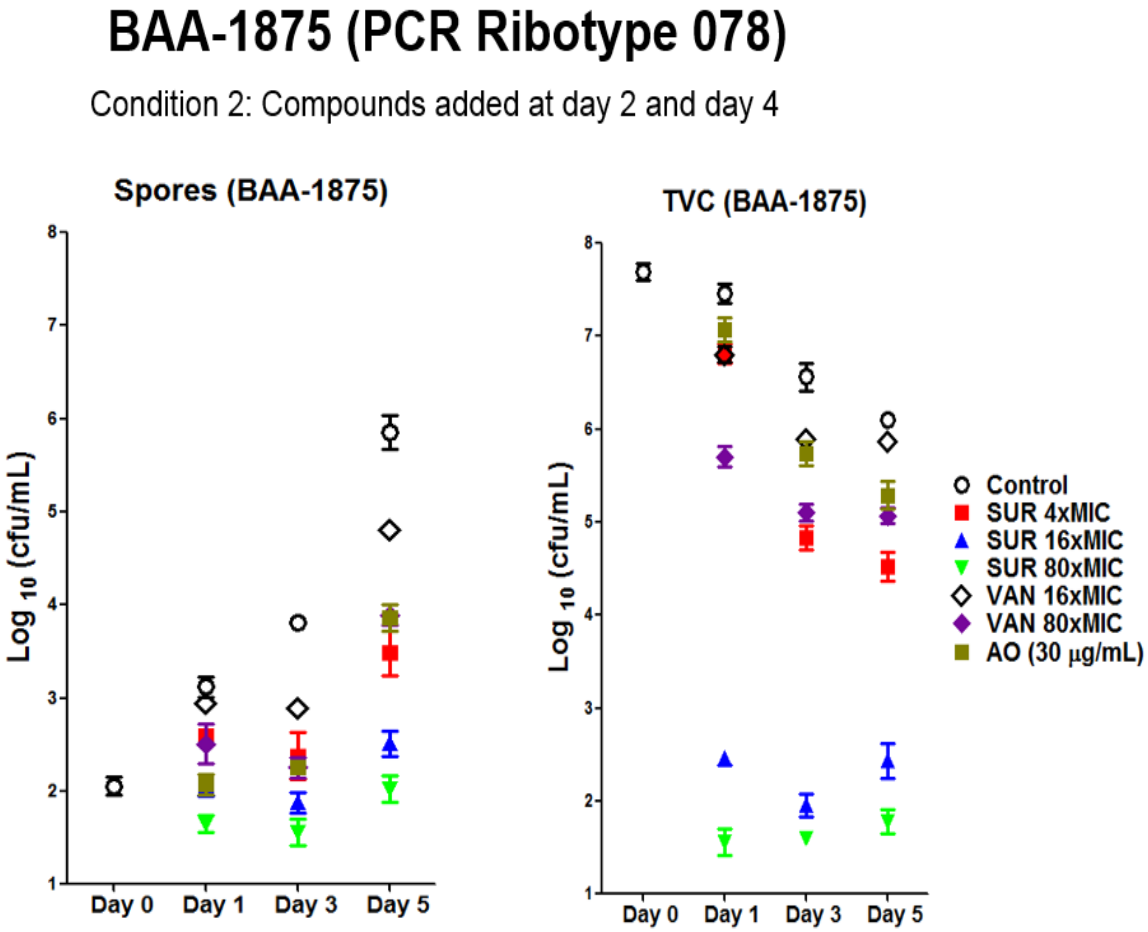


Figure 3-8: Effect of surotomycin on sporulation against BAA-1875 in condition 2, where the same concentrations of compounds were added at day 2 and at day 4. Compared to condition 1, more reduction in the spore numbers was observed. Surotomycin, even at the concentration of 16xMIC, significantly reduced spore numbers at day 3 and day 5. Results of vancomycin are also shown for the comparison. Total viable cell numbers, TVC, (right side of the graph) are also shown with the spore numbers Statistical significance was determined by one-way ANOVA at $P<0.01$. Data were plotted as mean (±SE), obtained from three independent cultures (n = 3). SUR: Surotomycin, VAN: Vancomycin, AO: Acridine orange.

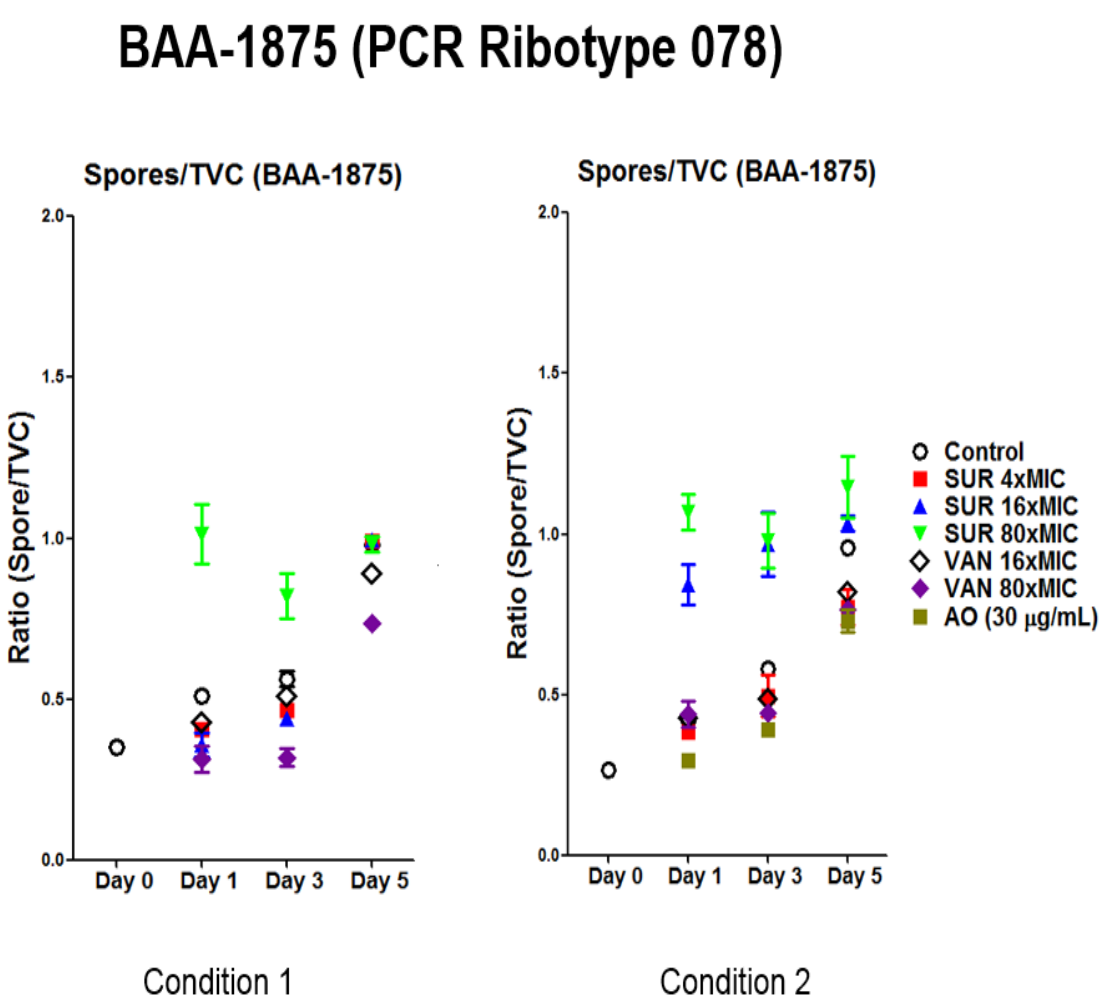


Figure 3-9: Ratio of spore numbers to total viable cells (TVC) in BAA-1875 strain. Left side of the figure shows the ratio in condition 1 and right side in condition 2. Data were plotted as mean (±SE), obtained from three independent cultures (n = 3). SUR: Surotomycin, VAN: Vancomycin, AO: Acridine orange.

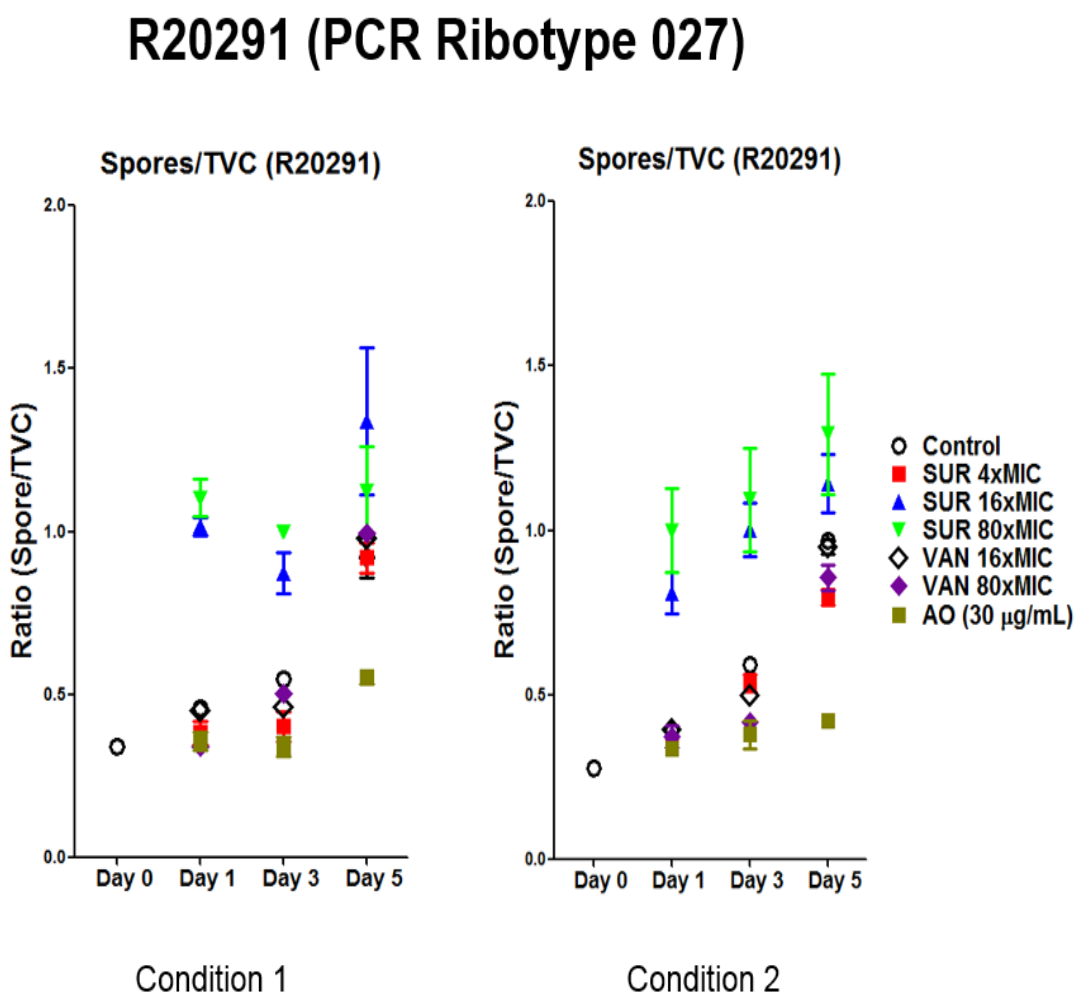


Figure 3-10: Ratio of spore numbers to total viable cells (TVC) in R20291 strain. Left side of the figure shows the ratio in condition 1 and right side in condition 2. Data were plotted as mean (±SE), obtained from three independent cultures (n = 3). SUR: Surotomycin, VAN: Vancomycin, AO: Acridine orange.

3.3.3 Surotomycin's activity decreases with prolong incubation

In condition 1 (Figure 3-5 and figure 3-7), where no extra compounds were added for the whole period of incubation, MIC was tested from the filtered supernatant at day 0, day 3, and at day 5. At day 3,

surotomycin's MIC changed by 2-fold, in two cultures, but notably, at day 5, the MIC raised by almost 8-fold, compared to the day 0 values. In condition 2 (Figure 4-6 and figure 4-8), where extra compounds were added (same concentrations as day 0) at day 2 and day 4, no change of MIC was observed at day 3; however, at day 5, only 2-fold increase was observed, compared to day 0 MIC values (Table 3-1).

Table 3-1: Bioassay (µg/mL) of surotomycin and other corresponding drugs over the period of sporulation assay. The values indicate crude measurement of the available bioactive concentrations of compounds, which were determined against the wild type, over-nightly grown culture of *C. difficile*. SUR: Surotomycin, VAN: Vancomycin.

	Day 0	Day 3	Day 5	Day 0	Day 3	Day 5
			BAA-1875			
SUR	0.5, 0.5, 0.5	1, 0.5, 1	4, 2, >4	0.5, 0.5, 0.5	0.5, 0.5, 1	1, 1, 0.5
VAN	0.5, 0.5, 1	1, 2, 1	4, 2, 4	2, 2, 1	2, 4, 4	2, 4, 2
			R20291			
SUR	1, 0.5, 1	>4, >4, >4	>4, >4, >4	0.5-1	0.5, 1, 0.5	1, 1, 1,
VAN	2, 1, 2	>4, 2, 2	>4, >4, >4	2, 2, 1	2, 2, 2	2, 2, 2

For metronidazole and vancomycin, in condition 1, significant changes in MIC values have been observed only at day 5 (not at day 3), which was almost 4-fold increase in MIC, for both of the compounds, compared to day 0. However, at condition 2, no changes in MIC were observed, even at day 5.

3.4 Discussion

Epidemic strains, which are believed to produce more toxins and sporulate heavily (3, 18, 112, 128), increase the severity and the recurrence of CDI. Moreover, they are refractory to the standard drug regimen (vancomycin and metronidazole) (2, 51). Metronidazole, after oral administration, absorbs rapidly through

the intestinal epithelium leaving low concentration in the gut to kill the stationary-phase cells (56, 116). On the other hand, vancomycin can achieve high concentration in the gut locally; however, it is not bactericidal to the stationary-phase cells, due to its mode of action, which inhibits the peptidoglycan biosynthesis in rapidly multiplying cells (51, 66). As a result, vancomycin is not effective enough to clear the gut from *C. difficile*, before it produces sufficient amounts of toxins (129). Unfortunately, toxins are mainly produced at the stationary-phase and also this is the stage, where bacteria prepare itself for sporulation (112, 130). Therefore, it is plausible that agents that can kill the stationary-phase cells could reduce the severity and prevent the recurrence of CDI.

Additionally, certain antibiotics used in CDI treatment having specific targets in the macromolecular biosynthetic process, are not able to eradicate non-growing *C. difficile*, though they can reduce the virulence *in vitro*. For example, fusidic acid, which can reduce the toxin production from the stationary-phase cells and can reduce the severity of the primary infection; however, it is bacteriostatic and cannot prevent the persistence of the *C. difficile* spores (131). Hence, in the search of the potential target that could rapidly kill the dormant cells, the membrane could be a promising one, as because the proper physiology of membrane is essential not only for the rapidly dividing cells, but also for the dormant cells. Moreover, the efficacy and the therapeutic role of membrane-targeting agents, for example, daptomycin and other compounds, have already been observed in treating recalcitrant infections (99, 132, 133).

A study by Wu et al. (71) showed that *C. difficile* is very sensitive to the membrane-active agents. Quick death with concurrent reduction of the ATP was observed after the treatment with most of the membrane-active compounds (71). This observation suggests the role of the membrane-targeting agents in disturbing the ATP synthase activities of the membrane that ultimately affect all the biosynthetic process of the cell. Surotomycin, which causes membrane potential disruption in *Staphylococcus aureus* (109) as well in the *C. difficile* (70), found to be effective in killing the stationary-phase cells of *C. difficile*. Depolarization of the Clostridial membrane by surotomycin happens at high concentration, which bodes well with the killing

concentration (against both the logarithmic and the stationary-phase) of the *C. difficile*. This observation leads us to hypothesize that surotomycin will also cause the reduction of toxin production and sporulation. The hypothesis is supported by our findings, where significant inhibition of the toxin production and sporulation occurs, at the bactericidal concentration, but not at the minimum inhibitory concentration of surotomycin. Also, in the qualitative toxin inhibition assay (measure both toxin A and toxin B) that was performed to validate the data of the quantitative assay, using C. diff. toxins A or B quanti kit (tgcBIOMICS), surotomycin demonstrated similar pattern in reducing the total concentrations of toxin A and toxin B. Moreover, in *in vitro* gut model, a recent study done by Wilcox et al., significant inhibitions of toxin production and spore numbers were observed within 3 days of surotomycin installation, at a concentration that mimic the fecal concentration measured in phase 1 clinical trials (134).

For the treatment of CDI, which is primarily a gastrointestinal infection, drugs need to be developed that have less absorption across the intestinal wall and can achieve high concentration in the gut, locally. In our *in vitro* study, we found that at the bactericidal concentration (and more), surotomycin causes significant inhibition of toxin production and sporulation. However, *in vivo*, the conditions might be different, and probably higher concentration is needed. Notably, in a double-blind study (phase1), performed on healthy volunteers after giving them 1g of surotomycin twice a day (data on file at Cubist Pharmaceuticals, Lexington, MA, USA), it was observed that in the stool, at day 5, the mean concentration of surotomycin can achieve at the concentration of 6,394 ±3,104 μg/g (109), which is more than 200-fold higher (minimum value) than the bactericidal concentration observed in our study. Furthermore, surotomycin, also found to be selective against the *C. difficile*, and has little effect on the bacteria of the normal commensals (62). The selectiveness is important in preventing the recurrence of the infection. One of the potential reasons why vancomycin and metronidazole have poor effects in preventing the recurrence of CDI is due to their broad-spectrum activities (61, 135). Although, these antibiotics can reduce the signs and symptoms of primary CDI, they also cause prolong dysbiosis by disrupting the normal gut flora. The dysbiosis contributes to the recurrence of the disease. Importantly, surotomycin has no effect on the normal gut flora, primarily against

Bacteroidetes spp, even at higher concentration (6,394 ±3,104 μg/g). In a study where a broad-range of gastrointestinal bacteria were tested against surotomycin and other traditional drugs, Citron et al. (62) observed that surotomycin has no effect against 21 strains of *Bacteroides fragilis* (MIC_{90} is >8,192 μg/mL).

Chapter IV

In vivo evaluation of metronidazole-tetramic acid (MTZ-TA) hybrid compounds as the treatment for *C. difficile* infection

4.1 Introduction

Clostridium difficile, which primarily infects the distal colon of the human intestine, is associated with 50-75% cases of the antibiotic-associated colitis at the hospital settings (20, 136). Toxigenic strains of *C. difficile* produce toxin A and toxin B, which cause inflammation of the intestinal epithelium and tissue damage (1). Traditionally, for the last three decades, CDI is treated by metronidazole or vancomycin, based on the clinical signs and symptoms that range from mild diarrhea to severe complicated infections (9, 20, 51).

According to the guidelines of the Society for Healthcare Epidemiology of America (SHEA) and Christina et al., the initial episodes of CDI can be classified into three classes (20, 23). The first class is the mild to moderate infection, where diarrhea is associated with white blood cell count $\leq$15,000/ mm^3, and serum creatinine level is less than 1.5-times of the premorbid level. The second class is the severe infection, where diarrhea is associated with WBC count $\geq$15,000/mm^3, and serum creatinine level is more than 1.5 times of the premorbid level. And the third class is the severe complicated infection, where the manifestations of the severe infections are associated with shock, hypotension, pseudomembranous colitis, or toxic mega-colon.

Oral metronidazole is the treatment of choice for the mild to moderate infection, while vancomycin is for the severe infection (20). Vancomycin is not recommended in mild to moderate infection due to the risk of the emergence of vancomycin-resistant enterococci (VRE) as well due to its cost (9, 137, 138). Except for pregnant and lactating women, metronidazole is the treatment of choice for the mild to moderate CDI (139).

Metronidazole is in use for the last three decades for treating CDI. Although, in *in vitro* studies, *C. difficile* is sensitive to metronidazole, there are evidences of decreasing the therapeutic effectiveness of metronidazole in clinical settings (60, 64, 140). In a prospective observational study performed by Musher DM et al., 22% of the patient observed to have the persistent infection after the 10 days treatment of oral metronidazole at the dose of 500 mg three times daily (56). In a review paper by Konstantinos et al., which analyzes the effectiveness of the standard drug therapy against CDI in last 10 years (2001 to 2010), it was reported that the rate of treatment failure and the recurrence of CDI was 22.4% and 27.1%, respectively, after the treatment with metronidazole (98). It is widely believed that, one of the main reasons why metronidazole is less effective in treating CDI patients, is its poor oral pharmacokinetics. After oral administration, metronidazole completely absorbs across the intestinal epithelium and achieves low concentration, locally, in the human gut to kill the vegetative cells of *C. difficile* (59, 60).

The tetramic acid, composed of 2,4 pyrolidiodinone ring system and a key structural unit in many natural products, exhibits a spectrum of biological activities (141, 142). For instance, in addition of antibacterial effect, it also demonstrates antiviral, cytotoxic, antiulcerative, and antifungal properties. Although the biological activities of tetramic acid were known since the early twentieth century, it was not before 1972, when the tetramic acid was first synthesized (141, 143). As an antibacterial, tetramic acid demonstrates a narrow spectrum of activities and are effective, mainly, against the gram-positive bacteria (142, 144). Interestingly, various mechanisms of actions have been proposed related to tetramic acid's antibacterial activities. Many investigators believe that tetramic acid demonstrates its bactericidal effect against gram-positive bacteria by inhibiting the cell wall biosynthesis, by disrupting the membrane potential, by inhibiting the RNA polymerase and also by inhibiting the topoisomerase enzyme (142, 145, 146). Recently, Cherian and his colleagues demonstrate that reutericyclin, a derivative of the tetramic acid, naturally obtained from *Lactobacillus reuteri*, dissipates the membrane-potential of *Staphylococcus aureus* as a concentration-dependent manner (146). Additionally, reutericyclin exhibits potent bactericidal activities against *C. difficile*, as demonstrated by Hurdle et al (130). Interestingly, as detected by the Caco-2

permeability assay (147), reutericyclin analogs show less permeability across the intestinal epithelium suggesting they can achieve high concentration in the gut locally, the property that makes this compound suitable for the treatment of the *C. difficile* infection.

In this study, in collaboration with the medicinal chemistry research group led by Dr. Richard E. Lee, St. Jude Children's Research Hospital, an effort was made to hybridize the metronidazole with the tetramic acid group, and to obtain the minimally absorbed MTZ-TA hybrid compounds (Figure 5-1), whose therapeutic efficacy and the gastrointestinal pharmacokinetics were tested using hamster model of *C. difficile* infection. Table 5-1 shows the chemical structures and the *in vitro* activities of these MTZ-TA hybrid compounds against two strains of *C. difficile* (R20291 and BAA-1875).

4.2 Materials and methods

4.2.1 Compounds and bacterial strain

The metronidazole-tetramic acid hybrid compounds (2344, 2345, 1971 and 2490) were synthesized by the medicinal chemistry research group at the SJCRH. Other compounds, metronidazole and clindamycin were purchased from the Sigma-Aldrich. Polyethylene glycol 400 (PEG 400:water, 85:15) was the vehicle used for the oral delivery of the compounds. *C. difficile* ATCC 43596, which is a metronidazole-susceptible toxigenic strain, was used for the hamster study. *C. difficile* ATCC 43596 was grown in the sporulation media, and before infecting to the hamsters, culture was washed once with pre-reduced PBS, and was given orally at an inoculum of 10^6 cfu/mL.

4.2.2 Hamster model of CDI

While performing the *in vivo* studies using hamsters, animals were treated by following the rules and regulations as approved by the Institutional Animal Care and Use Committee of the University of Texas at Arlington. This hamster model of CDI study was performed with some modifications as the protocol described by Mathur et al. and Anton et al. (148, 149). Golden male Syrian hamsters, each weight ≈100 gram, were purchased from the Charles River Laboratories. Hamsters were housed individually in autoclaved cages with sterile food and water. On day -1, each hamster was given clindamycin injection (subcutaneously) to disrupt the normal gut flora. On day 0, hamsters were infected with *C. difficile* (strain ATCC 43596) by oral gavaging. From day 1, treatment was started (n=8) with the test compounds at the dose of 50 mg/Kg/day in a vehicle containing 85% of PEG-400. Treatment was continued for five days. Metronidazole was included in this study and administered at the dose of 50 mg/Kg/day. After the end of treatment, hamsters were monitored daily until day 30 for any signs and symptoms of CDI as described by Anton et al. (148). During this monitoring, hamsters that were found in moribund condition were euthanized with CO_2 exposure followed by cervical dislocation. After the study period, all the survival hamsters were euthanized, and fecal-cecal materials were collected and stored in -80°C for the further study.

4.2.3 Pharmacokinetic studies

Pharmacokinetic studies were performed using hamsters having pre-implanted jugular vein cannula. These hamsters, each weight 100 gram, were also purchased from the Charles River Laboratories. Briefly, hamsters were fasted overnight as well as during the period (7 hour) of blood sample collection. After collecting the time 0 blood sample (200 μL), hamsters were dosed with the test compounds (100 mg/Kg) in the same vehicle (PEG-400: Water, 85:15) used in the hamster model of CDI. Metronidazole was used as the control drug. Blood was drawn (200 μL) at certain time points using the implanted jugular vein cannula and collected in the heparin-coated tubes, which were then immediately centrifuged at 3000 rpm for 10 minutes to separate the serum from the remaining blood components. Serum was collected and stored

at −20°C. To determine the fecal and plasma concentrations of test compounds, LCMS was performed at the Department of Chemical Biology and Therapeutics of the St. Jude Children's Research Hospital in Memphis.

4.2.4 Statistical analysis

Kaplan-Meier survival curve was prepared to compare the efficacy of metronidazole with the metronidazole-tetramic acid hybrid compounds. Statistical analysis was performed by the Log-rank test (Mantel-Cox) using GrapPad Prism (version 5) software, and the *P* value less than 0.05 was considered statistically significant.

4.3 Results

4.3.1 MTZ-TA compounds demonstrate better efficacy compared to metronidazole in hamster model of CDI

The hamster model of CDI has been adopted to test the hypothesis that reducing the gastrointestinal absorption of metronidazole (by hybridizing with the tetramic acid moiety, figure: 4-1) could improve the *in vivo* efficacies of this compound. Interestingly, four of the lead hybrid compounds (Table: 4-1), which have a diverse array of substitutions at the 5-position of the tetramic core (1971-isobutyl, 2345-biphenyl, 2344-napthyl and 2490-n-methyl indole), demonstrated better therapeutic efficacy, compared to metronidazole. All the hamsters from the metronidazole group died at day 10, while the hamsters from the hybrid compound groups had improved survival by addition 1 to 5 days of post-infection (Figure 4-2)

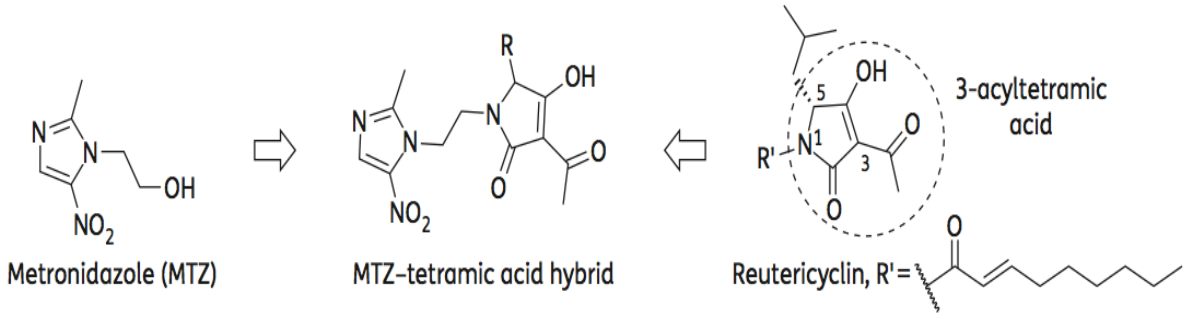
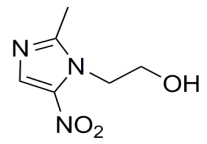
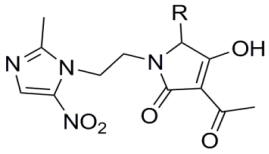
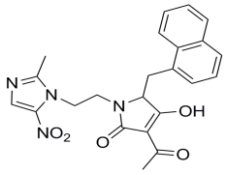
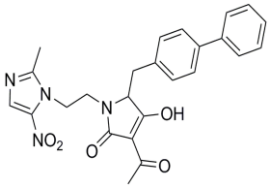
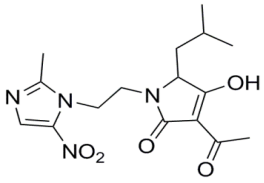
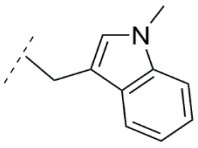


Figure 4-1: Hybridization of metronidazole to the tetramic acid motif of reutericyclin. Image obtained from Cherian et al. JAC. 2015 (147).

We believe that this differences in *in vivo* efficacies of the hybrid compounds are not due to the better activities, compared to the metronidazole as because the *in vitro* activities are similar against the test strain of *C. difficile* ATCC 43596 used in this model (MIC values: 0.125 µg/mL for metronidazole, 0.50 µg/mL for 1971 and 0.25 µg/mL for 2344, 2345 and 2490). However, we believe that the better efficacy of the hybrid compounds compared to metronidazole is due to their decrease absorption across the intestinal epithelium of the hamster gut. The decrease in absorption helps the hybrids to achieve high concentration in the gut locally to kill the *C. difficile*.

Table 4-1: MTZ-TA hybrids

Compound	**Structure**	**MIC (µg/mL)**	
		BAA-1875	R20291
MTZ		0.5	0.125
Basic Structure of MTZ-TA			
2344		1	1
2345		0.25	0.5
1971		2	1
2490		2	1

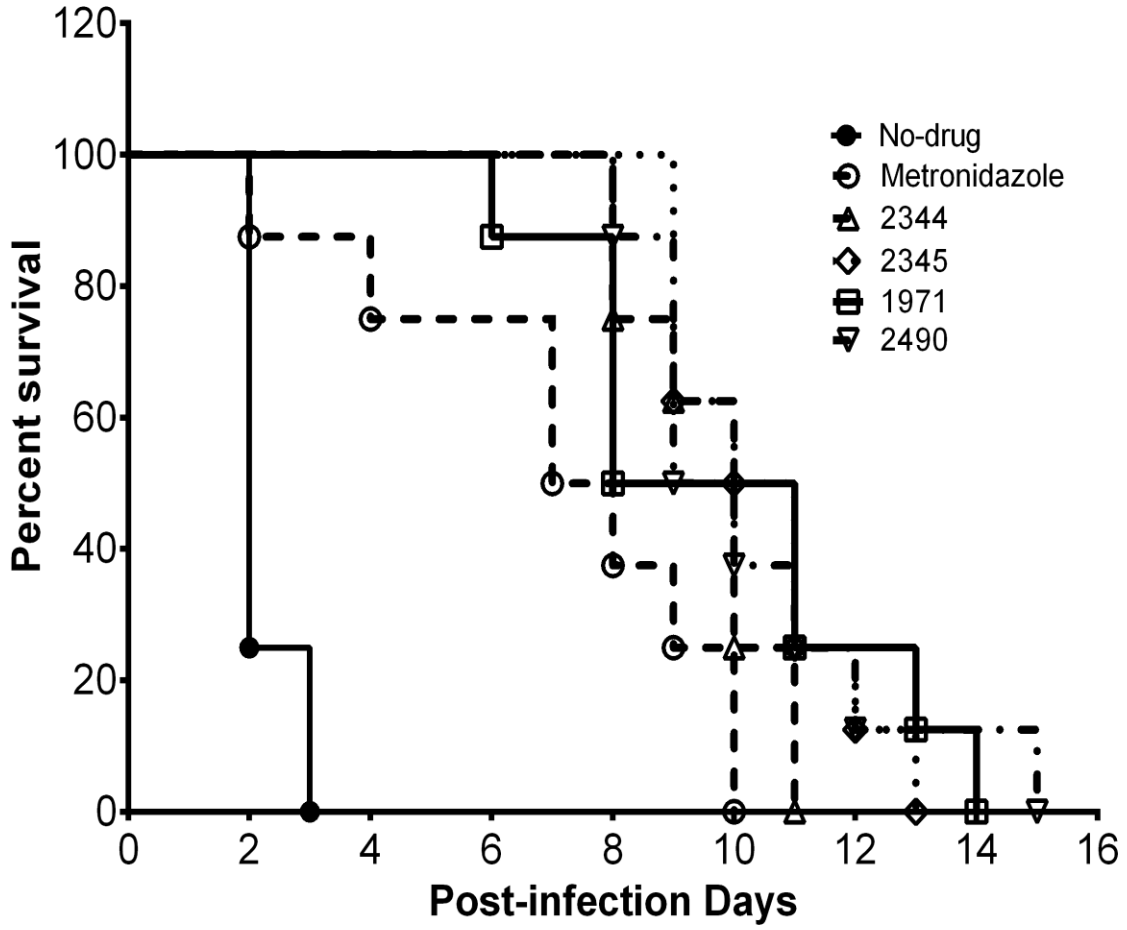


Figure 4-2: *In vivo* efficacy of metronidazole-tetramic acid (MTZ-TA) hybrid compounds compared to metronidazole in hamster model of infection. After treatment with the test compounds and MTZ, at the concentration of 50 mg/kg/day, hamsters (n = 8) were observed twice daily for the sign and symptoms of CDI. Comparison of the survival curves demonstrated statistical significance for all hybrids, compared to metronidazole. Statistical analysis was performed by the Log-rank test (Mantel-Cox) using GrapPad Prism (version 5) software, and the *P* value less than 0.05 was considered statistically significant. (With assistance and guidance of Dr. Julian G. Hurdle and Dr. Xiaoqian Wu, I performed the *in vivo* efficacy study).

4.3.2 MTZ-TA hybrids achieved very low concentration in plasma and high concentration in fecal materials, compared to metronidazole, after oral administration

To test the hypothesis that the improvement in the *in vivo* efficacies of the hybrid compounds is due to the decreased absorption across the intestinal epithelium, plasma and fecal concentration of the test compounds were determined at various time points after the oral administration of test compounds. The plasma concentrations of the hybrids (2344 and 2345) were much lower than the metronidazole (Figure 4-3).

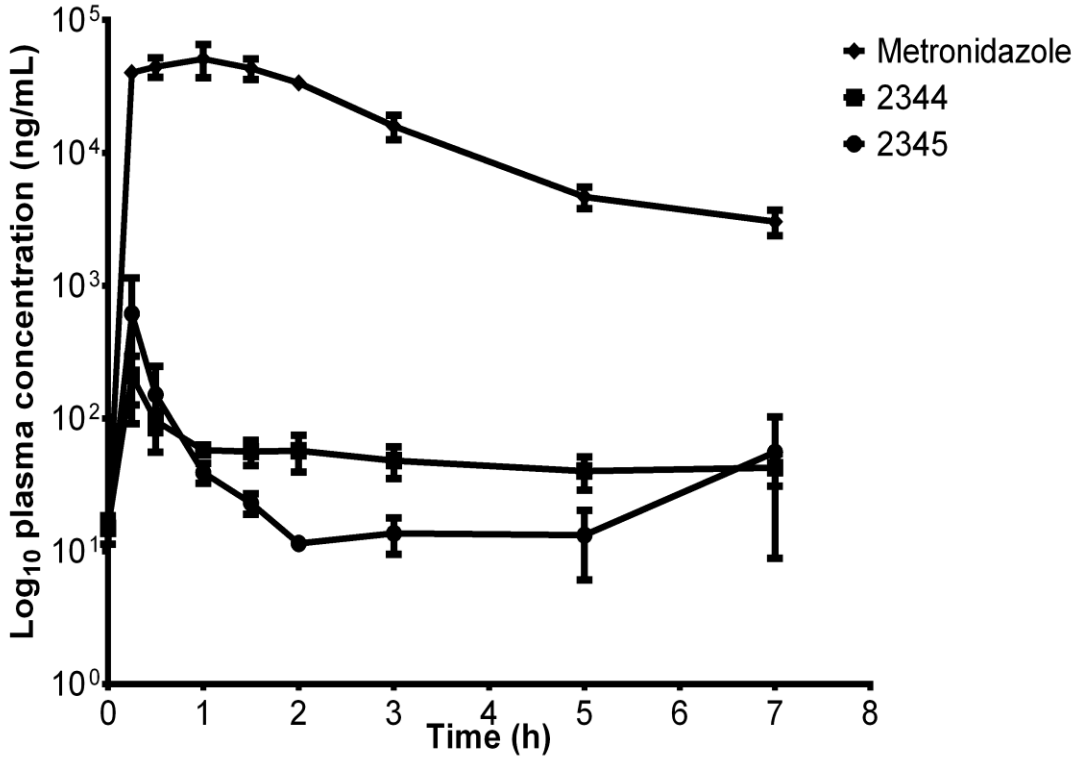


Figure 4-3: The pharmacokinetic properties of MTZ-TA and MTZ after oral administration of single dose of compounds (100 mg/kg). MTZ achieved very high concentration compared to the hybrids. Peak plasma concentration of MTZ was 51.04 mg/L, while the plasma concentration of 2344 and 2345 were 0.21 mg/L and 0.61 mg/L, respectively. This study was performed by Yizhe Chenand and his colleagues at the Department of Chemical Biology and Therapeutics of the SJCRH.

The plasma C_{max} for compound 2344 and 2345 were 241 and 82 times lower, in comparison to metronidazole (metronidazole: 51,036 ng/mL; 2344: 211 ng/mL; 2345: 616 ng/mL). In addition, the T_{max} for metronidazole was 1 hour, while the T_{max} for the 2344 and 2345 was 0.25 hour (Figure 5-3).

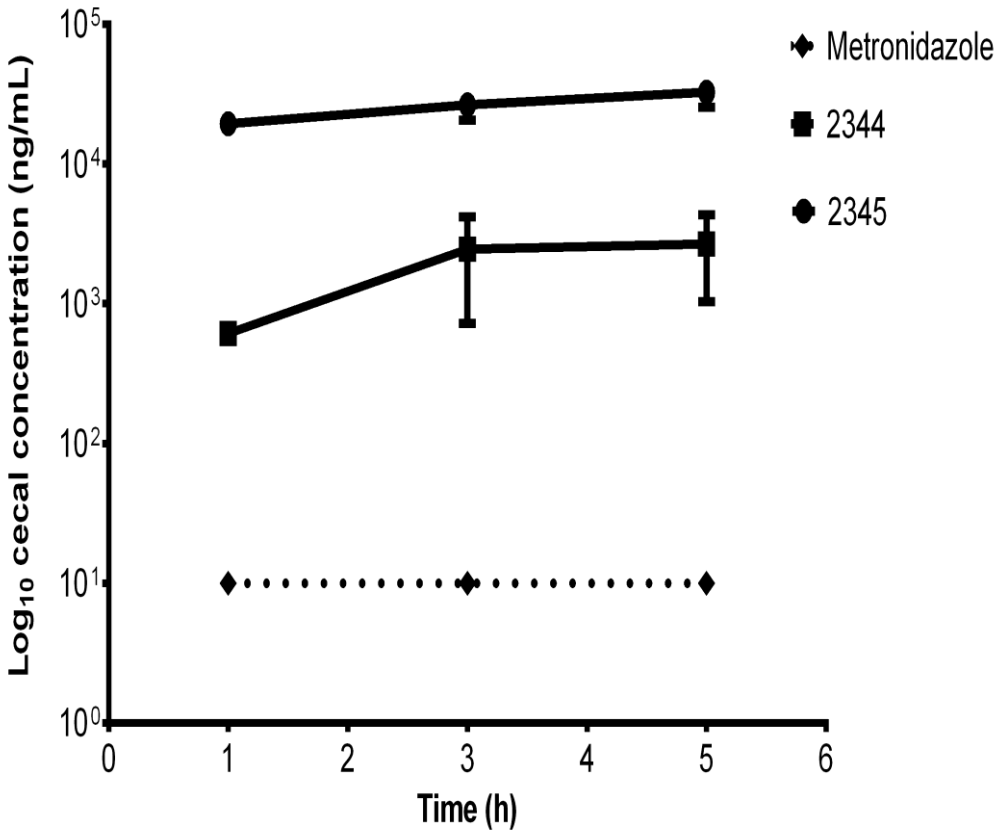


Figure 4-4: Fecal-concentration of MTZ and other hybrid compounds after single oral dosing at the concentration of 100 mg/kg. As expected, based on the data of the *in vitro* Caco-2 cell assay, hybrid compounds demonstrated less absorption across the hamster-gut epithelium. Peak fecal concentration of 2344 and 2345 were 2.68 mg/L and 32.65 mg/L, respectively; while the fecal concentration of MTZ was undetectable in LLOQ assay (<0.01 mg/L). This study was performed by Yizhe Chenand and his colleagues at the Department of Chemical Biology and Therapeutics of the SJCRH.

There might be two possibilities for the low plasma concentration of the hybrid compounds– high first pass metabolism or the decrease in gastrointestinal absorption.

To determine the *in vivo* fecal concentration of the test compounds, cecal contents were collected from hamsters at 1, 3 and 5-hour time points after oral dosing of the compounds. As expected, the fecal concentration of metronidazole was not detectable, while the peak cecal concentration of 2344 and 2345 were found much high, which were 32,649 ng/mL and 2,678 ng/mL, respectively (Figure 4-4).

4.4 Discussion

Nitroheterocyclic compounds are attractive choices for a long time in treating the infections caused by protozoa and anaerobic bacteria (150). Metronidazole, a nitroheterocyclic pro-drug, is used for three decades for the treatment of CDI. Due to its low-price and effectiveness in mild to moderate infection, it is adopted as the first-line treatment option for CDI (9, 51). However, due to high absorption rate across the gastrointestinal epithelium, metronidazole cannot achieve increased concentration in the gut, locally, to kill *C. difficile* (59). A recent study (2014) performed by Stuart Johnson and his colleagues, has shown a clear difference in the treatment outcomes between metronidazole and vancomycin, while treating severe CDI (151). In their randomized controlled trials, they observed that metronidazole is inferior to vancomycin, which has long been contemplated due to the poor oral pharmacokinetic properties of metronidazole. However, there are the lack of efforts to retain metronidazole and other nitroheterocyclic pro-drugs in the gut locally for localized treatment. This study, for the first time, provides the evidence that in CDI treatment, the low efficacy of metronidazole arises owing to its high absorption across the gastrointestinal tract; and interestingly, high concentration can be achieved by hybridizing the metronidazole with tetramic acid moiety resulting in improved efficacy of hybrids MTZ-TA), compared to metronidazole, in hamster model of CDI.

This study speculates that the tetramic acid moieties did not demonstrate any anti-difficile activities, as because they do not possess the required charge like reutericyclin, to be membrane-active (146). Therefore, the anti-difficile properties of these hybrid compounds primarily arise from the nitro group of metronidazole, and tetramic acid moieties help to prevent the absorption of these hybrids. This study also observed that though, MTZ-TA hybrids are non-absorbable, they demonstrated lower efficacy, compared to vancomycin, in the hamster model of CDI. This probably due to the sensitivity of the animal models used, as because in CDI, hamsters respond exceptionally well to vancomycin (152). The differences in antimicrobial susceptibilities among various animal models is further supported by the observation that metronidazole is superior to vancomycin in the murine model of CDI, as reported by Warren et al. (153). This observation indicates the limitation of animal models in studying the anti-difficile activities of compounds. Like metronidazole, the MTZ-TA hybrid compounds demonstrate activities against the gut anaerobe, *Bacteroidetes* spp, which is a major bacterium in normal gut flora and helps to prevent the recurrence of CDI (147). Therefore, this could be a challenging factor in developing MTZ-TA hybrids as an effective treatment for CDI.

Chapter V

Conclusions, limitation of the studies, future directions and prospects of membrane-active agents for the treatment of *C. difficile* infection

5.1 Conclusions

The action of surotomycin against various strains of *C. difficile* results in bactericidal activities against logarithmic and stationary phase cultures. More importantly, surotomycin demonstrates good therapeutic potency against the stationary-phase cells of *C. difficile*, which are refractory to traditional drugs and play a pivotal role in the pathogenesis of CDI. The rapid bactericidal effect of surotomycin results from the disruption of *C. difficle*'s membrane potential, which affects several key biosynthetic processes (DNA, RNA, protein and cell wall) of cells. This non-specific inhibition is consistent and well described while the bacterial membrane is the primary target. In addition, as a direct consequence of the bactericidal effect, surotomycin inhibits toxin production and sporulation from the toxigenic strain of C. difficile. Nevertheless, as detected by the bioassay, surotomycin's effect decreases over a long period (at day 5) of incubation at 37°C, and reduces the spore numbers significantly only at high concentration (80×MIC), which suggests the use of high concentration (5× bactericidal concentration) of surotomycin for the cure of CDI. However, this effect was observed in in vitro assay, and further studies are warranted to get more understanding about the role of surotomycin in reducing the virulence factors of *C. difficile*.

In hamster model of CDI study, the MTZ-TA hybrid compounds demonstrate better efficacy compared to metronidazole. This better efficacy is due to the decreased absorption of MTZ-TA hybrids across the intestinal epithelium that results in achieving high concentration in the gut locally, as observed by the plasma and fecal concentration of MTZ-TAs, to kill the stationary phase cells of *C. difficile*.

5.2 Limitation of the *in vitro* assays

There are certain limitations of the *in vitro* assays performed in this study. These *in vitro* assays, like, MIC, MBC, time-kill assay, toxin inhibition assay, etc., were done by simply incubating the bacterial cells with compounds; however, in gastrointestinal infection (*in vivo*), the milieu of the human gut is different and pathogens are associated with the mobile fecal-cecal materials. Therefore, *in vivo*, the finding might not be the same as the *in vitro*. For example, in the time-kill assay, surotomycin caused more than three log10 reductions in the viable cell counts against logarithmic-phage cells of *C. difficile* in 6 hour; while in human, for the treatment of CDI, when surotomycin is administered orally, the killing might not be like the *in vitro* time-kill assay. This is also true for the *in vitro* toxin inhibition and the sporulation assay. Therefore, in the perspective of this problem, the triple-stage chemostat human gut model (154), developed by Mark H. Wilcox and his colleagues to study the interaction between the antimicrobial agents and the complex gut environment, need to be used.

5.3 Lack of the LCMS data

The sporulation assay was performed using two conditions. In condition 1, no extra compound was added during the five-day period of the assay; however, in condition 2, extra compounds (the same concentration as added at day 0) were added at day 2 and day 4. The main purpose of using these conditions was to see the stability of surotomycin over the long period of incubation (five day) at the 37° Celsius. In condition 1, we observed the spike of the spore numbers at day 3 and day 5 against both of the strains of *C. difficile* (BAA-1875 and R20291) tested; while in condition 2, no spike of spore numbers were detected. Moreover, in the bioassay, at condition 1, increased surotomycin's MIC was observed. From these observations, we hypothesized that surotomycin loose its activity (due to breakdown, or may be utilized by the cells) in culture over a long period of incubation (≥48 hours). To confirm this hypothesis, in collaboration with the chemistry department of the UTA, we planned for LCMS study. Our plan was to detect the concentration of surotomycin and other compounds in the liquid media and also in culture. Unfortunately, we had some technical issues while performing this assay, and the assay was not completed.

5.4 Sensitivity of the ELISA kit

The toxin assay, which detects the toxin A and toxin B using *C. difficile* Tox A/B ELISA kit, has the specificity around 80% (155). It is primarily used as a screening test to detect the toxin from the stool samples and to exclude or include the patient in the hospital-based study (156, 157). In addition, this assay, which detects the absorbance at the wavelength of 450 nm, is not sensitive enough to detect the very low or the too high concentration of toxins present in the samples. Therefore, this ELISA test gives the quantitative analysis of the toxins rather than the qualitative analysis. To overcome this limitation and also to validate the quantitative assay, qualitative assay using the tgcBIOMICS-ELISA kit (tgcBIOMICS GmbH: Bingen) was performed. In addition, cell culture assay using the vero-cell line, which gives the qualitative analysis of toxin production as well, will be performed.

5.5 Limitation of the hamster model of CDI

Although hamster model of CDI is routinely used for the evaluation of the *in vivo* efficacies of compounds, there remain certain drawbacks and limitations in using this model. A major drawback of this model is that in this model, most of the time, hamsters do not typically develop diarrhea (152, 158). Hamsters occasionally show wet tail, lethargy, and irritability that ultimately lead to death. Therefore, many investigators observe this model as the prevention of death model rather than as the treatment model (158). In addition, hamsters are very sensitive to *C. difficile*, and if left untreated, the disease is rapidly fatal, and hamsters die within 2 to 3 days of infection (152, 158, 159). Therefore, the hamster model of CDI does not represent the usual course and spectrum of CDI observed in human. Moreover, several investigators believe that hamster model of CDI is not appropriate to study the relapse of CDI, as because hamsters are treated with the test compounds just after the infection, and there remain problems in determining the true relapse of CDI, using this model (160, 161). To overcome this limitation, many investigators use mice model of CDI (160).

5.6 Future direction

Elucidation of biological target

From the FACS study it was observed that surotomycin caused disruption of the membrane potential without causing any gross pore formations. Non-specific inhibition of macromolecular biosynthesis (DNA, RNA, protein and cell wall) further supports this mechanism of action, as because membrane plays a central role in energy production, which governs all the intracellular synthetic activities. In addition, in RT-PCR assay, which included a limited number of genes that have the effect on membrane function, significantly influenced by the surotomycin treatment. Therefore, based on the preliminary assays and the literature review, the suggestive mode of action of surotomycin in *C. difficile* is the disruption of the membrane potential. However, this does not give the proper understanding of interaction with the biological target (e.g., the cell membrane) of the compound. Transcriptional profiling experiments in *S. aureus* and *B. subtilis* suggests that daptomycin, a lipopeptide that possesses similar structure like surotomycin, induces cell wall stress response, which is typically induced by the cell wall active antibiotics (e.g., ampicillin, vancomycin, etc.) (115). Although daptomycin is believed to disrupt primarily bacterial membrane potential, this observation suggests that it causes the damage of cell wall. Besides, in another study performed by Jared A. Silverman and his colleagues, it was observed that daptomycin has an effect on the cell division proteins and induces abnormal septation events in many microorganisms (162). Additionally, it was also observed that alteration in the *yycG* gene product, a histidine kinase, which confers daptomycin resistance in *S. aureus*, also play a regulatory role in peptidoglycan biosynthesis, as well as in cell division (163, 164). Therefore, due to the closeness in structure and similarity in membrane depolarizing effect, it is reasonable to think that surotomycin might have affect in other cellular targets. Moreover, it is still not clear what the primary site of binding of surotomycin is and how it exerts its membrane disrupting affects. Furthermore, the effect of surotomycin in the whole bacterial transcriptomics, as well as in developing drug-resistance is not clearly understood.

Therefore, to fill the gaps in understanding the proper mode of action, following experimental plans have been proposed.

In the first experimental plan, to ascertain the cellular site of binding, surotomycin can be fluorescently labeled, which can be tracked on the cell surface (cell wall or cell membrane) or inside the cell. This method is routinely used for the precise understanding of the drug-targets. For example, daptomycin-BODIPY, a fluorescently labeled daptomycin, is used to determine the cellular targets of daptomycin (162). The fluorescently labeled surotomycin could be traced using fluorescence microscopy following the fractionation of the cells in the subcellular components. This assay should give clear understanding regarding the site of drug binding in bacterial cells.

The second experimental plan could be to determine the whole transcriptomic analysis of the *C. difficile*'s genome after treating the cells with surotomycin. This might help to understand the effect of surotomycin on the global gene expression. Microarray hybridization can be performed after treating (for 30 minutes) *C. difficile* with surotomycin, and other comparator drugs. After characterizing the global gene expression, based on the level of expression, particular groups of genes can be selected to get proper understanding in the specific targets, as well as in identifying the drug-resistance mechanism of surotomycin.

5.7 Prospects of membrane-active agents

In addition of clinical efficacy against recalcitrant biofilm producing gram-positive bacteria, membrane-active agents demonstrate excellent activities against C. *difficile*, including the epidemic strains (71, 109). There are certain other membrane-active compounds, in addition of surotomycin, which are in the ongoing clinical trials. For example, oritavancin, a semisynthetic lipoglycopeptide, which primarily acts by

disrupting membrane potential (165), is now in phase 3 clinical trials for CDI treatment (51). In *in vitro* gut model study, oritavancin demonstrated therapeutic effectiveness in reducing spore numbers and toxin productions from the epidemic strain of *C. difficile*, compared to vancomycin(166). This efficacy in reducing the virulence is believed due to the bactericidal effect of oritavancin against stationary-phase cells. Besides, due to the action in the membrane, oritavancin causes rapid killing of biofilms and dormant cells of Staphylococci (167). Another membrane-active agent, daptomycin, a lipopeptide identical to surotomycin, is already in use for the treatment of multidrug-resistant biofilm forming infection, including bacterial endocarditis (101-103, 132, 168). However, due to its acid-digestion, daptomycin cannot be given orally for the treatment gastrointestinal infections (109). In contrast, surotomycin, due to the presence of the aromatic ring in its chemical structure, can resist stomach acid digestion and is effective against the intestinal pathogens (109). The clinical effectiveness of surotomycin, oritavancin, etc., in reducing the severity of primary infections and the recurrence of CDI, have been validated in several hamster model, as well as in the clinical trials (109, 120, 166, 169, 170). Therefore, targeting Clostridial membrane is an emerging novel approach for the cure of CDI. Moreover, this approach holds a huge prospect for the discovery of successful antibacterial drugs to treat multidrug-resistant systemic infections.

Appendix

Published papers

Md. Zahidul Alam, Xiaoqian Wu, Carmela Mascio, Laurent Chesnel and Julian G. Hurdle. 2015. Mode of action and bactericidal properties of surotomycin against growing and non-growing *Clostridium difficile.* Antimicrobial Agents and Chemotherapy. Vol 59.

Xiaoqian Wu, Md. Zahidul Alam, Li Feng, Lissa S. Tsutsumi, Dianqing Sun and Julian G. Hurdle. 2013. Prospects for flavonoid and related phytochemicals as nature-inspired treatments for *Clostridium difficile* infection. Journal of Applied Microbiology. Vol 23 (31).

Li Feng, Marcus M. Maddox, Md. Zahidul Alam, Lissa S. Tsutsumi, Gagandeep Narula, David F. Bruhn, Xiaoqian Wu, Shayna Sandhaus, Charles J. Simmons, Yuk-Ching Tse-Dinh, Hurdle G. Hurdle, Richard E. Lee and Dianqing Sun. 2014. Synthesis, structure-activity relationship studies, and antibacterial evaluation of 4-chromanones and chalcones, as well as olympicin A and derivatives. Journal of Medicinal Chemistry. Vol 57 (20).

Philip T. Cherian, Xiaoqian Wu, Lei Yang, Jerrod S. Scarborough, Aman P. Singh, Md. Zahidul Alam, Richard E. Lee and Julian G. Hurdle. 2015. Gastrointestinal localization of metronidazole by a lactobacilli-inspired tetramic acid motif improves treatment outcomes in the hamster model of *Clostridium difficile* infection. Journal of Antimicrobial Chemotherapy. doi:10.1093/jac/dkv231.

References

1. **Rupnik M, Wilcox MH, Gerding DN.** 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol **7:**526-536.
2. **Kelly CP, LaMont JT.** 2008. *Clostridium difficile* — More Difficult Than Ever. New England Journal of Medicine **359:**1932-1940.
3. **Deneve C, Janoir C, Poilane I, Fantinato C, Collignon A.** 2009. New trends in *Clostridium difficile* virulence and pathogenesis. Int J Antimicrob Agents **33 Suppl 1:**S24-28.
4. **Borriello SP.** 1998. Pathogenesis of *Clostridium difficile* infection. Journal of Antimicrobial Chemotherapy **41:**13-19.
5. **Leffler DA, Lamont JT.** 2015. *Clostridium difficile* Infection. New England Journal of Medicine **372:**1539-1548.
6. **Knoop FC, Owens M, Crocker IC.** 1993. *Clostridium difficile*: clinical disease and diagnosis. Clinical Microbiology Reviews **6:**251-265.
7. **Freeman J, Bauer MP, Baines SD, Corver J, Fawley WN, Goorhuis B, Kuijper EJ, Wilcox MH.** 2010. The Changing Epidemiology of *Clostridium difficile* Infections. Clinical Microbiology Reviews **23:**529-549.
8. **Lessa FC, Gould CV, McDonald LC.** 2012. Current Status of *Clostridium difficile* Infection Epidemiology. Clinical Infectious Diseases **55:**S65-S70.
9. **Hedge DD, Strain JD, Heins JR, Farver DK.** 2008. New advances in the treatment of *Clostridium difficile* infection (CDI). Therapeutics and Clinical Risk Management **4:**949-964.
10. **Collins DA, Hawkey PM, Riley TV.** 2013. Epidemiology of *Clostridium difficile* infection in Asia. Antimicrobial Resistance and Infection Control **2:**21-21.
11. **Centers for Disease Control and Prevention.** 2013. Antibiotic Resistance Threat in The United States, 2013. Accessed

12. **He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, Connor TR, Harris SR, Fairley D, Bamford KB, D'Arc S, Brazier J, Brown D, Coia JE, Douce G, Gerding D, Kim HJ, Koh TH, Kato H, Senoh M, Louie T, Michell S, Butt E, Peacock SJ, Brown NM, Riley T, Songer G, Wilcox M, Pirmohamed M, Kuijper E, Hawkey P, Wren BW, Dougan G, Parkhill J, Lawley TD.** 2013. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat Genet **45:**109-113.

13. **Miller BA, Chen LF, Sexton DJ, Anderson DJ.** 2011. Comparison of the burdens of hospital-onset, healthcare facility-associated *Clostridium difficile* Infection and of healthcare-associated infection due to methicillin-resistant *Staphylococcus aureus* in community hospitals. Infect Control Hosp Epidemiol **32:**387-390.

14. **Pepin J, Valiquette L, Alary ME, Villemure P, Pelletier A, Forget K, Pepin K, Chouinard D.** 2004. *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. Cmaj **171:**466-472.

15. **Rupnik M, Wilcox M, Gerding D.** 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol **7:**526 - 536.

16. **Åkerlund T, Persson I, Unemo M, Norén T, Svenungsson B, Wullt M, Burman LG.** 2008. Increased Sporulation Rate of Epidemic *Clostridium difficile* Type 027/NAP1. Journal of Clinical Microbiology **46:**1530-1533.

17. **Foster NF, Collins DA, Ditchburn SL, Duncan CN, van Schalkwyk JW, Golledge CL, Keed ABR, Riley TV.** 2014. Epidemiology of *Clostridium difficile* infection in two tertiary-care hospitals in Perth, Western Australia: a cross-sectional study. New Microbes and New Infections **2:**64-71.

18. **Cartman ST, Heap JT, Kuehne SA, Cockayne A, Minton NP.** 2010. The emergence of 'hypervirulence' in *Clostridium difficile*. Int J Med Microbiol **300:**387-395.

19. **Brenda A. Wilson AA, Dixie D; Malcolm E,.** 2011. Bacterial Pathogenesis; A Molecular Approach, Third ed.

20. **Surawicz CM, Brandt LJ, Binion DG, Ananthakrishnan AN, Curry SR, Gilligan PH, McFarland LV, Mellow M, Zuckerbraun BS.** 2013. Guidelines for diagnosis, treatment, and prevention of *Clostridium difficile* infections. Am J Gastroenterol **108:**478-498; quiz 499.

21. **Paredes-Sabja D, Shen A, Sorg JA.** 2014. *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. Trends Microbiol **22:**406-416.

22. **Gupta A, Khanna S.** 2014. Community-acquired *Clostridium difficile* infection: an increasing public health threat. Infection and Drug Resistance **7:**63-72.

23. **Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH.** 2010. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). Infect Control Hosp Epidemiol **31:**431-455.

24. **Anonymous.** 2008. Surveillance for community-associated *Clostridium difficile*--Connecticut, 2006. MMWR Morb Mortal Wkly Rep **57:**340-343.

25. **Khanna S, Pardi DS, Aronson SL, Kammer PP, Orenstein R, St. Sauver JL, Harmsen WS, Zinsmeister AR.** 2012. The Epidemiology of Community-acquired *Clostridium difficile* infection: A population-based study. The American Journal of Gastroenterology **107:**89-95.

26. **Wilcox MH, Mooney L, Bendall R, Settle CD, Fawley WN.** 2008. A case–control study of community-associated *Clostridium difficile* infection. Journal of Antimicrobial Chemotherapy **62:**388-396.

27. **Karlstrom O, Fryklund B, Tullus K, Burman LG.** 1998. A prospective nationwide study of Clostridium difficile-associated diarrhea in Sweden. The Swedish *C. difficile* Study Group. Clin Infect Dis **26:**141-145.

28. **Bauer MP, Veenendaal D, Verhoef L, Bloembergen P, van Dissel JT, Kuijper EJ.** 2009. Clinical and microbiological characteristics of community-onset *Clostridium difficile* infection in The Netherlands. Clin Microbiol Infect **15:**1087-1092.

29. **Alam MJ, Anu A, Walk ST, Garey KW.** 2014. Investigation of potentially pathogenic *Clostridium difficile* contamination in household environs. Anaerobe **27:**31-33.

30. **Visser M, Sepehrim S, Olson N, Du T, Mulvey MR, Alfa MJ.** 2012. Detection of *Clostridium difficile* in retail ground meat products in Manitoba. The Canadian Journal of Infectious Diseases & Medical Microbiology **23:**28-30.

31. **Gould LH, Limbago B.** 2010. *Clostridium difficile* in food and domestic animals: a new foodborne pathogen? Clin Infect Dis **51:**577-582.

32. **Khanna S, Pardi DS, Aronson SL, Kammer PP, Baddour LM.** 2012. "Outcomes in community–acquired *Clostridium difficile* infection". Alimentary pharmacology & therapeutics **35:**613-618.

33. **Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC.** 2005. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet **366:**1079-1084.

34. **McDonald LC, Killgore GE, Thompson A, Owens RC, Kazakova SV, Sambol SP, Johnson S, Gerding DN.** 2005. An Epidemic, Toxin Gene–Variant Strain of *Clostridium difficile*. New England Journal of Medicine **353:**2433-2441.

35. **Aktories K, Lang AE, Schwan C, Mannherz HG.** 2011. Actin as target for modification by bacterial protein toxins. Febs j **278:**4526-4543.

36. **Durai R.** 2007. Epidemiology, pathogenesis, and management of *Clostridium difficile* infection. Dig Dis Sci **52:**2958-2962.

37. **Sekirov I, Russell SL, Antunes LC, Finlay BB.** 2010. Gut microbiota in health and disease. Physiol Rev **90:**859-904.

38. **Stecher B, Hardt W-D.** 2008. The role of microbiota in infectious disease. Trends in Microbiology **16:**107-114.

39. **Guarner F, Malagelada J-R.** 2003. Gut flora in health and disease. The Lancet **361:**512-519.

40. **Bengmark S.** 2013. Gut microbiota, immune development and function. Pharmacological Research **69:**87-113.

41. **Round JL, Mazmanian SK.** 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol **9:**313-323.

42. **Dupont A, Heinbockel L, Brandenburg K, Hornef MW.** 2014. Antimicrobial peptides and the enteric mucus layer act in concert to protect the intestinal mucosa. Gut Microbes **5:**761-765.

43. **Kamada N, Chen GY, Inohara N, Nunez G.** 2013. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol **14:**685-690.

44. **Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ.** 2000. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol **1:**113-118.

45. **O'Horo JC, Jindai K, Kunzer B, Safdar N.** 2014. Treatment of recurrent *Clostridium difficile* infection: a systematic review. Infection **42:**43-59.

46. **Keller JJ, Kuijper EJ.** 2015. Treatment of Recurrent and Severe *Clostridium difficile* Infection. Annual Review of Medicine **66:**373-386.

47. **Abujamel T, Cadnum JL, Jury LA, Sunkesula VCK, Kundrapu S, Jump RL, Stintzi AC, Donskey CJ.** 2013. Defining the Vulnerable Period for Re-Establishment of *Clostridium difficile* Colonization after Treatment of *C. difficile* Infection with Oral Vancomycin or Metronidazole. PLoS One **8:**e76269.

48. **Figueroa I, Johnson S, Sambol SP, Goldstein EJ, Citron DM, Gerding DN.** 2012. Relapse versus reinfection: recurrent *Clostridium difficile* infection following treatment with fidaxomicin or vancomycin. Clin Infect Dis **55 Suppl 2:**S104-109.

49. **Olsen MA, Yan Y, Reske KA, Zilberberg MD, Dubberke ER.** 2015. Recurrent *Clostridium difficile* infection is associated with increased mortality. Clin Microbiol Infect **21:**164-170.

50. **Duke PS, Fardy J.** 2014. Recurrent *Clostridium difficile* infection treated with home fecal transplantation: a case report. J Med Case Rep **8:**393.

51. **Tsutsumi LS, Owusu YB, Hurdle JG, Sun D.** 2014. Progress in the Discovery of Treatments for *C. difficile* Infection: A Clinical and Medicinal Chemistry Review. Current Topics in Medicinal Chemistry **14:**152-175.

52. **Goldman P.** 1982. The development of 5-nitroimidazoles for the treatment and prophylaxis of anaerobic bacterial infections. J Antimicrob Chemother **10 Suppl A:**23-33.

53. **Löfmark S, Edlund C, Nord CE.** 2010. Metronidazole Is Still the Drug of Choice for Treatment of Anaerobic Infections. Clinical Infectious Diseases **50:**S16-S23.

54. **Freeman CD, Klutman NE, Lamp KC.** 1997. Metronidazole. A therapeutic review and update. Drugs **54:**679-708.

55. **Muller M.** 1983. Mode of action of metronidazole on anaerobic bacteria and protozoa. Surgery **93:**165-171.

56. **Musher DM, Aslam S, Logan N, Nallacheru S, Bhaila I, Borchert F, Hamill RJ.** 2005. Relatively poor outcome after treatment of *Clostridium difficile* colitis with metronidazole. Clin Infect Dis **40:**1586-1590.

57. **Lau AH, Lam NP, Piscitelli SC, Wilkes L, Danziger LH.** 1992. Clinical pharmacokinetics of metronidazole and other nitroimidazole anti-infectives. Clin Pharmacokinet **23:**328-364.

58. **Lamp KC, Freeman CD, Klutman NE, Lacy MK.** 1999. Pharmacokinetics and pharmacodynamics of the nitroimidazole antimicrobials. Clin Pharmacokinet **36:**353-373.

59. **Bolton RP, Culshaw MA.** 1986. Faecal metronidazole concentrations during oral and intravenous therapy for antibiotic associated colitis due to *Clostridium difficile*. Gut **27:**1169-1172.

60. **Kuijper EJ, Wilcox MH.** 2008. Decreased Effectiveness of Metronidazole for the Treatment of *Clostridium difficile* Infection? Clinical Infectious Diseases **47:**63-65.

61. **Chen C, Dolla NK, Casadei G, Bremner JB, Lewis K, Kelso MJ.** 2014. Diarylacylhydrazones: Clostridium-selective antibacterials with activity against stationary-phase cells. Bioorg Med Chem Lett **24:**595-600.

62. **Citron DM, Tyrrell KL, Merriam CV, Goldstein EJC.** 2012. *In Vitro* Activities of CB-183,315, Vancomycin, and Metronidazole against 556 Strains of *Clostridium difficile*, 445 Other Intestinal Anaerobes, and 56 Enterobacteriaceae Species. Antimicrobial Agents and Chemotherapy **56:**1613-1615.

63. **Al-Nassir WN, Sethi AK, Li Y, Pultz MJ, Riggs MM, Donskey CJ.** 2008. Both oral metronidazole and oral vancomycin promote persistent overgrowth of vancomycin-resistant enterococci during treatment of *Clostridium difficile*-associated disease. Antimicrob Agents Chemother **52:**2403-2406.

64. **Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, Kuijper EJ, Wilcox MH.** 2008. Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*. J Antimicrob Chemother **62:**1046-1052.

65. **Reynolds PE.** 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. Eur J Clin Microbiol Infect Dis **8:**943-950.

66. **Nagarajan R.** 1991. Antibacterial activities and modes of action of vancomycin and related glycopeptides. Antimicrobial Agents and Chemotherapy **35:**605-609.

67. **Pepin J.** 2008. Vancomycin for the Treatment of *Clostridium difficile* Infection: For Whom Is This Expensive Bullet Really Magic? Clinical Infectious Diseases **46:**1493-1498.

68. **Johnson S, Homann SR, Bettin KM, Quick JN, Clabots CR, Peterson LR, Gerding DN.** 1992. Treatment of asymptomatic *Clostridium difficile* carriers (fecal excretors) with vancomycin or metronidazole. A randomized, placebo-controlled trial. Ann Intern Med **117:**297-302.

69. **Leeds JA, Sachdeva M, Mullin S, Barnes SW, Ruzin A.** 2014. *In vitro* selection, via serial passage, of *Clostridium difficile* mutants with reduced susceptibility to fidaxomicin or vancomycin. Journal of Antimicrobial Chemotherapy **69:**41-44.

70. **Alam MZ, Wu X, Mascio C, Chesnel L, Hurdle JG.** 2015. Mode of Action and Bactericidal Properties of Surotomycin against Growing and Non-growing *Clostridium difficile*. Antimicrobial Agents and Chemotherapy.

71. **Wu X, Cherian PT, Lee RE, Hurdle JG.** 2013. The membrane as a target for controlling hypervirulent *Clostridium difficile* infections. J Antimicrob Chemother **68:**806-815.

72. **Lancaster JW, Matthews SJ.** 2012. Fidaxomicin: The Newest Addition to the Armamentarium Against *Clostridium difficile* Infections. Clinical Therapeutics **34:**1-13.

73. **Golan Y, Epstein L.** 2012. Safety and efficacy of fidaxomicin in the treatment of *Clostridium difficile*-associated diarrhea. Therapeutic Advances in Gastroenterology **5:**395-402.

74. **Tannock GW, Munro K, Taylor C, Lawley B, Young W, Byrne B, Emery J, Louie T.** 2010. A new macrocyclic antibiotic, fidaxomicin (OPT-80), causes less alteration to the bowel microbiota of *Clostridium difficile*-infected patients than does vancomycin. Microbiology **156:**3354-3359.

75. **Miller M.** 2010. Fidaxomicin (OPT-80) for the treatment of *Clostridium difficile* infection. Expert Opinion on Pharmacotherapy **11:**1569-1578.

76. **Goldstein EJC, Babakhani F, Citron DM.** 2012. Antimicrobial Activities of Fidaxomicin. Clinical Infectious Diseases **55:**S143-S148.

77. **O'Connor JR, Galang MA, Sambol SP, Hecht DW, Vedantam G, Gerding DN, Johnson S.** 2008. Rifampin and Rifaximin Resistance in Clinical Isolates of *Clostridium difficile*. Antimicrobial Agents and Chemotherapy **52:**2813-2817.

78. **Cornely OA, Miller MA, Louie TJ, Crook DW, Gorbach SL.** 2012. Treatment of First Recurrence of *Clostridium difficile* Infection: Fidaxomicin Versus Vancomycin. Clinical Infectious Diseases **55:**S154-S161.

79. **Goldstein EJ, Citron DM, Sears P, Babakhani F, Sambol SP, Gerding DN.** 2011. Comparative susceptibilities to fidaxomicin (OPT-80) of isolates collected at baseline, recurrence, and failure from patients in two phase III trials of fidaxomicin against *Clostridium difficile* infection. Antimicrob Agents Chemother **55:**5194-5199.

80. **Cornely OA, Crook DW, Esposito R, Poirier A, Somero MS, Weiss K, Sears P, Gorbach S.** 2012. Fidaxomicin versus vancomycin for infection with *Clostridium difficile* in Europe, Canada,

and the USA: a double-blind, non-inferiority, randomised controlled trial. Lancet Infect Dis **12:**281-289.

81. **Louie TJ, Miller MA, Mullane KM, Weiss K, Lentnek A, Golan Y, Gorbach S, Sears P, Shue Y-K.** 2011. Fidaxomicin versus Vancomycin for *Clostridium difficile* Infection. New England Journal of Medicine **364:**422-431.

82. **Orenstein R.** 2012. Fidaxomicin Failures in Recurrent *Clostridium difficile* Infection: A Problem of Timing. Clinical Infectious Diseases.

83. **Mattila E, Arkkila P, Mattila PS, Tarkka E, Tissari P, Anttila VJ.** 2013. Rifaximin in the treatment of recurrent *Clostridium difficile* infection. Aliment Pharmacol Ther **37:**122-128.

84. **Adachi JA, DuPont HL.** 2006. Rifaximin: A Novel Nonabsorbed Rifamycin for Gastrointestinal Disorders. Clinical Infectious Diseases **42:**541-547.

85. **Curry SR, Marsh JW, Shutt KA, Muto CA, O'Leary MM, Saul MI, William PA, Harrison LH.** 2009. High Frequency of Rifampin Resistance Identified in an Epidemic *Clostridium difficile* Clone from a Large Teaching Hospital. Clinical Infectious Diseases **48:**425-429.

86. **Huang H, Weintraub A, Fang H, Nord CE.** 2009. Antimicrobial resistance in *Clostridium difficile*. International Journal of Antimicrobial Agents **34:**516-522.

87. **O'Horo J, Safdar N.** 2009. The role of immunoglobulin for the treatment of *Clostridium difficile* infection: a systematic review. Int J Infect Dis **13:**663-667.

88. **Lowy I, Molrine DC, Leav BA, Blair BM, Baxter R, Gerding DN, Nichol G, Thomas WD, Leney M, Sloan S, Hay CA, Ambrosino DM.** 2010. Treatment with Monoclonal Antibodies against *Clostridium difficile* Toxins. New England Journal of Medicine **362:**197-205.

89. **Sharp M.** A study of MK-3415 and other monoclonal antibodies.

90. **Aboudola S, Kotloff KL, Kyne L, Warny M, Kelly EC, Sougioultzis S, Giannasca PJ, Monath TP, Kelly CP.** 2003. *Clostridium difficile* Vaccine and Serum Immunoglobulin G Antibody Response to Toxin A. Infection and Immunity **71:**1608-1610.

91. **Abougergi MS, Kwon JH.** 2011. Intravenous immunoglobulin for the treatment of *Clostridium difficile* infection: a review. Dig Dis Sci **56:**19-26.

92. **Goldenberg JZ, Ma SS, Saxton JD, Martzen MR, Vandvik PO, Thorlund K, Guyatt GH, Johnston BC.** 2013. Probiotics for the prevention of *Clostridium difficile*-associated diarrhea in adults and children. Cochrane Database Syst Rev **5:**Cd006095.

93. **McFarland LV.** 2006. Meta-Analysis of Probiotics for the Prevention of Antibiotic Associated Diarrhea and the Treatment of *Clostridium difficile* Disease. Am J Gastroenterol **101:**812-822.

94. **Eiseman B, Silen W, Bascom GS, Kauvar AJ.** 1958. Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. Surgery **44:**854-859.

95. **Kassam Z, Lee CH, Yuan Y, Hunt RH.** 2013. Fecal Microbiota Transplantation for *Clostridium difficile* Infection: Systematic Review and Meta-Analysis. Am J Gastroenterol **108:**500-508.

96. **Kelly CP.** 2013. Fecal microbiota transplantation--an old therapy comes of age. N Engl J Med **368:**474-475.

97. **Gough E, Shaikh H, Manges AR.** 2011. Systematic Review of Intestinal Microbiota Transplantation (Fecal Bacteriotherapy) for Recurrent *Clostridium difficile* Infection. Clinical Infectious Diseases **53:**994-1002.

98. **Vardakas KZ, Polyzos KA, Patouni K, Rafailidis PI, Samonis G, Falagas ME.** 2012. Treatment failure and recurrence of *Clostridium difficile* infection following treatment with vancomycin or metronidazole: a systematic review of the evidence. Int J Antimicrob Agents **40:**1-8.

99. **Hurdle JG, O'Neill AJ, Chopra I, Lee RE.** 2011. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol **9:**62-75.

100. **Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL.** 2007. Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat Rev Drug Discov **6:**29-40.

101. **Seaton RA, Malizos KN, Viale P, Gargalianos-Kakolyris P, Santantonio T, Petrelli E, Pathan R, Heep M, Chaves RL.** 2013. Daptomycin use in patients with osteomyelitis: a preliminary report from the EU-CORE(SM) database. J Antimicrob Chemother **68:**1642-1649.

102. **Straus SK, Hancock REW.** 2006. Mode of action of the new antibiotic for Gram-positive pathogens daptomycin: Comparison with cationic antimicrobial peptides and lipopeptides. Biochimica et Biophysica Acta (BBA) - Biomembranes **1758:**1215-1223.

103. **Mascio CTM, Alder JD, Silverman JA.** 2007. Bactericidal Action of Daptomycin against Stationary-Phase and Nondividing *Staphylococcus aureus* Cells. Antimicrobial Agents and Chemotherapy **51:**4255-4260.

104. **Micklefield J.** 2004. Daptomycin Structure and Mechanism of Action Revealed. Chemistry & Biology **11:**887-888.

105. **Khanna S, Pardi DS, Aronson SL, Kammer PP, Orenstein R, St Sauver JL, Harmsen WS, Zinsmeister AR.** 2012. The Epidemiology of Community-Acquired *Clostridium difficile* Infection: A Population-Based Study. Am J Gastroenterol **107:**89-95.

106. **Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, Holzbauer SM, Meek JI, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago BM, Fridkin SK, Gerding DN, McDonald LC.** 2015. Burden of *Clostridium difficile* infection in the United States. N Engl J Med **372:**825-834.

107. **Bolton RP, Culshaw MA.** 1986. Faecal metronidazole concentrations during oral and intravenous therapy for antibiotic associated colitis due to *Clostridium difficile*. Gut **27:**1169-1172.

108. **Louie TJ, Cannon K, Byrne B, Emery J, Ward L, Eyben M, Krulicki W.** 2012. Fidaxomicin Preserves the Intestinal Microbiome During and After Treatment of *Clostridium difficile* Infection (CDI) and Reduces Both Toxin Reexpression and Recurrence of CDI. Clinical Infectious Diseases **55:**S132-S142.

109. **Mascio CT, Mortin LI, Howland KT, Van Praagh AD, Zhang S, Arya A, Chuong CL, Kang C, Li T, Silverman JA.** 2012. *In vitro* and *in vivo* characterization of CB-183,315, a novel lipopeptide antibiotic for treatment of *Clostridium difficile*. Antimicrob Agents Chemother **56:**5023-5030.

110. **Pogliano J, Pogliano N, Silverman JA.** 2012. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. J Bacteriol **194:**4494-4504.

111. **Mascio CT, Alder JD, Silverman JA.** 2007. Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. Antimicrob Agents Chemother **51:**4255-4260.

112. **Merrigan M, Venugopal A, Mallozzi M, Roxas B, Viswanathan VK, Johnson S, Gerding DN, Vedantam G.** 2010. Human Hypervirulent *Clostridium difficile* Strains Exhibit Increased Sporulation as Well as Robust Toxin Production. Journal of Bacteriology **192:**4904-4911.

113. **Wu X, Alam MZ, Feng L, Tsutsumi LS, Sun D, Hurdle JG.** 2014. Prospects for flavonoid and related phytochemicals as nature-inspired treatments for *Clostridium difficile* infection. Journal of Applied Microbiology **116:**23-31.

114. **Nuding S, Fellermann K, Wehkamp J, Mueller HA, Stange EF.** 2006. A flow cytometric assay to monitor antimicrobial activity of defensins and cationic tissue extracts. J Microbiol Methods **65:**335-345.

115. **Muthaiyan A, Silverman JA, Jayaswal RK, Wilkinson BJ.** 2008. Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. Antimicrob Agents Chemother **52:**980-990.

116. **Kumar M, Adhikari S, Hurdle JG.** 2014. Action of nitroheterocyclic drugs against *Clostridium difficile*. Int J Antimicrob Agents **44:**314-319.

117. **Davlieva M, Zhang W, Arias CA, Shamoo Y.** 2013. Biochemical characterization of cardiolipin synthase mutations associated with daptomycin resistance in enterococci. Antimicrob Agents Chemother **57:**289-296.

118. **Adams HM, Li X, Mascio C, Chesnel L, Palmer KL.** 2015. Mutations associated with reduced surotomycin susceptibility in *Clostridium difficile* and *Enterococcus* species. Antimicrob Agents Chemother **59:**4139-4147.

119. **Approvals Nd.** 2010. Surotomycin fact sheet.

120. **Cubist Pharmaceticals.** 2013. Surotomycin fact sheet.

121. **Hobbs JK, Miller K, O'Neill AJ, Chopra I.** 2008. Consequences of daptomycin-mediated membrane damage in *Staphylococcus aureus*. J Antimicrob Chemother **62:**1003-1008.

122. **Silverman JA, Perlmutter NG, Shapiro HM.** 2003. Correlation of Daptomycin Bactericidal Activity and Membrane Depolarization in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy **47:**2538-2544.

123. **Zheng C-J, Sohn M-J, Kim W-G.** 2006. Atromentin and Leucomelone, the First Inhibitors Specific to Enoyl-ACP Reductase (FabK) of *Streptococcus pneumoniae*. J Antibiot **59:**808-812.

124. **Kuijper EJ, Barbut F, Brazier JS, Kleinkauf N, Eckmanns T, Lambert ML, Drudy D, Fitzpatrick F, Wiuff C, Brown DJ, Coia JE, Pituch H, Reichert P, Even J, Mossong J, Widmer AF, Olsen KE, Allerberger F, Notermans DW, Delmee M, Coignard B, Wilcox M, Patel B, Frei R, Nagy E, Bouza E, Marin M, Akerlund T, Virolainen-Julkunen A, Lyytikainen O, Kotila S, Ingebretsen A, Smyth B, Rooney P, Poxton IR, Monnet DL.** 2008. Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. Euro Surveill **13**.

125. **News C.** 2011. *Clostridium difficile* outbreak over in 3 Ontario hospitals. doi:http://www.cbc.ca/news/canada/toronto/c-difficile-outbreak-over-in-3-ontario-hospitals-1.995941.

126. **Janoir C, Denève C, Bouttier S, Barbut F, Hoys S, Caleechum L, Chapetón-Montes D, Pereira FC, Henriques AO, Collignon A, Monot M, Dupuy B.** 2013. Adaptive Strategies and Pathogenesis of *Clostridium difficile* from In Vivo Transcriptomics. Infection and Immunity **81:**3757-3769.

127. **Garneau J, Valiquette L, Fortier L-C.** 2014. Prevention of *Clostridium difficile* spore formation by sub-inhibitory concentrations of tigecycline and piperacillin/tazobactam. BMC Infectious Diseases **14:**29.

128. **Heinlen L, Ballard JD.** 2010. *Clostridium difficile* Infection. The American journal of the medical sciences **340:**247-252.

129. **Pepin J, Valiquette L, Gagnon S, Routhier S, Brazeau I.** 2007. Outcomes of *Clostridium difficile*-Associated Disease Treated With Metronidazole or Vancomycin Before and After the Emergence of NAP1//027. Am J Gastroenterol **102:**2781-2788.

130. **Hurdle JG, Heathcott AE, Yang L, Yan B, Lee RE.** 2011. Reutericyclin and related analogues kill stationary phase *Clostridium difficile* at achievable colonic concentrations. J Antimicrob Chemother **66:**1773-1776.

131. **Norén T, Wullt M, Åkerlund T, Bäck E, Odenholt I, Burman LG.** 2006. Frequent Emergence of Resistance in Clostridium difficile during Treatment of *C. difficile*-Associated Diarrhea with Fusidic Acid. Antimicrobial Agents and Chemotherapy **50:**3028-3032.

132. **Luther MK, Arvanitis M, Mylonakis E, LaPlante KL.** 2014. Activity of daptomycin or linezolid in combination with rifampin or gentamicin against biofilm-forming *Enterococcus faecalis* or *E. faecium* in an in vitro pharmacodynamic model using simulated endocardial vegetations and an *in vivo* survival assay using *Galleria mellonella* larvae. Antimicrob Agents Chemother **58:**4612-4620.

133. **Humphries RM, Pollett S, Sakoulas G.** 2013. A current perspective on daptomycin for the clinical microbiologist. Clin Microbiol Rev **26:**759-780.

134. **Chilton CH, Crowther GS, Todhunter SL, Nicholson S, Freeman J, Chesnel L, Wilcox MH.** 2014. Efficacy of surotomycin in an in vitro gut model of *Clostridium difficile* infection. J Antimicrob Chemother **69:**2426-2433.

135. **Zanella Terrier MC, Simonet ML, Bichard P, Frossard JL.** 2014. Recurrent *Clostridium difficile* infections: The importance of the intestinal microbiota. World Journal of Gastroenterology : WJG **20:**7416-7423.

136. **Bartlett JG.** 2002. Clinical practice. Antibiotic-associated diarrhea. N Engl J Med **346:**334-339.

137. **Gerding DN, Johnson S, Peterson LR, Mulligan ME, Silva J, Jr.** 1995. *Clostridium difficile*-associated diarrhea and colitis. Infect Control Hosp Epidemiol **16:**459-477.

138. **Gerding DN.** 2005. Metronidazole for *Clostridium difficile*-Associated Disease: Is It Okay for Mom? Clinical Infectious Diseases **40:**1598-1600.

139. **Schaefer C.** 2007. Drugs During Pregnancy and Lactation: Treatment Options and Risk Assessment.125.

140. **Al-Nassir WN, Sethi AK, Nerandzic MM, Bobulsky GS, Jump RL, Donskey CJ.** 2008. Comparison of clinical and microbiological response to treatment of *Clostridium difficile*-associated disease with metronidazole and vancomycin. Clin Infect Dis **47:**56-62.

141. **Royles BJL.** 1995. Naturally Occurring Tetramic Acids: Structure, Isolation, and Synthesis. Chemical Reviews **95:**1981-2001.

142. **Ganzle MG.** 2004. Reutericyclin: biological activity, mode of action, and potential applications. Appl Microbiol Biotechnol **64:**326-332.

143. **Holtzel A, Ganzle MG, Nicholson GJ, Hammes WP, Jung G.** 2000. The First Low Molecular Weight Antibiotic from Lactic Acid Bacteria: Reutericyclin, a New Tetramic Acid. Angew Chem Int Ed Engl **39:**2766-2768.

144. **Schobert R, Schlenk A.** 2008. Tetramic and tetronic acids: an update on new derivatives and biological aspects. Bioorg Med Chem **16:**4203-4221.

145. **Ondeyka JG, Smith SK, Zink DL, Vicente F, Basilio A, Bills GF, Polishook JD, Garlisi C, McGuinness D, Smith E, Qiu H, Gill CJ, Donald RG, Phillips JW, Goetz MA, Singh SB.** 2014. Isolation, structure elucidation and antibacterial activity of a new tetramic acid, ascosetin. J Antibiot (Tokyo) **67:**527-531.

146. **Cherian PT, Wu X, Maddox MM, Singh AP, Lee RE, Hurdle JG.** 2014. Chemical Modulation of the Biological Activity of Reutericyclin: a Membrane-Active Antibiotic from Lactobacillusreuteri. Sci Rep **4**.

147. **Cherian PT, Wu X, Yang L, Scarborough JS, Singh AP, Alam ZA, Lee RE, Hurdle JG.** 2015. Gastrointestinal localization of metronidazole by a lactobacilli-inspired tetramic acid motif improves treatment outcomes in the hamster model of *Clostridium difficile* infection. J Antimicrob Chemother doi:10.1093/jac/dkv231.

148. **Anton PM, O'Brien M, Kokkotou E, Eisenstein B, Michaelis A, Rothstein D, Paraschos S, Kelly CP, Pothoulakis C.** 2004. Rifalazil treats and prevents relapse of *Clostridium difficile*-associated diarrhea in hamsters. Antimicrob Agents Chemother **48:**3975-3979.

149. **Mathur T, Kumar M, Barman TK, Kumar GR, Kalia V, Singhal S, Raj VS, Upadhyay DJ, Das B, Bhatnagar PK.** 2011. Activity of RBx 11760, a novel biaryl oxazolidinone, against *Clostridium difficile*. J Antimicrob Chemother **66:**1087-1095.

150. **Raether W, Hanel H.** 2003. Nitroheterocyclic drugs with broad spectrum activity. Parasitol Res **90 Supp 1:**S19-39.

151. **Johnson S, Louie TJ, Gerding DN, Cornely OA, Chasan-Taber S, Fitts D, Gelone SP, Broom C, Davidson DM.** 2014. Vancomycin, Metronidazole, or Tolevamer for *Clostridium difficile* Infection: Results from Two Multinational, Randomized, Controlled Trials. Clinical Infectious Diseases.

152. **Hutton ML, Mackin KE, Chakravorty A, Lyras D.** 2014. Small animal models for the study of *Clostridium difficile* disease pathogenesis. FEMS Microbiol Lett **352:**140-149.

153. **Warren CA, van Opstal EJ, Riggins MS, Li Y, Moore JH, Kolling GL, Guerrant RL, Hoffman PS.** 2013. Vancomycin treatment's association with delayed intestinal tissue injury, clostridial overgrowth, and recurrence of *Clostridium difficile* infection in mice. Antimicrob Agents Chemother **57:**689-696.

154. **Crowther GS, Chilton CH, Todhunter SL, Nicholson S, Freeman J, Baines SD, Wilcox MH.** 2014. Development and Validation of a Chemostat Gut Model To Study Both Planktonic and Biofilm Modes of Growth of *Clostridium difficile* and Human Microbiota. PLoS One **9:**e88396.

155. **O'Connor D, Hynes P, Cormican M, Collins E, Corbett-Feeney G, Cassidy M.** 2001. Evaluation of Methods for Detection of Toxins in Specimens of Feces Submitted for Diagnosis of *Clostridium difficile*- Associated Diarrhea. Journal of Clinical Microbiology **39:**2846-2849.

156. **Rüssmann H, Panthel K, Bader RC, Schmitt C, Schaumann R.** 2007. Evaluation of three rapid assays for detection of *Clostridium difficile* toxin A and toxin B in stool specimens. European Journal of Clinical Microbiology & Infectious Diseases **26:**115-119.

157. **Chapin KC, Dickenson RA, Wu F, Andrea SB.** 2011. Comparison of Five Assays for Detection of *Clostridium difficile* Toxin. The Journal of Molecular Diagnostics : JMD **13:**395-400.

158. **Best EL, Freeman J, Wilcox MH.** 2012. Models for the study of *Clostridium difficile* infection. Gut Microbes **3:**145-167.

159. **Buckley AM, Spencer J, Candlish D, Irvine JJ, Douce GR.** 2011. Infection of hamsters with the UK *Clostridium difficile* ribotype 027 outbreak strain R20291. Journal of Medical Microbiology **60:**1174-1180.

160. **Sun X, Wang H, Zhang Y, Chen K, Davis B, Feng H.** 2011. Mouse Relapse Model of *Clostridium difficile* Infection. Infection and Immunity **79:**2856-2864.

161. **Babcock GJ, Broering TJ, Hernandez HJ, Mandell RB, Donahue K, Boatright N, Stack AM, Lowy I, Graziano R, Molrine D, Ambrosino DM, Thomas WD.** 2006. Human Monoclonal Antibodies Directed against Toxins A and B Prevent *Clostridium difficile*-Induced Mortality in Hamsters. Infection and Immunity **74:**6339-6347.

162. **Pogliano J, Pogliano N, Silverman JA.** 2012. Daptomycin-Mediated Reorganization of Membrane Architecture Causes Mislocalization of Essential Cell Division Proteins. Journal of Bacteriology **194:**4494-4504.

163. **Fukushima T, Szurmant H, Kim E-J, Perego M, Hoch JA.** 2008. A sensor histidine kinase coordinates cell wall architecture with cell division in Bacillus subtilis. Molecular microbiology **69:**621-632.

164. **Friedman L, Alder JD, Silverman JA.** 2006. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother **50:**2137-2145.

165. **Allen NE, Nicas TI.** 2003. Mechanism of action of oritavancin and related glycopeptide antibiotics. FEMS Microbiol Rev **26:**511-532.

166. **Baines SD, O'Connor R, Saxton K, Freeman J, Wilcox MH.** 2008. Comparison of oritavancin versus vancomycin as treatments for clindamycin-induced *Clostridium difficile* PCR ribotype 027 infection in a human gut model. Journal of Antimicrobial Chemotherapy **62:**1078-1085.

167. **Belley A, Neesham-Grenon E, McKay G, Arhin FF, Harris R, Beveridge T, Parr TR, Moeck G.** 2009. Oritavancin Kills Stationary-Phase and Biofilm *Staphylococcus aureus* Cells In Vitro. Antimicrobial Agents and Chemotherapy **53:**918-925.

168. **Pranav H. Nakhate VKY.** 2013. A Review on Daptomycin; the first US-FDA approved Lipopeptide antibiotics. Journal of Scientific and Innovative Research **2**.

169. **Freeman J, Marquis M, Crowther GS, Todhunter SL, Fawley WN, Chilton CH, Moeck G, Lehoux D, Wilcox MH.** 2012. Oritavancin does not induce *Clostridium difficile* germination and toxin production in hamsters or a human gut model. J Antimicrob Chemother **67:**2919-2926.

170. **Chilton CH, Freeman J, Crowther GS, Todhunter SL, Wilcox MH.** 2012. Effectiveness of a short (4 day) course of oritavancin in the treatment of simulated *Clostridium difficile* infection using a human gut model. Journal of Antimicrobial Chemotherapy **67:**2434-2437.

Biography

Md. Zahidul Alam was born in Chandpur, Bangladesh on September 15, 1983. During his higher secondary education, he attended the Notre Dame College, Dhaka, Bangladesh. He earned his medical graduate degree, bachelor of medicine and bachelor of surgery (M.B.B.S.) from Dhaka Medical College, Bangladesh in the year of 2008. After then, he completed his clinical postgraduate fellowship training in the department of internal medicine and cardiology of the Dhaka Medical College Hospital. He started his Ph.D., which primarily focused in the development of antibacterial drugs for the treatment of *C. difficile* infection, at the department of biology of the University of Texas at Arlington in fall 2011. He is graduating on November 12, 2015. After the graduation, his aim is to work as a postdoctoral fellow, and continue the research in the field of drug-development. His long-term future goal is to be a faculty in the field of biomedical sciences, where he can teach as well as can do independent research.