A Clinical and Molecular Analysis of *Clostridium difficile* Strains Isolated from Groote Schuur Hospital

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PLAGIARISM DECLARATION

I know the meaning of plagiarism and declare that all of the work in the dissertation, save for that which is properly acknowledged, is my own.

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CONTENTS

ABSTRACT

A clinical and molecular analysis of *Clostridium difficile* isolated from symptomatic patients at Groote Schuur Hospital was conducted in order to gain insight into the identity, epidemiology and pathogenesis of the various strains. *C. difficile* was detected and isolated by selective culture from stool specimens from 34 of the 162 symptomatic patients (20%). Three toxigenic-types were distinguished by PCR: A+B+ (47%), A-B+ (47%) and A-B- (6%), none of which harboured the binary toxin genes. Compared to the direct culture method, enzyme immunoassay-based detection tests (Meridian ImmunoCard and bioMérieux MiniVidas) were found to be lacking clinical sensitivity, while nucleic acid amplification tests (Hain Lifescience CDiff and Cepheid GeneXpert) were far more sensitive in the local clinical setting. PCR ribotyping identified all the A-B+ strains as PCR ribotype 017, which was the prevalent strain type (47%). Genotyping based on the *tcdC* gene and MLVA both grouped the ribotype 017 strains in a single clade. The antimicrobial susceptibility of all the isolates to metronidazole (MET), vancomycin (VAN), moxifloxacin (MOX) and erythromycin (ERY) were determined by the Etest method. All were sensitive to MET and VAN; however, four ribotype 017 strains displayed reduced susceptibility to MET. With regard to MOX, reduced susceptibly and full resistance were observed in 32% and 12% of the isolates, respectively, with all of these belonging to ribotype 017. MOX-resistant strains had a Thr82→Ile amino acid substitution in the GyrA enzyme and strains displaying reduced susceptibility to MOX had an Asp426→Asn amino acid substitution in GyrB. High-level resistance to ERY was observed in 47% of the isolates, which were primarily ribotype 017. ERY-resistant strains all harboured the *ermB* gene, suggesting that this was the genetic basis of the observed phenotype. Auto-aggregation analysis revealed that the ribotype 017 strains were significantly stronger auto-aggregators than the other ribotypes examined. Results of

semi-quantitative RT-PCR analysis suggest that the expression level of the *cwpV* gene, encoding the CwpV protein, may play a role in auto-aggregation. In conclusion, this pilot study revealed that the GeneXpert method was the most accurate and sensitive technique for diagnosing CDI in the clinical setting at Groote Schuur Hospital. Ribotype 017 was the most prevalent strain type, and the antimicrobial resistance profile and increased auto-aggregation capacity of this ribotype may contribute to its high prevalence.

ABBREVIATIONS

aa	amino acids
bp	base pair(s)
cDNA	complementary DNA
CDT/cdt	*C. difficile* toxin (binary toxin)
CFU	colony forming unit(s)
cm	centimetre(s)
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetra-acetic acid
g	gram(s)
GSH	Groote Schuur Hospital
M	molar
mg	milligram(s)
MIC	minimum inhibitory concentration
min	minute(s)
ml	millilitre(s)
mM	millimolar
MW	molecular weight
NAP1	North American pulse-field gel electrophoresis type 1
ng	nanogram(s)
nm	nanometer(s)
OD	optical density
p	P-value (statistical significance level)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

RNA	ribonucleic acid
rRNA	ribosomal RNA
s	seconds
SDS	sodium dodecyl sulfate
SE	Swedish PCR ribotype library
Tris	tris(hydroxymethyl)aminomethane
U	unit(s)
UDP-glucose	uracil-diphosphate glucose
UK	United Kingdom
USA	United States of America
vol	volume(s)
v/v	volume per volume (in ml per 100 ml)
w/v	weight per volume (in grams per 100 ml)
α	alpha
β	beta
γ	gamma
Δ	delta
μ	micro

CHAPTER ONE

LITERATURE REVIEW

Contents

1.1 Introduction

Clostridium difficile is a Gram positive, anaerobic bacterium, which has emerged as the leading cause of healthcare- and antimicrobial-associated diarrhoeas. Disease symptoms range from mild diarrhoea to potentially fatal pseudomembranous colitis and are collectively known as *Clostridium difficile* infection (CDI) or *C. difficile*-associated disease (CDAD). CDI caused by various *C. difficile* strains has reached epidemic proportions in hospitals worldwide and numerous studies have been conducted in order to characterise this pathogenic bacterium and to understand its ability to persist and cause disease. The pathogenesis and the epidemiology of *C. difficile,* as well as the diagnosis and treatment of CDI, the typing of *C. difficile*, and some aspects of its physiology that are relevant to infection will be reviewed below.

1.2 Pathogenesis

1.2.1 Risk factors in the development of CDI

Antimicrobials

Antimicrobial therapy is the primary risk factor for developing CDI (Baxter *et al.*, 2008; Spigaglia *et al.*, 2011). Antimicrobial use disturbs the normal enteric microbiota and selects for the growth of *C. difficile* (De La Cochetière *et al.*, 2008; Sullivan *et al.*, 2001). *C. difficile* spores and/or vegetative cells can remain, in low numbers, as a part of the enteric microbiota in asymptomatic individuals (Kyne *et al.*, 2000). However, there is a difference in the abundance and diversity of the microbiota between asymptomatic and healthy individuals (Zhang *et al.*, 2015). Disruption of the microbiota and mucosa provides *C. difficile* with the opportunity to proliferate and colonise (Pérez-Cobas *et al.*, 2014). In addition, antimicrobials

have been shown to promote bacterial growth and toxin production by resistant epidemic-associated *C. difficile* strains (Adams *et al.*, 2007; Freeman *et al.*, 2007).

Broad-spectrum antimicrobials such as penicillins, clindamycin, fluoroquinolones and cephalosporins are commonly administered in hospital environments and have been associated with the development of CDI and associated outbreaks (Biller *et al.*, 2007; Dubberke *et al.*, 2015; Johnson *et al.*, 1999; Pépin *et al.*, 2005). In the 1970's, clindamycin was considered the highest-risk antimicrobial to cause CDI (Tedesco *et al.*, 1974). Thereafter, the decreased use of clindamycin in hospitals in Europe and the USA led to fewer cases of CDI (Climo *et al.*, 1998; Pear *et al.*, 1994). Clindamycin use is still suggested as a potential risk factor for CDI caused by A-B+ strains of *C. difficile* (Kim *et al.*, 2012a; Kuijper *et al.*, 2001) (see section 1.2.3). During the 1980's and 1990's, the use of cephalosporins, such as cefuroxime, ceftriaxone and cefotaxime, was identified as a risk factor for CDI acquisition (de Lalla *et al.*, 1989; Nelson *et al.*, 1994). Reducing the use of cephalosporins has been shown to reduce the incidence of CDI (Thomas & Riley, 2003). In addition, fluoroquinolones have been identified as a potential risk factor for developing CDI (Pépin *et al.*, 2005).

Other risk factors

Immunosuppression as the result of old age, autoimmune disease and immune deficiency is a risk factor for CDI due to the disruption of the enteric microbiota associated with these conditions (Kyne *et al.*, 2000, 2001, 2002). The pathophysiology of the intestine associated with Hirschsprung's Disease and inflammatory bowel disease are also risk factors for developing CDI (Rodemann *et al.*, 2007; Schechter *et al.*, 1999). Another contributing risk factor is mucosal damage of the intestine by chemotherapy, radiation and gastrointestinal surgery (Hautmann *et al.*, 2011; Husain *et al.*, 1998; Zerey *et al.*, 2007).

Some studies suggest that the use of proton pump inhibitors (PPI) that suppress gastric acidity can increase the risk of CDI (Aseeri *et al.*, 2008; Dial *et al.*, 2004). However, this is disputed with some other studies having described a lack of association between PPI and CDI (Freedberg *et al.*, 2013).

1.2.2 The role of *C. difficile* toxins in pathogenesis

Large clostridial toxins A and B

The diseases caused by *C. difficile* are largely mediated by toxin A (TcdA) and toxin B (TcdB) (Just *et al.*, 1995a, b). These toxins are glucosyltransferases that irreversibly inactivate host cell Rho GTPases using UDP-glucose as the donor (Jank & Aktories, 2008; Voth & Ballard, 2005). GTPases, such as Rho proteins are involved in the regulation of the cellular actin cytoskeleton and paracellular permeability at the tight junctions. Modification of these GTPases can result in cell apoptosis, degradation of the actin cytoskeleton or interruption in cell signalling of intoxicated cells. These modifications can induce inflammation and degrade the intestinal epithelial tissue, manifesting as diarrhoea and pseudomembranous colitis, the primary symptoms of CDI.

Binary toxin

An additional toxin, the binary toxin, contributes to the disease caused by *C. difficile* (Perelle *et al.*, 1997). Two components make up the toxin. CdtA is the enzymatic component, which is an actin-modifying ADP-ribosyltransferase. CdtB is a separate component that binds to the targeted host cell and transports CdtA into the cell. Binary toxins stimulate the excessive growth of microtubules resulting in enterocytes with protruding microtubule structures (Schwan *et al.*, 2009, 2014). These cell surface protrusions are postulated to contribute to the virulence of *C. difficile* by assisting the bacterium to adhere to the enterocytes and colonise

the intestine. While toxins A and B have been described as the main virulence factors of *C. difficile*, there have been reports of suspected CDI cases being caused by strains that only produced the binary toxin, and isogenic toxin A-B-Cdt+ mutants were able to cause disease in a hamster model (Eckert *et al.*, 2015; Kuehne *et al.*, 2014).

Toxin genetics and regulation

Pathogenic strains of *C. difficile* harbour a 19.6 kb pathogenicity locus (PaLoc) (Figure 1.1) composed of the *tcdA* and *tcdB* genes, which encode toxin A and toxin B, respectively, as well as *tcdR*, *tcdC* and *tcdE*, which are known as accessory genes (Hammond & Johnson, 1995; Hundsberger *et al.*, 1997).

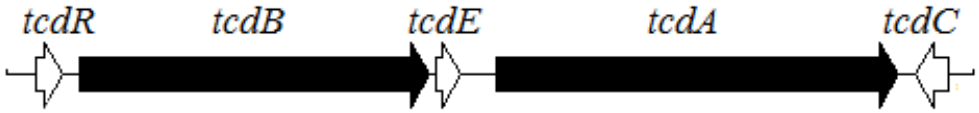


Figure 1.1 The arrangement of the genes in the *C. difficile* pathogenicity locus. The *tcdA* and *tcdB* genes are highlighted in black, while the accessory genes, *tcdD*, *tcdE* and *tcdC*, are highlighted in white. (Adapted from Hundsberger *et al.*, 1995).

The binary toxin components are also chromosomally encoded, but the *cdtA* and *cdtB* genes are not located in the PaLoc and are only found in some *C. difficile* strains (Metcalf & Weese, 2011; Perelle *et al.*, 1997; Popoff *et al.*, 1988). There are various deletions in the *tcdA* gene that can result in the non-production of toxin A. These strains only encode functional toxin B and are referred to as toxin A-negative, toxin B-positive (A-B+) strains, whereas strains that can encode both toxins are toxin A-positive, toxin B-positive (A+B+) strains (Kato *et al.*, 1998; Lyerly *et al.*, 1992). The most frequently isolated A-B+ strains have a deletion of

1.8 kb in the repeat regions of the *tcdA* gene, resulting in the non-production of toxin A (Alfa *et al.*, 2000; Kato *et al.*, 1999; Moncrief *et al.*, 2000). A few other types of A-B+ strains have been described including one with a large deletion of 5.9 kb from the 3' end of the *tcdA* gene extending to the 5' end of the *tcdC* gene (Rupnik *et al.*, 2003; Soehn *et al.*, 1998). In addition to the lack of toxin A, pathogenic A-B+ strains usually encode a variant toxin B that appears to be a hybrid between the TcdB toxin from the reference strain, *C. difficile* VPI 10463, and the TclS lethal toxin from *Clostridium sordellii* (Chaves-Olarte *et al.*, 1999; Lyerly *et al.*, 1992; Torres, 1991). While toxin B from the reference strain glucosylates Rho, Rac and Cdc42 small GTPases, the variant toxin B from A-B+ only targets Rac GTPases and shows a differential cytopathic effect (Huelsenbeck *et al.*, 2007). Nevertheless, A-B+ strains have been responsible for several outbreaks and are still capable of severe and lethal disease (Alfa *et al.*, 2000; Komatsu *et al.*, 2003). Finally, some strains of *C. difficile* do not harbour the PaLoc, and they are referred to as A-B- or non-toxigenic strains (Fluit *et al.*, 1991).

The PaLoc also includes three accessory genes: *tcdR*, *tcdE* and *tcdC*. The *tcdE* gene encodes a putative holin-like protein that is proposed to facilitate the release of *C. difficile* toxins; however, a study has shown that the inactivation of TcdE did not significantly affect toxin release from *C. difficile* (Govind & Dupuy, 2012; Olling *et al.*, 2012; Tan *et al.*, 2001). The *tcdR* gene, previously referred to as *tcdD*, encodes an alternative RNA polymerase sigma factor that functions as a positive regulator in the expression of toxins A and B (Mani & Dupuy, 2001). The *tcdC* gene is proposed to encode a negative modulator of toxin expression and certain deletions in the gene have been found in some *C. difficile* strains, which show increased and/or prolonged toxin production (Carter *et al.*, 2011; Curry *et al.*, 2007; Matamouros *et al.*, 2007; Warny *et al.*, 2005). The notion that TcdC is a negative regulator, however, lacks strong supportive evidence and remains a subject of debate, since recent studies have shown that TcdC does not significantly inhibit toxin expression (Bakker *et al.*,

2012; Cartman *et al.*, 2012). The roles of TcdE, TcdC and the mutations in *tcdC* require further investigation to determine their exact contribution, if any, to the pathogenesis of *C. difficile*. An additional mechanism of toxin regulation, independent of TcdC, has been proposed, which involves a quorum-signalling system mediated by thiolactone, which can be detected in stool specimens of CDI patients (Darkoh *et al.*, 2015).

1.3 Molecular typing of *C. difficile* strains

A variety of molecular typing methods have been developed to study the epidemiology of *C. difficile*. Initially, *C. difficile* strains were differentiated and identified based on phenotypic characteristics (Delmee *et al.*, 1985; Mulligan *et al.*, 1988). Phenotyping methods are, however, not reproducible and are of a low discriminatory power. They have, therefore, been replaced by a variety of genotypic typing methods.

1.3.1 Restriction-based typing methods

Pulsed-field gel electrophoresis (PFGE) and restriction endonuclease analysis (REA) involve the digestion of whole genomic DNA using *Sma*I and *Hind*III, respectively (Clabots *et al.*, 1993; Gal *et al.*, 2005). Using REA, the digested DNA fragments are separated by agarose gel or polyacrylamide gel electrophoresis. The PFGE technique involves DNA fragment separation using agarose gel electrophoresis with the electric field orientation repeatedly changing in two different directions and is able to resolve the sizes of the larger fragments. In both techniques, the banding pattern of the resulting DNA fragments determines the strain type.

1.3.2 PCR amplification-based typing methods

The amplified fragment length polymorphism (AFLP) method uses a combination of restriction enzyme digestion of DNA and amplification of sets of the resultant restriction fragments. Gel analysis of the amplified restriction fragments is then used to visualise the different *C. difficile* strain types (Klaassen *et al.*, 2002). The arbitrarily primed polymerase chain reaction (AP-PCR) method involves DNA amplification using a single short primer, which binds non-specifically to the genome sequence. Several DNA fragments of different lengths are amplified creating a banding pattern when separated by agarose gel electrophoresis (McMillin & Muldrow, 1992; Tang *et al.*, 1995).

PCR ribotyping differentiates *C. difficile* strains based on the amplification of the 16S-23S rRNA intergenic spacer regions, which vary in both number and length among strains, thereby, creating specific banding patterns when resolved using agarose gel or capillary electrophoresis (Bidet *et al.*, 1999; Stubbs *et al.*, 1999). Each unique banding pattern is assigned a ribotype number. Three sets of primers have been proposed for PCR ribotyping. The first set of primers is widely used and a large library of PCR ribotypes has been produced (Stubbs *et al.*, 1999). These primers have been revised and a second set of primers of greater specificity for *C. difficile* PCR ribotyping have been proposed (Bidet *et al.*, 1999). More recently, a third set of primers of increased specificity for *C. difficile* have been designed for direct PCR ribotyping from stool specimens (Janezic *et al.*, 2011). However, they are not as widely used as the first two sets of primers. With three sets of primers available, PCR ribotyping protocols are not standard worldwide. In addition, this method is of a low resolution, and requires data-portability and inter-laboratory comparability. Poor resolution can be improved by using the capillary gel-based PCR ribotyping method (Indra *et al.*, 2008). This method involves labelling the primers with fluorescent tags and analysing the PCR

fragments using capillary gel-based electrophoresis. Recently, there have been attempts to develop internationally standardised capillary gel-based protocols that should allow a more efficient exchange of data between laboratories (Fawley *et al.*, 2015).

Multilocus variable-number tandem-repeat analysis (MLVA) is a highly discriminatory genotyping method that can further subtype strains within a single PCR ribotype (van den Berg *et al.*, 2007; Manzoor *et al.*, 2011; Marsh *et al.*, 2006). MLVA involves the amplification of variable-number tandem-repeat loci in the genome using primers with fluorescent tags. The PCR products are analysed by multi-coloured fluorescence capillary electrophoresis. The data can be used to construct a minimum spanning tree, which depicts the genetic relatedness between isolates based on the summed tandem repeat differences.

1.3.3 Sequence-based methods

Multilocus sequence typing (MLST) is a preferred method for typing pathogens and has been applied to typing *C. difficile* (Griffiths *et al.*, 2010; Lemée *et al.*, 2004a). This method involves the PCR amplification of several reference genes followed by the nucleotide sequence analysis of these PCR fragments to differentiate strains. A unique combination of alleles is assigned its own sequence type number. The data produced by MLST can be applied to investigating the genetic relatedness between *C. difficile* isolates and the population structure. Online databases are available allowing laboratories to compare data and allowing global investigations to be conducted more easily (Griffiths *et al.*, 2010).

Toxinotyping distinguishes strain types based on polymorphisms in the toxin and regulatory genes of the PaLoc (Rupnik *et al.*, 1998, 2001). This technique involves the PCR amplification of six regions in the PaLoc, followed by an RFLP analysis of the amplicons. The sequencing of the variable region of the *slpA* gene, which encodes a surface-layer protein A, and the entire *tcdC* gene have also been used as genotyping methods for *C. difficile* (Curry

et al., 2007; Joost *et al.*, 2009; Kato *et al.*, 2010; Spigaglia & Mastrantonio, 2002). Sequence-based typing methods are reproducible and allow inter-laboratory data exchange.

The most recent method, whole genome sequencing (WGS) involves determining the nucleotide sequence of an organism's entire genome (Eyre *et al.*, 2013a, b). This technique can distinguish *C. difficile* isolates belonging to the same genotype based on single nucleotide polymorphisms (SNPs) in the non-repetitive core region of the genome. The data generated by WGS also provides information regarding gene content and genetic differences among *C. difficile* strains. WGS can be used to study the population structure of *C. difficile* and to investigate the transmission of CDI.

1.4 Epidemiology of various *C. difficile* strains and outbreaks

There are several *C. difficile* strains that are currently prevalent and responsible for hospital outbreaks. Outbreaks occurred in Canada and the USA from the year 2000 onward, in the United Kingdom in 2004, and other European countries thereafter, and were found to be caused by the spreading of PCR ribotype 027 (Kuijper *et al.*, 2006; Labbé *et al.*, 2008; McDonald *et al.*, 2005; Smith, 2005). Asian countries have also reported the isolation of ribotype 027 from CDI patients (Cheng *et al.*, 2009; Kim *et al.*, 2011). The ribotype 027 clade, also known as the NAP1 group (as identified by PFGE) and the BI group (as identified by REA) has been investigated extensively. Clinical isolates belonging to the ribotype 027 clade have been associated with high-level production of toxin A and toxin B over a prolonged period of time during infection contributing to severe disease. They have been associated with an 18 bp deletion and a single nucleotide deletion at position 117 in the *tcdC* gene, which introduces a frameshift mutation in the *tcdC* gene. This results in a truncated version of the TcdC protein being encoded (Warny *et al.*, 2005). The ribotype 027 clade has

also been associated with the production of the additional binary toxin, increased rate of sporulation and resistance to several antimicrobials, such as erythromycin and fluoroquinolone antimicrobials (Akerlund *et al.*, 2008; Warny *et al.*, 2005).

More recently, studies have reported the emergence of clinical isolates belonging to the PCR ribotype 017 clade in North America and Europe, and several outbreaks caused by this strain have been recorded (Dobreva *et al.*, 2013; Drudy *et al.*, 2007a; Pituch *et al.*, 2011; Tenover *et al.*, 2011). It is also prevalent in various Asian countries where the free use of antimicrobials without prescription may promote the spread of this strain (Hawkey *et al.*, 2013; Lee *et al.*, 2014). Ribotype 017 strains do not produce a functional toxin A but are nonetheless of clinical interest due to their ability to still cause severe cases of CDI (Drudy *et al.*, 2007a; Kim *et al.*, 2012a).

The emergence of the various *C. difficile* strains, some of which have caused severe disease and/or have reached epidemic proportions, has led to the need for rapid and reliable diagnostic tests for early detection and subsequent infection control, as unreliable tests can result in an under-representation of the number of CDI cases (Davies *et al.*, 2014).

1.5 CDI diagnosis

Several clinical and laboratory diagnostic methods have been developed for CDI, each based on different *C. difficile* targets. Detection methods include targeting the *C. difficile* toxins A and/or B genes or proteins, the *C. difficile* glutamate dehydrogenase, or by isolation of the organism itself. The success rate of these detection methods can vary due to the strain type or geographical location (Tenover *et al.*, 2010).

1.5.1 Enzyme-immunoassay based tests

Enzyme-immunoassay (EI-) based tests detect toxins A and B antigens in stool specimens. They have been the standard method for detection since the mid 1980's and are widely available (Lyerly *et al.*, 1983). However, analysis of the performances of various EI-based tests has shown that their diagnostic sensitivity is often poor (Eastwood *et al.*, 2009; Hernández-Rocha *et al.*, 2013; Swindells *et al.*, 2010). Despite the knowledge of their poor sensitivity, EI-based tests are rapid to perform and remain as the standard diagnostic method in many hospitals worldwide.

1.5.2 Cell culture neutralisation assay

The cell culture neutralisation assay (CCNA) detects the presence of toxin B directly in stool specimens. An observed cytopathic effect on the cells and neutralisation with a *C. difficile* toxin B antibody indicate a positive result. The CCNA has often been used as a reference method when evaluating the clinical performance of different diagnostic tests. However, certain studies have also reported on its poor diagnostic sensitivity (Barbut *et al.*, 2009; Hernández-Rocha *et al.*, 2013; Stamper *et al.*, 2009; Swindells *et al.*, 2010). In addition, this method is time consuming (24 – 48 hrs to perform) and requires tissue culture facilities, experience in tissue culture techniques and a reliable antitoxin for neutralisation, which are not available to many routine diagnostic laboratories.

1.5.3 Toxigenic culture

Toxigenic culture of *C. difficile* involves isolating the organism from stool specimens using selective media and then determining if the isolate is toxigenic. The culturing of *C. difficile* on selective media was described in the 1970's and employed an egg-yolk fructose base, with either cycloserine or both D-cycloserine and cefoxitin added as the selective agents (George

et al., 1979). Since then, variations of the medium include reducing the antimicrobial concentration, including taurocholate to enhance spore germination, and replacing the egg-yolk with blood (Levett, 1985; Wilson *et al.*, 1982). Heat- or alcohol-shock, as well as broth enrichment, prior to plating on agar have been shown to enhance the recovery of *C. difficile* isolates (Arroyo *et al.*, 2005; Marler *et al.*, 1992; Riley *et al.*, 1987).

Once the bacterium has been identified, the isolate's ability to produce biologically active toxins is determined. This involves growing the isolate in broth and using the CCNA or an enzyme-linked immunosorbent assay (ELISA) to test for active toxins in the culture supernatant. Alternatively, PCR screening for the toxin genes has been used to determine whether the isolates are potentially toxigenic (Lemée *et al.*, 2004b).

To combine the isolation and determination of toxin production in a single step, chromogenic media have been developed. One such medium contains cycloserine and cefoxitin as selective agents, as well as a chromogenic substrate (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside) (Darkoh *et al.*, 2011a). This substrate has similar stereochemical characteristics to UDP-glucose, which is naturally used by toxins A and B as a co-substrate to inactivate proteins. Hydrolysis of the substrate during the growth of toxin-producing strains produces blue colonies, while the colonies of non-toxigenic strains remain white.

1.5.4 Nucleic acid amplification tests

In recent years diagnostic tests have developed considerably and there has been a shift to using nucleic acid amplification tests (NAATs). These show a high level of diagnostic sensitivity when compared to the CCNA and toxigenic culture methods, but tend to be expensive and are not able to identify whether *C. difficile* strains are actively producing toxin during an infection (Swindells *et al.*, 2010). With the gradual replacement of EI-based tests

with NAATs in various laboratories, the reported detection of *C. difficile* has increased (Grein *et al.*, 2014; Longtin *et al.*, 2013). This is not necessarily a reflection of an increase in the incidence of *C. difficile*, but may be a representation of the formerly undetected cases of CDI.

1.5.5 Diagnostic test algorithms

While some of the abovementioned diagnostic methods are regarded as more reliable than the others, accurate diagnosis still remains a problem. There has been debate whether detection of the various species-specific genes using NAATs is an indication of a true disease state, since CDI is mediated by the encoded toxins and the presence of toxin genes alone does not necessarily imply sufficient levels of the organism to cause disease (Baker *et al.*, 2013). Several laboratories have adopted two-step or three-step algorithms for the accurate diagnosis of CDI (Gilligan, 2008; Goldenberg *et al.*, 2010; Novak-Weekley *et al.*, 2010; Sharp *et al.*, 2010). The first test in the algorithm is often the detection of *C. difficile* glutamate dehydrogenase (GDH) to confirm the presence of the organism in stool specimens. However, screening for GDH does not differentiate between non-toxigenic (A-B-) and toxigenic (A-B+ or A+B+) strains. Screening for GDH is, therefore, followed by an assay screening for the toxin genes and/or biologically active toxins. Combining diagnostic tests is more time consuming and labour intensive, but it produces more accurate diagnostic results and has the potential to reduce the overall number of tests required. Repeating a diagnostic test is discouraged in many countries, therefore, it is imperative that diagnostic methods are as accurate as possible.

1.6 Treatment of CDI

1.6.1 Antimicrobial treatment - metronidazole and vancomycin

Controlled use of broad-spectrum antimicrobials and the use of effective treatment methods are necessary to avoid and control outbreaks of CDI. Metronidazole and vancomycin have been, and still are, the most commonly administered antimicrobials for the treatment of CDI (Debast *et al.*, 2014). Metronidazole is used to clinically treat a wide range of anaerobic infections and is the preferred treatment option for patients with mild to moderate cases of CDI (Zar *et al.*, 2007). Metronidazole is a 5-nitroimidazole agent, which is activated within anaerobic bacterial cells during bacterial anaerobic metabolism to form a reactive nitrogen radical. The active metronidazole causes DNA strand breakage resulting in bacterial cell death (Dachs *et al.*, 1995; Edwards & Mathison, 1970). Vancomycin is produced by *Streptomyces orientalis* and it inhibits the biosynthesis of peptidoglycan (Hammes & Neuhaus, 1974). Vancomycin has been shown to be superior to metronidazole and more effective in severe cases of CDI (Zar *et al.*, 2007). However, it is significantly more expensive and its use has led to the emergence of vancomycin-resistant *Enterococcus* strains (Al-Nassir *et al.*, 2008a; Nerandzic *et al.*, 2012). It is, therefore, reserved for treatment failure or severe cases (Baines *et al.*, 2009; Al-Nassir *et al.*, 2008b).

1.6.2 Other antimicrobials

Fidaxomicin is a recently approved antimicrobial for the treatment of CDI. Fidaxomicin, also known as OPT-80, belongs to a new class of macrocyclic antimicrobials that is secreted by *Dactylosporangium aurantiacum* (Theriault *et al.*, 1987). It is a narrow-spectrum antimicrobial, which displays activity against *C. difficile*, by inhibiting RNA polymerase during transcription, and causes minimal disruption of the enteric microbiota (Artsimovitch *et al.*, 2012; Finegold *et al.*, 2004; Tannock *et al.*, 2010). Compared to vancomycin,

fidaxomicin has been found to be as effective in the treatment of CDI and superior in reducing the recurrence of CDI (Crook *et al.*, 2012; Louie *et al.*, 2011). In a human gut model, fidaxomicin was able to reduce the total viable count of *C. difficile* within a shorter period of time than vancomycin, while metronidazole failed to do so (Chilton *et al.*, 2014). In addition, fidaxomicin was able to reduce *C. difficile* spores in the gut model and the antimicrobial was able to persist over a longer period of time than both vancomycin and metronidazole. However, the high cost of fidaxomicin prevents it from being used widely.

Tigecycline is a broad-spectrum tetracycline derivative that has shown activity against *C. difficile in vitro* and has been successfully used to treat patients with severe cases of CDI (Britt *et al.*, 2014; Herpers *et al.*, 2009). Rifaximin, belonging to the rifamycin class of antimicrobials, has also been shown as a possible treatment option for severe cases of CDI (Kokkotou *et al.*, 2008; Mattila *et al.*, 2013). While these antimicrobials may be effective treatment options, there are insufficient clinical data at present to support their routine use.

1.6.3 Antimicrobial resistance

Reported reduced susceptibility or resistance of *C. difficile* to antimicrobials in association with CDI has raised interest in investigating the mechanisms responsible for these observations. Reduced susceptibility and resistance to metronidazole have been reported in a few *C. difficile* strains (Baines *et al.*, 2008; Brazier *et al.*, 2001; Peláez *et al.*, 2008). Metronidazole concentrations that can be achieved in the intestine are as low as approximately 9.5 μg/g faeces, which, may not be able to inhibit strains with reduced susceptibility and could lead to treatment failure (Bolton & Culshaw, 1986). This has raised concern regarding the effective treatment of CDI and warrants the need to monitor antimicrobial susceptibility *in vitro*. The exact mechanism for metronidazole resistance in *C. difficile* is poorly understood. In *Bacteroides fragilis*, an anaerobic bacterium that causes

gastrointestinal infections, various nitroimidazole genes (*nimA-J*) encode nitroreductases, which are thought to inactivate metronidazole (Gal & Brazier, 2004; Haggoud *et al.*, 1994; Husain *et al.*, 2013). PCR screening has shown that *B. fragilis nim* homologues are present in other species including one strain of *Clostridium bifermentans* (Lubbe *et al.*, 1999). While *nim* homologues have not yet been detected in *C. difficile*, a recent proteomic study reported the increased expression of several proteins, including putative 5-nitroimidazole reductases in a stable metronidazole resistant *C. difficile* strain even in the absence of metronidazole (Chong *et al.*, 2014).

There are very few reports of reduced susceptibility or resistance to vancomycin in *C. difficile* (Chia *et al.*, 2013). This may be explained by the high vancomycin concentrations (1000-5000 μg/ml) that can be maintained in the intestine (Edlund *et al.*, 1997). While *in vitro* induction methods have allowed the identification of several mutations that lead to a reduced susceptibility to vancomycin in *C. difficile*, the clinical significance of these mutations is unclear (Leeds *et al.*, 2014). Recurrence of CDI after the use of metronidazole or vancomycin has been reported and has prompted the development of alternative therapeutic methods (Pépin *et al.*, 2007).

Clindamycin and erythromycin are macrolide–lincosamide–streptogramin B (MLS_B) agents, which inhibit protein synthesis (Tenson *et al.*, 2003). The most widely described resistance mechanism is the dimethylation of the 23S rRNA gene by a 23S rRNA methylase encoded by the erythromycin ribosomal methylase (*ermB*) gene. High-level resistance to these antimicrobials in *C. difficile* is associated with the presence of the *ermB* gene, which is usually located on a mobilisable genetic element such as the transposon Tn*5398* (Johnson *et al.*, 1999).

Moxifloxacin has been shown to promote bacterial growth of, and toxin production by, fluoroquinolone-resistant epidemic *C. difficile* strains (Adams *et al.*, 2007). This class of antimicrobials is selective for highly resistant *C. difficile* strains. Ciprofloxacin and moxifloxacin are second and third generation fluoroquinolones, respectively, which interact with the DNA gyrase subunits A and B that are involved in DNA replication (Hooper, 1999). The interaction results in DNA breakage. *C. difficile* fluoroquinolone resistance has been associated with *gyrA* and *gyrB* mutations, which result in amino acid substitutions in the encoded proteins (Dridi *et al.*, 2002).

Tetracycline is another broad-spectrum antimicrobial that inhibits protein synthesis (Chopra & Roberts, 2001). Resistance to tetracycline is mediated by the *tetM* gene, which is usually located on Tn*916*-like transposons such as Tn*5397* (Mullany *et al.*, 1990). Tetracycline resistance is commonly associated with erythromycin resistance, and certain studies have observed a physical link between the *ermB* and *tetM* genes (Spigaglia *et al.*, 2007). Having these genetic resistance determinants located on mobilisable genetic elements allows intra- and interspecies exchange, aiding in the spread of antimicrobial resistance (Mullany *et al.*, 1990; Wasels *et al.*, 2014).

Alternative antimicrobial resistance mechanisms in bacteria involve efflux systems, some of which are specific to MLS_B and fluoroquinolone antimicrobials (Ambrose *et al.*, 2005; Martinez-Garriga *et al.*, 2007). Bacterial genes encode efflux proteins that pump the antimicrobial out of the cell keeping intracellular concentrations below lethal levels (Lin *et al.*, 2015; McMurry *et al.*, 1980; Saiful *et al.*, 2008). However, very few studies have investigated these systems in *C. difficile*, and so their contribution to antimicrobial resistance in CDI is not known (Dridi *et al.*, 2004; Lebel *et al.*, 2004).

1.6.4 Alternative treatment methods

Problems related to antimicrobial resistance and the recurrence of CDI after antimicrobial treatment have led to the development of alternative treatment methods. Faecal transplantation, which involves the transfer of stool from a healthy individual into the colon of an infected individual, has been shown to be an effective treatment option for patients with recurrent CDI (Kelly *et al.*, 2012; van Nood *et al.*, 2013). If successful, this treatment option restores a healthy balance to the enteric microbiota, thus supressing the prevalence of *C. difficile*. Alternatively, the colon of a patient with recurrent CDI can be infused with a combination of pure cultured bacterial species that make up the normal and protective microbiota of a healthy individual (Tvede & Rask-Madsen, 1989). Other non-antimicrobial treatment options have been suggested for the treatment of CDI including the use of probiotics to provide a protective barrier of low-virulence microorganisms against *C. difficile*, the use of toxin-binding polymers, or the use of immunotherapy whereby neutralising antibodies target *C. difficile* toxins (van Dissel *et al.*, 2005; Lawrence *et al.*, 2005; Mogg *et al.*, 1982). These treatment options require further research to validate their efficacy and safety.

1.7 Sporulation

C. difficile forms endospores, which survive and may remain dormant in the host intestine and are subsequently shed by CDI patients. *C. difficile* spores are resistant to many environmental and antimicrobial factors (Dawson *et al.*, 2011; Fawley *et al.*, 2007; Goldenberg *et al.*, 2012). This enables them to persist in hospital environments over a long period of time creating a reservoir for transmission (Sjöberg *et al.*, 2014). Transmission in hospital environments can also be aided by the contamination of healthcare workers, and this

has increased the importance of washing hands and disinfecting hospital surfaces regularly (Bobulsky *et al.*, 2008; Guerrero *et al.*, 2012; Landelle *et al.*, 2014). However, disinfectants have also been reported to increase sporulation and antimicrobial treatment can reportedly induce super-shedding of spores by CDI patients (Lawley *et al.*, 2009; Wilcox & Fawley, 2000).

The rate of sporulation can vary between *C. difficile* strains. Published studies have reported that isolates of epidemic clades, such as ribotypes 001 and 027, have an increased sporulation frequency *in vitro*, which may enable them to persist and spread more easily (Akerlund *et al.*, 2008; Merrigan *et al.*, 2010; Vohra & Poxton, 2011; Wilcox & Fawley, 2000). These studies, however, analysed only a few isolates and, therefore, do not provide a true representation of sporulation characteristics in the various strains. More recent studies, using a larger sample size, have reported that the sporulation frequency of ribotype 027 strains was not higher than non-027 strains. In addition, there was variation in sporulation among isolates belonging to ribotype 027, suggesting that the frequency was not associated with the strain type (Burns *et al.*, 2010, 2011).

1.8 Colonisation of the host intestine

In order to cause disease, *C. difficile* must first colonise the host intestine. Colonisation is initiated by the germination of ingested spores. This is followed by the proliferation of vegetative cells, which then adhere to colonic mucus, and likely, to the host enteric cells and colonise the intestine. Very little is known about the colonisation mechanism of *C. difficile*. However, some strains may bind more robustly to human intestinal epithelial cells than other strains, providing an advantage during colonisation (Merrigan *et al.*, 2013). Several other

factors have also been proposed to play a role in adherence and colonisation and it is likely that the process is a multi-factorial one.

Certain cell surface proteins of *C. difficile* have been identified and may facilitate adherence to other bacterial cells and/or to the host. The *C. difficile* flagellar proteins, FliC and FliD, have been proposed to play a role in the adherence to mucus, which is the first barrier encountered before reaching the intestinal tissue (Tasteyre *et al.*, 2001). Recently, *C. difficile* R20291 *fliC* and *fliD* mutant strains have been shown to display decreased adherence to Caco-2 cells when compared to the wild type strain (Baban *et al.*, 2013). However, similar *C. difficile* 630 mutant strains were able to adhere better to Caco-2 cells than the wild type strain, suggesting that the role of flagella in adherence may vary across strains (Dingle *et al.*, 2011b). Other flagellar modifications may also play a role in adherence and cell aggregation (Faulds-Pain *et al.*, 2014).

Adhesins are an attractive target for vaccine development and, therefore, the characterisation of the various adhesins and their roles in bacterial attachment would be of value. A fibronectin-binding protein, Fbp68, and a heat shock protein, GroEL, of *C. difficile* reportedly play a role in adherence (Hennequin *et al.*, 2003). The *C. difficile* lipoprotein CD0873 is a surface protein shown to adhere to Caco-2 cells, while a non-CD0873-expressing mutant was unable to do so (Kovacs-Simon *et al.*, 2014). Another factor suggested to contribute to adherence and aggregation is cyclic diguanylate, through regulation of the expression of type IV pili (Bordeleau *et al.*, 2015; Purcell *et al.*, 2012).

A large family of cell wall proteins (CWPs) of *C. difficile* have been identified and may play a role in adherence. One major CWP is surface layer protein SlpA, which is encoded by the *slpA* gene and post-translationally processed to form the high- and low-molecular weight subunits. These assemble together on the surface of the cell to form a paracrystalline lattice

(Takeoka *et al.*, 1991). Purified SlpA has been shown to adhere to Caco-2 cells, and this binding was inhibited by anti-SlpA antibodies (Merrigan *et al.*, 2013). Other CWPs include Cwp66 and Cwp84. The Cwp66 is an adhesin that has been shown to facilitate bacterial adherence to Vero cells as the addition of two antibodies raised against the protein reduced adherence (Waligora *et al.*, 2001). The Cwp84 is a protease that processes immature SLPs into the high molecular weight and low molecular weight SLPs and may play a role in modulating biofilm formation (Kirby *et al.*, 2009; Pantaléon *et al.*, 2015). Cwp84 has also been shown to cleave proteins of the host-cell extracellular matrix thereby reducing the integrity of the host tissue (Janoir *et al.*, 2007).

CwpV is a recently identified adhesin of *C. difficile* (Emerson *et al.*, 2009; Reynolds *et al.*, 2011). The protein consists of an N-terminal domain, which is conserved between different strains and is proposed to anchor the protein to the cell wall. The C-terminal domain facilitates adherence between *C. difficile* cells, thereby promoting aggregation, which may contribute towards biofilm formation. The C-terminal domain consists of repeated amino acids, with the number of repeats varying between strains, and inducing different levels of auto-aggregation of *C. difficile* (Reynolds *et al.*, 2011). *C. difficile* has been shown to aggregate to form biofilms, which aids in survival and colonisation (Dapa *et al.*, 2013; Dawson *et al.*, 2012). However, CwpV has not yet been linked specifically to biofilm formation.

CwpV is encoded by the *cwpV* gene, and its expression is phase-variable. Previously it has been shown that only 10% of the cells within a population express CwpV *in vitro* (Emerson *et al.*, 2009; Reynolds *et al.*, 2011). Upstream of the start codon of *cwpV* is a 195 bp nucleotide sequence flanked by inverted repeats (Figure 1.2). DNA inversion of this region is

mediated by a recombinase, probably RecV, and allows expression of *cwpV* in one orientation (ON), while in the other orientation the gene is not transcribed (OFF).

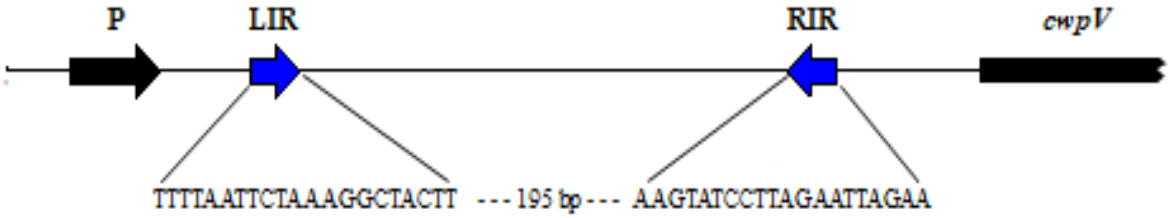


Figure 1.2 The inversion region upstream of the *cwpV* gene. A pair of 21 bp inverted repeats (IRs) separated by 195 bp and located between the promotor (P) and the *cwpV* gene. (Adapted from Emerson *et al.*, 2009)

A study investigating CwpV expression in various *C. difficile* strains identified a strain that did not express the protein (Emerson *et al.*, 2009). While this strain harboured the *cwpV* gene, PCR analysis targeting the inversion region revealed that only the OFF orientation was present, whereas, both the ON and OFF orientations were detected in *C. difficile* strains that expressed CwpV. Interestingly, the non-expressing strain had a two-nucleotide deletion in one of the inverted repeat sequences, which could potentially prevent processing by the recombinase responsible for the inversion, thus 'locking' the region in the OFF orientation.

The function of CwpV in auto-aggregation and colonisation still has to be verified. A recent study has shown that this protein, specifically the C-terminal domain, has anti-phage activity (Sekulovic *et al.*, 2015). In addition, cells with the ON orientation were expressing *cwpV* and, consequently, resistant to phage infection.

1.9 Project aim

Extensive research has been conducted in Canada, USA, various European countries, Australia and various Asian countries to gain insight into the pathogenicity of *C. difficile* and establish the regional epidemiology of CDI with a view to containing the severity and extent of the disease. Research has shown that strain prevalence differs geographically and over time. Therefore, it is necessary for each country to conduct local surveillance to order to understand the pathogenesis and epidemiology of *C. difficile* strains in their respective settings.

Very few studies regarding *C. difficile* in South Africa have been published. One study was conducted in the Vhembe district, in the Limpopo Province (Samie *et al.*, 2008), where the prevalence of toxigenic *C. difficile* strains in stool specimens provided by hospitalised patients and primary school children was estimated by PCR screening for the *tpi*, *tcdA*, *tcdB*, *tcdC* and binary toxin genes. Toxigenic *C. difficile* was detected in approximately 11% of diarrhoea specimens. Another study monitored *C. difficile* among diarrhoea patients attending the Steve Biko Academic Hospital in Pretoria, in the Gauteng Province, using an EI-based assay targeting toxin A only in stool specimens (Lekalakala *et al.*, 2010). However, the overall incidence of toxigenic *C. difficile* was not reported and the assay would not have detected pathogenic strains that did not produce toxin A. Another study, also using an EI-based assay targeting toxin A only, conducted at Groote Schuur Hospital in Cape Town, in the Western Province, reported a 9.2% incidence of CDI among symptomatic patients (Rajabally *et al.*, 2013). In addition, this study identified two CDI cases possibly caused by ribotype 027 isolates, based on the PCR detection of the 18 bp deleted version of the *tcdC* gene in the patient specimen. However, this 18 bp deletion is also present in non-027 strains

and is, therefore, not a valid marker for the identification of an isolate belonging to the ribotype 027 clade (Curry *et al.*, 2007).

With the spread and outbreaks of CDI worldwide and the increase in prevalence of antimicrobial resistance, it is essential to begin the monitoring and characterisation of South African *C. difficile* strains in order to administer active therapy and implement effective infection control protocols. Prior to the initiation of this project, Groote Schuur Hospital made use of the EI-based ImmunoCard Toxins A & B (Meridian Bioscience, Inc) diagnostic test. However, its clinical performance within the local setting had not been determined. Therefore, the aims of this project were to evaluate current local methods of diagnosing CDI and to conduct a molecular characterisation of *C. difficile* strains isolated from symptomatic patients at Groote Schuur Hospital. The study planned to isolate *C. difficile* from symptomatic patients and to type these bacteria by PCR ribotyping, sequencing of the *tcdC* gene and MLVA. The susceptibility of the *C. difficile* isolates to various antimicrobials, both those used in treatment and those known to be risk factors for the development of CDI, would be determined, as well as the underlying genetic resistance mechanisms. The abilities of the isolates to produce biologically active toxins and to sporulate would also be investigated. Finally, auto-aggregation, which may be important during biofilm formation and host colonisation, would be investigated in all the isolates.

This thesis, therefore, reports the results of a pilot study carried out to investigate *C. difficile* strains responsible for clinical infections at a major hospital in South Africa. The characterisation and analysis of pathogenic strains infecting patients attending Groote Schuur Hospital will provide a better understanding of the bacterium and how it causes disease. Information collected during this project can be further used to help guide improvements in

the prevention and treatment of CDI at Groote Schuur Hospital and other medical centres in South Africa.

CHAPTER TWO

THE VALIDITY OF CLINICAL DIAGNOSIS METHODS AS A SCREEN FOR *CLOSTRIDIUM DIFFICILE* IN STOOL SPECIMENS

Contents

2.0 Abstract

Clostridium difficile is a toxin-producing, anaerobic pathogen of increasing clinical interest worldwide. It is responsible for life-threatening gastrointestinal diseases, known as *C. difficile* infection (CDI), and is the leading cause of nosocomial, antimicrobial-associated diarrhoea. The prevalence of CDI in South Africa is not known at present. In this study, stool specimens were collected from 162 symptomatic patients at Groote Schuur Hospital. *C. difficile* was isolated from 34/162 specimens (20% prevalence) by toxigenic culture using selective media. A multiplex PCR identified the *C. difficile* species-specific *tpi* gene fragment and three toxigenic-types: A+B+ (47% of strains), A-B+ (47%) and A-B- (6%). The binary toxin genes were not present in any of the clinical isolates. Rapid and reliable diagnostic methods to identify CDI are necessary to help improve infection control. In this study, four clinical diagnostic tests were analysed and compared to the reference method of toxigenic culture described above. The enzyme immunoassay-based tests, Meridian ImmunoCard and bioMérieux MiniVidas, had low diagnostic sensitivities of 37.5% and 50%, respectively. The nucleic acid amplification tests, Hain Lifescience CDiff and Cepheid GeneXpert, had sensitivities of 87.5% and 90.6%, respectively. The GeneXpert performed the best and is recommended for the future clinical diagnosis of CDI at Groote Schuur Hospital.

2.1 Introduction

Clostridium difficile infection (CDI) is a significant problem affecting individuals within healthcare facilities, where it is responsible for approximately 15 to 25% of antimicrobial-associated diarrhoea cases (Bartlett & Gerding, 2008; Bauer *et al.*, 2011). Nosocomial outbreaks have been reported and studied in the last decade, especially in Canada, the USA and Europe (Labbé *et al.*, 2008; Warny *et al.*, 2005). In recent years, published studies have also begun to emerge from Asia, Australia and Latin America (Cheng *et al.*, 2009; King *et al.*, 2015; Quesada-Gómez *et al.*, 2010; Riley *et al.*, 2009). However, the occurrence and epidemiology of *C. difficile* in South Africa has yet to be determined.

Rapid and reliable detection methods are important for early detection and implementation of therapy and infection control. As discussed in Chapter 1, several options are available for the diagnosis of CDI. However, there have to date been no studies evaluating the diagnostic performance of any of the available tests in the local setting. The data obtained from such an evaluation would help identify the most appropriate diagnostic approach for local laboratories.

Enzyme-immunoassay (EI)-based tests are rapid and easy to use, however, they have been shown to have a poor diagnostic sensitivity (Eastwood *et al.*, 2009; Hernández-Rocha *et al.*, 2013; Swindells *et al.*, 2010). Two examples of EI-based tests that are in widespread use are the Meridian ImmunoCard Toxins A & B assay (Meridian Bioscience, Inc. Cincinnati, OH) and the bioMérieux MiniVidas assay (BioMerieux, Marcy l'Etoile, France), both of which use antitoxin antibodies. The ImmunoCard involves a chromogenic substrate which produces a blue product upon hydrolysis, which is analysed visually. The MiniVidas is an automated enzyme-linked fluorescent immunoassay assessed using the MiniVidas platform.

Several newer nucleic acid amplification tests (NAATs) have also been developed. They screen for the presence of *C. difficile* specific genes, and have been shown to have improved diagnostic sensitivity over EI-based tests (Eastwood *et al.*, 2009; Hernández-Rocha *et al.*, 2013; Swindells *et al.*, 2010). The Cepheid Xpert *C. difficile* assay (Cepheid, Sunnyvale, CA, United States) is a fully automated procedure performed in a GeneXpert instrument. The system involves DNA extraction by sonication, real-time PCR and detection of the toxin B gene (*tcdB*), the binary toxin *cdtB* gene and a version of the *tcdC* gene with a single nucleotide deletion at position 117. The positive detection of all these targets presumptively identifies the presence of *C. difficile* ribotype 027 in the specimen from the patient. An alternative NAAT is the Hain GenoType CDiff assay (Hain Lifescience SA (Pty) Ltd) which targets the *tpi*, *tcdA*, *tcdB*, *cdtA*, *cdtB*, *tcdC* and *gyrA* genes as well as a further *C. difficile*-specific target not disclosed by the manufacturer. DNA is extracted either manually or using an automated instrument, followed by conventional multiplex PCR using a primer mix supplied by the manufacturer. Detection of the various targets is performed by reverse hybridisation of the PCR products to a strip containing membrane-bound probes. This test can differentiate between non-pathogenic *C. difficile* isolates, various toxigenic strains and isolates belonging to the ribotype 027 clade.

A suitable reference method is important for the comparison of different diagnostic methods. Cell culture neutralisation assays (CCNA) have been used in the past for this purpose (Hernández-Rocha *et al.*, 2013; Swindells *et al.*, 2010). However they are difficult to perform and may lack sensitivity (Barbut *et al.*, 2009; Stamper *et al.*, 2009). An alternative reference method is toxigenic culture, which has the additional advantage of allowing for further characterisation and molecular tracking of isolates and is useful in epidemiological analyses. A variety of selective media for *C. difficile* are commercially available. One popular medium is the chromogenic medium, ChromID, which is manufactured by bioMérieux and yields

grey/black toxigenic *C. difficile* colonies. This medium has been found to be equal, and even superior, to other media in the isolation of spores and vegetative cells, respectively (Boseiwaqa *et al.*, 2013; Perry *et al.*, 2010).

Confirmation of potential *C. difficile* isolates from various selective media can be carried out by amplification of a 230 bp fragment of the *tpi* gene, which was found to be more discriminatory than using 16S rRNA gene amplification to differentiate *C. difficile* from other *Clostridium* species (Dhalluin *et al.*, 2003). To simultaneously confirm the identity of a *C. difficile* isolate and determine its toxigenic type, a multiplex PCR targeting the *C. difficile* species-specific fragment of the *tpi* gene, and internal fragments of the toxin A and B genes, has also been designed (Lemée *et al.*, 2004b). The primers designed to target the *tcdA* gene span the region commonly deleted in toxigenic A-B+ strains resulting in fragments of 110 bp and 369 bp for toxigenic A-B+ and A+B+ strains, respectively (Lemée *et al.*, 2004b).

Prior to the initiation of this project, the Groote Schuur Hospital (a large clinical tertiary institution in Cape Town, South Africa) made use of the EI-based ImmunoCard Toxins A & B (Meridian Bioscience, Inc). In a previous study, an alternative EI-based assay targeting toxin A only, reported a 9.2% incidence of *C. difficile* among symptomatic patients attending Groote Schuur Hospital (Rajabally *et al.*, 2013). The aim of the work reported in this chapter was to investigate the prevalence of toxigenic *C. difficile* in symptomatic patients attending Groote Schuur Hospital using selective culture methods and gene-specific PCR, and to evaluate the performance of two EI-based assays as well as two nucleic acid amplification tests (NAATs) in the clinical diagnostic setting.

2.2 Materials and Methods

2.2.1 Bacterial strains

A non-toxigenic *C. difficile* strain (ATCC 700057) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). *C. difficile* strain 709 is a toxigenic A+B+CDT+ strain, kindly provided by Professor Andrej Weintraub, originally obtained from a patient attending the Karolinska University Hospital, Stockholm, Sweden.

2.2.2 Clinical specimens

Stool specimens were collected from 162 symptomatic in- and out-patients attending Groote Schuur Hospital between March 2012 and March 2013. Symptoms were defined as diarrhoea, with two or more runny stools per day for at least two days (Barbut *et al.*, 2007).

2.2.3 Media and growth conditions

Three types of selective media were used in this project (Table 2.1). The ChromID C. difficile medium (CHR) was purchased as pre-poured plates from the manufacturer (bioMérieux). The Oxoid (OXO) medium was prepared as needed using the Clostridium difficile agar base manufactured by Oxoid. The Darkoh *et al.* (2011a) chromogenic selective medium (DCS) was also prepared as needed using published basic components (Darkoh *et al.*, 2011a). Both the OXO and DCS media were supplemented with defibrinated horse blood and the selective supplements D-cycloserine (250 µg/ml final concentration) and cefoxitin (8 µg/ml final concentration; Oxoid).

Table 2.1 Selective media

Medium	Selection method	Positive identity	Reference/Source
CHR	Selective agents are not disclosed by the manufacturer	Black colonies*	bioMérieux
DCS	Selection by D-cycloserine (250 µg/ml) and cefoxitin (8 µg/ml), and differentiation between toxin-positive and toxin-negative strains by chromogenic substrate (X-gal)	Blue colonies	Darkoh *et al.*, 2011
OXO	Selection by D-cycloserine (250 µg/ml) and cefoxitin (8 µg/ml)	Colourless colonies	Oxoid

* Some strains of *C. difficile* did not yield black colonies on the CHR medium (Boseiwaqa *et al.*, 2013; Perry *et al.*, 2010).

Stool specimens from symptomatic patients were streaked directly onto each of the selective media and the agar plates were incubated at 37ºC for 48 hours in an anaerobic chamber (Model 1024, Forma Scientific Inc., Marietta, Ohio) with atmospheric conditions of N_2, CO_2 and H_2 (85:10:5 by volume). Non-toxigenic *C. difficile* ATCC 700057 was used for media quality control every time a new batch of media was used. The bioMérieux medium was incubated for 48 hours instead of the manufacturer's recommendation of 24 hours to maximise the isolation of *C. difficile* (Eckert *et al.*, 2013). *C. difficile* was isolated based on colony morphology and/or colour change and sub-cultured on pre-reduced Brain Heart Infusion (BHI) (Difco) media supplemented with cysteine (0.5 mg/ml final concentration) (Oxoid).

2.2.4 *C. difficile* identification and toxigenic typing by PCR analysis

DNA template was prepared by suspending single colonies picked from BHI agar in 300 µl suspension buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing proteinase K (1% v/v). The suspended cells were incubated for 15 min at 37ºC, followed by 30 min at

80ºC. Cellular debris was removed by centrifugation (6000 x g for 2 min) and 4 µl supernatant was used as template in the various PCR experiments. Genomic DNA extracted from *C. difficile* strain 709 was used as a positive control in all reactions.

All isolates were screened for the *C. difficile* species-specific *tpi* fragment, the toxin A (*tcdA*) (full-length or truncated) and toxin B (*tcdB*) genes in a single multiplex PCR (Lemee *et al.*, 2004). In a separate multiplex PCR, isolates were screened for the binary toxin genes (*cdtA* and *cdtB*) (Stubbs *et al.*, 2000), using primers listed in Table 2.2.

Table 2.2 Primers used for *C. difficile* identification and toxigenic typing

Target gene	**Primer sequence (5' → 3')**	**Size* (bp)**	**Reference**
tpi	F: AAAGAAGCTACTAAGGGTACAAA R: CATAATATTGGGTCTATTCCTAC	230	Lemee *et al.*, 2004
tcdA	F: AGATTCCTATATTTACATGACAATAT R: GTATCAGGCATAAAGTAATATACTTT	369 or 110	Lemee *et al.*, 2004
tcdB	F: GGAAAAGAGAATGGTTTTATTAA R: ATCTTTAGTTATAACTTTGACATCTTT	160	Lemee *et al.*, 2004
cdtA	F: TGAACCTGGAAAAGGTGATG R: AGGATTATTTACTGGACCATTTG	375	Stubbs *et al.*, 2000
cdtB	F: CTTAATGCAAGTAAATACTGAG R: AACGGATCTCTTGCTTCAGTC	510	Stubbs *et al.*, 2000

* The 110 bp fragment refers to a truncated *tcdA* gene fragment.

All PCRs were performed in a 50 µl reaction volume containing Kapa Taq ReadyMix (Kapa Biosystems), 1 µM of each tcd primer and 0.5 µM of each tpi primer. Alternatively, 0.5 µM of each cdt primer was used in a separate reaction. All reactions in this study were performed on the GeneAmp PCR System 9700 (Applied Biosystems) unless stated otherwise. The reactions were subjected to thermal cycling conditions previously described (Lemee *et al.*, 2004b; Stubbs *et al.*, 2000) PCR products were resolved by electrophoresis on a 2% agarose

gel stained with ethidium bromide, and analysed with a ChemiDoc XRS+ with Image Lab Software (Bio-Rad).

2.2.5 Clinical diagnostic assays

Four clinical diagnostic tests were used to determine the presence of *C. difficile* in stool specimens (Table 2.3). The ImmunoCard, MiniVidas and GeneXpert were conducted in parallel to culture on selective media in the Microbiology Laboratory of the National Health and Laboratory Services (NHLS) at Groote Schuur Hospital. The GenoType CDiff assay (Hain Lifescience) was conducted by Dr Brian Kullin at the Department of Molecular and Cell Biology at the University of Cape Town. Each test was performed according to the manufacturers' instructions.

Table 2.3 Diagnostic tests

Diagnostic test	**Target**	**Comment**
ImmunoCard Toxins A & B (Meridian Bioscience, Inc)	*C. difficile* toxins A and B	
MiniVidas *C. difficile* Toxin A & B (bioMérieux)	*C. difficile* toxins A and B	
GeneXpert *C. difficile* (Cepheid)	The *tcdB* and *cdtA* genes, and the Δ117 variant of the *tcdC* gene	A positive result for all the targets is a presumptive indication of an isolate belonging to the ribotype 027 clade
GenoType CDiff (Hain Lifescience)	The *tpi*, *tcdA*, *tcdB*, *cdtA*, *cdtB*, *tcdC* and *gyrA* genes as well as a further *C. difficile*-specific target not disclosed by the manufacturer	Differentiates between non-pathogenic, virulent and ribotype 027 isolates

Sensitivity, specificity and predictive values for each of the tests were calculated relative to the positive identification of *C. difficile* by culture on selective media followed by the *tpi*, *tcdA* and *tcdB* multiplex PCR (Table 2.4) (Lalkhen & McCluskey, 2008).

Table 2.4 Formulae used to calculate Sensitivity, Specificity, Positive and Negative Predictive Values of the clinical diagnostic assays (Lalkhen & McCluskey, 2008)

Characteristic	Formulae
Sensitivity	$\frac{True\ positives}{True\ positives + False\ negatives}$
Specificity	$\frac{True\ negatives}{True\ negatives + False\ positives}$
Positive predictive value	$\frac{True\ positives}{True\ positives + False\ positive}$
Negative predictive value	$\frac{True\ negatives}{True\ negatives + False\ negatives}$

2.3 Results and Discussion

2.3.1 Prevalence of *C. difficile* in stool specimens at Groote Schuur Hospital as determined by bacterial culture

The prevalence of *C. difficile* in patients attending Groote Schuur Hospital was investigated. A total of 164 stool specimens from 162 symptomatic patients (two patients provided two specimens each) were tested using a combination of three selective media. The bacterium was isolated and positively identified from 34 stool specimens (listed in Table 2.5), of which 32 were positive for at least one of the toxin genes, representing 20% prevalence of toxigenic

C. difficile among the 162 patients. This is approximately double the prevalence observed in the previous study amongst diarrhoea patients at the same site between 2010 and 2011, which determined a 9.2% incidence of toxigenic *C. difficile* (Rajabally *et al.*, 2013). However, the previous study used an EI-based assay targeting toxin A only and, therefore, would not have detected A-B+ strains. There have only been two other published *C. difficile* detection studies performed in South Africa and these were both conducted using different study designs and in geographically distinct populations making inter-study comparisons difficult. A review regarding the detection of *C. difficile* using an EI-based assay targeting toxin A only in stool specimens from diarrhoea patients attending Steve Biko Academic Hospital in Pretoria, in the Gauteng Province, reported that 46 of 266 (17.2%) stool specimens tested positive during the study year (Lekalakala *et al.*, 2010). However, the 46 positive specimens were obtained as repeat specimens from only 26 patients and it is, therefore, not possible to calculate the overall prevalence of *C. difficile* for their study. The only published PCR-based study in South Africa to date was conducted in the Vhembe district, in the Limpopo Province, and reported a prevalence of 11.4% for toxigenic *C. difficile* in stool specimens from individuals with diarrhoea, as detected by direct PCR screening for the *tpi*, toxins A and B, and binary toxin genes (Samie *et al.*, 2008). The study population included hospitalised patients as well as patient members from the surrounding community with diarrhoea. Further studies are needed to determine the burden of *C. difficile* infection in South Africa.

Different selective media have been employed to isolate *C. difficile* from stool specimens. In this project, the stool specimens were cultured on each of three types of selective media to maximise the isolation of *C. difficile*, as represented in Figure 2.1. The bacterium was identified based on colony morphology and/or colour change according to the various selective media. The CHR medium was the most effective in selecting for *C. difficile* when isolating cells directly from non-heat- or -alcohol-shocked stool, and most isolates were

obtained from this medium. Fewer isolates were obtained from the DCS and OXO media, as there often was an overgrowth of non *C. difficile* isolates, suggesting that these media are not sufficiently selective to analyse stool specimens without prior heat- or alcohol-shock procedures.

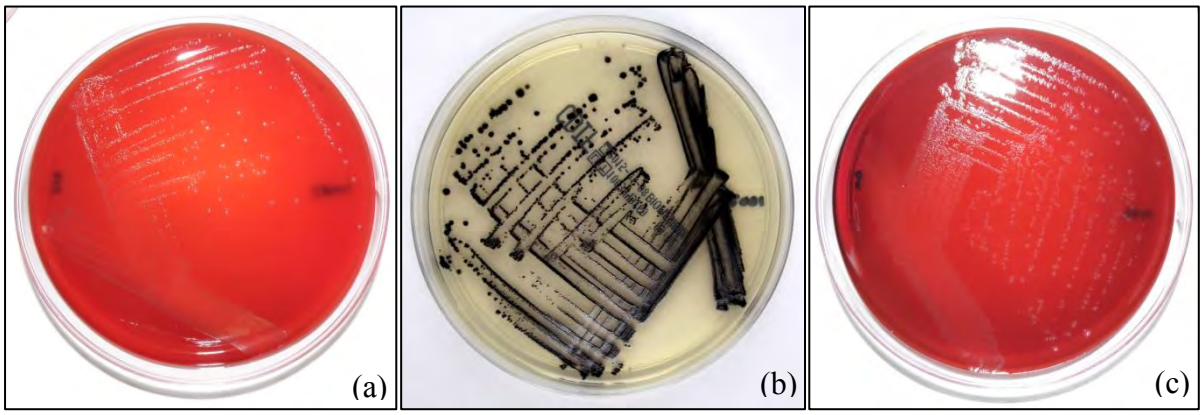


Figure 2.1 Representative agar plates of the three types of selective media used in this study: a) Darkoh chromogenic selective (DCS) medium*, b) ChromID (CHR) medium and c) Oxoid (OXO) medium.

* Toxigenic *C. difficile* isolates grow as blue colonies on the DCS medium, which is not represented in the figure above.

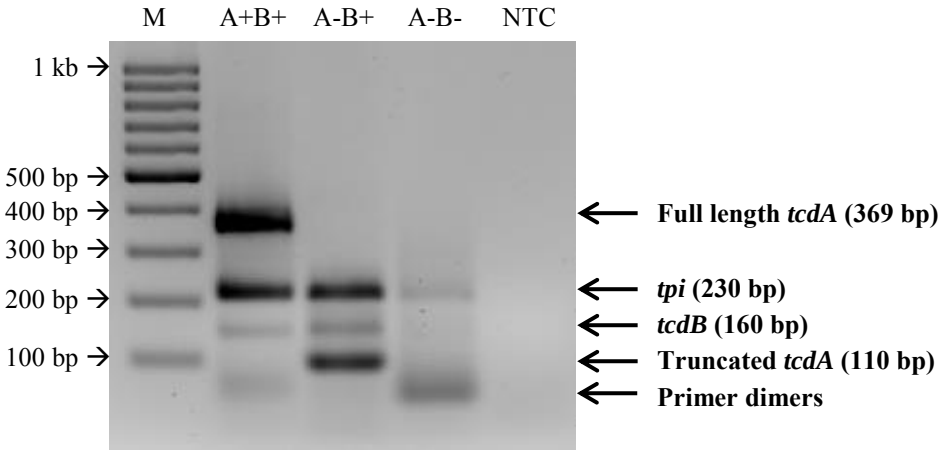
The variation in *C. difficile* yield from the three selective media may be attributed to differences in their content. Different concentrations of the selective agents D-cycloserine and cefoxitin have been recommended in order to balance diagnostic sensitivity and specificity of selective media (George *et al.*, 1979; Levett, 1985). While the concentration of the selective agents in the CHR medium is not disclosed by the manufacturers, both the DCS and OXO media contained D-cycloserine and cefoxitin at the same final concentration (250 µg/ml and 8 µg/ml respectively). These concentrations were introduced once it was established that some *C. difficile* strains were unable to grow when using the higher concentrations of the selective agents recommended in the original medium (Levett, 1985). However, this reduces the specificity of the medium, which is why heat- or alcohol-shock

steps are often included prior to plating to enhance the yield of *C. difficile* bacteria and reduce the growth of contaminating bacteria. The resistant *C. difficile* spores are able to survive this treatment, while vegetative cells are removed (Marler *et al.*, 1992; Riley *et al.*, 1987). Neither heat- nor alcohol-shock procedures were performed in the current study, which may explain the high level of contaminating growth on the DCS and OXO media. The addition of taurocholate to *C. difficile* selective media enhances spore germination (Wilson *et al.*, 1982). While the content of the CHR media is not known, taurocholate was not added to the DCS and OXO media in the current study, which may explain why fewer *C. difficile* isolates were obtained from these two media. The CHR media has been shown to be specific enough to isolate *C. difficile* cells directly from stool and this was again the case in the current study (Boseiwaqa *et al.*, 2013; Carson *et al.*, 2013). A recent media comparative study included the CHR medium and three other selective media. A Cycloserine-cefoxitin egg yolk agar (CCEY) was reported as being the most sensitive in the detection of *C. difficile* spores, followed by the CHR medium, which also had the best recovery rate of vegetative cells from stool specimens (Lister *et al.*, 2014).

2.3.2 Toxigenic typing of *C. difficile* pure strains using PCR

Multiplex PCR screening for the *C. difficile* species-specific *tpi* fragment and the toxin A and B genes was used to confirm the identity of the *C. difficile* strains isolated from the selective media. All isolates harboured the *C. difficile* species-specific *tpi* fragment of 230 bp (Figure 2.2 a). PCR screening for toxin genes revealed three toxigenic-types: A+B+, A-B+ and non-toxigenic A-B-. Representative examples of these are depicted in Figure 2.2 (a). Further toxigenic typing was done by screening for the binary toxin genes *cdtA* and *cdtB* (Figure 2.2 b). These binary toxin genes were absent in the clinical isolates from Groote Schuur Hospital.

(a)



(b)

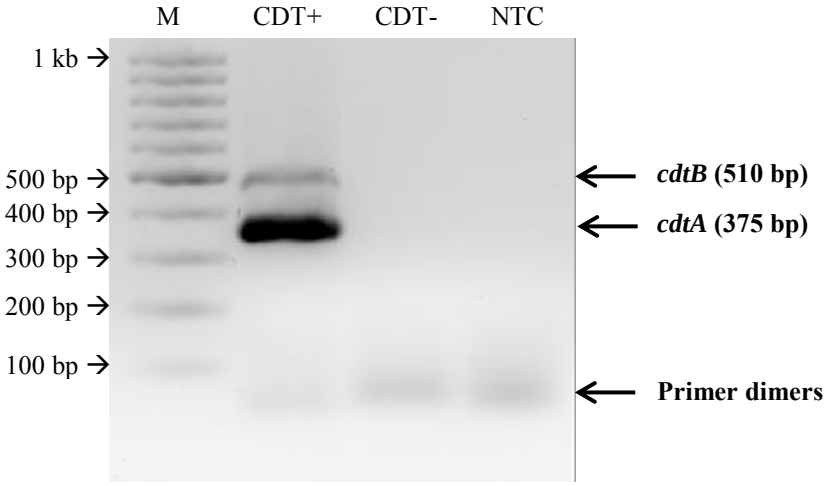


Figure 2.2 Representative gels showing toxigenic typing of *C. difficile*. (a) Multiplex PCR screening for *tpi*, *tcdA* and *tcdB* and (b) binary toxin genes *cdtA* and *cdtB*. M, 100 bp DNA Molecular weight ladder. NTC, no DNA template control. *C. difficile* strain 709 is a toxigenic A+B+CDT+ strain, and was used as positive controls in both reactions.

Toxigenic typing of *C. difficile* from Groote Schuur Hospital patients revealed an equal representation (47% each) of A+B+ and A-B+ strains among the 34 isolates (Table 2.5). *C. difficile* strains isolated from two stool specimens were identified as non-toxigenic type A-B- as only the *tpi* gene fragment was amplified (Figure 2.2 a). This represented 6% of the study group. None of the isolates harboured the binary toxin genes.

Table 2.5 Prevalence of toxigenic-types among clinical isolates

Toxigenic-type	Prevalence	Specimen			
A+B+CDT-	47%	GSH6553	GSH9316	GSH4696	GSH2753
		GSH8033	GSH8162	GSH9173	GSH1858
		GSH2702	GSH1795	GSH5681	GSH9354
		GSH5291	GSH1588	GSH1239	GSH4115
A-B+CDT-	47%	GSH6999	GSH4693	GSH2529	GSH5567
		GSH5556	GSH5760	GSH4722	GSH3758
		GSH8043	GSH8880	GSH3235	GSH1983
		GSH1709	GSH1526	GSH2797	GSH0616
A-B-CDT-	6%	GSH0574	GSH0541		

The high proportion of A-B+ strains amongst the Groote Schuur Hospital clinical isolates is interesting. This phenomenon, has also been observed in Asia (Kim *et al.*, 2008, 2010; King *et al.*, 2015; Komatsu *et al.*, 2003), but is not the case in North America and Europe where disease is primarily due to A+B+ strains (Labbé *et al.*, 2008; Warny *et al.*, 2005). However, the incidence of A-B+ strains is of clinical concern since they have caused outbreaks in these parts of the world (al-Barrak *et al.*, 1999; Drudy *et al.*, 2007b; Kuijper *et al.*, 2001). These strains contain a deletion in the *tcdA* gene and, therefore, only produce functional toxin B and yet they have been reported to cause severe disease (Drudy *et al.*, 2007b; Kim *et al.*, 2008; Kuijper *et al.*, 2001). The previous study conducted at Groote Schuur Hospital made use of an EI-based assay, which only detected toxin A. It can, therefore, be concluded that the A-B+ strains remained undetected by this assay method resulting in an under-representation of the actual *C. difficile* prevalence. This may explain the prevalence in the previous study as approximately half (9.2%) of that found in the current study (20%). It should be noted that this conclusion is based on the assumption that the prevalence and the strain pattern were

similar during the previous study. Subsequent to the findings of the study reported in this thesis, the diagnosis methodology of CDI at the hospital was updated and now includes tests that detect both toxin A and B (section 2.3.3).

None of the 34 isolates in this study had the binary toxin genes, which were present in the A+B+ ribotype 027 control strain (Figure 2.2 b). These genes were amplified among strains isolated from the Vhembe district, South Africa: 1.7% and 4% of symptomatic patients showed evidence of harbouring A-B-CDT+ and A+B+CDT+ strains, respectively (Samie *et al.*, 2008). The binary toxin has been associated with PCR ribotype 027 and 078 strains. This additional toxin stimulates excessive microtubule growth of enterocytes resulting in protrusions, which have been suggested to contribute towards increased disease severity. The 0% prevalence of *C. difficile* containing binary toxin genes suggests that it is likely that none of the isolates belong to ribotype 027, but this requires further confirmation (Chapter 3).

2.3.3 Evaluation of clinical diagnostic methods

The performances of two enzyme immunoassay (EI)-based and two nucleic acid amplification tests (NAATs) were evaluated in diagnosing *C. difficile* at Groote Schuur Hospital. The performance of each of the tests was evaluated relative to the following criteria: Any stool specimen that grew *C. difficile* on the combination of selective media with confirmed detection of the *C. difficile* species-specific *tpi* and *tcdB* gene fragments by PCR was considered a true positive. The performance of each of the tests is summarised in Table 2.6 as percentages, and the importance of the various parameters evaluated are discussed below.

Table 2.6 Performance of clinical diagnostic methods relative to true positives *

	ImmunoCard	MiniVidas	GeneXpert	Hain Lifescience
Sensitivity (%)	37.5	50.0	90.6	87.5
Specificity (%)	98.3	98.3	96.4	89.7
Positive predictive value (%)	85.7	88.2	87.9	70.0
Negative predictive value (%)	85.4	88.5	97.3	96.3

* A true positive is a specimen that grew *C. difficile* on selective media and confirmed by detecting the *C. difficile tpi* and *tcdB* fragments by PCR.

Sensitivity

The sensitivity of a clinical test is its ability to accurately identify a true-positive specimen as positive (Lalkhen & McCluskey, 2008). A sensitivity of 37.5% is interpreted as the ImmunoCard only being able to identify 37.5% of the specimens that did, in fact, harbour toxin-positive *C. difficile*. This indicates that the ImmunoCard lacked the ability to effectively detect toxins A and B immunologically in the stool specimens and, therefore, provided an under-representation of the intestinal presence of *C. difficile* within the hospital. The MiniVidas, another EI-based method, also had a low sensitivity and was able to detect toxins in only half of the *C. difficile* positive specimens. In contrast the NAATs had high sensitivity values, with the Hain Lifescience and GeneXpert being able to positively identify the majority (87.5 % and 90.6%, respectively) of the specimens harbouring *C. difficile*. These results suggest that the EI-based assays are not as effective in identifying *C. difficile* positive stool specimens as the NAATs. Several studies have also evaluated the validity of these two EI-based methods and have established that they are not as sensitive in detecting toxins A and B (Peterson *et al.*, 2011; Planche *et al.*, 2008). Studies comparing EI-based assays to NAATs

have also calculated a higher sensitivity for NAATs (Eastwood *et al.*, 2009; Hernández-Rocha *et al.*, 2013; Swindells *et al.*, 2010). The GeneXpert had the highest sensitivity (Table 2.6) suggesting that it is the most effective test of the clinical diagnostic methods investigated by this study.

The low detection rate of the EI-based assays could possibly be influenced by the dilution of stool toxin by the increased volume of stool due to diarrhoea, or to degradation of toxins by the time the assay was performed. Another plausible reason for a false-negative result is that *C. difficile* may not be producing toxins in the intestine when the stool specimen was collected. Faecal bacterial load can also affect the performance of a detection method. It has been proposed that tests with a high sensitivity are able to detect a lower faecal bacterial load. A few studies have investigated this and reported that specimens identified as positive by only a PCR-based method had the lowest bacterial load, while those identified by both PCR and EI-based methods had a significantly higher bacterial load (Dionne *et al.*, 2013; Leslie *et al.*, 2012).

The detection of free toxins within stool specimens compared to PCR detection of toxigenic *C. difficile* with the potential to produce toxins has raised debate (Su *et al.*, 2013). *C. difficile* toxins are the main virulence factors of CDI, but the actual proteins cannot be detected by NAATs. While NAATs have a high sensitivity, they are less specific as they do not differentiate between active CDI and patients that simply harbour the bacterium and have diarrhoea caused by alternative agents. Specimens determined as positive by the GeneXpert, but negative by the EI-based assays may contain toxigenic *C. difficile* strains that are not actively producing toxins at the time of sampling. Alternatively, toxins may be produced, but the EI-based assays are not sensitive enough to detect them. This warrants the investigation of toxin activity to determine whether *C. difficile* detected by GeneXpert is responsible for disease in symptomatic patients. To overcome this two-step and three-step diagnostic

algorithms have been proposed and published (Bamber *et al.*, 2012; Goldenberg *et al.*, 2010). This involves combining currently available diagnostic methods where the first test is employed for initial screening and the second and/or third test to confirm the diagnosis. Improved clinical performances have been reported using these algorithms; however, they can cost more and are more time consuming.

Specificity

Specificity is the ability of a clinical test to accurately identify a true-negative specimen as negative (Lalkhen & McCluskey, 2008). A low specificity would be the result of a high number of false-positive results. Both EI-based assays had high specificities, reflecting a low proportion of false positive results. The low sensitivity and high specificity of the EI-based assays in this study compares to the values reported by previous studies when compared to the toxigenic culture or cell culture cytotoxicity neutralisation assay methods (Eastwood *et al.*, 2009; Hernández-Rocha *et al.*, 2013; Swindells *et al.*, 2010). The Hain Lifescience test had the lowest specificity value (89.7%), which indicates that this test most often identified non-*C. difficile* targets in the stool specimen as *C. difficile* positive. Despite the lower specificity of the NAATs, they are rapid and their detection of toxigenic *C. difficile* is useful in the prevention of nosocomial transmission and the implementation of infection control.

Positive and negative predictive values

The positive predictive value (PPV) of a diagnostic test is the likelihood that a patient does have the disease given that the test result is positive, while the negative predictive value (NPV) is the likelihood that a patient is disease-free given that the test result is negative. The PPV and NPV vary depending on the prevalence of the disease, and are useful to clinicians by giving an indication of a given test's usefulness in a particular population, while sensitivity and specificity evaluates the detection capacity alone. Of the four diagnostic tests,

the Hain Lifescience test had the lowest PPV (70.0%), whereas the other tests had similar PPVs (85.7-88.2%) (Table 2.6). Therefore, in this particular population, the Hain Lifescience test has the highest risk of producing a false-positive among the four tests. The GeneXpert and Hain Lifescience tests had the highest NPV (97.3% and 96.3% respectively), which provides clinicians with the most confidence in these tests regarding the correct identification of CDI disease-free patients.

The low clinical sensitivity values of the EI-based assays suggest that they are not ideal as a stand-alone detection method at Groote Schuur Hospital, and should be replaced by a more sensitive one, such as a NAAT, for rapid and reliable diagnosis. Overall, the GeneXpert performed the best. It had the highest clinical sensitivity value, PPV and NPV, and it had a high specificity value; which all provides confidence in its results in the local setting. In addition, the GeneXpert results were in agreement with the PCR analysis targeting the *tpi*, *tcdB* and *cdtA* genes. It should be noted, however, that the GeneXpert is designed to presumptively ideally identify isolates belonging to the ribotype 027 clade, although it can detect the presence of other ribotypes as well. In addition, the genetic markers targeted by the GeneXpert to identify a ribotype 027 isolate are not only associated with this clade. Therefore, the GeneXpert is not specific to ribotype 027 isolates. Despite these drawbacks, the GeneXpert is recommended at this stage as the preferred diagnostic method in the local setting.

2.4 Conclusion

In conclusion, *C. difficile* was present in a significant proportion of symptomatic patients attending Groote Schuur Hospital during the study period. NAATs were more sensitive for detecting *C. difficile* than the EI-based tests and the results of these analyses have encouraged

the Microbiology Laboratory at the NHLS to adopt the GeneXpert for clinical diagnosis of CDI. While the NAATs only detect the presence of the various *C. difficile* genes future work should also establish whether the *C. difficile* isolates have the potential to actually produce toxins in order to confirm that the *C. difficile* detected by the NAATs is capable of causing disease.

The high proportion of toxigenic-type A-B+ strains in this study group is interesting and could be indicative of strain transmission between patients. However, in order to assess this, further genotyping methods are necessary to identify exactly which *C. difficile* strains are present amongst the study population. PCR ribotyping and multilocus variable-number tandem-repeat analysis (MLVA) are techniques that have been used previously to examine *C. difficile* strain diversity (van den Berg *et al.*, 2007; Stubbs *et al.*, 1999). These aspects were investigated and the results reported in Chapter 3 of this thesis.

CHAPTER THREE

THE IDENTIFICATION AND GROUPING OF *CLOSTRIDIUM DIFFICILE* ISOLATES BY GENOTYPING

Contents

3.0 Abstract

Various *C. difficile* strains have emerged which can cause disease. Genotyping methods developed for *C. difficile* have allowed the monitoring of the spread of the various strains, which may reach epidemic levels. In this study, three genotyping methods were used to identify *C. difficile* clinical isolates from patients at Groote Schuur Hospital and determine their relatedness. PCR ribotyping identified twelve different ribotypes among the clinical isolates. All the toxigenic A-B+ isolates belonged to PCR ribotype 017, which was the predominant ribotype (47%). Ribotypes 001 (14%), 015 (9%) and SE108 (6%) were also identified, as well as one isolate of each of the following ribotypes: 002, 012, 056, SA1, SE9, SE19m, SE21e and SE23a. Genotyping based on sequencing of the *tcdC* gene grouped the isolates into four *tcdC* genotypes, with PCR ribotype 017 isolates forming a separate clade. MLVA allowed further subtyping of isolates within a single ribotype. Isolates of the same ribotype were clustered together and ribotype 017 isolates again formed a separate clade. The predominance of ribotype 017 among the clinical isolates and the clustering of these isolates in separate clades by *tcdC* and MLVA genotyping suggest that this group is significantly different from the others and that there may be selective pressure promoting the high prevalence of this ribotype in the hospital.

3.1 Introduction

Several different strain types of *C. difficile* that cause disease have emerged and have reached epidemic proportions in many parts of the world (as reviewed in Chapter 1). In order to identify and monitor strains of concern, various molecular typing methods for *C. difficile* have been developed. These include restriction-based methods such as pulsed-field gel electrophoresis (PFGE) and restriction endonuclease analysis (REA) (Clabots *et al.*, 1993; Gal *et al.*, 2005), amplification-based methods such as amplified fragment length polymorphism (AFLP), arbitrarily primed PCR (AP-PCR) and PCR ribotyping (Klaassen *et al.*, 2002; McMillin & Muldrow, 1992; Stubbs *et al.*, 1999), and sequence-based methods such as multilocus sequence typing (MLST) and *slpA* typing (Griffiths *et al.*, 2010; Kato *et al.*, 2010). While these methods have improved the monitoring of outbreaks and transmission, and helped to reduce the incidence of CDI, many of them require specialised expertise and/or equipment, and inter-laboratory comparisons are often difficult.

PCR ribotyping is most frequently employed in Europe, and more recently in the USA, and an extensive PCR ribotype database has been generated allowing the identification of over 400 PCR ribotypes (Cardiff-European Centre for Disease Prevention and Control (ECDC) collection). Ribotyping involves PCR amplification of the 16S-23S rRNA gene intergenic spacer sequences and the resulting band patterns are visualised on agarose gels or analysed by capillary gel electrophoresis and compared to a web-based database such as Webribo (Indra *et al.*, 2008; Stubbs *et al.*, 1999). Two different sets of primers have been proposed (Bidet *et al.*, 1999; Stubbs *et al.*, 1999), with the O'Neil primers described by Stubbs *et al*., (1999) having a reportedly higher discriminatory power (van den Berg *et al.*, 2004).

With the aid of genotyping techniques, it has been observed that the distribution of *C. difficile* strain types has varied temporally and geographically. In the last decade, PCR ribotype 027,

also referred as NAP1 or BI, has been linked to the increase in the number of CDI cases and outbreaks in North America and Europe (Labbé *et al.*, 2008; McDonald *et al.*, 2005; Smith, 2005). More recently, ribotype 027 has also been reported to cause disease in further European countries, Asia, Australia and Latin America (Bacci *et al.*, 2009; Baldan *et al.*, 2010; Cheng *et al.*, 2009; Kato *et al.*, 2007; Lachowicz *et al.*, 2014; Oleastro *et al.*, 2014; Quesada-Gómez *et al.*, 2010; Riley *et al.*, 2009). Other PCR ribotypes reaching high proportions and/or causing outbreaks include ribotype 078 (Goorhuis *et al.*, 2008; Weber *et al.*, 2013); ribotype 001 (Borgmann *et al.*, 2008; Stubbs *et al.*, 1999); and ribotype 017 in North America (Tenover *et al.*, 2011), Europe (Dobreva *et al.*, 2013; Pituch *et al.*, 2011), and frequently in many Asian countries (Hawkey *et al.*, 2013; Lee *et al.*, 2014; Ngamskulrungroj *et al.*, 2015).

As an addition to PCR ribotyping, the sequences of numerous genes, including the *tcdC* gene, which encodes a putative negative regulator of toxin production, have been used for cluster analysis of *C. difficile* to determine the relatedness of strains belonging to different PCR ribotypes (Kurka *et al.*, 2014). Several polymorphisms in the *tcdC* gene have been identified and found to be associated with various PCR ribotypes (Curry *et al.*, 2007; Spigaglia & Mastrantonio, 2002).

In order to investigate closely related strains, thereby allowing the determination of strain transmission during an outbreak, additional genotyping methods of a higher discriminatory power are required. Multilocus variable-number tandem-repeat analysis (MLVA) and whole genome sequencing (WGS) have been previously used for this purpose (van den Berg *et al.*, 2007; Cairns *et al.*, 2015; Eyre *et al.*, 2013a). WGS can be used to distinguish *C. difficile* strains based on single-nucleotide variants between the isolates. MLVA involves PCR amplification and distinguishes *C. difficile* strains based on the varying sizes of short tandem repeats distributed throughout the genome (van den Berg *et al.*, 2007; Marsh *et al.*, 2006). MLVA is a cheaper method than WGS and does not require the extensive bioinformatics

analysis required for the latter. Moreover, the technique has been shown to be almost as discriminatory as WGS (Eyre *et al.*, 2013b).

Monitoring the spread and emergence of *C. difficile* by identifying strains is important for the implementation of effective infection control. By determining the epidemiology of circulating strains, targeted interventions can be applied when an epidemic strain is identified. When employed in conjunction with national surveillance schemes, accurate strain typing and monitoring have previously been associated with a decline in the prevalence of epidemic strains (Kanerva *et al.*, 2013; Wilcox *et al.*, 2012).

Prior to this study, the types of *C. difficile* infecting patients at Groote Schuur Hospital, and potentially causing disease, were not known. Therefore, the aim of the work reported in this chapter was to apply molecular genotyping techniques to determine the identity and distribution of *C. difficile* PCR ribotypes. The genetic relatedness of the clinical isolates to one another and to international *C. difficile* reference strains was determined based on the *tcdC* gene sequence and multilocus variable-number tandem repeats distributed throughout the genome.

3.2 Materials and Methods

3.2.1 Bacterial strains and plasmids

C. difficile strains listed in Table 3.1 were used as control strains for genotyping. The cloning of the *tcdC* PCR product was done in *E. coli* JM109 (Sambrook *et al.*, 1989; Yanisch-Perron *et al.*, 1985).

Table 3.1 *C. difficile* reference strains used for genotyping analysis

Strain	Ribotype	Reference/Origin
C. difficile 709	Ribotype 027	Gift from Prof Andrej Weintraub, Karolinska Institute, Stockholm, Sweden
C. difficile 728	Ribotype 078	
C. difficile 630	Ribotype 012	Gift from Prof Brendan Wren, London School of Hygiene and Tropical Medicine, London, UK
C. difficile CD305	Ribotype 023	
C. difficile M120	Ribotype 078	
C. difficile M68	Ribotype 017	
C. difficile CF5	Historic ribotype 017	
C. difficile CD196	Historic ribotype 027	
C. difficile R20291	Hyper-virulent ribotype 027	

3.2.2 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from 16 hour cultures grown in BHI broth (Chapter 2) using the ISOLATE II Genomic DNA Kit (Bioline). A pre-lysis step of the broth culture, using 200 µl resuspension buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing lysozyme (2% w/v), proteinase K (1% v/v) and Triton X-100, was included. A total of 100 ng gDNA was used as template for the MLVA and *tcdC* PCRs. PCR ribotyping made use of DNA template obtained by cell lysis as described in Chapter 2 (section 2.2.4).

3.2.3 PCR ribotyping

PCR ribotyping was performed using previously described primers (Stubbs *et al.*, 1999). Genomic DNA of *C. difficile* 709 and 728 were used as positive PCR controls. PCRs were performed in a 100 µl reaction volume containing 0.5 µM of each primer (5'-CTGGGGTGAAGTCGTAACAAGG-3' and 5'-GCGCCCTTTGTAGCTTGACC-3'). The reactions were denatured at 95ºC for 5 min, and then subjected to 35 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 2 min, followed by a final extension at 72ºC for 5 min. Reactions were performed using a Primus 25 thermo cycler (PEQLAB). PCR products were concentrated

by heating at 75ºC for 105 min and resolved by electrophoresis on a 3% agarose gel stained with ethidium bromide (Stubbs *et al.*, 1999). Banding patterns were compared visually to a laboratory database of known PCR ribotypes. Isolates with ribotype patterns that could not be identified using this database were submitted to Andrej Weintraub at Karolinska Institute for further analysis. PCR ribotypes annotated with 'SE' were identified using a Swedish library, and remaining ribotypes were identified according to the Cardiff-ECDC nomenclature.

3.2.4 Amplification and sequencing of the *tcdC* gene

The full-length *tcdC* gene of all toxigenic clinical isolates was amplified using previously described primers (Spigaglia & Mastrantonio, 2002). PCRs were performed in 25 µl reaction volumes containing 0.3 µM of each primer (5'-TTAATTAATTTTCTCTACAGCTATCC-3' and 5'-TCTAATAAAAGGGAGATTGTATTATG-3') in Kapa HiFi HotStart ReadyMix (Kapa Biosystems). The reactions were denatured at 95ºC for 5 min, and then subjected to 25 cycles of 98ºC for 20 s, 50ºC for 30 s and 72ºC for 80 s, followed by a final extension at 72ºC for 5 min. PCR products were cloned into the pJet1.2 vector (Thermo Scientific) and transformed into competent *E. coli* JM109 using standard protocols (Sambrook *et al.*, 1989). Plasmid DNA was extracted using the Plasmid DNA Extraction Kit (BioFlux) according to the manufacturer's instructions, and the presence of insert confirmed by both PCR using the pJet1.2F/R primer set and by restriction mapping. DNA sequencing was performed by Macrogen Inc., Seoul, South Korea. Nucleic acid sequences were analysed using DNAMAN (v. 4.13) and compared to the National Centre for Biotechnology Information (NCBI) database using the BLAST algorithm (Altschul *et al.*, 1997). Published *tcdC* sequences for strains 630, CF5, M68, CD305, CD196, R20291 and M120 were obtained from the NCBI database. Nucleic acid sequences were aligned and a dendrogram was constructed using the Neighbour Joining method as previously described (Kurka *et al.*, 2014), using MEGA (v. 5.2) (Tamura *et al.*, 2011).

3.2.5 Multilocus variable-number tandem-repeat analysis (MLVA)

The clinical isolates and the reference strains (Table 3.1) were typed using MLVA as previously described (van den Berg *et al.*, 2007). PCR amplifications of seven loci, designated A6, B7, C6, E7, F3, G8 and H9 (Table 3.2), were performed as three separate duplex reactions (A_6 – H_9, B_7 – F_3, and C_6 – E_7) and one single reaction (G_8). PCRs were performed in a 50 µl reaction volume containing Kapa HiFi HotStart ReadyMix (Kapa Biosystems) and 0.2 µM of each primer. The reactions were denatured at 95ºC for 5 min, and then subjected to 35 cycles of 98ºC for 20 s, 51ºC for 30 s and 72ºC for 30 s, followed by a final extension at 72ºC for 10 min.

Table 3.2 Primers used for *C. difficile* MLVA genotyping (van den Berg *et al.*, 2007)

Marker*	**Repeat motif**	**Primer sequence (5' → 3')**	**Label**
A_6	AAGAGC	F: TTAATTGAGGGAGAATGTTAAA R: AAATACTTTTCCCACTTTCATAA	FAM
B_7	ATCTTCT	F: CTTAATACTAAACTAACTCTAACCAGTAA R: TTATATTTTATGGGCATGTTAAA	HEX
C_6	TATTGC	F: GTTTAGAATCTACAGCATTATTTGA R: ATTGGAATTGAATGTAACAAAA	TET
E_7	ATAGATT	F: TGGAGCTATGGAAATTGATAA R: CAAATACATCTTGCATTAATTCTT	HEX
F_3	TTA	F: TTTTTGAAACTGAACCAACATA R: ACAAAAGACTGTGCAAATATACTAA	TET
G_8	TAAAAGAG	F: TGTATGAAGCAAGCTTTTTATT R: AATCCAGCAATCTAATAATCCA	FAM
H_9	TCTTCTTCC	F: GTTTTGAGGAAACAAACCTATC R: GATGAGGAAATAGAAGAGTTCAA	Cy5

*The numbers written in subscript indicate the size of the repeat motif of each loci targeted.

The forward primer for each marker was labelled on the 5' end with either 6-carboxyfluorescein (FAM), hexachlorofluorescein (HEX), 5-tetrachloro-fluorescein (TET) or

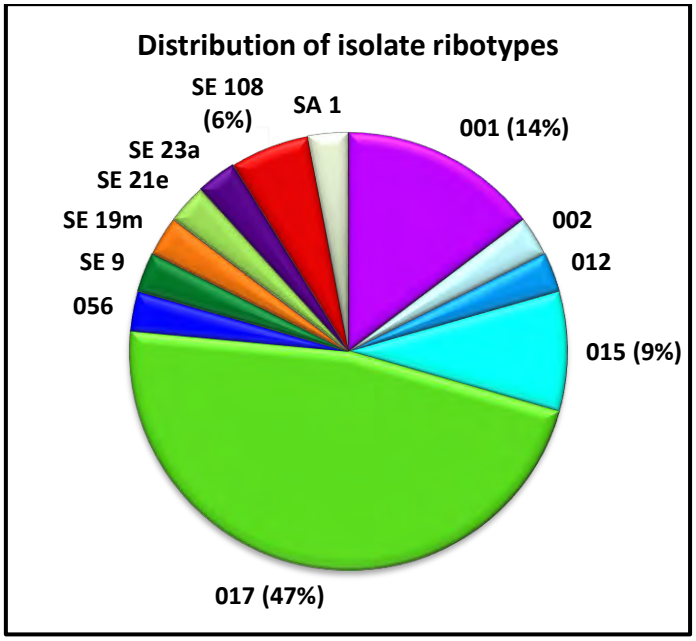
Cyanine 5 (Cy5) (Table 3.2). PCR fragments were analysed using multi-coloured capillary gel electrophoresis on an ABI 3130xl genetic analyser, with a ROX500 marker as an internal size standard for each sample. The size of each locus was determined using Peak Scanner version 1.0 software (Applied Biosystems). The repeat numbers for each locus were calculated manually and the isolate profiles compared to each other using the Manhattan coefficient to construct a minimum spanning tree in BioNumerics version 7.1 software (Applied Maths, Kortrijk, Belgium). Isolates with a summed tandem repeat difference (STRD) of ≤ 2 across the seven loci were identified as genetically related.

3.3 Results and Discussion

3.3.1 Typing of *C. difficile* isolates by PCR ribotyping

This study used PCR ribotyping to identify the *C. difficile* clinical isolates and compare them to international strains. A total of twelve PCR ribotypes were identified among the 34 clinical isolates. The ribotype distribution is summarised in Figure 3.1 (a) and representative gels of PCR product pattern for each ribotype are depicted in Figure 3.1 (b). The PCR product patterns correspond to the Swedish and Cardiff databases. PCR ribotype 017 was the predominant strain (47%), followed by ribotypes 001 (14%), 015 (9%) and SE 108 (6%). The remaining isolates each belonged to a different ribotype: 002, 012, 056, SA1, SE9, SE19m, SE21e and SE23a (3% each). PCR ribotype 027 was included as a reference PCR product (Figure 3.1 b); however, none of the clinical isolates had banding patterns identical to this ribotype. All the A-B+ isolates belonged to ribotype 017. The non- toxigenic isolates, GSH0574 and GSH0541, belonged to ribotypes SE9 and SA1, respectively. The A+B+ isolates belonged to the remaining various PCR ribotypes.

(a)



(b)

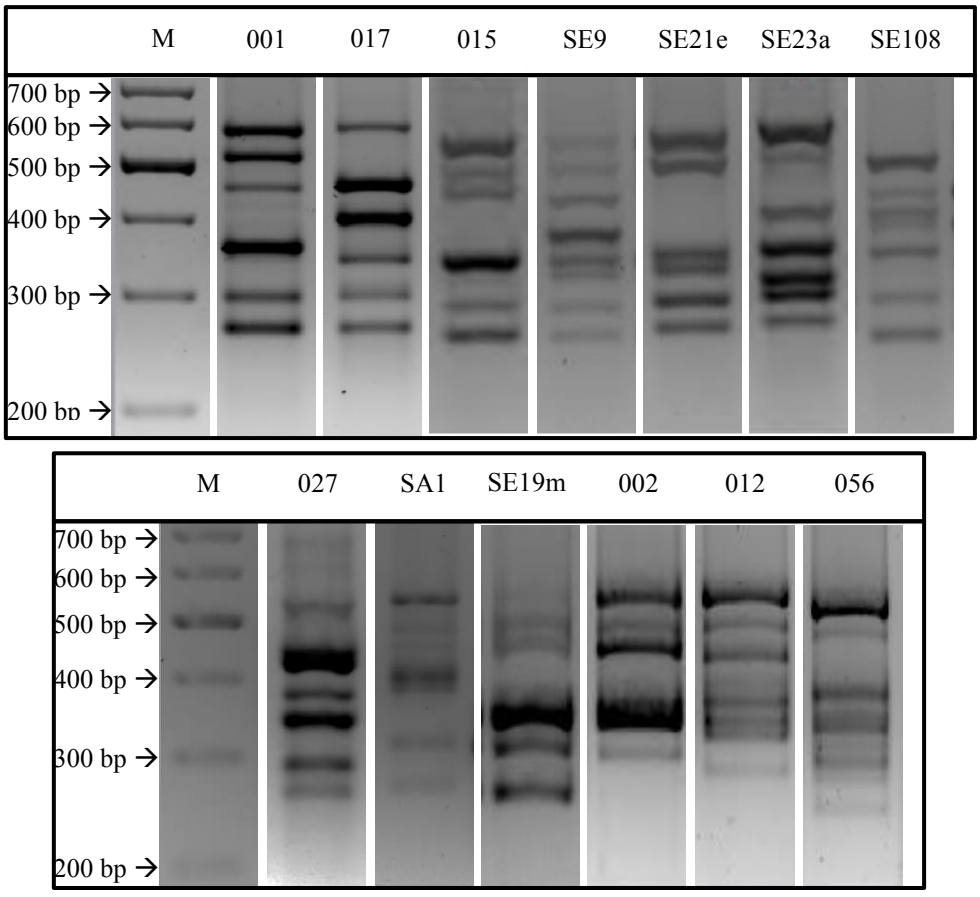


Figure 3.1 PCR ribotyping. (a) Distribution of PCR ribotypes among the 34 clinical isolates. (b) Representative gel images showing examples of the PCR ribotype banding patterns. M, 100 bp DNA Molecular weight ladder (200 – 700 bp).

This study is the first to identify PCR ribotypes of *C. difficile* within South Africa; therefore, a comparison to other sites in the country cannot be made. Approximately half of the isolates, all toxigenic-type A-B+, belonged to PCR ribotype 017. Ribotype 017 isolates are typically characterised by a 1.8 kb deletion in the *tcdA* gene, but are still capable of causing severe disease (Alfa *et al.*, 2000). Many ribotype 017 isolates have also been characterised as resistant to multiple antimicrobials (Kim *et al.*, 2012b; Pituch *et al.*, 2011). It is difficult to accurately determine the prevalence of ribotype 017 strains in other parts of the world on the basis of toxigenic-types alone since not all A-B+ strains belong to ribotype 017 and non-017 A-B+ strains have been reported in Asia (Rupnik *et al.*, 2003). In addition, several published studies from sites in Asia report a high prevalence of A-B+ strains, but they do not always specifically identify the ribotype of these isolates (Chia *et al.*, 2013; Rupnik *et al.*, 2003). However, studies that report the PCR ribotype have identified ribotype 017 as the leading strain at hospitals in Korea, China, Poland and Bulgaria (Dobreva *et al.*, 2013; Hawkey *et al.*, 2013; Kim *et al.*, 2010a, b; Pituch *et al.*, 2011). The epidemic proportions that this ribotype has reached in some parts of the world and the high prevalence of this ribotype reported by this study suggest it may have the potential to cause significant problems at the Groote Schuur Hospital study site.

PCR ribotype 001 was the second most prevalent ribotype among the clinical isolates. This ribotype has become frequently isolated in Europe and North America (Bauer *et al.*, 2011; Borgmann *et al.*, 2008; Cheknis *et al.*, 2009; Stubbs *et al.*, 1999). The worldwide prevalence of ribotype 001 has reduced in recent years; however, this is the ribotype that is most often reported to display reduced susceptibility to metronidazole, which is the antimicrobial recommended for first-line treatment of CDI. Continued molecular typing of South Africa isolates and determination of antimicrobial susceptibility is necessary to determine whether this trend is also observed locally.

PCR ribotypes 002, 012, 015 and 056 were of a low prevalence in this study. These ribotypes have been isolated in Europe, but also at lower frequencies (Bauer *et al.*, 2011). The hyper-virulent ribotypes 027 and 078 were not identified among any of the clinical isolates from GSH. These results are in agreement with the absence of binary toxin genes in the isolates, which are characteristic of these two ribotypes, and in agreement with the results of GeneXpert (Cepheid), which did not presumptively identify ribotype 027 in any of the stool specimens (Chapter 2). Thus, the prevalence of PCR ribotypes in this study is similar to some Asian hospitals where there is a high prevalence of ribotype 017 and scarcity of ribotype 027, relative to the high proportions of the latter in other parts of the world (Cheknis *et al.*, 2009; Hawkey *et al.*, 2013; Labbé *et al.*, 2008).

The disadvantage of PCR ribotyping is the need to compare and differentiate banding patterns visually, under identical conditions and preferably on the same gel. This requires inter-laboratory exchange of the actual bacterium or gDNA. To overcome this problem, capillary gel electrophoresis-based PCR ribotyping has been developed, for which a web-based database has been created (Indra *et al.*, 2008). While the Cardiff database has grown to over 400 PCR ribotypes, there remain several ribotypes yet to be identified and, therefore, the identification of some strains was not always possible in this study. Ribotypes SE9, SE19m, SE21e, SE23a and SE108 were identified according to a library at the Karolinska Institute in Sweden (with thanks to Dr Andrej Weintraub). One isolate could not be identified by comparative analysis with any other available control and was assigned a temporary PCR ribotype, SA1, until the identity is verified. Alternative molecular typing methods were used in an attempt to overcome these limitations.

3.3.2 Genotyping based on the *tcdC* gene

Molecular genotyping based on the *tcdC* gene was performed to differentiate toxigenic *C. difficile* isolates and determine the association between *tcdC* genotype and PCR ribotype. Four different *tcdC* genotypes were identified among the clinical isolates, all of which have been reported previously (Curry *et al.*, 2007). When aligned to one another and plotted as a dendrogram, the clinical isolates were grouped into four clades with ribotypes 027 and 078 control strains creating an additional two clades (Figure 3.2). Ribotypes 002, 015, 056, SE19m and SE108 grouped together and belonged to the *tcdC-0*, or the wild type genotype.

Ribotype 001 belonged to *tcdC-sc3*, which is characterised by a single nucleotide substitution at position 148 leading to an alanine to serine change in the translated protein. Ribotypes 012, SE21e and SE23a belonged to *tcdC-sc9*, characterised by a single nucleotide substitution at position 21 leading to a glutamate to aspartate change. Ribotype 017 diverged from the A+B+ clinical isolates and belonged to *tcdC-sc7*, characterised by five different nucleotide substitutions, of which two lead to amino acid changes in the translated protein. None of the clinical isolates were grouped with the ribotype 023, 027 or 078 control strains, which belong to the *tcdC-sc16*, *tcdC-sc1* and *tcdC-A*, respectively, which are characterised by nonsense mutations that lead to truncated proteins (Curry *et al.*, 2007; Spigaglia & Mastrantonio, 2002). The two non-toxigenic isolates belonging to ribotypes SE9 and SA1, respectively, do not contain the *tcdC* gene and, therefore, could not be typed by this method.

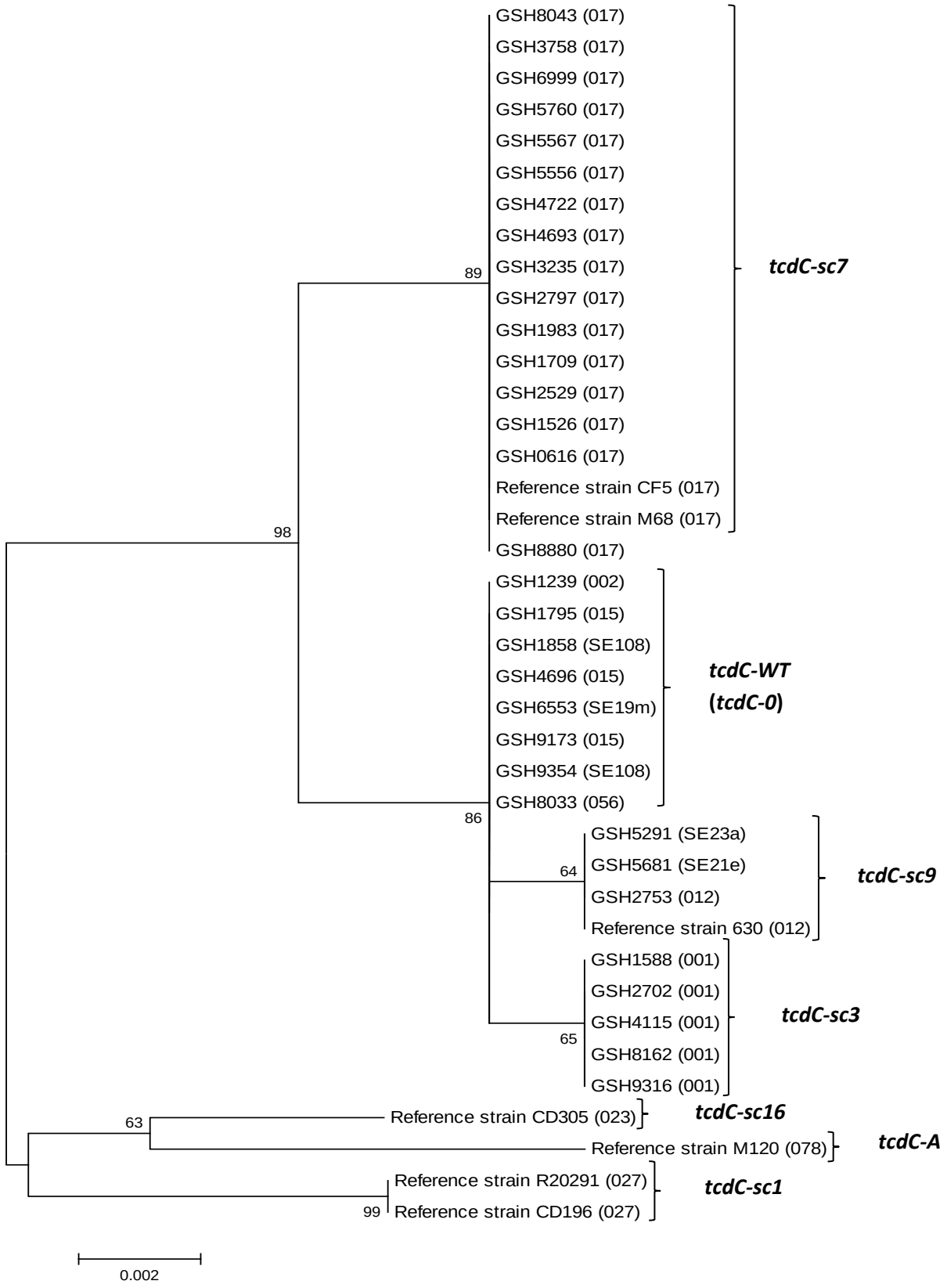


Figure 3.2 A Dendrogram based on the nucleotide sequence of the *tcdC* gene of *C. difficile* constructed using the Neighbour Joining method. The ribotype is indicated in brackets. Numbers at the branch points represent the bootstrap values. The *tcdC* genotype is written in bold.

Previously published studies report that *C. difficile* differentiates into five clades based on MLST and whole genome sequencing (Dingle *et al.*, 2011a; Griffiths *et al.*, 2010; Stabler *et al.*, 2012). Clade 1 is heterogeneous consisting of ribotypes 001, 002, 012, 015, 056 and others. Clade 2 consists of ribotype 027, clade 3 of ribotype 023, clade 4 of ribotype 017, and clade 5 of ribotype 078. In this study, isolates differentiated based on *tcdC* nucleotide sequences clustered in a similar manner. The clustering of isolates belonging to the same PCR ribotype in the *tcdC* dendrogram correspond with published studies that show that PCR ribotype is not only a reflection of variation in 16S-23S rRNA intergenic spacer sequences but also a reflection of differences in the *tcdC* sequence (Kurka *et al.*, 2014). A published study demonstrated this correlation between sequence diversity of the *tcdC* gene, as well as other *C. difficile* conserved genes, and PCR ribotype (Kurka *et al.*, 2014), thus demonstrating that PCR ribotyping as a suitable method for grouping *C. difficile*. However, while *tcdC* genotyping groups isolates belonging to the same ribotype together, typing based on this single gene does not differentiate isolates further within a PCR ribotype, demonstrating *tcdC* genotyping as a typing method of a low discriminatory power. A more discriminatory molecular genotyping technique was, therefore, attempted.

3.3.3 Subtyping by MLVA

MLVA, a highly discriminatory molecular typing method, was performed to subtype all the isolates further (Killgore *et al.*, 2008). Amongst the 34 clinical isolates 31 unique MLVA types were observed (Figure 3.3). Seven control strains belonging to ribotypes 012, 017, 023, 027 and 078 were included in the analysis and generated an additional seven MLVA types. Isolates belonging to the same ribotype clustered together. The sixteen clinical isolates belonging to ribotype 017 formed a distinct clade differentiating them from the toxigenic A+B+ strains.

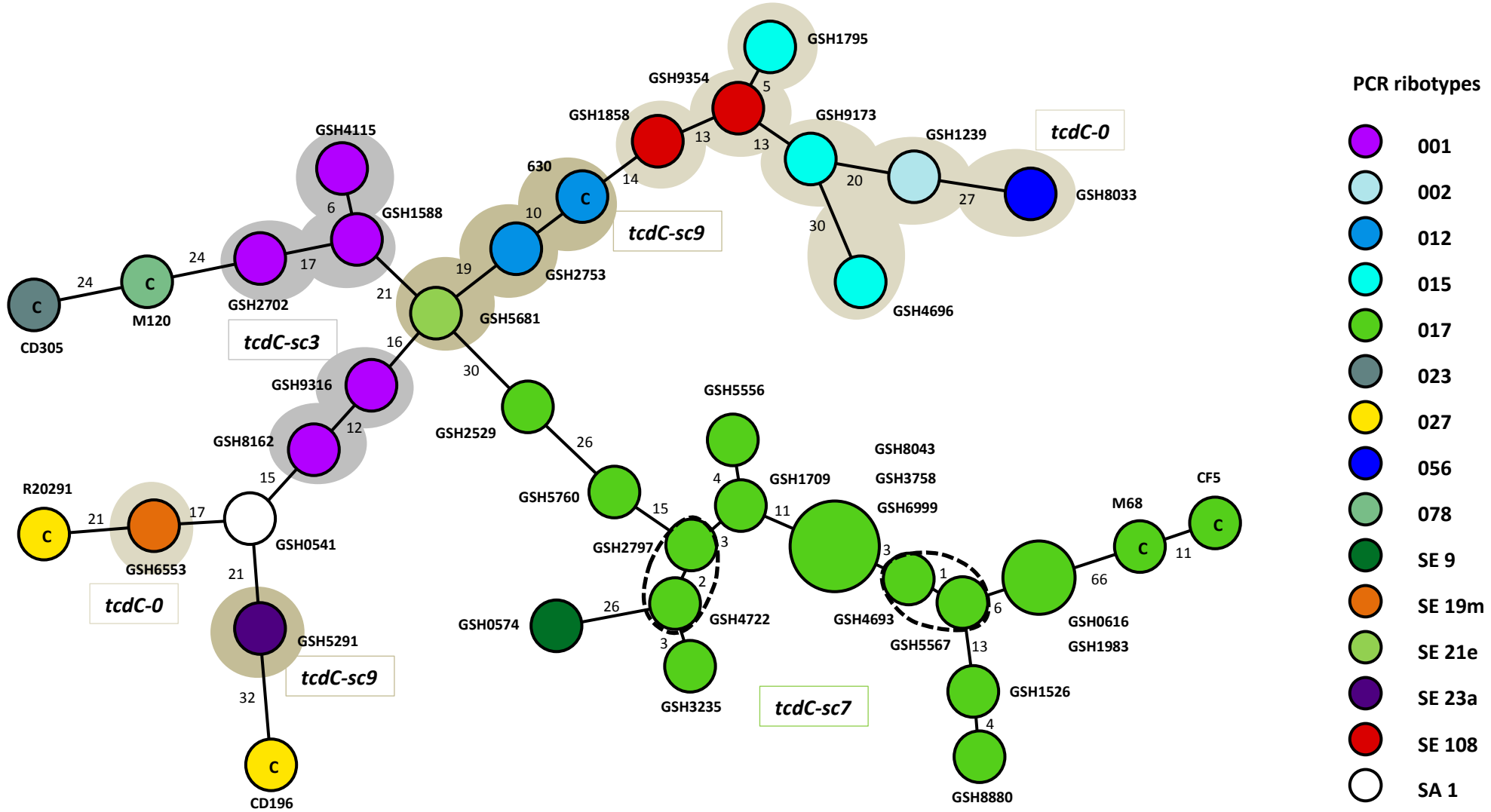


Figure 3.3 Minimum spanning tree representation of MLVA data for *C. difficile* isolates. Unique MLVA types are repesented by indvidual circles and the size of the circle represents the number of isolates per MLVA type. Numbers between the circles represent the STRDs between isolates and isolates with a STRD ≤ 2 are linked by a dotted line. The colour of the circle represents the PCR ribotpe. Circles labelled C represent published strains and serve as controls in this analysis. The grey shading is indicative of MLVA types belonging to the same *tcdC* genotype. The tree has been redrawn for ease of viewing and is not to scale.

A group of two isolates and a group of three isolates were indistinguishable by MLVA, as represented by enlarged circles in Figure 3.3, and were regarded as the same bacterial clone. Therefore, a group of two and a group of three patients were infected with the same *C. difficile* strain, respectively. In addition, two groups of two isolates, encircled by a dotted line in Figure 3.3, had a summed tandem repeat difference of (STRD) of one and two, respectively. Isolates having a STRD of ≤ 2 have previously been regarded as genetically related (van den Berg *et al.*, 2007) and may be indicative of transmission between these two sets of patients. MLVA showed genetic relatedness and differentiation among isolates that was not apparent by PCR ribotyping.

Interestingly, some ribotype 001 isolates were more closely related to ribotype SE21e than to one another. In addition, two ribotype 015 isolates, GSH1795 and GSH9173, were more closely related to ribotype SE108 than the third ribotype 015 isolate, GSH4696. The clustering of ribotypes can be improved using the extended MLVA (eMLVA) approach (Manzoor *et al.*, 2011), which involves increasing the number of targeted loci to fifteen. This method has been shown to further differentiate within a single ribotype and to create tighter ribotype clusters with greater STRDs between ribotype clusters. However, the cost of increasing the number of loci can be prohibitively expensive, due to the large numbers of labelled primers and extra capillary gel analyses required. In this study, the STRDs between different ribotypes were as low as five (Figure 3.3), while eMLVA generated STRDs ≥ 23 between different ribotypes (Manzoor *et al.*, 2011). Alternatively, WGS sequencing can be used. A recent study reported three diverging sub-lineages of ribotype 017 strains using WGS (Cairns *et al.*, 2015).

The clustering of ribotypes based on MLVA is similar to *tcdC* genotyping (Figure 3.2) as represented by the shaded areas in Figure 3.3 with the exception of ribotypes SE19m and SE23a, which belonged to *tcdC-0* and *tcdC-sc9*, respectively. Other than these exceptions, the

data generated by MLVA and *tcdC* genotyping are in agreement. The clustering of *tcdC* genotypes could also perhaps be improved by eMLVA.

MLVA can be used to investigate *C. difficile* transmission and outbreaks (Dingle *et al.*, 2011a; Eckert *et al.*, 2011; Goorhuis *et al.*, 2009). The data generated by MLVA can be used in conjunction with information such as hospital admission or the ward a patient resided in to demonstrate patient-to-patient transmission and determine whether MLVA types are restricted to wards. Alternatively, information such as the date of admission and date of specimen collection could be used to track the spread over time. A published study using eMLVA, reported smaller STRDs between isolates from the same time period (Manzoor *et al.*, 2011). In this study, a group of three ribotype 017 isolates, GSH6999, GSH3758 and GSH8043, were of the same MLVA type (Figure 3.3); however; GSH6999 was isolated approximately five months prior to the other two isolates. In addition, GSH3758 and GSH8043 were isolated two days apart, however, from different wards. This is suggestive of this strain type being able to persist in the hospital.

3.4 Conclusion

This is the first study to identify the PCR ribotypes of *C. difficile* in South Africa providing a platform for future epidemiological studies. In general, PCR ribotyping, *tcdC* genotyping and MLVA genotyping agreed with one another even though the typing methods rely on different targets in the genome. PCR ribotyping identified twelve different ribotypes. With the exception of ribotype SA1, which could not be successfully identified using the available libraries, the remaining eleven PCR ribotypes identified were not novel. Genotyping based on the *tcdC* gene grouped the isolates according to four genotypes with ribotype 017 isolates forming a separate clade. MLVA genotyping was able to subtype isolates belonging to the

same PCR ribotype and genetic diversity was reflected by PCR ribotyping with ribotype 017 isolates forming a distinct clade.

The predominance of ribotype 017 suggests that it may be more virulent and/or selected for by antimicrobial or environmental pressure. The clustering of ribotype 017 in a single and entirely separate clade based on *tcdC* and MLVA genotyping indicates that this strain is different. Published studies have described ribotype 017 as a high-level and multi-drug resistant pathogen (Davies *et al.*, 2014; Drudy *et al.*, 2007a; Kim *et al.*, 2012a). In this study, the antimicrobial resistance of the Groote Schuur Hospital isolates was, therefore, investigated and the results reported in Chapter 4 of this thesis.

CHAPTER FOUR

THE CHARACTERISATION OF *CLOSTRIDIUM DIFFICILE* ISOLATES BY DETERMINATION OF THEIR ANTIMICROBIAL RESISTANCE PROFILES

Contents

4.0 Abstract

C. difficile displays resistance to various antimicrobials, and their use can, therefore, result in an increased risk of developing CDI. Despite the evolving resistance profiles of some *C. difficile* strain types, metronidazole (MET) and vancomycin (VAN) remain the current treatments of choice for CDI in South Africa. In this study, antimicrobial susceptibility profiles for each of the clinical isolates were determined. Etests showed that all the clinical isolates were sensitive to both MET and VAN, although some ribotype 017 isolates displayed reduced susceptibility to MET. None of the strains displaying reduced susceptibility to MET harboured the previously characterised *nim* gene resistance determinants. Eleven isolates (32%), all belonging to ribotype 017, were resistant to moxifloxacin (MOX) and had a Thr82→Ile amino acid substitution in GyrA. Four isolates (12%), also ribotype 017, displayed reduced susceptibility to MOX and had an Asp426→Asn amino acid substitution in GyrB. Sixteen isolates (47%), primarily ribotype 017, were highly resistant to erythromycin (ERY) (MIC ≥ 256 μg/ml) and harboured the *ermB* gene, which is known to contribute towards ERY resistance. Another two isolates (6%) were resistant to ERY (48 μg/ml), but did not harbour the *ermB* gene. Disc diffusion analysis revealed potential resistance to clindamycin, ciprofloxacin, cefotaxime, ceftriaxone and tetracycline, and ribotype 017 strains showed a trend towards multi-drug resistance. The resistance profile of the ribotype 017 isolates may contribute to the high prevalence of this strain type at Groote Schuur Hospital.

4.1 Introduction

A major risk factor for developing CDI is the use of broad-spectrum antimicrobials (Baxter *et al.*, 2008), which disrupt the enteric microbiota allowing the overgrowth of resistant *C. difficile* strains (Sullivan *et al.*, 2001). Clindamycin, penicillin, cephalosporins, and fluoroquinolones have mostly been implicated in the development of CDI and outbreaks of the disease (Biller *et al.*, 2007; Dubberke *et al.*, 2015; Johnson *et al.*, 1999; Kim *et al.*, 2012a; Pépin *et al.*, 2005). Different levels of resistance to these antimicrobials in *C. difficile* have been reported worldwide.

Clindamycin is a macrolide-lincosamide-streptogramin B (MLS_B) agent, which inhibits protein synthesis by targeting the 23S rRNA gene (Tenson *et al.*, 2003). Resistance to MLS_B agents in *C. difficile* is usually mediated by an erythromycin ribosomal methylase (*ermB*) gene, the product of which dimethylates the 23S rRNA gene (Farrow *et al.*, 2001). Fluoroquinolones inhibit DNA replication by interacting with DNA gyrase subunits A and B (Hooper, 1999). Resistance is associated with mutations in the *gyrA* and *gyrB* genes resulting in amino acid substitutions in the encoded proteins (Dridi *et al.*, 2002; Drudy *et al.*, 2006).

Despite the evolving resistance profile of *C. difficile*, metronidazole or vancomycin remain the preferred treatment options for CDI. However, recent published studies report reduced susceptibility to metronidazole in some *C. difficile* strains, and reports of metronidazole or vancomycin resistance have also been published (Baines *et al.*, 2008; Brazier *et al.*, 2001; Chia *et al.*, 2013; Peláez *et al.*, 2008). The developing trend towards reduced susceptibility to these agents indicates that the monitoring of *in vitro* antimicrobial susceptibility is important.

In *B. fragilis*, resistance to metronidazole is, in certain cases, associated with the presence of various nitroimidazole genes (*nimA-J*) (Gal & Brazier, 2004; Haggoud *et al.*, 1994; Husain *et al.*, 2013). These *nim* genes encode nitroreductases, which inactivate metronidazole. A

published study that made use of "universal" primers targeted to conserved sequences in the *B. fragilis nimA-H* genes reported the presence of these genes in other species including one strain of *Clostridium bifermentans* (Lubbe *et al.*, 1999). However, *nim* homologues in *C. difficile,* have not yet been reported (Brazier *et al.*, 2001; Peláez *et al.*, 2008). A role for *nim* genes in *C. difficile* has, therefore, not yet been conclusively established and the exact mechanism of metronidazole resistance has yet to be determined.

Prior to this study, *C. difficile* strains had not been isolated from patients at Groote Schuur Hospital and so the antimicrobial resistance in this bacterium had also not been determined. The aim of the work presented in this chapter was to determine the antimicrobial susceptibility profiles of the previously identified *C. difficile* isolates, to examine a potential association of resistance with PCR ribotype, and to identify the possible underlying genetic resistance mechanisms of the resistant isolates.

4.2 Materials and Methods

4.2.1 Bacterial strains

The Groote Schuur Hospital *C. difficile* isolates listed in Table 2.5 (Chapter 2) and the *C. difficile* reference strains listed in Table 3.1 were included for antimicrobial susceptibility characterisation. However, the *gyrA* and *gyrB* genes from only *C. difficile* isolates displaying some resistance to moxifloxacin, as well as three selected sensitive isolates, were sequenced. *C. difficile* strains listed in Table 4.1 were used as controls, and *B. fragilis* strains were used as PCR controls when screening for the various *nim* genes.

Table 4.1 Control strains

Strain	Genotype/phenotype	Reference/source
C. difficile 11/11	Reduced susceptibility to metronidazole	Baines *et al.*, 2008
C. difficile SU1244	Resistant to fluoroquinolones	Gift from Prof Andrej Weintraub, Karolinska University Hospital, Stockholm, Sweden
B. fragilis GBR7	*B. fragilis nimA* gene present	Löfmark *et al.*, 2005
B. fragilis 638R	*B. fragilis nimA-H* gene absent	Privitera *et al.*, 1979
B. fragilis 615nimJ	*B. fragilis nimJ* present	Husain *et al.*, 2013
B. fragilis 616nimJ		

4.2.2 Antimicrobial susceptibility testing

Susceptibilities to clindamycin (CLI 10 μg), ciprofloxacin (CIP 5 μg), tetracycline (TET 30 μg), cefotaxime (CTX 30 μg) and ceftriaxone (CRO 30 μg) were determined by the disc diffusion method according to the manufacturer's instructions (Oxoid). Three biological repeats for each antimicrobial were performed. The minimum inhibitory concentrations (MICs) of metronidazole (MET), vancomycin (VA), erythromycin (ERY) and moxifloxacin (MOX) were determined by the Etest method according to the manufacturer's instructions (bioMérieux) in single tests. For both methods, the initial bacterial inoculum was prepared in 20 ml Wilkins Chalgren (WC) broth (Oxoid) and after an 18 hour incubation period the broth culture suspensions were normalised to 0.1 OD_{600} in WC medium. The disc diffusion method was performed on WC agar as previously described (Barbut *et al.*, 1999). The diameter of the zones of inhibition surrounding the discs was measured with a ruler. In addition, the Etests were performed on Brucella agar supplemented with blood as previously described (Baines *et al.*, 2008). The metronidazole Etest was also performed on WC agar. Agar plates were incubated anaerobically at 37ºC for 24 hours. The MIC breakpoints for the Etest method were defined as VA ≥ 32 μg/ml, MET ≥ 32 μg/ml, ERY ≥ 8 μg/ml and MOX ≥ 8 μg/ml according to the Clinical and Laboratory Standards Institute (CLSI, 2014). Clinical breakpoints for the

disc diffusion method have not been defined for *C. difficile*. The metronidazole MICs determined on the two types of media were analysed for statistical significance using a Mann-Whitney test performed using SOFAstats (v. 1.4.3.).

4.2.3 Genetic determination of antimicrobial resistance

DNA template, obtained by cell lysis as described in Chapter 2 (section 2.2.4), was used for PCR screening of strains for *nim* and *ermB* genes. Purified genomic DNA, obtained as described in Chapter 3 (section 3.2.2) was used for PCR amplification of *gyrA* and *gyrB*.

Table 4.2 Primers used for genetic-based analysis of antimicrobial resistance

Gene target	Primer sequence (5' → 3')	Size (bp)	Source
C. difficile putative *nim*	F: CCAAATAGCTTCTCTTTCACTACC R: ACATGGAG AGGAGAATTAGC	863	CD630_14590 (AM180355)
B. fragilis nim A-H	F: ATGTTCAGAGAAATGCGGCGTAAGCG R: GCTTCCTTGCCTGTCATGTGCTC	458	Trinh & Reysset, 1996
B. fragilis nimJ	F: GCGCAAACGCCAACAGC* R: GTCGAAACGAATCATCAGCA	498	Husain *et al.*, 2013
ermB	F: AATAAGTAAACAGGTAACGTT R: GCTCCTTGGAAGCTGTCAGTAG	688	Johnson *et al.*, 1999
gyrA	F: AATGAGTGTTATAGCTGGACG R: TCTTTTAACGACTCATCAAAGTT	390	Dridi *et al.*, 2002
gyrB	F: AGTTGATGAACTGGGGTCTT R: TCAAAATCTTCTCCAATACCA	390	Dridi *et al.*, 2002

*The *nimJ* F-primer was designed by Dr R Meggersee, a co-author of the published study (Husain *et al.*, 2013).

4.2.3.1 PCR screening for various *nim* genes

PCR screening for the *B. fragilis nimA-H* genes, using the published "universal" *nim* primers (Table 4.2), was performed (Trinh & Reysset, 1996). *B. fragilis* 638R and *B. fragilis* GBR7 strains were used as negative and positive controls, respectively. PCRs were performed in

25 µl reaction volumes containing KAPA ReadyMix (Kapa Biosystems) and 0.2 µM of each primer. PCR screening for a putative *B. fragilis nimJ* gene, using previously designed primers (Table 4.2), was performed. *B. fragilis* strains *615nimJ* and *616nimJ* strains were used as positive controls (Husain *et al.*, 2013). A PCR using published primers designed to detect a putative *C. difficile nim*-like gene, as published in the genome sequence of *C. difficile* 630 (CD630_14590), was performed. The *C. difficile* 11/11 control strain was included in the screening for the putative *C. difficile nim*-like gene. These PCRs were performed as mentioned above for the *Bacteroides nimA-H* genes, except that reactions contained 0.4 µM of each primer. All reaction mixtures were initially denatured at 95ºC for 5 min, and subjected to 30 cycles of 95ºC for 45 s, 53ºC for 45 s and 72ºC for 1 min, followed by 72ºC for 7 min.

4.2.3.2 PCR screening for the *C. difficile ermB* gene

PCR screening for the *ermB* gene, using published primers (Table 4.2), was performed (Johnson *et al.*, 1999). PCRs were performed in 50 µl reaction volumes containing KAPA ReadyMix (Kapa Biosystems) and 0.7 µM of each primer. A single modification to the published thermal cycling conditions was made: 72ºC for 40 s instead of 3 min.

4.2.3.3 PCR amplification and sequencing of the *C. difficile gyrA* and *gyrB* genes

Published primers targeting the *gyrA* and *gyrB* genes (Table 4.2) were added to separate 25 µl reactions with 100 ng of template and 0.3 µM of each primer in Kapa HiFi HotStart ReadyMix (Kapa Biosystems). *C. difficile* SU1244 is resistant to fluoroquinolones and was used as a PCR positive control for both the *gyrA* and *gyrB* genes. PCRs previously described were modified (Dridi *et al.*, 2002). The reactions were denatured at 95ºC for 5 min, and then subjected to 25 cycles of 98ºC for 20 s, 55ºC for 15 s and 72ºC for 30 s, followed by a final extension at 72ºC for 5 min.

PCR products of *gyrA* and *gyrB* were cloned into the pJet1.2 vector (Thermo Scientific) and sequenced using the pJet1.2-F/R primer pair as described in Chapter 3 (section 3.2.4). Nucleic acid sequences were analysed and amino acid sequences were derived using DNAMAN (v. 4.13). The published nucleic acid sequences for the *gyrA* and *gyrB* genes of the wild type *C. difficile* 630 strain were obtained from the NCBI database (CD630_00060 and CD630_00050, respectively), as well as three Groote Schuur Hospital isolates sensitive to moxifloxacin (GSH9316, GSH4696 and GSH5760) were included in the analysis as controls. Amino acid alignments for GyrA and GyrB were performed using MEGA (v. 5.05) (Tamura *et al.*, 2011). Substitutions were searched for manually.

4.3 Results and Discussion

4.3.1 Antimicrobial susceptibility profiles

The antimicrobial susceptibility profiles of the *C. difficile* clinical isolates were characterised by the Etest and disc diffusion methods. The distributions of minimum inhibitory concentrations (MICs) of vancomycin, metronidazole, erythromycin and moxifloxacin for *C. difficile* isolates belonging to different ribotypes are shown in Figure 4.1 and Table 4.3. In this study, none of the clinical isolates were resistant (≥ 32 µg/ml) to metronidazole or vancomycin (Figure 4.1 a, b and c); however, according to the epidemiological cut-off values (ECOFFs) published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, v. 5.0, valid 2015.01.01), four or five isolates displayed reduced susceptibility to metronidazole (ECOFF ≥ 2 µg/ml), depending on the type of media used. Resistance to moxifloxacin and erythromycin (≥ 8 µg/ml) was found in 32% and 53% of the isolates (Figure 4.1 d and e), respectively, while four isolates (12%) displayed reduced susceptibility to moxifloxacin.

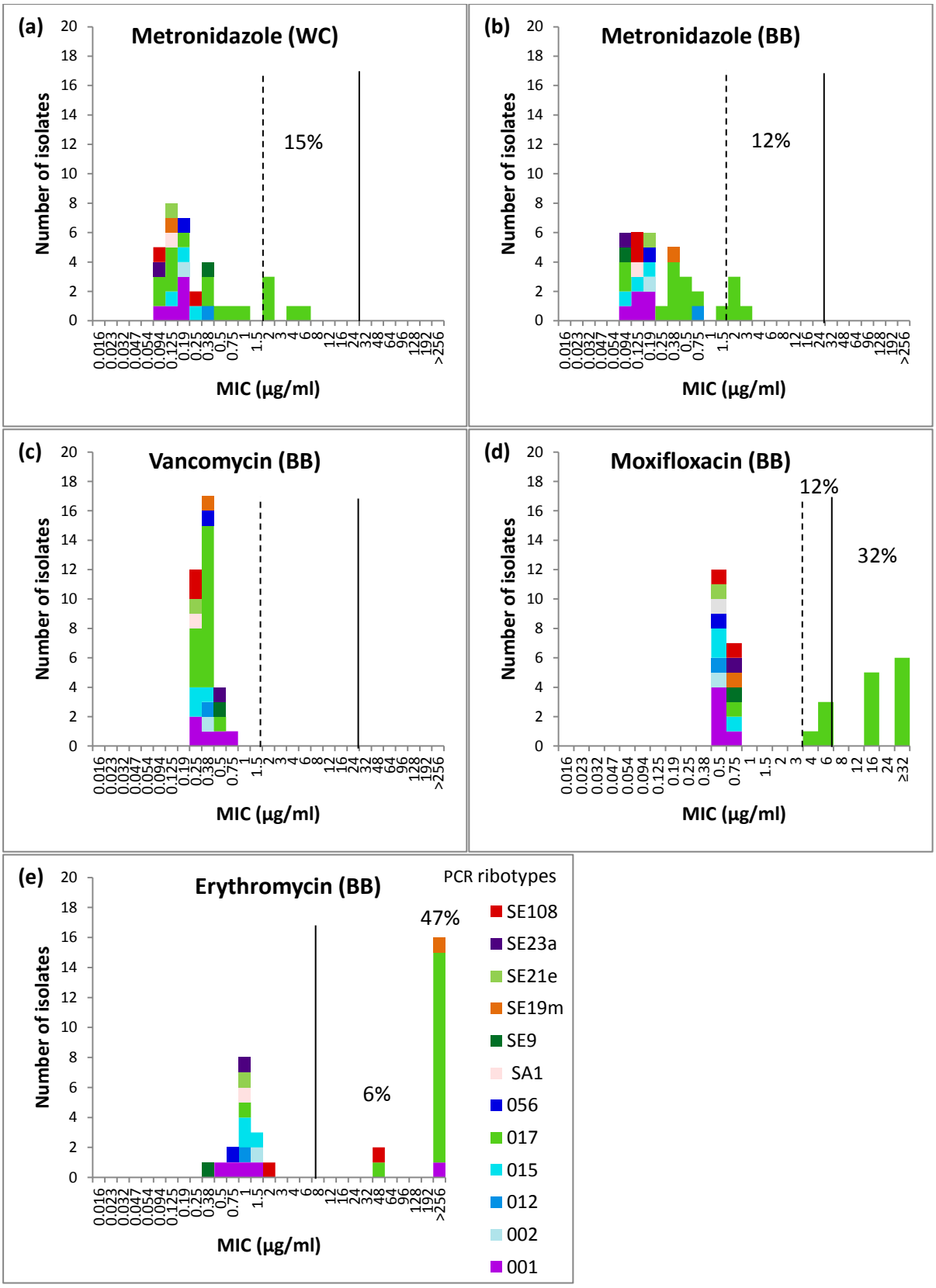


Figure 4.1 The distribution of MICs determined by Etests for clinical isolates belonging to different PCR ribotypes. (a) Metronidazole tested on Wilkins Chalgren (WC) agar, and (b) metronidazole, (c) vancomycin, (d) moxifloxacin and (e) erythromycin tested on Brucella blood (BB) agar. The key to PCR ribotype colours is shown in (e). The ECOFFs are represented by a dashed line. The resistance breakpoints are represented by a solid line.

Table 4.3 Minimum inhibitory concentrations of antimicrobials and the presence of resistance genes or mutations in the GSH clinical isolates

Isolate	Ribotype	MET (µg/ml)	VA (µg/ml)	ERY (µg/ml)	MOX (µg/ml)	*ermB*	*gyrA*/*gyrB* mutation (amino acid substitution)
GSH0574	SE 9	0.064	0.5	0.38	0.75	N	-
GSH1588	001	0.19	0.5	0.5	0.5	N	-
GSH8033	056	0.19	0.38	0.75	0.5	N	-
GSH4115	001	0.125	0.25	0.75	0.5	N	-
GSH0541	SA 1	0.125	0.25	1	0.5	N	-
GSH5681	SE 21e	0.19	0.25	1	0.5	N	-
GSH2753	012	0.75	0.38	1	0.5	N	-
GSH1795	015	0.094	0.25	1	0.5	N	-
GSH9173	015	0.19	0.25	1	0.5	N	-
GSH8162	001	0.094	0.25	1	0.5	N	-
GSH5291	SE 23a	0.094	0.5	1	0.75	N	-
GSH1239	002	0.19	0.38	1.5	0.5	N	-
GSH4696	015	0.125	0.5	1.5	0.75	N	none
GSH9316	001	0.125	0.75	1.5	0.75	N	none
GSH9354	SE 108	0.125	0.25	2	0.5	N	-
GSH1858	SE 108	0.125	0.25	48	0.75	N	-
GSH2702	001	0.19	0.38	>256	0.5	Y	-
GSH6553	SE 19m	0.38	0.38	>256	0.75	Y	-
GSH5760	017	**3**	0.25	>256	0.75	Y	none
GSH5556	017	0.38	0.38	1	**4**	N	*gyrB* (Asp426→Asn)
GSH4693	017	0.5	0.38	>256	**6**	Y	*gyrB* (Asp426→Asn)
GSH5567	017	0.38	0.38	>256	**6**	Y	*gyrB* (Asp426→Asn)
GSH1526	017	0.094	0.38	>256	**6**	Y	*gyrB* (Asp426→Asn)
GSH2529	017	0.094	0.25	48	16	N	*gyrA* (Thr82→Ile)
GSH4722	017	**2**	0.38	>256	16	Y	*gyrA* (Thr82→Ile)
GSH1709	017	**2**	0.38	>256	16	Y	*gyrA* (Thr82→Ile)
GSH2797	017	**2**	0.38	>256	16	Y	*gyrA* (Thr82→Ile)
GSH6999	017	0.75	0.5	>256	16	Y	*gyrA* (Thr82→Ile)
GSH3758	017	0.5	0.25	>256	>32	Y	*gyrA* (Thr82→Ile, Asp71→Glu)
GSH8043	017	0.38	0.25	>256	>32	Y	*gyrA* (Thr82→Ile, Asp71→Glu)
GSH8880	017	0.25	0.38	>256	>32	Y	*gyrA* (Thr82→Ile)
GSH3235	017	1.5	0.38	>256	>32	Y	*gyrA* (Thr82→Ile)
GSH1983	017	0.5	0.38	>256	>32	Y	*gyrA* (Thr82→Ile)
GSH0616	017	0.38	0.38	>256	> 32	Y	*gyrA* (Thr82→Ile)

Circled values indicate resistance and values shown in bold indicate reduced susceptibility. The MIC resistance breakpoints for antimicrobials according to the CLSI (2007) are as follows: MET ≥ 32 µg/ml, VA ≥ 32 µg/ml, ERY ≥ 8 µg/ml and MOX ≥ 8 µg/ml. The MIC breakpoints of reduced susceptibility for antimicrobials according to EUCAST (2015) are as follows: MET ≥ 2 µg/ml, VA ≥ 2 µg/ml and MOX ≥ 4 µg/ml. The MIC breakpoint of reduced susceptibility for ERY is not available. Y and N indicate the presence and absence of the *ermB* gene, respectively. 'None' indicates the absence of mutations in the *gyrA* and *gyrB* genes. All the ribotype 017 strains had the Ser366→Ala substitution in GyrB. A dash (-) indicates that the respective experiment was not conducted as it was not applicable.

Table 4.4 Minimum inhibitory concentrations of antimicrobials and the presence of resistance genes or mutations in the *C. difficile* reference and control strains

Reference strain	Ribotype	MET (µg/ml)	VA (µg/ml)	ERY (µg/ml)	MOX (µg/ml)	*ermB*	*gyrA*/*gyrB* mutation
M120	078	0.125	0.38	0.75	0.5	N	-
CD305	023	0.19	0.38	1	0.5	N	-
CD196	027	0.25	0.38	1	0.5	N	-
CF5	017	0.25	0.38	1	0.75	N	-
630	012	0.094	0.25	(>256)	0.5	Y	none
M68	017	1	0.38	(>256)	**6**	Y	*gyrB* (Asp426→Val)
R20291	027	1	0.25	(>256)	(12)	Y	*gyrA* (Thr82→Ile)
11/11	001	6 - 8	0.38	(>256)	(> 32)	-	-
700057	038	<0.016	0.5	2	<0.002	-	-

Circled values indicate resistance and values shown in bold indicate reduced susceptibility. The MIC resistance breakpoints for antimicrobials according to the CLSI (2007) are as follows: MET ≥ 32 µg/ml, VA ≥ 32 µg/ml, ERY ≥ 8 µg/ml and MOX ≥ 8 µg/ml. The MIC breakpoints of reduced susceptibility for antimicrobials according to EUCAST (2015) are as follows: MET ≥ 2 µg/ml, VA ≥ 2 µg/ml and MOX ≥ 4 µg/ml. The MIC breakpoint of reduced susceptibility for ERY is not available. Y and N indicate the presence and absence of the *ermB* gene, respectively. 'None' indicates the absence of mutations in the *gyrA* and *gyrB* genes. All the ribotype 017 strains had the Ser366→Ala substitution in GyrB. A dash (-) indicates that the respective experiment was not conducted as it was not applicable. *C. difficile* 11/11 was used as a reference strain for metronidazole resistance and, therefore, the *ermB* gene and mutations in the *gyrA* or *gyrB* genes were not screened for.

Metronidazole

The MIC distribution of metronidazole tested on Wilkins Chalgren (WC) ranged from 0.094 – 2 µg/ml (Figure 4.1 a), whereas MICs tested on Brucella blood (BB) agar ranged from 0.094 – 6 µg/ml (Figure 4.1 b). The distribution of MICs for the various ribotypes differed between the two media. However, the difference in MICs between them was not statistically significant ($p = 0.762$). Published studies have reported that the composition of the agar can affect the magnitude of the MIC (Baines *et al.*, 2008). While all the isolates in this study were sensitive to metronidazole (≤ 32 µg/ml), five isolates (15% of the study group) displayed reduced susceptibility (ECOFF ≤ 2 µg/ml) when tested on WC agar (Figure 4.1 a), and four isolates (12%) displayed reduced susceptibility when tested on BB agar (Figure 4.1 b). The *C. difficile* 11/11 control strain (ribotype 001), known to display reduced susceptibility had an MIC of 6 – 8 µg/ml (Table 4.4). Published studies have reported reduced susceptibility to metronidazole in *C. difficile* belonging to ribotypes 001 and 010 (Baines *et al.*, 2008; Moura

et al., 2013; Peláez *et al.*, 2008). In this study, all isolates belonging to ribotype 001 were sensitive to metronidazole (0.064 – 0.19 μg/ml) (Figure 4.1). Ribotype 027 has also been reported to display reduced metronidazole susceptibility (Martin *et al.*, 2008); however, the ribotype 027 reference strains in this study were sensitive. Interestingly, all the isolates displaying reduced susceptibility on both media belonged to ribotype 017. Reduced susceptibility of ribotype 017 strains to metronidazole has not been previously reported. The MICs of these 4 – 5 isolates are clinically relevant as they are near the upper limit of the metronidazole concentration that can be achieved in the intestine during therapy (approximately 9.5 μg/g faeces) (Bolton & Culshaw, 1986).

Vancomycin

MICs of vancomycin were distributed over a narrow range (0.25 – 0.75 μg/ml) as shown in Figure 4.1 (c) and all the clinical isolates were sensitive to vancomycin (ECOFF ≤ 2 μg/ml). This sensitivity to vancomycin has also been reported by European-wide studies (Barbut *et al.*, 2007; Freeman *et al.*, 2015). A few studies have reported reduced susceptibility or resistance (≥ 32 μg/ml) to vancomycin. However, different antimicrobial testing methods were used and it is, therefore, not possible to compare those MICs to the current study (Chia *et al.*, 2013; Ngamskulrungroj *et al.*, 2015). Unlike the case for metronidazole, it is possible to achieve vancomycin concentrations as high as 1000 – 5000 μg/ml in the intestine (Edlund *et al.*, 1997). The low MICs of vancomycin reported by this study suggest that vancomycin remains an effective antimicrobial for treating CDI within this setting.

Moxifloxacin

Only ribotype 017 isolates displayed resistance or reduced susceptibility to moxifloxacin. The occurrence of resistance to moxifloxacin (≥ 8 μg/ml) was 32% (Figure 4.1 d) and four isolates (12%) displayed reduced susceptibility (ECOFF ≥ 4 μg/ml), as well as the *C. difficile* M68

and R20291 reference strains (Table 4.4). Some published studies report that resistance to moxifloxacin has been associated significantly with previous use of fluoroquinolones while other studies disagree (Ackermann *et al.*, 2003; Barbut *et al.*, 2007). It was not possible to gather data regarding previous antimicrobial use for patients enrolled in the current study. Such data is important in future work to determine whether fluoroquinolone use is a risk factor for developing CDI at Groote Schuur Hospital. In this study, only one ribotype 017 isolate, GSH5760, was sensitive to moxifloxacin (Table 4.3). Within the MLVA minimum spanning tree (Chapter 3), three isolates (GSH4693, GSH5567 and GSH1526) out of the four that displayed reduced susceptibility to moxifloxacin were clustered together. A further common phenotype amongst these three was their resistance to erythromycin (≥ 256 μg/ml) while the fourth isolate (GSH5556) showing reduced susceptibility to moxifloxacin clustered separately and was sensitive to erythromycin (1 μg/ml) (Table 4.3).

Erythromycin

The occurrence of resistance to erythromycin (≥ 8 μg/ml) was 53%. The MICs for two isolates were 48 μg/ml and it was ≥ 256 μg/ml for the remaining resistant isolates. These latter values were regarded as highly resistant (Table 4.3). An ECOFF value for erythromycin has not been published for *C. difficile* by EUCAST, and so isolates displaying reduced susceptibility could not be determined. The highly resistant isolates were ribotype 017 strains with the exception of isolates GSH6553 and GSH2702, which were ribotypes SE19m and 001, respectively (Table 4.3). In the MLVA minimum spanning tree (Chapter 3), the ribotype SE19m isolate has the least genetic distance from ribotype 027 reference strain R20291, which also displayed high-level resistance to erythromycin (Table 4.4).

Eleven isolates (32%) displayed co-resistance to erythromycin and moxifloxacin and their MICs are circled in Table 4.3. These isolates were all ribotype 017. Moxifloxacin resistance

has been commonly found to be together with erythromycin and/or clindamycin resistance in *C. difficile* (Ackermann *et al.*, 2003; Lachowicz *et al.*, 2014), although there seems to be no genetic link between the two resistance mechanisms. Published studies have also described ribotype 017 as a multi-drug resistant pathogen (Drudy *et al.*, 2007a; Kim *et al.*, 2012b; Pituch *et al.*, 2011). Although the ribotype 017 isolates appeared to be more resistant than the non-017 isolates, statistical analysis to determine whether this was significant could not be done as the study group was not large enough.

Disc diffusion

The susceptibility to clindamycin, ciprofloxacin, ceftriaxone, cefotaxime and tetracycline was determined by the disc diffusion method due to the high costs of Etest strips. The distribution of antimicrobial susceptibilities, defined by the diameter of the zone of inhibition, for Groote Schuur Hospital clinical isolates is summarised in Figure 4.2. Clinical breakpoints for the disc diffusion method have not been defined for *C. difficile*. Therefore, the proportion of clinically resistant isolates to these antimicrobials could not be determined. However, 50%, 44% and 71% of the GSH isolates gave no zone of inhibition around the discs containing clindamycin, ciprofloxacin and cefotaxime, respectively (Figure 4.2 a, b and d), suggesting resistance to these antimicrobials. Overall, clinical isolates showed no zones or smaller zones of inhibition to cefotaxime than to ceftriaxone, both of which are third-generation cephalosporins (Figure 4.2 c and d). One isolate, ribotype 012, formed a zone much smaller than the rest of the study group to tetracycline (Figure 4.2 e). A diameter of 14 mm may be indicative of resistance. A previous European study reported tetracycline resistance primarily in ribotype 012 and 048 strains (Spigaglia *et al.*, 2011). Overall, in this study, isolates forming smaller zones of inhibition were primarily ribotype 017.

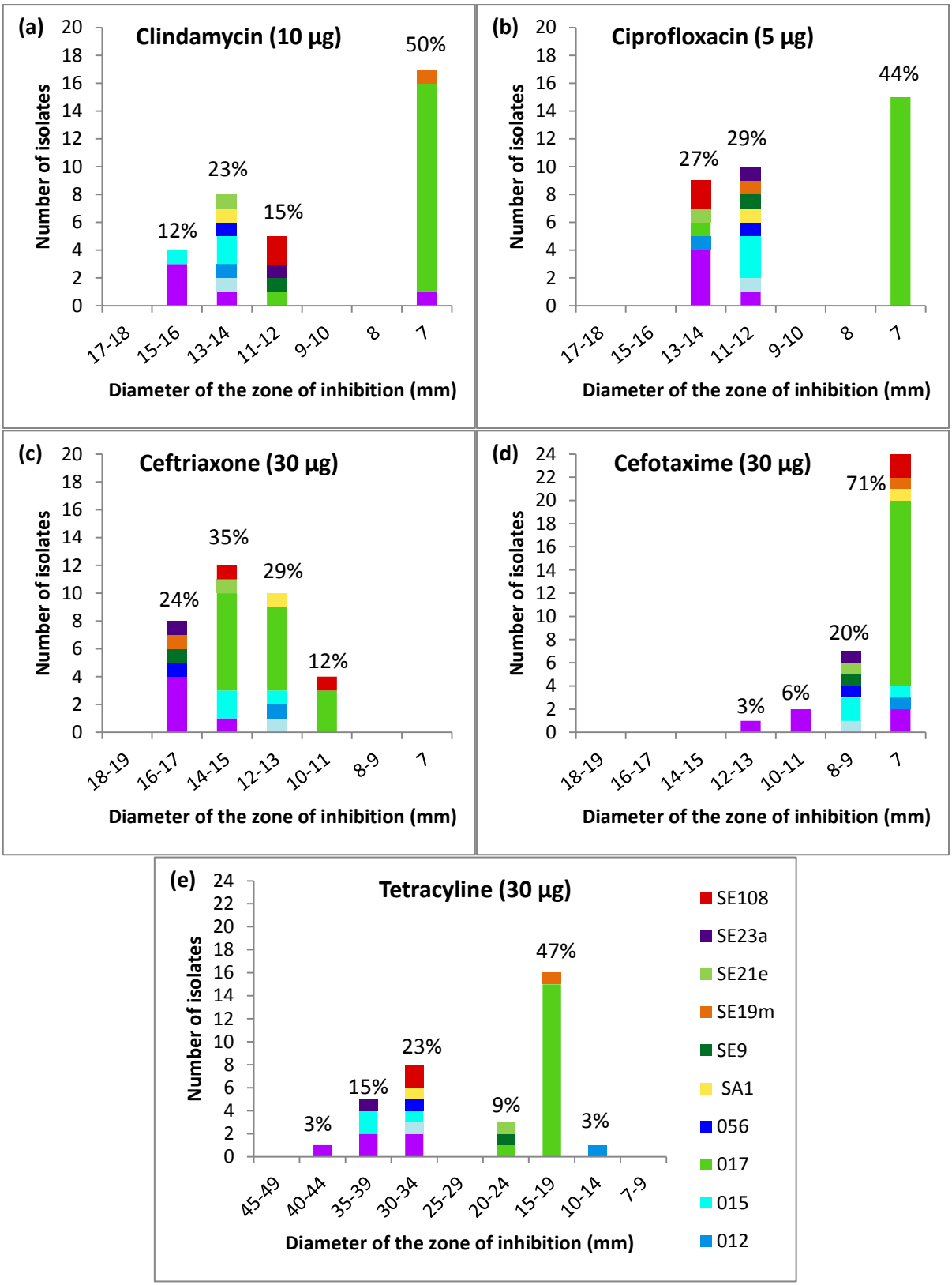


Figure 4.2 The distribution of antimicrobial susceptibilities determined by disc diffusion. (a) Clindamycin, (b) ciprofloxacin, (c) ceftriaxone, (d) cefotaxime and (e) tetracycline tested on Wilkins Chalgren (WC) agar. The key to PCR ribotype colours is shown in (e). The graphs are oriented according to increasing antimicrobial resistance, with smaller zones of inhibition plotted on the right. A diameter of 7 mm represents the diameter of the disc (i.e. no zone of inhibition/full antimicrobial resistance to the disc content).

The Etest method is an effective method of determining the MIC for particular antimicrobials, however it is expensive and, therefore, not all antimicrobials could be tested by this method. The disc diffusion method is much cheaper, and was used for older generation antimicrobials, none of which are used to treat CDI. A published study correlated the diameters produced by disc diffusion to MICs produced by Etests and demonstrated disc diffusion as a reliable method for distinguishing susceptible and resistant *C. difficile* strains (Erikstrup *et al.*, 2012). The data presented here cannot be compared to the published study as only vancomycin, metronidazole and moxifloxacin were investigated. The clinical breakpoint values specific for *C. difficile* have yet to be established. However, the data reported serves as an indication of potential resistance displayed by various isolates, especially those belonging to ribotype 017. Future work on potentially resistant strains could include Etests to confirm true resistance.

4.3.2 Genetic determinants of antimicrobial resistance

To investigate the possible genetic mechanism of the reduced susceptibility to metronidazole observed in *C. difficile*, PCRs were performed to screen for the various published *nim* genes, which often play a role in metronidazole resistance in *B. fragilis*. The *B. fragilis* "universal" *nim* primers that target conserved sequences in the *Bacteroides nimA-H* genes and the specific *B. fragilis nimJ* primers, successfully amplified targets in the respective *B. fragilis* positive control strains. However, none of the *C. difficile* clinical isolates were shown to harbour homologues of these genes (4.3 a and b). An additional putative chromosomal *nim* gene, which had been annotated in the *C. difficile* genome sequence, was screened for by PCR using gene-specific primers. This *C. difficile nim*-like gene was found in all the *C. difficile* clinical isolates regardless of their metronidazole sensitivity or reduced susceptibility (Figure 4.3 c).

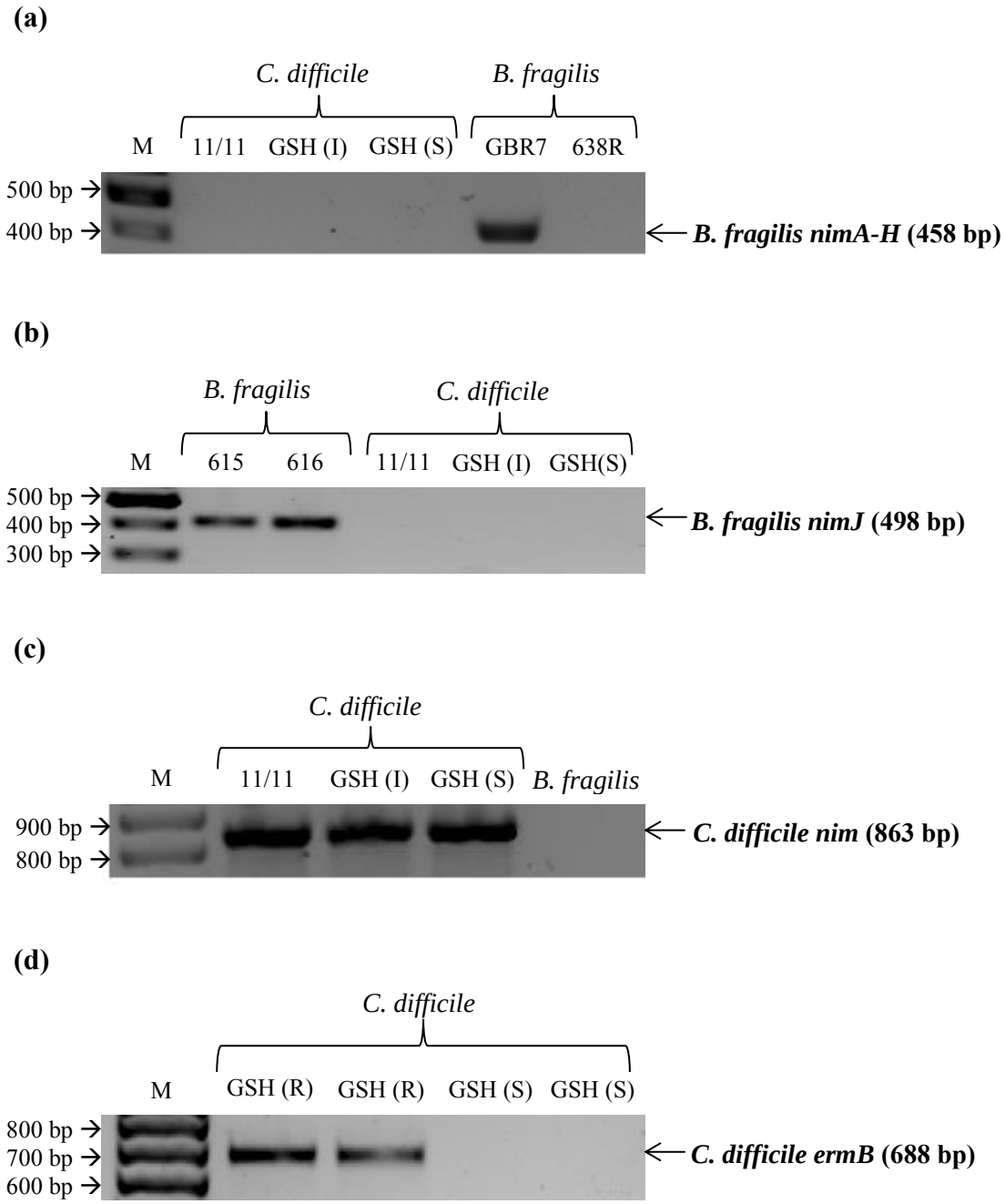


Figure 4.3 Representative gel images showing PCR screening for the various *nim* genes and the *C. difficile ermB* gene: (a) The *B. fragilis nimA* gene present in the *B fragilis* GBR7 control strain, (b) the *B. fragilis nimJ* gene present in the *B. fragilis 615nimJ* and *616nimJ* control strains, (c) the putative *C. difficile nim*-like gene present in the *C. difficile* 11/11 control strain and all the GSH clinical isolates, and (d) the *C. difficile ermB* gene present in the erythromycin resistant *C. difficile* isolates. S = sensitive, I = reduced susceptibility and R = resistant. M, 100 bp DNA Molecular weight ladder.

The primers targeting the *B. fragilis nimA-H* genes have successfully amplified homologous *nim* genes in several species including one strain of *Clostridium bifermentans* (Lubbe *et al.*, 1999); however, it has not been possible to detect *nim* genes in *C. difficile* using these primers (Brazier *et al.*, 2001; Peláez *et al.*, 2008). Annotated genome sequences for *C. difficile* have revealed several putative *nim*-like genes amongst different strains, although functional characterisation of these genes is lacking. The *C. difficile* gene-specific primers, designed to detect a chromosomally annotated putative *nim*-like gene, recognised it in all the clinical isolates, irrespective of whether they were sensitive or resistant to metronidazole. Its presence in the isolates sensitive to metronidazole suggests that it is not involved in the observed phenotype of reduced susceptibility to metronidazole.

The underlying genetic mechanism for the reduced susceptibility to metronidazole observed in some of the *C. difficile* clinical isolates has, therefore, yet to be determined. A recent study, using whole genome sequencing, reported SNPs in several genes involved in essential metabolic pathways of a stably metronidazole resistant strain that may confer resistance (Lynch *et al.*, 2013). A recent proteomic study reported the increased expression of several proteins, such as putative 5-nitroimidazole reductases, and proteins involved in DNA repair, in a stably metronidazole resistant *C. difficile* strain (Chong *et al.*, 2014). This was observed even in the presence of metronidazole, which reduced the cell density and sporulation of the resistant strain. These putative 5-nitroimidazole reductases are expressed by genes other than the *nimA-H* or *nimJ* genes that were screened for in this current study, and could be targets in future studies. The identification of these proteins suggests that several factors and pathways may be associated with metronidazole resistance in *C. difficile* and this warrants further investigation.

Primers targeting the *C. difficile ermB* gene were used to investigate the underlying genetic basis for erythromycin resistance. The *ermB* gene was found in *C. difficile* isolates displaying

high-level resistance (HR) to erythromycin (≥ 256 µg/ml), which represents 47% of the clinical isolates (Figure 4.3 d and Table 4.5). *C. difficile* reference strains 630, M68 and R20291 also displayed high-level resistance to erythromycin and harboured the *ermB* gene (Table 4.4). These results suggest that the *ermB* gene is a common determinant for high-level resistance to erythromycin among the Groote Schuur Hospital clinical isolates. Resistant isolates with an MIC of 48 µg/ml did not harbour the *ermB* gene. The mechanism by which these isolates display some level of resistance has yet to be determined. Alternative erythromycin resistance mechanisms could include other *erm* genes, efflux pumps or a mutation in the target sequence in the 23S rRNA (Ambrose *et al.*, 2005; Eady *et al.*, 1993; Wittmann *et al.*, 1973). Future investigations of efflux pump activity could include examining the effect of efflux pump inhibitors, such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP), reserpine or sodium orthovanadate, on antimicrobial susceptibility (Drudy *et al.*, 2006; Spigaglia *et al.*, 2011).

Table 4.5 A summary of erythromycin susceptibility and the presence of the *ermB* gene among the clinical isolates

Susceptibility	Etest MIC (µg/ml)	Prevalence	*ermB* gene
S	0.38 - 1.5	47%	N
R	48	6%	N
HR	≥ 256	47%	Y

S = sensitive, R = resistant and HR = high level resistance. Y and N indicate the presence and absence of the *ermB* gene.

The *gyrA* and *gyrB* genes of all the ribotype 017 isolates were sequenced as only strains of this ribotype displayed some level of resistance to moxifloxacin. The results are shown in

Table 4.3 and summarised in Table 4.6. All clinical isolates with a moxifloxacin MIC of 16 or ≥ 32 µg/ml had a mutation in the *gyrA* gene resulting in an amino acid substation of Thr82→Ile. Isolates GSH3758 and GSH8043 had an additional mutation in *gyrA* leading to an Asp71→Glu substitution. All the clinical isolates with an MIC of 4 or 6 µg/ml had a mutation in the *gyrB* gene leading to an Asp426→Asn amino acid substitution, while the *C. difficile* M68 reference strain (ribotype 017) with an MIC of 6 µg/ml had an Asp426→Val substitution in GyrB (Table 4.4). All the ribotype 017 strains, including the *C. difficile* M68 reference strain and the single sensitive ribotype 017 isolate (GSH5760), had the Ser366→Ala substitution in GyrB.

Table 4.6 A summary of moxifloxacin susceptibility and the presence of resistance-causing amino acid substitutions in GyrA or GyrB subunits of the ribotype 017 isolates

Susceptibility*	**E-test MIC (µg/ml)**	**Prevalence**	**Amino acid substitution**	
			GyrA	**GyrB‡**
S	0.5 or 0.75	3%	none	none
I	4 or 6	25%	none	Asp426→Asn
R	16	69%	Thr82→Ile	none
HR	≥32		Thr82→Ile Asp71→Glu†	none

*S = sensitive, I = reduced susceptibility, R = resistant and HR = high level resistance.
†Asp71→Glu was observed in only two isolates, GSH3758 and GSH8043, as shown in Table 4.3.
‡All the ribotype 017 isolates had the Ser366→Ala substitution in GyrB.

None of the mutations found in the clinical isolates were novel. The Thr82→Ile and Asp71→Glu substitutions in GyrA, and the Asp426→Asn substitution in GyrB have been previously published and associated with fluoroquinolone resistance (Dridi *et al.*, 2002; Drudy *et al.*, 2006). The Asp426→Val substitution in GyrB of the *C.difficile* M68 has also

been previously published, and was found in all the ribotype 017 strains displaying high-level resistance to fluoroquinolone (Drudy *et al.*, 2006). The Ser366→Ala substitution in GyrB has been previously published; however, it does not play a role in fluoroquinolone resistance (Drudy *et al.*, 2006). This is in agreement with the presence of the Ser366→Ala in a moxifloxacin sensitive ribotype 017 isolate. Two sensitive non-ribotype 017 isolates, GSH4696 and GSH9316, were also selected for sequencing. Like the sequences of the wild type *C. difficile* 630 strain obtained from NCBI, they did not harbour any substitutions in GyrA or GyrB (Tables 4.3 and 4.4).

The minimum spanning tree (Chapter 3) depicts GSH6999, GSH3758 and GSH8043 as isolates of the same MLVA type; however, isolate GSH6999 does not have the Asp71→Glu amino acid substitution in GyrA. As discussed in Chapter 3, using extended MLVA (eMLVA) may be able to differentiate isolate GSH6999 as a separate MLVA type from the other two. In this study, the *gyrA* mutation resulting in a single amino acid substitution of Thr82→Ile was associated with high-level resistance to moxifloxacin, whereas the *gyrB* mutation resulting in Asp426→Asn was associated with intermediate-level resistance.

4.4 Conclusion

Antimicrobial susceptibility testing revealed resistance among the clinical isolates. All the clinical isolates were sensitive to vancomycin, while a few ribotype 017 isolates displayed reduced susceptibility to metronidazole, although this did not reach the levels of clinical significance. Erythromycin resistance was associated with *ermB*-positive isolates, while moxifloxacin resistance was associated with a Thr82→Ile amino acid substitution in GyrA. These resistant characteristics were observed in 47% and 32% of the clinical isolates, respectively; and co-resistance was observed in 32% of the clinical isolates.

Antimicrobial pressure may explain the high prevalence of the 017 ribotype among the clinical isolates. The high prevalence of fluoroquinolone resistant ribotype 017 isolates may be a reflection of the high use of moxifloxacin to treat respiratory tract infections in Groote Schuur Hospital. In Korea, the increase in ribotype 017 strains occurred as moxifloxacin use was introduced (Lee *et al.*, 2014). The majority of the ribotype 017 strains were resistant to moxifloxacin, as well as clindamycin, erythromycin and ciprofloxacin. While the data regarding specific antimicrobial treatment of the patients enrolled in this study were not available, the data reported by this study are of value to the hospital in the overall future monitoring of antimicrobial resistance among the patients and can be used to inform antimicrobial prescribing policies. Restriction of high-risk antimicrobials can be used as a strategy to reduce CDI.

Antimicrobial characterisation revealed that ribotype 017 isolates were primarily high-level and multi-drug resistant. This resistance profile may provide them with an advantage over non-017 isolates to persist in the host intestine and lead to infection. Colonisation is another important step in the development of CDI. In order to determine whether ribotype 017 isolates have an advantage over the non-017 isolates, aspects of colonisation potential were investigated and the results reported in Chapter 5 of this thesis.

CHAPTER FIVE

THE CHARACTERISATION OF THE TOXIN PRODUCTION, SPORULATION AND AUTO-AGGREGATION CAPACITIES OF *CLOSTRIDIUM DIFFICILE* ISOLATES

Contents

5.0 Abstract

Colonisation of the host intestine by *C. difficile* together with the production of toxins are important steps in the development of infection. Sporulation then allows CDI patients to shed spores, which aid in the transmission of *C. difficile*. Several factors are thought to play a role in colonisation. These include the presence of adhesins, such as the phase-variable CwpV protein. Adhesins are expressed on the bacterial cell surface and may induce cellular aggregation of *C. difficile* and subsequently contribute towards colonisation. In the study reported here, attempts were made to determine the sporulation frequency and toxin production levels of the isolated strains. However, it was not possible to evaluate them or their possible relationship to colonisation due to the high physiological variation between replicate experiments. However, an auto-aggregation assay revealed that the ribotype 017 strains were significantly better auto-aggregators when compared to the other ribotypes investigated. A PCR targeting an inversion region upstream of the *cwpV* gene revealed that all the clinical isolates contained individuals within the population carrying the region in either orientation regardless of the differing auto-aggregating capacities of the population as a whole. Semi-quantitative RT-PCR was performed to determine the transcription level of *cwpV* in three isolates of different auto-aggregation phenotypes. These showed differences in the transcription level between the isolates, suggesting that the expression level of CwpV may play a role in auto-aggregation.

5.1 Introduction

The development of disease caused by *C. difficile* is influenced by several factors. The ability to efficiently colonise the human host intestine and produce biologically active toxins during infection, and the capacity to produce spores have all been proposed to play a role in the acquisition and development of CDI. While the predominance of PCR ribotype 017 strains in this current study may be related to their resistance to multiple antimicrobials, additional factors such as their toxin levels, sporulation capacity, and ability to auto-aggregate and form biofilms may also play a role in their increased prevalence. These aspects were investigated in this study.

The disease caused by *C. difficile* is mainly mediated by toxins A and B. There is variation in the amount of toxin produced by different strains. For example, isolates belonging to the ribotype 027 clade have been associated with increased and/or prolonged toxin production, thereby causing more severe disease (Akerlund *et al.*, 2008; Vohra & Poxton, 2011; Warny *et al.*, 2005). Conversely, A-B+ strains only produce functional toxin B, but are still able to cause severe disease (Drudy *et al.*, 2007b; Kim *et al.*, 2008; Kuijper *et al.*, 2001). As discussed in Chapter 2, all but two of the *C. difficile* isolates investigated in this study harboured the toxin genes; however, the poor clinical sensitivities of the EI-based tests did not successfully establish the presence of toxins in many of the stool specimens. In order to determine whether the *C. difficile* isolated from the stool specimens have the potential to cause the disease experienced by the symptomatic patients, it is important to determine whether the various *C. difficile* strains have the ability to produce biologically active toxins. Toxins can be detected and quantitated using a cell culture neutralisation assay (CCNA) or an enzyme-linked immunosorbent assay (ELISA) (Hernández-Rocha *et al.*, 2013; Lyerly *et al.*, 1983; Nguyen *et al.*, 1990). Alternatively, Darkoh *et al.* (2011b) developed a quantitative assay that detects toxin A and B activity. The assay involves using a chromogenic substrate, *p*-nitrophenyl-ß-D-

glucopyranoside (PNPG), which is stereochemically similar to UDP-glucose, the substrate naturally targeted by toxins A and B. Cleavage of PNPG can be measured spectrophotometrically. This assay does not require specialised facilities and is cost effective.

The ability of the bacteria to form spores plays an important role in CDI, since these structures are highly resistant to many environmental and antimicrobial factors enabling them to persist in the environment aiding in disease transmission (Fawley *et al.*, 2007; Goldenberg *et al.*, 2012; Sjöberg *et al.*, 2014). An increased sporulation frequency of some strain types, such as ribotype 027 strains, has been proposed to aid in their survival and spread (Akerlund *et al.*, 2008; Fawley *et al.*, 2007). However, other studies have also reported that sporulation frequency is not associated with the strain type (Burns *et al.*, 2010, 2011).

Colonisation of the intestine requires the bacterium to interact with, and adhere to, the host tissue. This is mediated by cell surface-associated adhesion proteins. Amongst these, cell wall protein V (CwpV) has been reported to potentially play a role in colonisation by promoting auto-aggregation of *C. difficile* (Emerson *et al.*, 2009; Reynolds *et al.*, 2011). CwpV has a conserved N-terminal domain involved in cell wall anchoring and a variable C-terminal domain which mediates aggregation. The protein is encoded by the *cwpV* gene and expressed in a phase-variable manner, which is mediated by inversion of an upstream DNA region. One orientation allows the expression of *cwpV* (ON), while in the other orientation the gene is not transcribed (OFF).

To further characterise the local clinical *C. difficile* isolates, their abilities to produce toxins, their sporulation frequency, and their auto-aggregation capacity were investigated and reported in this chapter.

5.2 Materials and Methods

5.2.1 Bacterial strains

The Groote Schuur Hospital *C. difficile* isolates listed in Table 2.5 (Chapter 2) and the *C. difficile* reference strains listed in Table 3.1 (Chapter 3) were included in the various analyses.

5.2.2 Quantitative toxin activity assay

Toxin A and B activities were detected by the Cdifftox activity assay for clinical *C. difficile* isolates using 30 mM PNPG, as previously described (Darkoh *et al.*, 2011b). Cleavage of the PNPG substrate was measured spectrophotometrically at an absorbance of 405 nm since the filter for the published wavelength of 410 nm was not available to this study. The *C. difficile* R20291 and CD196 reference strains were included in the analysis as positive controls for the production of both toxins A and B.

5.2.3 Sporulation frequency assay

Sporulation frequency was measured using a published method (Burns *et al.*, 2011). Starter cultures were prepared by growing cells for 18 hour in 20 ml BHI broth. Fresh broth (20 ml) was inoculated with 200 µl starter culture and cultures were incubated until the OD_{600} was between 0.4 and 0.6 to allow any spores to germinate. Finally, this was used to inoculate fresh broth for the sporulation assay (time = 0) and incubated at 37ºC up to 120 hours. Total viable counts, as well as spore formation, were enumerated at 0 hour, 24 hours, 48 hours and 120 hours incubation of culture. At each time point, a volume of 500 µl culture was subjected to heat shock at 65ºC for 25 min, and then serially diluted in PBS. At each time point, another volume of 500 µl culture was serially diluted in PBS (without heat-shock), for the total viable count. A volume of 100 µl was spread plated onto BHI agar supplemented with the bile salt

taurocholate (0.1% w/v, Sigma). The R20291 reference strain was used as a control as its sporulation frequency has been published (Burns *et al.*, 2011).

5.2.4 Auto-aggregation assay

A previously published assay method was used (Purcell *et al.*, 2012). The *C. difficile* clinical isolates and reference strains were grown in 15 ml BHI broth in test tubes at 37ºC for 18 hours. Without mixing the cultures, the optical density at 600 nm was measured at 1 cm below the surface of the broth culture. The cultures were then vortexed until the aggregates were fully dispersed, and the optical densities were measured again. The auto-aggregation percentage was calculated as [(ODpost-vortex – ODpre-vortex) / ODpost-vortex] × 100. Five biological repeats were performed. The auto-aggregating capacities between the ribotype 017 and the non-017 strains were analysed for statistical significance using a Mann-Whitney test performed using SOFAstats (v. 1.4.3.).

5.2.5 Orientation-specific PCR targeting the inversion region upstream of the *cwpV* gene

The orientation of the inverted region upstream of the *cwpV* gene was determined by PCR analysis. Two separate reactions amplifying the ON and OFF orientations were performed using published primers (Table 5.1) (Emerson *et al.*, 2009). A total of 100 ng gDNA and 0.2 µM of each primer were used in each 50 µl reaction using KAPA ReadyMix (Kapa Biosystems). Reactions were denatured at 95ºC for 5 min, and then subjected to 35 cycles of 95ºC for 30 s, 45ºC for 30 s and 72ºC for 30 s, followed by a final extension at 72ºC for 7 min. All the *C. difficile* clinical isolates and reference strains were included in the analyses.

PCR products of the ON and OFF orientation of the *C. difficile* 630 reference strain were cloned into the pJet1.2 vector (Thermo Scientific) and sequenced using the pJet1.2-F/R primer pair as described in Chapter 3 (section 3.2.4). The nucleic acid sequence of the inversion

region of the published *C. difficile* 630 strain as well as R20291, CD196, M68 and CF5 were obtained from the NCBI database and were included for nucleic acid sequence alignment using MEGA (v. 5.05) (Tamura *et al.*, 2011).

Table 5.1 Primers used to investigate the *cwpV* gene

Gene Target	**Primer sequence (5' → 3')**	**Size (bp)**	**Reference**
ON-cwpV	F: TTTAAGGTAAGTTTGATTTTTATGTTAATGAAT R: GCATTTTTCCATCCATCTTG	300	Emerson *et al.*, 2009
OFF-cwpV	F: ATTCATTAACATAAAAATCAAACTTACCTTAAA R: GCATTTTTCCATCCATCTTG*	400	
cwpV	F: TGGTGAAAAAGGACTTGCAG R: CATCACCATTAGGCATGTTG	700	
16S rRNA	R907: AAACTCAAAGGAATTGACGG R1391: GACGGGCGGTGTGTACAA	502	Metcalf *et al.*, 2010

* The same reverse primer was used for the amplification of both the ON and OFF orientation of the region upstream from *cwpV*.

5.2.6 RNA extraction and reverse transcription PCR of the *cwpV* gene

The expression of the *cwpV* gene in *C. difficile* was investigated by reverse-transcription PCR (RT-PCR). The 16S rRNA gene was used as a reference gene to normalise the expression level of *cwpV* (Metcalf *et al.*, 2010). Three biological replicates were analysed.

Total RNA was extracted from *C. difficile* strains using a modified hot phenol method (Rafudeen, 2001). Briefly, *C. difficile* was cultured in 10 ml BHI broth until the OD_{600} was between 0.4 and 0.6 and harvested by centrifugation (2500 x g for 10 min). Cell pellets were resuspended in 500 µl resuspension buffer (0.3 M sucrose, 0.01 M sodium acetate, pH 4). To lyse the cells, 500 µl of lysis buffer (2% SDS, 0.01 M sodium acetate, pH 4) was added and the mixture was incubated at 65ºC for 1.5 min. A volume of 1 ml water-saturated phenol

(pH 4) was added. The samples were mixed, incubated further at 65ºC for 3 min and then cooled at -80ºC for 1 min. The aqueous phase was recovered by centrifugation (8000 x g for 5 min). Hot phenol extractions were repeated until the aqueous phase was clear. RNA was precipitated by the addition of 1/6.6 vol of 3 M sodium acetate (pH 5.5) and 3 vol absolute ethanol, and left at -80ºC for 30 min. After centrifugation (10 000 rpm for 10 min) the pellet was resuspended in 200 µl 1X DNase I Buffer (Thermo Scientific). DNase I (Thermo Scientific) was added (10 U), and the samples were left at 37ºC for 3 hours. Finally, DNase-treated RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

RNA was reverse transcribed into cDNA using the Tetro cDNA Synthesis Kit (Bioline) according to the manufacturer's instructions, using 5 µg total RNA and random hexamers. A 5 µl volume of cDNA was used to amplify the *cwpV* and 16S rRNA genes in a multiplex PCR using published primers (Table 5.1) (Emerson *et al.*, 2009; Metcalf *et al.*, 2010). PCRs were performed in 50 µl reaction volumes containing KAPA ReadyMix (Kapa Biosystems) and 0.2 µM of each *cwpV* and 16S rRNA primers. The reactions were denatured at 95ºC for 5 min, and then subjected to 30 cycles of 95ºC for 30 s, 45ºC for 30 s and 72ºC for 40 s, followed by a final extension at 72ºC for 7 min. Genomic DNA from the M68 reference strain was used as a PCR control.

The expression level of *cwpV* was semi-quantified by determining the band intensity of the gene-specific PCR product relative to that of the 16S rRNA gene using Image Lab Software (Bio-Rad).

5.3 Results and Discussion

5.3.1 Toxin activity of the clinical isolates

The quantitative toxin assay of Darkoh *et al.*, (2011b) was attempted in order to determine whether the clinical isolates produce biologically active toxins and whether some strains produce more toxins than others. Four *C. difficile* strains were selected to test the toxin assay: a non-toxigenic strain (GSH0541), a ribotype 017 strain (GSH6999), and the CD196 and R20291 positive control reference strains. However, toxin production of the clinical isolates could not be quantitated as the toxin activity could not be detected, even in the published R20291 ribotype 027 reference strain, which is known to have increased toxin production (Akerlund *et al.*, 2008; Vohra & Poxton, 2011; Warny *et al.*, 2005). The assay involved cleavage of a substrate by the toxins in the culture supernatant resulting in a colour change, which was quantified using a spectrophotometer. The OD_{405} of the clinical isolates did not differ from the negative control, containing no culture supernatant. Using a wavelength of 405 nm instead of 410 nm did not pose a problem as a Nanodrop analysis showed no significant difference in the absorbance of *p*-nitrophenol between these two wavelengths. This toxin assay was used as it did not require the specialised facilities that are required for other types of assay and were not available to this study. It is unclear why the assay was not successful and after several attempts the assay was discontinued. An alternative more sensitive toxin assay method is currently being performed by a USA-based collaborator (Merrigan *et al.*, 2013).

5.3.2 Sporulation frequency of the clinical isolates

The sporulation assay was performed in order to determine whether some isolates had a higher sporulation frequency, and whether there was an association between sporulation frequency and ribotype. The more frequent production of spores by certain ribotype groups may lead to a

larger reservoir of spores belonging to those strains in the hospital environment, allowing them to spread more easily, which may help to explain the increased prevalence of various ribotypes. The following strains were used to test the sporulation assay: a non-toxigenic strain (GSH0574), two ribotype 017 strains (GSH6999 and GSH3758), a ribotype 002 strain (GSH1239), and the R20291 ribotype 027 reference strain as a positive control. Sporulation was measured based on colony forming units (CFU) derived from spores after heat shock. However, an accurate sporulation frequency for the clinical isolates could not be determined as the assay posed certain complications. Firstly, the number of heat-resistant CFU at time = 0 could not be normalised as was required by the method (Burns *et al.*, 2011). In order to accurately follow the production of spores, it is essential to begin the assay with zero spores at the time of inoculation (time = 0). In an attempt to do this, starter broth cultures were prepared by inoculating 18 hour broth cultures into fresh broth and incubating until the OD_{600} was between 0.4 and 0.6 to allow any residual spores to germinate. These were then inoculated into fresh broth for the sporulation assay. However, aliquots removed at this time point still showed evidence of heat-resistant spores. This may be the result of slow germinating spores from the starter culture that only began to germinate once plated onto the taurocholate-containing agar medium. Secondly, the sporulation frequency varied greatly between biological repeats. The bacterial growth rate or the rate of sporulation of the various strains may be sensitive to unknown factors in the growth conditions and media. Additionally, spore resistance and germination ability may vary even within strain types, and all these factors may explain the variation across the repeated experiments. The assay was discontinued after several biological and technical repeat experiments failed to give reproducible results.

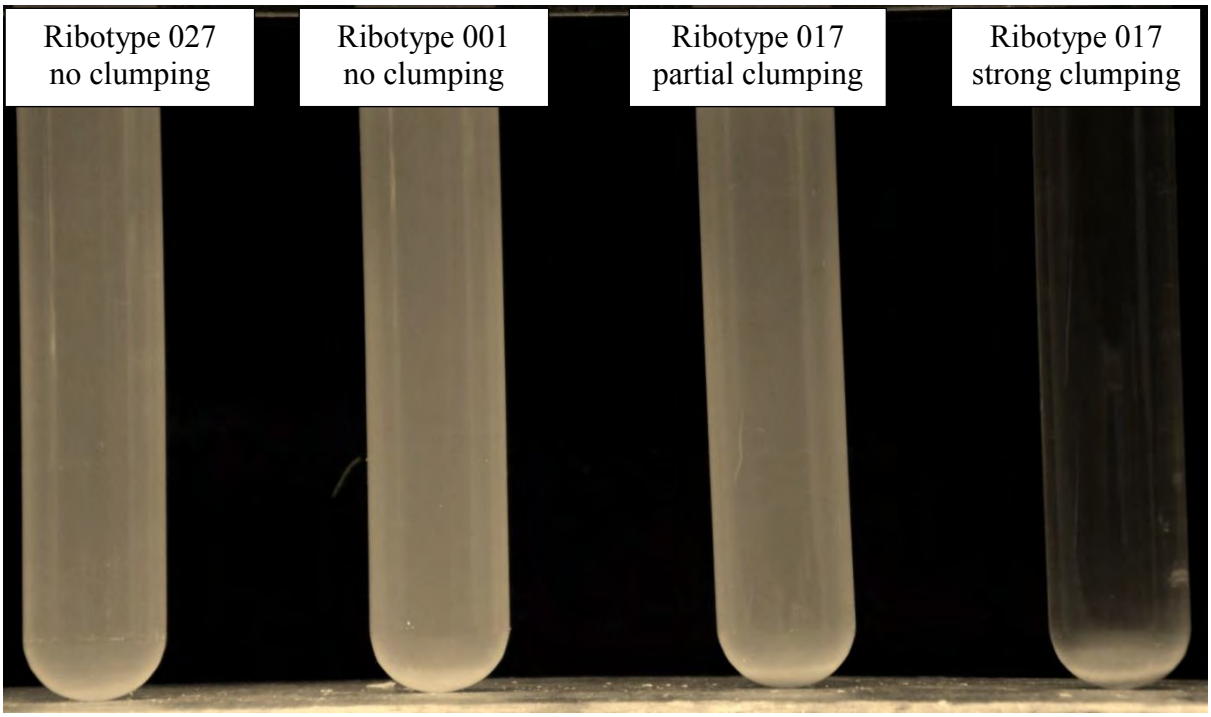
Published studies have reported conflicting data as to whether there is a direct correlation between sporulation and ribotype, with some reporting that the sporulation frequency can vary among isolates belonging to the same ribotype (Burns *et al.*, 2010, 2011; Lynch *et al.*, 2013).

Even studies that included the same *C. difficile* strains for analyses have reported differing results, suggesting that there is too much variation using the current methods. An alternative method to measure sporulation involves the use of phase-contrast microscopy (Burns *et al.*, 2011). This method is simpler as it does not require treatment of bacterial broth culture and it is easy to distinguish spores from vegetative cells (the difference can be further enhanced by staining) (Fawley *et al.*, 2007; Underwood *et al.*, 2009). Unfortunately, there is no "preferred" method for determining sporulation frequency and the development of an improved method is necessary to determine the sporulation of *C. difficile* more accurately.

5.3.3 Auto-aggregation

When the *C. difficile* isolates were cultured in broth throughout the study, the ribotype 017 strains showed a strong tendency to auto-aggregate and settle at the bottom of the growth container. The settled cells formed a tight clump that required vigorous vortexing for cell dispersal. An assay was performed to determine whether the *C. difficile* strains were able to aggregate to one another within a population, and whether there was an association between the auto-aggregating capacity and ribotype. A greater auto-aggregating capacity by certain ribotypes may enhance their ability to colonise the host intestine, thereby out-competing other ribotypes during the colonisation process. Various auto-aggregating phenotypes in bacterial broth culture were observed among the *C. difficile* clinical isolates and reference strains (Figure 5.1). The auto-aggregation percentage of the various strains was calculated and is an indication of the proportion of the bacterial population within the broth culture that was involved in auto-aggregation (Figure 5.2).

(a)



(b)

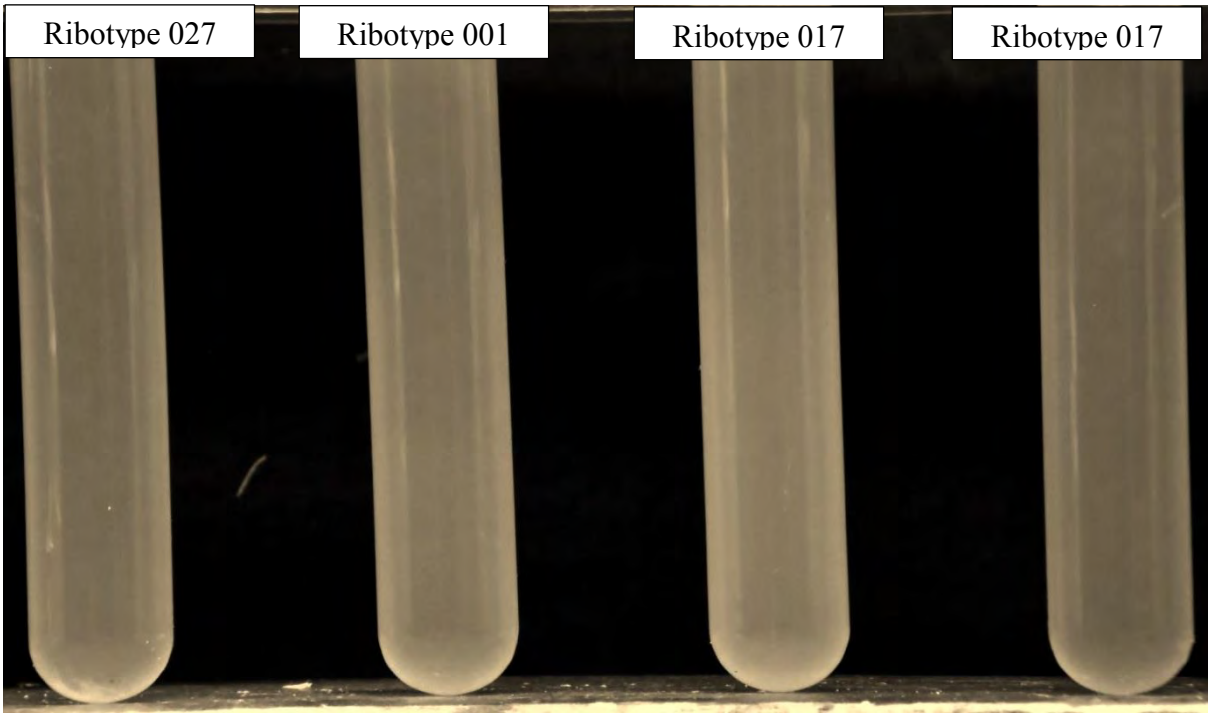


Figure 5.1 Auto-aggregation of *C. difficile* bacterial cells. (a) A representation of the various auto-aggregating phenotypes observed in BHI broth culture. The turbidity of the broth culture is evenly distributed and no clumping is observed in ribotype 027 and 001. Partial and complete clumping is observed in the ribotype 017 strains. (b) A representation of broth cultures after vortexing to disperse bacterial aggregates.

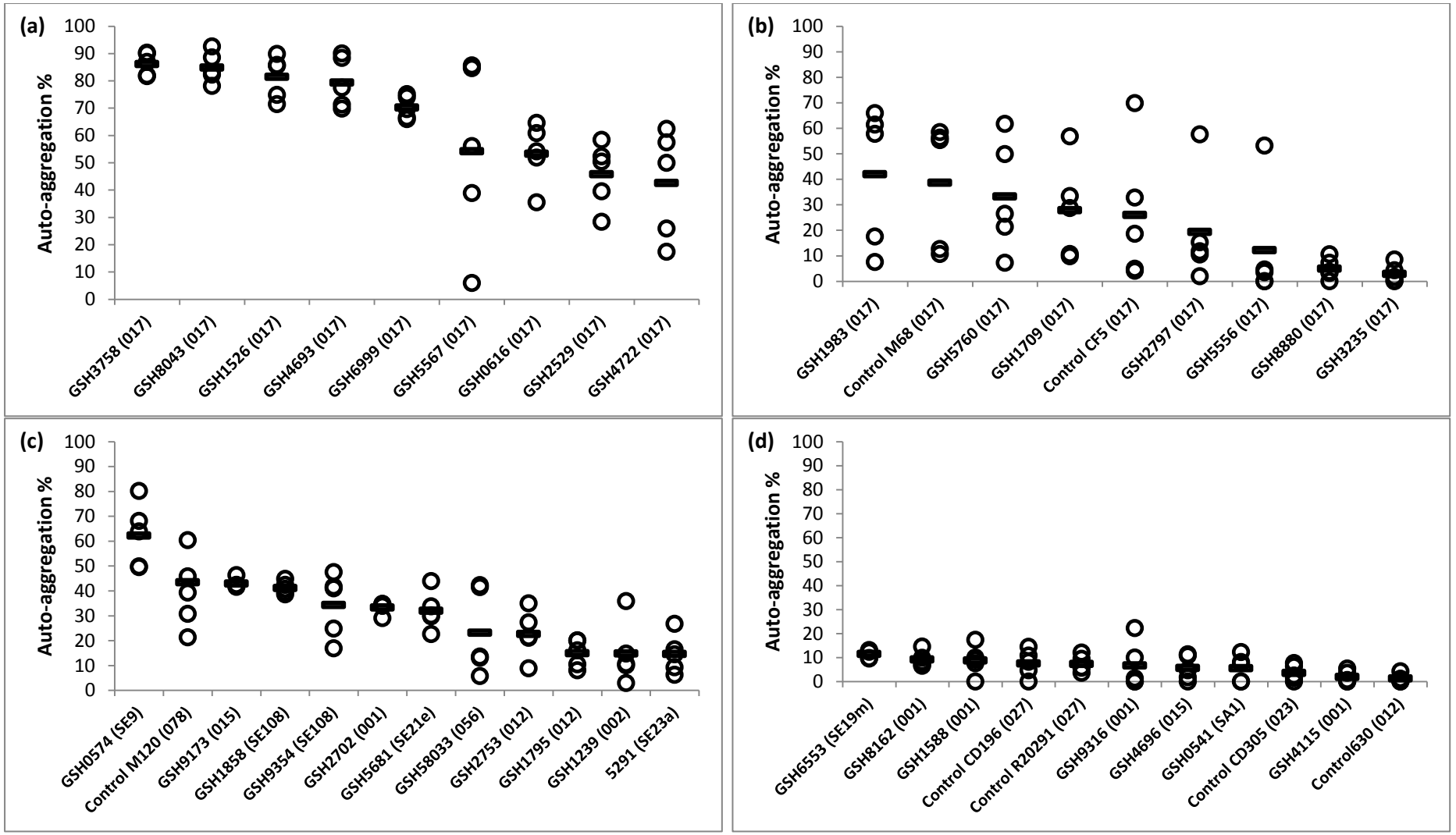


Figure 5.2 Auto-aggregation percentages. The distribution of auto-aggregation percentages of the *C. difficile* isolates and control strains. Ribotypes are given in parentheses. Each circle represents one of five biological repeats. The bold line represents the mean of the five biological replicate experiments.

C. difficile strains that produced bacterial clumps at the bottom of the test tube with clear broth before vortexing; represented by the strong clumping of a ribotype 017 strain in Figure 5.1 (a), had auto-aggregation percentages as high as 80%. Isolates that displayed very little auto-aggregation (< 15%) are represented by ribotypes 027 and 001 in Figure 5.1 (a), where the turbidity of the broth culture was evenly distributed and no clumping was observed. Other phenotypes were also observed, where only some bacterial clumping occurred (as represented by the ribotype 017 partial clumping in Figure 5.1 a) or the turbidity of the broth culture was concentrated towards the bottom of the test tube (not shown). Overall, the clinical isolates belonging to the ribotype 017 group (Figure 5.2 a and b) had significantly higher auto-aggregating capacities (p = 0.011) than the non-017 isolates (Figure 5.2 c and d). Auto-aggregation was found to be consistently highest in isolate GSH3758 (ribotype 017), and consistently lowest in isolate GSH4115 (ribotype 001) and the control strain 630 (ribotype 012). A few isolates, including GSH5567 (ribotype 017), GSH5760 (ribotype 017) and GSH9354 (ribotype SE108), displayed a wide range of auto-aggregating percentages across the various replicate experiments.

Very few published studies have investigated auto-aggregation of *C. difficile*, but those that have done so also observed different auto-aggregation phenotypes (Faulds-Pain *et al.*, 2014; Purcell *et al.*, 2012; Reynolds *et al.*, 2011). The significantly higher auto-aggregating capacity of the ribotype 017 strains in the study reported in this thesis is interesting, since this phenotype may aid the bacterial cells during colonisation. Auto-aggregation could allow *C. difficile* to adhere to those *C. difficile* cells that are already attached to the host tissue, thereby possibly enhancing their ability to colonise the intestine. Aggregation of *C. difficile* is also important for biofilm formation, which plays a role in survival, persistence, resistance to antimicrobials and colonisation of the host intestine (Dawson *et al.*, 2012; Flemming & Wingender, 2010). Bacterial motility, conferred by flagella, plays a role in colonisation

(Eaton *et al.*, 1992; Ottemann & Lowenthal, 2002). However, according to preliminary analysis of the *C. difficile* isolates, motility did not appear to be a factor in the cell clumping as both motile and non-motile isolates showed strong auto-aggregation phenotypes (results not shown).

The CwpV protein reportedly promotes auto-aggregation of *C. difficile* (Reynolds *et al.*, 2011). The expression of this protein is phase variable, which may explain the various auto-aggregating phenotypes observed between the *C. difficile* clinical isolates, as well as variation within a strain in replicate experiments.

5.3.4 Orientation of the inversion region upstream from the *cwpV* gene

In order to investigate a possible genetic basis for the various auto-aggregating phenotypes observed in the clinical strains isolated in this study, the inversion region upstream of the *cwpV* gene was amplified using primers that separately targeted the ON and OFF transcription orientations (Figure 5.3).

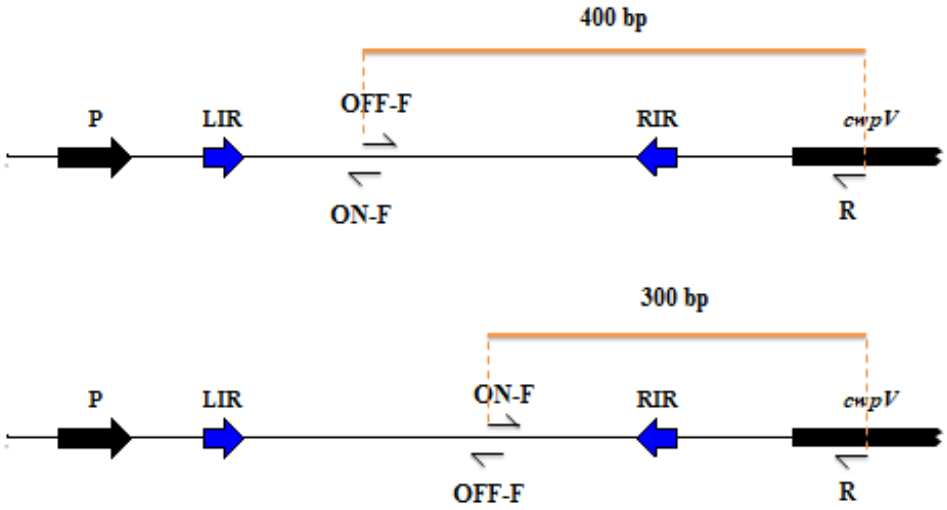


Figure 5.3 Representation of the annealing sites of the primers to probe the orientation of the inversion region upstream of the *cwpV* gene. The R primer anneals outside the inverted repeats (LIR and RIR). The OFF-F and ON-F primers anneal inside the inversion region in opposite orientations. A 400 bp fragment was amplified when the inversion region was in the OFF orientation, and a 300 bp fragment was amplified when the sequence was inverted (ON orientation) (Adapted from Emerson *et al.*, 2009).

Both the ON and OFF orientations of this region were successfully amplified in all the clinical isolates and reference strains regardless of their differing auto-aggregating phenotypes. A representative gel showing these two PCR products is shown in Figure 5.4.

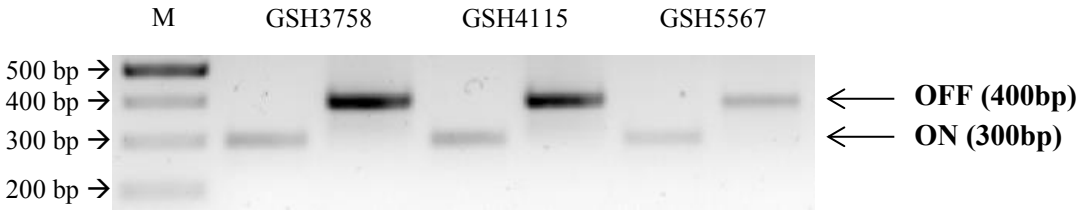


Figure 5.4 PCR detection of the orientation of the inversion region. A representative gel image showing fragments of the ON and OFF orientation of the region upstream from the *cwpV* gene amplified from GSH3758 (a highly-aggregating isolate) GSH4115 (a non-aggregating isolate) and GSH5567 (an isolate of varying auto-aggregating capacity).

The PCR products of both orientations of the *C. difficile* 630 reference strain were sequenced and compared to the nucleotide sequences of the inversion region in the published *C. difficile* 630 genome sequence obtained from NCBI. Nucleotide sequence alignments of both orientations confirmed that the correct region was amplified from the genome of the clinical isolates.

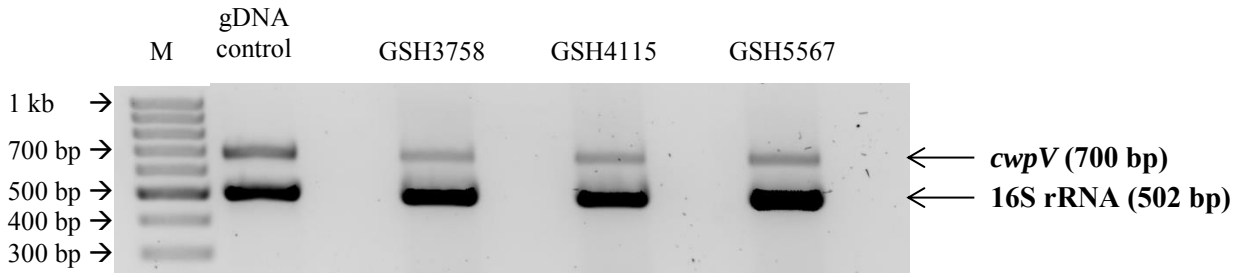
A published study reported that a non-aggregating strain only had the OFF orientation of the inversion region and did not express CwpV due to a mutation in the region (Emerson *et al.*, 2009). All the non-aggregators in this current study had the ON orientation of the inversion region in addition to the OFF orientation. While the ON orientation was present in the non-aggregating isolates of this current study, often the band intensity of the OFF PCR product appeared to be stronger than the ON PCR product (Fig 5.4). It is possible that differences in expression of the *cwpV* gene may play a role in the observed different auto-aggregation phenotypes. Published studies have reported that throughout the growth period only 5 – 10% of cells within a population express CwpV (Emerson *et al.*, 2009; Reynolds *et al.*, 2011). This percentage has been shown to increase (up to 95% of the cell population expressing the gene) in lysogenic *C. difficile* R20291 cells carrying the ΦCD38-2 prophage, possibly due to interference with the RecV-mediated recombination process responsible for the ON-OFF inversion (Sekulovic & Fortier, 2014).

5.3.5 Semi-quantification of *cwpV* gene expression by RT-PCR

A semi-quantitative RT-PCR pilot investigation was done to determine the approximate levels of transcription of the *cwpV* gene in strains showing different auto-aggregation phenotypes. Three *C. difficile* isolates of different auto-aggregating capacities were selected: GSH3758, which had the highest auto-aggregation percentage, GSH4115, which had the

lowest, and GSH5567, which displayed the widest range of auto-aggregating phenotypes (Figure 5.2). The transcription level of *cwpV* was evaluated semi-quantitatively by analysing the band intensity of the *cwpV* PCR product in the agarose gel image as compared with the corresponding 16S rRNA PCR product as an internal reference gene to normalise the values of the expression levels. Based on the band intensity, presented as a ratio, the highest expression level was observed in the high auto-aggregating strain, GSH3758, while various expression levels were observed among the three biological repeats of GSH5567 which was previously shown to have variable auto-aggregation capacity (Figure 5.5).

(a)



(b)

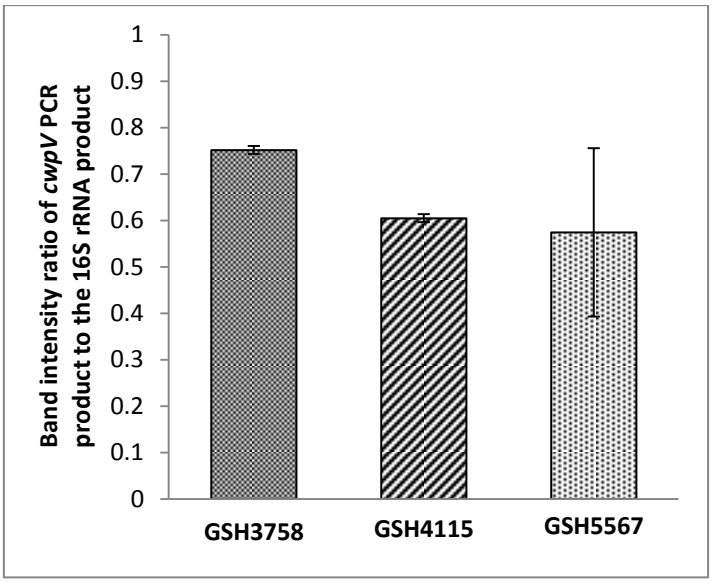


Figure 5.5 Semi-quantification of the *cwpV* gene expression by RT-PCR. (a) RT-PCR of the *cwpV* and 16S rRNA gene transcripts. The cDNA of the *cwpV* and 16S rRNA gene were amplified in a multiplex reaction. Genomic DNA of GSH3758 was used as a PCR control. (b) The band intensity of the *cwpV* PCR product as a measure of gene expression. The mean band intensity and standard error of the *cwpV* PCR product expressed as a ratio to the 16S rRNA PCR product. Error bars represent standard error of 3 biological replicates.

The potentially varying *cwpV* expression levels and the varying auto-aggregating phenotypes displayed by GSH5567, and the uniform *cwpV* expression levels of GSH3758 and GSH4115, which both also displayed consistent auto-aggregating percentages, suggest that the expression level of the *cwpV* gene may have played a role in the observed auto-aggregation

phenotypes. However, RT-PCR was performed only on three *C. difficile* isolates as a preliminary investigation of *cwpV* expression levels and this can only be considered a pilot experiment since the sample set was small and the statistical significance of the differences between the expression levels of the various isolates could not be determined. Additionally, end-point RT-PCR analysis, as opposed to fully quantitative real-time PCR (qRT-PCR), is only able to provide an indication of major differences in the expression level of genes and does not take account of possible differences in the primer amplification efficiencies across the three strains. However, the results do warrant further investigation. Quantitation of *cwpV* expression can be improved using qRT-PCR and including validated reference genes in the analysis (Bakker *et al.*, 2012; Metcalf *et al.*, 2010). In addition, immunolabeling using antibodies raised to the CwpV protein could be used to visually assess the number of cells expressing the protein (Sekulovic & Fortier, 2014).

The different auto-aggregation phenotypes may also be attributed to the nature of the C-terminal domain of the CwpV protein, which has been shown to vary across *C. difficile* strain types and to promote different auto-aggregation phenotypes (Reynolds *et al.*, 2011). A published study over-expressed the CwpV protein from several *C. difficile* strains in a *C. difficile* 630 Δ*cwpV* mutant and found that they affected the ability of the strain to auto-aggregate (Reynolds *et al.*, 2011). The CwpV protein from the *C. difficile* 630 strain performed the best at inducing auto-aggregation, followed by the CwpV protein from a ribotype 027 strain, while the same protein from a ribotype 017 strain did not induce aggregation in the *C. difficile* 630 Δ*cwpV* mutant. In the study reported in this thesis (Figure 5.2), the *C. difficile* 630 reference strain had the least auto-aggregating ability among the entire sample set, and the ribotype 027 reference strains also had low auto-aggregating percentages. Compared to these strains, the ribotype 017 strains were significantly better auto-aggregators. However, it should be noted that the two studies are not directly

comparable since slightly different assay methods were used. The auto-aggregation assay in the published study examined cell clumping over time in a dense suspension of cells, starting with an OD_{600nm} of 10 (Reynolds *et al.*, 2011), whereas, the current study examined clumping after 16 hours of growth in broth medium (Purcell *et al.*, 2012).

Further research investigating the genetic basis for *cwpV* expression and its possible role in auto-aggregation is necessary to support these results. Future work should include making a *cwpV* mutation in a strong auto-aggregating ribotype 017 strain and observing any physiological changes. Mutation using ClosTron is not an option as the strong auto-aggregators all contained the *ermB* gene and are resistant to erythromycin and lincomycin, which are the antimicrobials used to select for intron insertion during the mutation process (Heap *et al.*, 2007, 2010). However, recently developed allelic exchange-based methods can be used to create *cwpV* mutants (Ng *et al.*, 2013).

Additional candidate proteins or factors that are known to contribute to or regulate *C. difficile* auto-aggregation should also be investigated. These include cyclic diguanylate and flagellar modifications (Bordeleau *et al.*, 2015; Faulds-Pain *et al.*, 2014; Purcell *et al.*, 2012). These were not investigated in the current study and offer alternative targets for analysis. To determine whether any of these proteins potentially play a role in auto-aggregation, a proteomics analysis can be conducted whereby the expression of these proteins during auto-aggregation can be compared between a strong aggregating and a weak aggregating clinical strain (Chong *et al.*, 2014).

5.4 Conclusion

The data reported here showed that the ribotype 017 strains were significantly better auto-aggregators than the other ribotypes tested. The literature suggests that this may play a role in colonisation, and further experiments should aim at assessing whether this strong auto-aggregation phenotype is associated with enhanced adherence to intestinal cells and/or more robust biofilm formation. If this is so, the *cwpV* expression levels of GSH3758, GSH4115 and GSH5567 should be fully characterised to determine whether they were in agreement with their auto-aggregation phenotypes. In this preliminary study, semi-quantitative RT-PCR of a subset of strains suggested that the expression level of *cwpV* may play a role in auto-aggregation and subsequently a potential role in colonisation. Future studies should include determining whether mutations in or the knockout of *cwpV* in a strong auto-aggregating strain reduces its aggregation and biofilm formation ability. Alternative accurate quantitative methods for determining the expression level, such as qRT-PCR or immunolabeling of the protein, should also be included.

CHAPTER SIX

GENERAL CONCLUSIONS

Prior to this study, very little was known about the epidemiology or the pathogenesis of *C. difficile* strains potentially spreading and causing disease in patients attending Groote Schuur Hospital. At the initiation of this study, an EI-based toxin protein detection diagnostic method was used routinely at this hospital to detect *C. difficile* in stool specimens from diarrhoea patients. However, it was not known whether this diagnostic method was clinically valid. This project was designed as a pilot study to determine the most appropriate, accurate and sensitive method for the detection of *C. difficile* in the hospital setting. In addition, *C. difficile* was isolated and characterised at the molecular and phenotypic levels in order to gain insight into the identity, epidemiology and pathogenesis of these strains.

C. difficile was detected by selective culture and gene-specific PCR in a significant proportion of symptomatic patients in the local setting. However, the toxin detection assay did not successfully determine whether these isolates could produce biologically active toxins. In addition, the EI-based methods designed to detect the *C. difficile* toxins A and B in stool specimens were not clinically sensitive, and were, therefore, not recommended as a stand-alone assay in the local setting. Based on the evaluation conducted in this study, the Microbiology Laboratory of the NHLS at Groote Schuur Hospital adopted the GeneXpert *C. difficile* detection method as it performed the best in confirming the presence of *C. difficile* DNA. However, the limitation of the GeneXpert technique is that, although it targets some *C. difficile* toxin-encoding genes (which can indicate the presence of the organism in stool specimen) it cannot diagnose the patients as actively CDI-positive since it cannot detect the disease-causing toxin proteins themselves. Future studies could make use of CCNA or

ELISA to detect actual *C. difficile* toxin production in addition to using the GeneXpert for DNA detection.

Through strain typing of the *C. difficile* isolates and through molecular and phenotypic characterisation, PCR ribotype 017 was identified as the prevalent strain among the isolates. *C. difficile* ribotype 017 strains differed significantly from the other strain types. Genotyping based on both MLVA and sequencing of the *tcdC* gene grouped the ribotype 017 strains in a single and separate clade from the other *C. difficile* isolates with a toxin profile of A-B+. The PCR ribotype 017 strain has reached epidemic proportions in European and Asian hospitals, and it is overall the most prevalent strain type in Asia (Dobreva *et al.*, 2013; Hawkey *et al.*, 2013; Lee *et al.*, 2014; Pituch *et al.*, 2011), but is less common at other international sites (Cheknis *et al.*, 2009; Tenover *et al.*, 2011). By expanding the pilot study to other hospitals in Cape Town and the rest of the country, it could be determined whether the prevalence of ribotype 017 is the norm within the larger Cape Town area and/or South Africa as a whole. If this ribotype predominates in South Africa, a large cohort comparison of this strain type from all these hospitals could determine whether they have pathogenic characteristics in common. In addition, a comparison of the patient risk factors in the hospital settings could reveal why and how these ribotype 017 strains are predominant in these particular hospital settings. Other genotyping methods such as MLST or WGS could also be used in future studies to determine the source and/or the transmission of these ribotype 017 isolates within the local setting, as well as other hospitals (Cairns *et al.*, 2015; Didelot *et al.*, 2012; Eyre *et al.*, 2013a, b; Walker *et al.*, 2012). These two typing techniques can be used to track the accumulation of changes in the respective targeted regions of the genome over time, which could be used to reconstruct the transmission pattern of *C. difficile* strains. Obtaining samples from hospital surfaces or the hands of healthcare workers could be used to determine whether environmental contamination is a source and/or contributes to the transmission of CDI. A

recent study involved WGS analysis of ribotype 017 strains isolated from patients and the hospital environment (Cairns *et al.*, 2015). Data generated was used in conjunction with patient and hospital information to trace which strains were transmitted between patients or via ward contamination, which were community-acquired or which persisted on hospital surfaces over a long period of time. All this information would be useful in implementing appropriate infection control procedures.

The GeneXpert *C. difficile* detection method was developed based on the emergence of the hyper-virulent ribotype 027 strain in North America and Europe. With the emergence of ribotype 017 strains worldwide with a high prevalence in some European and Asian hospitals, as well as the local setting of this study, it would be more useful for epidemiological purposes to use a diagnostic test that could differentiate between toxinogenic A+B+ and A-B+ strains or presumptively detect PCR ribotype 017. While the Hain Lifescience assay evaluated in this study was not as sensitive as the GeneXpert, it may have value in the local setting as it targets both toxin A and B genes; however it also has a longer turnaround time than the GeneXpert leading to delays in the implementation of appropriate therapy for patients. Based on the molecular characterisation conducted in this study, a NAAT could additionally screen for the *tcdA* gene with the 1.8 kb deletion, the *tcdC-sc7* genotype, and the various mutations in the *gyrA* and *gyrB* genes in order to presumptively identify ribotype 017 strains.

Antimicrobial susceptibility profiling revealed that the ribotype 017 strains were generally resistant to high-levels of antimicrobials, and were often multi-drug resistant. Reduced susceptibility to metronidazole and resistance to moxifloxacin were characteristic of only ribotype 017 strains, while resistance to erythromycin, clindamycin and ciprofloxacin were primarily a characteristic of these strains, but was also observed in some of the other ribotypes. The high-level antimicrobial resistance profile may explain the high prevalence of ribotype 017 isolated from patients attending the Groote Schuur Hospital where these

antimicrobials are generally in common use for the treatment of infections (CDI and other) and may favour selection of these bacteria. A cohort study could be conducted in the Groote Schuur Hospital to investigate specifically whether certain antimicrobials, such as moxifloxacin, are in fact risk factors for the development of CDI in the local setting. This study would involve monitoring the development of CDI in symptomatic patients and collecting data regarding administration of antimicrobials, other treatment received or procedures performed, as well as their diagnosis (Pépin *et al.*, 2005).

The presence of the *ermB* gene among the erythromycin-resistant strains and the presence of previously characterised mutations in the *gyrA* and *gyrB* genes among the strains displaying some level of resistance to moxifloxacin suggest that these are the genetic determinants responsible for resistance to these antimicrobials. The basis for the reduced susceptibility to metronidazole observed amongst some ribotype 017 strains could not be determined, although this is likely to be a result of several contributing factors rather than a single underlying genetic mechanism (Chong *et al.*, 2014; Lynch *et al.*, 2013).

Auto-aggregation analysis revealed that the ribotype 017 strains isolated in this study were significantly stronger auto-aggregators than the other ribotypes. Auto-aggregation may be important for biofilm formation, which plays a role in colonisation. Future studies could involve an adherence assay to determine whether the strong auto-aggregating strains are also more efficient in forming biofilms *in vitro* on abiotic surfaces (Dawson *et al.*, 2012). This increased auto-aggregation capacity may enable the ribotype 017 strains to out-compete other ribotypes in the colonisation process. To investigate this, a gut model could be infected with a strong auto-aggregating strain and a non-aggregating strain (Baines *et al.*, 2013; Crowther *et al.*, 2014).

The study reported in this thesis was not able to determine whether *cwpV* and its expression level, as determined by semi-quantitative RT-PCR, contributed to the auto-aggregation capacity. In future studies, quantitative RT-PCR (qRT-PCR) or immunolabelling of the CwpV protein could be used to determine the mRNA and protein expression level of *cwpV*, respectively. In addition, mutation of *cwpV* in a strong auto-aggregating strain, using a gene specific insertion or deletion strategy, could be used to determine whether CwpV contributes towards the strong auto-aggregation phenotype of the ribotype 017 strains in this study (Chapter 5). *C. difficile* Δ*cwpV* mutants could also be compared to wild-type strong auto-aggregators in the adherence assay, or co-infected in the gut model.

In summary, this pilot study successfully identified and investigated the presence of *C. difficile* in approximately 20% of symptomatic patients at Groote Schuur Hospital. It provided data on the most appropriate diagnostic method that could be used to detect its presence, information on the strain types potentially causing disease, and their antimicrobial resistance and auto-aggregation phenotypes. Hypotheses were also developed which suggested how various strain types, primarily ribotype 017, were able to persist and spread in the hospital, and identified a candidate gene, which may be involved in pathogenesis, for future study. The results produced by this study provide a platform for future studies to gain a better understanding of the *C. difficile* strains causing disease in South Africa. This would allow the future implementation of effective infection control procedures and treatment.

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