PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF *CLOSTRIDIUM DIFFICILE*

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Phenotypic and genotypic characterisation of *Clostridium difficile*

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SUMMARY

Clostridium difficile is at present one of the most common nosocomial infections in the developed world. Hypervirulent strains (PCR ribotype 027) of *C. difficile* which produce enhanced levels of toxins have also been associated with other characteristics such as a greater rate of sporulation and resistance to fluoroquinolones. Infection due to *C. difficile* PCR ribotype 027 has also been associated with greater rates of morbidity and mortality. The aim of this thesis was to investigate both the phenotypic and genotypic characteristics of two populations of toxigenic clinical isolates of *C. difficile* which were recovered from two separate hospital trusts within the UK. Phenotypic characterisation of the isolates was undertaken using analytical profile indexes (APIs), minimum inhibitory concentrations (MICs) and S-layer protein typing. In addition to this, isolates were also investigated for the production of a range of extracellular enzymes as potential virulence factors. Genotypic characterisation was performed using a random amplification of polymorphic DNA (RAPD) PCR protocol which was fully optimised in this study, and the gold standard method, PCR ribotyping. The discriminatory power of both methods was compared and the similarity between the different isolates also analysed. Associations between the phenotypic and genotypic characteristics and the recovery location of the isolate were then investigated. Extracellular enzyme production and API testing revealed little variation between the isolates; with S-layer typing demonstrating low discrimination. Minimum inhibitory concentrations did not identify any resistance towards either vancomycin or metronidazole; there were however significant differences in the distribution of antibiogram profiles of isolates recovered from the two different trusts. The RAPD PCR protocol was successfully optimised and alongside PCR ribotyping, effectively typed all of the clinical isolates and also identified differences in the number of types defined between the two locations. Both PCR ribotyping and RAPD demonstrated similar discriminatory power; however, the two genotyping methods did not generate amplicons that mapped directly onto each other and therefore clearly characterised isolates based on different genomic markers. The RAPD protocol also identified different subtypes within PCR ribotypes, therefore demonstrating that all isolates defined as a particular PCR ribotype were not the same strain. No associations could be demonstrated between the phenotypic and genotypic characteristics observed; however, the location from which an isolate was recovered did appear to influence antibiotic resistance and genotypic characteristics. The phenotypic and genotypic characteristics observed amongst the *C. difficile* isolates in this study, may provide a basis for the identification of further targets which may be potentially incorporated into future methods for the characterisation of *C. difficile* isolates.

Keywords: *Clostridium difficile*, RAPD, PCR ribotyping, phenotypic, genotypic.

For grandma

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AAD	Antibiotic associated diarrhoea
ATCC	American Type Culture Collection
bp	Base pairs
BSAC	British Society for Antimicrobial Chemotherapy
CDAD	*Clostridium difficile* associated disease
CDI	*Clostridium difficile* infection
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediamine-tetraacetic acid
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable number tandem repeat analysis
MRSA	Methicillin resistant *Staphylococcus aureus*
NCTC	National collection of type cultures
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMC	Pseudomembranous colitis
RAPD	Random amplification of polymorphic DNA
REA	Restriction enzyme analysis
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
TAE	Tris acetic acid EDTA buffer
Taq	DNA polymerase from *Thermus aquaticus*
Tris	Tris [hydroxymethyl] aminomethane
UPGMA	Unweighted pair-group method using arithmetic averages

CHAPTER 1 INTRODUCTION

1.1 *Clostridium difficile*

Clostridium difficile belongs to the family *Costridiaceae* and genus Clostridium. This genus consists of Gram positive, spore forming bacilli that possess low G+C content chromosomal DNA. Clostridia are frequently described as motile obligate anaerobes however there is variability in aerotolerance and motility between different species (Washington *et al.*, 2005). Over 100 species of Clostridium have been identified to date; only thirteen species are considered to be seriously pathogenic towards humans or animals (Dupuy *et al.*, 2006). The Clostridia produce more protein toxins than any other genus (Johnson, 1999) and unlike other species of pathogenic bacteria, the main cause of their pathology is through the action of these toxins which are some of the most potent in nature (Johnson, 1999).

Vegetative cells of *C. difficile* are typically larger than other bacterial cells measuring 3-16.9 µm in length, 0.5 -1.9µm in width and producing subterminal spores (Hatheway, 1990) that are highly resistant to most standard forms of sterilization and disinfection. *C. difficile* is a heterotrophic organism with an optimal growth temperature of 37°C, most strains are motile and possess petrichious flagella. Colonies of *C. difficile* following 48 hours incubation in anaerobic conditions at 37°C are typically large, flat and slightly grey in colour with a 'ground glass appearance'. *C. difficile* also has a distinctive 'elephant house' odour due to the production of iso-valeric acid, iso-caproic acid and p-cresol, which are the products of various metabolic pathways within the organism (Levett, 1984).

First isolated in 1935 from stool samples of newborn children and named *Bacillus difficilis*, *C. difficile* was initially identified as a commensal organism of the digestive tract of young infants (Hall and O' Toole, 1935) and this thought remained for over the next forty years.

In 1977 a clostridial toxin was isolated from cases of pseudomembranous colitis (PMC) (Larson & Price, 1977) but it was not until 1978 that *C. difficile* was identified as a cause of antibiotic associated PMC and acknowledged as a human pathogen (Bartlett *et al.*, 1978, George *et al.*, 1978, Larson *et al.*, 1978).

1.2 Virulence and pathogenesis

1.2.1 Cell associated virulence factors

Some of the cell associated proteins that *C. difficile* possess contribute to successful colonisation within the gut. Some strains of *C. difficile* possess flagella which are reported to be involved in cell adhesion with strains lacking flagella being unable to adhere as closely (Tasteyre *et al.*, 2000). Capsules have also been observed in some strains of *C. difficile* which may provide evasion from the host immune system (Davies and Borriello, 1990).The surface layer proteins of *C. difficile* have been proposed to have immunoreactive properties (Ausiello *et al.*, 2006) and also be involved in adhesion to host cells (Calabi *et al.*, 2002). Other cell surface factors reported to have adhesive properties include fibronectin binding proteins (Hennequin *et al.*, 2003), Cwp66 (Waligora *et al.*, 2001) and the heat shock protein GroEL (Hennequin *et al.*, 2001b). It has also been reported that some of these proteins also stimulate an immune response (Pechine *et al.*, 2005).

1.2.2 Toxin A (TcdA) and Toxin B (TcdB)

The virulence of *C. difficile* is attributed to the production of two major toxins; Toxin A and Toxin B. Strains of *C. difficile* that do not produce toxin A and B are not associated with disease (Kelly *et al.*, 1994). Both toxins are high molecular weight glucosyltransferases (308kDa and 270kDa respectively) and originally both were characterised as cytotoxic however, only Toxin A was also regarded as an enterotoxin. In

recent years however it has been established that both toxins are also enterotoxic to human intestinal cells (Savidge *et al.*, 2003, Pothoulakis and Lamont, 2001). Toxin A and B are encoded for by the genes *tcdA* and *tcdB* respectively, which reside on the 19.6kb pathogenicity locus (PaLoc) in addition to the genes *tcdC*, *tcdD* and *tcdR* (Figure 1.1). The genes *tcdC* and *tcdD* are the respective negative and positive regulators of the toxin genes (Hundsberger *et al.*, 1997) with evidence suggesting that *tcdE* is responsible for holin function (Tan *et al.*, 2001); facilitating the release of the toxins from the cell.

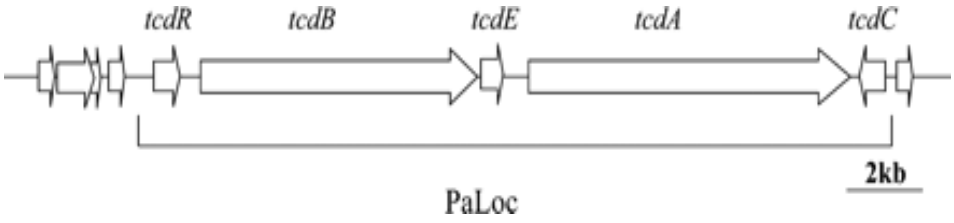


Figure 1.1 Structure of the PaLoc locus, arrows depict the direction in which genes are transcribed. Adapted from (Dupuy *et al*., 2008)

When the association between *C. difficile* and PMC was first discovered, initial work implicated that there was only one toxin (Toxin B) responsible for the symptoms associated with *C. difficile* infection (CDI); it was not until later that Toxin A was also isolated (Taylor *et al.*, 1981). Initial research into both toxins later suggested that Toxin A was the more potent of the two toxins and that Toxin B did not have the capacity to cause disease unless Toxin A was also produced, with Toxin A providing entry into the cells; this was based on work carried out in animal models (Lyerly *et al.*, 1985, Mitchell *et al.*, 1986, Torres *et al.*, 1990, Triadafilopoulos *et al.*, 1987). The isolation of the first Toxin A-/B+ strain and its characterisation prompted investigations on mammalian cells lines using Toxin B alone. The findings from such studies established that Toxin B had the ability to cause a disease state independent of Toxin A; thereby demonstrating that Toxin A-/B+

strains do have the capacity to cause symptomatic disease (Pothoulakis *et al.*, 1986, Hecht *et al.*, 1992). The isolation of Toxin A-/B+ strains from patients with CDI are now increasing in frequency (Voth and Ballard, 2005) despite being rare in previous years, it is also apparent that these strains can cause disease of equal severity as strains that produce both toxins (Drudy *et al.*, 2007). Research into the independent action of both toxins has long been hindered as *C. difficile* is hard to genetically manipulate and therefore the virulence of strains that only produce Toxin A could not be investigated as these strains do not naturally occur in nature (Lyras *et al.*, 2009). New methodologies however using genetically manipulated strains of *C. difficile* have recently allowed novel studies to be carried out that investigate the independent action of both toxins in a hamster model. In contrast to earlier work, these results have suggested that Toxin B is essential for virulence with genetically altered strains that only produce Toxin A markedly losing the ability to cause disease (Lyras *et al.*, 2009); such evidence also conflicts with earlier work that suggested Toxins A and B work synergistically (Lima *et al.*, 1988, Lyerly *et al.*, 1985).

Both toxins display a high degree of similarity to each other at the amino acid level (63%) (Von Eichel-Streiber *et al.*, 1992) and this is reflected in the structure. Both toxins can be divided into three domains: a receptor binding domain, a catalytic or enzymatic domain and a translocation domain as depicted in figure 1.2. The region with the greatest similarity between the two toxins is seen in the catalytic domain (Voth and Ballard, 2005); this is the region that monoglycosylates Rho GTPases within the cell and is responsible for the changes in cell physiology. The cytotoxic effect of both toxins is the same, with differences between the two originally reported to be the ability of Toxin A to cause fluid

accumulation (Borriello, 1998) however there is now evidence that contradicts this (Savidge *et al.*, 2003). Other apparent differences between the toxins are the variability that

occurs in the receptor binding domain and it is this that is likely to govern the differences in receptor binding (Jank *et al.*, 2007).

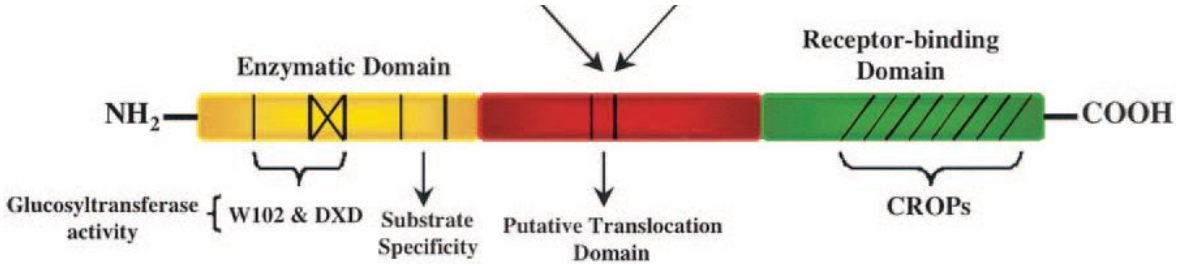


Figure 1. 2 Proposed protein domain structures of Toxin A and Toxin B of *C. difficile*. Adapted from (Voth and Ballard, 2005)

Both toxins are produced during the late log and stationary phases of growth (Voth and Ballard, 2005), allowing cells to have become established within the host gut before toxin production begins. Toxins are taken up by host cells through receptor mediated endocytosis; the receptors for both toxins differ, with the receptor for Toxin A better characterised than that for Toxin B. The receptor for Toxin A is the disaccharide Galß1-4GlcNac found on I, X and Y blood antigens that are expressed on several types of cell including intestinal epithelial cells (Tucker and Wilkins, 1991); the receptor for Toxin B has not yet been identified but its ability to infiltrate a variety of cells suggests a common receptor (Voth and Ballard, 2005). Once both the toxin and receptor has been internalised, the endosome enclosing them is acidified, this allows the toxin to undergo structural transformations upon which the active portions of the toxin (catalytic domain) are released into the cytosol. Both toxins exert their effect on cells by glycosylating the Rho family of proteins; proteins which are essential for many processes within the cell including regulation of the actin cytoskeleton, disruption of tight junctions and parts of the cell cycle (Etienne-Manneville and Hall, 2002, Giesemann *et al.*, 2008). Glycosylation of the Rho GTPases leads to inactivation and inhibition of their regulatory activity within the cell, most notably leading to depolymerisation of the actin cytoskeleton and rounding of the

cells and ultimately apoptosis. Cell rounding also leads to the disruption of tight junctions due to both the loss in the structure of the actin cytoskeleton but also because Rho proteins also regulate tight junctions. The loss of tight junctions then leads to increased permeability causing the diarrhoea that is characteristic of CDI (Poxton *et al.*, 2001). Both toxins have the capacity to cause changes in host cell physiology and although both toxins have the same cytopathic effect on cells, Toxin B is reported to be a more potent cytotoxin in comparison to Toxin A (Poxton *et al.*, 2001).

1.2.3 Binary toxin

In addition to TcdA and TcdB, some strains of *C. difficile* also produce a binary toxin (CDT) which has been identified as an actin-specific ADP-ribosyltransferase. This toxin is similar to other clostridial iota toxins which act specifically on actin within the cell (Popoff *et al.*, 1988) The role of the binary toxin in CDI is still unknown at present but this toxin has been shown to have a cytopathic effect on vero cells *in vitro* (Perelle *et al.*, 1997). Not all strains of *C. difficile* produce a binary toxin which indicates that this toxin is not essential to the virulence of the organism. The production of this toxin is most frequently seen alongside Toxins A and B and is produced by the hypervirulent PCR ribotype 027 strains (Carter *et al.*, 2007); there have however been reports of strains that produce binary toxin only (Stubbs *et al.*, 2000, Geric *et al.*, 2006, Geric *et al.*, 2003).

1.2.4 Additional secreted virulence factors

Unlike many other bacteria, *C. difficile* does not produce a range of additional virulence factors such as extracellular enzymes. Although toxin production is well documented as the major virulence factor of *C. difficile*, limited studies have been undertaken to investigate the production of other virulence factors such as hydrolytic and proteolytic enzymes (Popoff and Dodin, 1985, Seddon and Borriello, 1992, Seddon *et al.*, 1990, Hafiz

and Oakley, 1976, Steffen and Hentges, 1981). Studies have indicated that *C. difficile* may produce some extracellular enzymes such as heparinase and hyaluronidase however the number of strains investigated has been small and levels of enzyme have been found to vary between strains (Hafiz and Oakley, 1976, Steffen and Hentges, 1981). The variation in extracellular enzyme production indicates that *C. difficile* does not produce obvious additional secreted virulence factors but where present are more likely to facilitate its survival within the digestive tract (Poxton *et al.*, 2001).

1.2.5 Spore production

The ability of *C. difficile* to produce highly resilient endospores enables effective transmission and survival within an environment; they also allow the organism to persist within the gut despite antibiotic treatment thereby providing a type of resistance. The nosocomial transmission of *C. difficile* can be largely attributed to the ingestion of spores that have been picked up from contaminated surfaces or through aerial transmission (Underwood *et al.*, 2009); spore formation allows *C. difficile* to spread efficiently which is why they are so important in its transmission (Lawley *et al.*, 2009). Spores are also effectively expelled from patients with CDI through the profuse diarrhoea that is associated with the infection; it has been demonstrated that approximately 10^5 spores can be expelled in each gram of faeces from a *C. difficile* patient in addition to vegetative cells, further enhancing transmissibility (Jump *et al.*, 2007). Sporulation occurs when vegetative cells of *C. difficile* are exposed to unfavourable conditions such as nutritional deprivation (Sorg and Sonenshein, 2008). In such environments a spore is formed within the mother cell; this ensures the preservation of the strain until conditions are such that the spore will be stimulated to germinate into its vegetative cell state where it can produce toxin and cause disease. Spores of *C. difficile* germinate in the presence of certain bile salts which are found in the small intestine of humans (Wilson, 1983); and it is therefore likely that this is

where germination occurs in the human body. There are several bile salts that induce the germination of *C.difficile* however sodium taurocholate is the most effective and well documented, glycine and thioglycolate also act as co-germinants (Sorg and Sonenshein, 2008, Wheeldon *et al.*, 2008a). Once spores have been formed they are very hard to eliminate as traditional cleaning agents are often ineffective (Wheeldon *et al.*, 2008c); sodium hypochlorite eliminates spores (Kaatz *et al.*, 1988) but is also hazardous to use (Rutala and Weber, 1997).The high level of relapse associated with CDI may also be attributable to spores (Tang-Feldman *et al.*, 2003) as they can remain unaffected in the gut during antibiotic treatment; because spores are not capable of causing disease, symptoms will resolve and patients will appear clear of infection. When antibiotic therapy has concluded and conditions within the gut again become favourable, spores then germinate into vegetative cells, producing toxins and causing a return to a disease state. Without effective spore production, the transmission of *C. difficile* would be considerably more difficult.

1.2.6 Hypervirulent strains of *C. difficile*

Strains of *C. difficile* that produce elevated levels of toxin are described as hypervirulent; these strains are often associated with severe cases of infection, complications, and higher morbidity and relapse rates (Cookson, 2007). Depending on the typing method used, the predominant hypervirulent strain is known as PCR ribotype 027/ PFGE type NAP-1/ toxinotype III and REA group B1 and was first isolated and reported in 1985 (Popoff., 1988); at the time however it was not regarded as a significant strain especially as it was very rare. It was not until 2002 in Quebec that this strain began to emerge with regularity leading to outbreaks and a significant number of deaths throughout Montreal. By 2005, this hypervirulent strain had been isolated from patients in several other countries including the United States, England and the Netherlands (Van Steenbergen *et al.*, 2005). This strain is

now widespread across many countries in Europe (Hensgens *et al.*, 2009) and there have been reports from many countries across the world including Australia, Korea, Japan and Hong Kong (Riley *et al.*, 2009, Tae *et al.*, 2009, Kato *et al.*, 2007, Cheng *et al.*, 2009). Not only does the PCR ribotype 027 strain produce excess toxin but the additional binary toxin in addition to reports of increased sporulation (Akerlund *et al.*, 2008) and increased antimicrobial resistance, most notably towards fluoroquinolones (Mcdonald *et al.*, 2005). The reason for the excess production of toxins was initially shown to be due to a specific 18bp deletion within the *tcdC* gene; the negative regulator of toxin production with deletions then leading to truncated proteins. There is now evidence that suggests that not only does this deletion not affect regulation of the toxin (Verdoorn *et al.*, 2010), but also that the genomes of other strains of *C. difficile* contain similar deletions and mutations with no detrimental effect to the protein and toxin regulation (Matamouros *et al.*, 2007, Spigaglia and Mastrantonio, 2002, Maccannell *et al.*, 2006). Recent analysis has also shown that PCR ribotype 027 strains posses an additional 234 genes in comparison to the *C.difficile* strain 630 (PCR ribotype 012) (Stabler *et al.*, 2009) which may account for the differences observed in virulence and antibiotic resistance. The hypervirulence of PCR ribotype 027 therefore appears less straightforward than previously thought and may be due to a combination of several factors.

There are other hypervirulent strains that have begun to emerge and these are PCR ribotypes 017 and 078. Both of these strains have been associated with serious outbreaks of CDI and were also previously quite rare (Dawson *et al.*, 2009). PCR ribotype 078 produces toxins A and B and has deletions in the *tcdC* gene, it also produces binary toxin (Goorhuis et al., 2008). Isolates belonging to PCR ribotype 017 have displayed high levels of resistance to fluoroquinolones however do not produce Toxin A (Dawson *et al.*, 2009) ; another indicator that Toxin B is equally as important in disease as Toxin A. The general

trend of hypervirulent strains is they appear to go from almost obscurity in a population to quite rapid emergence; this was seen with the PCR ribotype 027 strains in several countries and an increase in the isolation of PCR ribotype 078 from patients have recently been reported in the UK and the Netherlands (Hensgens *et al.*, 2009).

1.3 Epidemiology, transmission and prevention

1.3.1 Epidemiology

Clostridium difficile is the most common cause of nosocomial diarrhoea in developed countries (Bacci *et al.*, 2009). Carriage and colonisation rates vary between age groups with carriage estimated to be less than 3% in the healthy adult population but as high as 70% in infants and neonates (Bartlett, 1994). Colonisation rates have also been found to be higher amongst hospital employees and caregivers (Giannasca et al., 2004) and those who have been hospitalised (Barbut and Petit, 2001).

Prior to 2003, rates of CDI were significantly lower across North America and Europe. The rise in CDI that has been reported in recent years appears to have been triggered by the emergence of the PCR ribotype 027 strain which, based on recent evidence, appears to have evolved into a more virulent and transmissible strain than had been seen previously (Stabler *et al.*, 2009). When outbreaks of CDI began to occur in 2003, those most susceptible were hospitalised elderly patients often receiving antibiotic treatment. Although this is still the case, reports are increasing where people who do not appear to belong to belong to obvious risk groups such as pregnant women and children are developing CDI (Klein *et al.*, 2006, Rouphael *et al.*, 2008, Kuijper and Van Dissel, 2008).

1.3.1.1 Surveillance in England, Wales and Northern Ireland

Voluntary reporting of *C. difficile* infection in England and Wales was introduced in 1990 and in Northern Ireland in 2001. Reports from this time period indicate that rates of CDI

had steadily risen since 1990 with a significant increase between 2001 and 2007 (HPA, 2008). Reporting became mandatory in hospital Trusts throughout England in January 2004; initially reporting was only mandatory in those over 65, but in April 2007 mandatory reporting was extended to all patients over two years of age. Despite the introduction of the mandatory reporting scheme, the voluntary reporting scheme is still in place and therefore data is still collected for Wales and Northern Ireland and additional Trusts in England. Data obtained from the voluntary and mandatory surveillance schemes are not comparable for several reasons including different participation rates, and the difference in isolation methods, with a positive toxin result not being required for inclusion in the voluntary reporting scheme (HPA, 2008). Reports of CDI in England peaked in 2006 and 2007 with 55,635 and 58,176 cases reported respectively however 2007 also included reports of infections in those aged between two and sixty four years old for the first time. In 2008 cases of CDI fell significantly to 40,704 and have continued to fall in 2009 although there have been alterations made to the reporting guidelines (HPA, 2009a).

1.3.1.2 *Clostridium difficile* ribotyping network (CDRN)

The large increase in the number of CDI cases led to an increase in demand for ribotyping services and so in April 2007 the *Clostridium difficile* Ribotyping Network for England (CDRNE) was established; the addition of a laboratory in Northern Ireland in April 2009 has seen the service renamed the *Clostridium difficile* Ribotyping Network (CDRN). At present there are eight laboratories that are part of the CDRN and in addition to ribotyping also provide an enhanced fingerprinting service using Multi-locus Variable Number Tandem Repeat Analysis (MLVA) and in some cases antibiotic susceptibility testing. An enhanced fingerprinting technique was introduced as a higher discriminatory method to PCR ribotyping in order to try to discriminate further within PCR ribotypes. Such typing is required in order to gather more accurate information about the spread and transmission of

C. difficile, especially as reports from 2007/2008 indicated that there were three predominant PCR ribotypes (001, 027 and 106) in England and these alone were responsible for 70% for *C. difficile* infections (HPA, 2009b). Characterisation by both ribotyping and MLVA is strictly controlled and certain criteria have to be fulfilled in order to get *C. difficile* isolates typed, therefore a large proportion of isolates still remain uncharacterised. The CDRN has allowed a larger number of isolates to be typed providing more accurate epidemiological information about the PCR ribotype of isolates across a large geographical area.

1.3.1.3 Random sampling scheme

As most laboratories do not culture for *C. difficile* there has been little surveillance of antibiotic resistance amongst *C. difficile* isolates. The random sampling scheme was first introduced in 2005 to try and monitor the epidemiology of PCR ribotypes and also antibiotic susceptibility patterns of *C. difficile* isolates from across England. The sampling scheme includes only acute NHS Trusts in England where a specified number of *C. difficile* toxin positive samples collected during an allocated week have to be sent to the Anaerobic Reference Laboratory (ARL) in Cardiff, UK. Here isolates are both ribotyped and tested for antibiotic resistance against a panel of eight antimicrobials (vancomycin, metronidazole, erythromycin, moxifloxacin, co-amoxiclav, penicillin, imipenem and piperacillin-tazobactem) using E test methodology (HPA, 2008). The random sampling scheme has identified a rise in minimum inhibitory concentrations (MICs) towards metronidazole amongst isolates belonging to the three most common ribotypes of *C. difficile* in England (001, 027 and 106) in comparison to previous years; these MICs are also significantly higher amongst these ribotypes in comparison to the less commonly isolated ribotypes (HPA, 2008). The more common PCR ribotypes also display higher

MICs towards erythromycin and moxifloxacin, with isolates belonging to PCR ribotype 027 displaying resistance to both of these antibiotics (HPA, 2008).

1.3.1.4 European surveillance

Similar surveillance systems to that in England have been set up in France, Belgium and the Netherlands for the reporting of CDAD. Prior to the outbreaks and the increase in the incidence of CDAD; the European Study Group for *Clostridium difficile* (ESGCD) was established in 2001 to promote awareness of *C. difficile* and provide surveillance on its spread throughout Europe. This collaborative surveillance has provided important information about the changing epidemiology of *C. difficile* and in particular that of PCR ribotype 027 (Kuijper *et al.*, 2008). The collection of such large pools of data allows patterns and trends to be established where they may have gone unnoticed with smaller data sets.

1.3.1.5 Strain epidemiology

The emergence of PCR ribotype 027 led to enhanced typing, reporting and surveillance of *C. difficile* isolates across Europe and North America. Following the emergence of this hypervirulent strain, PCR ribotype 027 has predominated in several countries in recent years; there are now reports however of a decline in the incidence of this ribotype in some countries (Hensgens *et al.*, 2009). Despite the predominance of PCR ribotype 027 in some countries, variations do occur between countries in the frequency of the different PCR ribotypes isolated (Kuijper *et al.*, 2008); for example PCR ribotype 106 is one of the most frequently isolated PCR ribotypes in the UK however it is rarely isolated in other countries (Brazier *et al.*, 2008). In addition to variations in predominant PCR ribotype strains between countries and species, PCR ribotypes vary over time as has been demonstrated by the rapid and widespread emergence of PCR ribotype 027.

1.3.1.6 *C. difficile* in animals

Isolation of *C. difficile* from animals has also shown that different PCR ribotypes predominate in animal species with PCR ribotype 078 the most frequently isolated strain from pigs, calves and horses (Keel *et al.*, 2007, Goorhuis *et al.*, 2008, Rupnik *et al.*, 2008). Studies have also shown that retail meat can also be contaminated with *C. difficile* (Broda *et al.*, 1996, Weese *et al.*, 2005, Rodriguez-Palacios *et al.*, 2007, Songer *et al.*, 2009, Weese *et al.*, 2009, Simango and Mwakurudza, 2008). In studies that have investigated the isolation of *C. difficile* from meat products, PCR ribotype 078 is often recovered (Songer *et al.*, 2009, Weese *et al.*, 2009). Isolates of *C. difficile* have also been recovered from other food products (Al Saif and Brazier, 1996, Bakri *et al.*, 2009) The increase in the isolation of *C. difficile* from food products humans raises questions about possible transmission between animals and humans (Weese, 2010, Rupnik, 2007) although there is at present no direct evidence of this; there is evidence that the same strains can cause symptomatic disease in both pigs and humans (Debast *et al.*, 2009).

1.3.2 Transmission of *C. difficile*

The faecal-oral route is the route of transmission for *C. difficile*, through the ingestion of spores and cells. Although it is possible that cells may also be ingested and contribute to the transmission of CDI, their inability to survive in aerobic environments for sufficient amounts of time and the acidic environment of the stomach make this unlikely; spores however can survive in aerobic environments and can also survive the low pH conditions of the stomach making them the most likely and more effective route of transmission. When spores of *C. difficile* are expelled from an infected patient, aerial dissemination of spores into the environment occurs where they can persist on a variety of surfaces, thus acting as a reservoir of infection (Mulligan *et al.*, 1980). In addition to this, spores are not

only shed from symptomatic patients; shedding can occur for up to four weeks following the cessation of treatment (Sethi *et al.*, 2010). Healthcare environments are associated with the acquisition of several infections including *C. difficile*; this is not surprising as many studies have reported that *C. difficile* can contaminate many areas of the clinical setting (Mulligan *et al.*, 1980, Malamou-Ladas *et al.*, 1983, Fawley and Wilcox, 2001, Samore *et al.*, 1996, Kim *et al.*, 1981, Fekety *et al.*, 1981, Cohen *et al.*, 1997, Titov *et al.*, 2000, Cohen, 2000) including non clinical areas (Dumford *et al.*, 2009). Contamination has also been reported on blood pressure cuffs (Walker *et al.*, 2006) and also manual handling equipment (Barnett *et al.*, 1999). In addition to hospital surfaces, *C. difficile* spores can also be isolated from the air (Roberts *et al.*, 2008), providing a greater problem in regards to cleaning and infection control. Healthcare workers are also often implemented in the transmission of *C. difficile* (Fekety *et al.*, 1981, Mcfarland *et al.*, 1989) and this is often attributed to inadequate hand washing, in addition to this it is proposed that uniforms could also be a source (Perry *et al.*, 2001).

1.3.2.1 Community acquired *C. difficile*

Community acquired *C. difficile* is defined as CDI when a patient has not been hospitalised prior to infection; in those who have been recently hospitalised, community acquired CDI is defined as infection after twelve weeks of discharge from hospital (HPA, 2009a). If CDI presents between four and twelve weeks following discharge from hospital, it is often difficult to determine whether this is a hospital or community acquired infection. Incidences of *C. difficile* outside of the healthcare setting appear to be increasing (Dial *et al.*, 2006, Wilcox *et al.*, 2008, Bauer *et al.*, 2008, Paltansing *et al.*, 2007) although the reasons for this are not known.

1.3.3 Prevention of *C. difficile* infection

1.3.3.1 Isolation of patients infected with *C. difficile*

Patients suspected of having CDI should be immediately removed from the main ward and placed in isolation. In a clinical setting which is experiencing outbreaks of CDI, cohort wards should be established to isolate clusters of infected patients. Preventative measures including single and cohort isolation have been shown to reduce transmission and also control outbreaks (National *Clostridium diffic*ile Standards Group, 2004, HPA, 2009).

1.3.3.2 Cleaning of the hospital environment and equipment

Efficient removal of spores from the hospital environment either through physical removal or the use of sporicidal agents including hypochlorite is the most effective way to reduce the rate of infection and transmission between patients. There are limited cleaning agents which posses sporicidal activity most notably chlorine, peracetic acid, acidified nitrite and gluteraldhyde, however not all of these agents are suitable for use within a clinical setting (Wullt *et al.,* 2003c). It is important that such agents are used as the use of some cleaning agents have been found to increase sporulation when applied to vegetative cells and can therefore contribute to the problem (Fawley *et al.,* 2007). Chlorine and peracetic acid are more frequently found in hospital cleaning agents and although chlorine is known to kill spores more efficiently, there are fewer hazards associated with agents containing peracetic acid (Wheeldon *et al.*, 2008b). In addition to the application of chemicals to hard surfaces, the antimicrobial properties of surfaces such as copper have also been investigated and it has been shown that spores of *C. difficile* are more susceptible to killing under certain conditions when on a copper surface (Weaver *et al.,* 2008, Wheeldon *et al.*, 2008c). Not only do *C. difficile* spores contaminate the hospital environment but also health care equipment that is designed for repeated use. Equipment such as thermometers and blood

pressure cuffs have been found to contribute to the transmission of *C. difficile* and it has been demonstrated that replacing items such as traditional thermometers with single use alternatives can significantly reduce the incidence of CDI (Brooks *et al.*, 1992, Jernigan *et al.*, 1998). The Department of Health (DoH) recommends that hospital rooms occupied by *C. difficile* patients should be cleaned daily with a chlorine based hard surface disinfectant (at least 1000 ppm available chlorine) (HPA, 2009a). When a *C. difficile* patient permanently vacates a room the mattress, bed linen and curtains should also be changed (HPA, 2009a). The aerial dissemination of *C. difficile* spores means that not only do they contaminate surfaces but also remain in the air (Roberts *et al.*, 2008) and these are harder to eliminate with hydrogen peroxide vaporisation being the most effective way to eliminate such spores (Barbut *et al.*, 2009, Shapey *et al.*, 2008, Boyce *et al.*, 2008).

1.3.3.3 Handwashing

Not only have spores been found to contaminate the hospital environment but also the hands of healthcare workers (Mcfarland *et al.*, 1989, Fekety *et al.*, 1981), therefore thorough and regular handwashing with soap and water reduces the risk of further infection. All healthcare workers should wash their hands both before and after contact with CDI patients; it is also recommended that disposable gloves and aprons are worn when dealing with *C. difficile* patients (HPA, 2009a).

1.3.3.4 Antibiotic prescribing

Prescribing of antibiotics that are most commonly implicated in the cause of CDI, in addition to other broad spectrum antibiotics should be restricted in order to limit the number of cases. The antibiotics most frequently associated with the onset of CDI are fluoroquinolones, third generation cephalosporins and clindamycin, although virtually all antibiotics have been implicated (Kelly *et al.*, 1994, Fekety, 1997). Broad spectrum

antibiotics are more frequently associated with CDI as they are not specific in the bacteria which they target and therefore effectively sterilise the gut making the patient vulnerable to CDI if they are in an environment where they are likely to ingest *C. difficile* spores. Broad spectrum antibiotics are usually administered as prophylactic therapy or when the cause of an infection is unknown. Restrictions in the prescribing of broad spectrum antibiotics can significantly reduce the number of *C. difficile* cases; (Thomas and Riley, 2003, Kallen *et al.*, 2009, Brown *et al.*, 1990, Pear *et al.*, 1994, Gaynes *et al.*, 2004, Mcnulty *et al.*, 1997, Khan and Cheesbrough, 2003, Valiquette *et al.*, 2007, Carling *et al.*, 2003) this not only controls the unnecessary prescribing of antibiotics but also encourages the prescription of antibiotics that are less likely to disrupt the commensal gut flora.

1.4 *Clostridium difficile* carriage and infection

1.4.1 Asymptomatic carriage

The asymptomatic carriage of *C. difficile* in adults is reported to be due to previous infection (Riggs *et al.*, 2007), prior hospitalisation (Barbut and Petit, 2001) and also possible carriage of non-toxin producing isolates (Delmee *et al.*, 2005). The high rates of asymptomatic carriage amongst neonates are believed to be due to immaturity of gut receptors to which *C. difficile* toxins bind (Wilson, 1993); the introduction of normal healthy gut flora and colonization resistance then eradicates *C. difficile* prior to receptor maturity (Eglow *et al.*, 1992). The significant difference in the reported carriage rates of *C. difficile* between infant and adult populations indicates that colonization resistance and the immunity that the commensal gut bacteria provide are very influential in CDI and may be a factor in carriage also. Due to the nature by which CDI is diagnosed through toxin detection alone, actual carriage rates of *C. difficile* are unknown. Although it is speculated

that carriage is due to non toxigenic strains, carriage of toxin producing strains may occur but any pathogenic effect inhibited by the presence of commensal bacteria.

1.4.2 *Clostridium difficile* infection (CDI)

Antibiotic associated diarrhoea can be described as unexplained episodes of diarrhoea that begin during or up to two months following cessation of antibiotic therapy (Fekety, 1997). Most antibiotics have been reported to cause AAD (Barbut and Petit, 2001) with different antibiotics having different occurrence rates (Bignardi, 1998); broad spectrum antibiotics and those with activity on gut flora are however more frequently implicated (Bignardi, 1998). Infectious AAD is due to the disruption of the gut flora, allowing the overgrowth of opportunist pathogenic bacteria. Overgrowth of *C. difficile* is the predominant cause of infectious AAD (CDI); other infectious causes include *Staphylococcus aureus*, *Klebsiella oxytoca* and *Clostridium perfringens*. A large proportion of AAD cases however are not due to infection and are often the result of varied physiological responses to antibiotics within the gut (Hogenauer *et al.*, 1998).

Symptoms of CDI include mild to moderate diarrhoea, sometimes accompanied by abdominal pain, fever, nausea, lethargy and dehydration. In uncomplicated cases, CDI can often be resolved by discontinuation of the offending antibiotic and rehydration therapy if required. In more serious cases of CDI, antibiotic treatment with metronidazole or vancomycin is required to eliminate *C. difficile* from the gut.

1.4.3 Pseudomembranous colitis

Pseudomembranous colitis (PMC) was first described in 1893 (Finney, 1986). Primarily caused by *C. difficile*, cases of PMC due to another causative agent are rare and therefore the terms PMC and *C. difficile* colitis are often used interchangeably. Cases of PMC were rarely reported until antibiotic use became widespread with clindamycin being implicated

as the cause of PMC in 1974 (Tedesco *et al.*, 1974). Pseudomembranous colitis occurs in 10% of AAD cases (Mcfarland, 1998) however in over 90% of these PMC cases, *C. difficile* is the cause (Surawicz and Mcfarland, 1999).

Symptoms of PMC are similar to those in CDI yet more pronounced with profuse watery diarrhoea and severe abdominal pain, often accompanied by fever and swelling and tenderness of the abdomen (Kelly *et al*., 1994). Internal examination reveals the presence of yellow pseudomembranous plaques (Figure 1.3) that consist of dead mucosal cells, mucous, fibrin and neutrophils with the extent of plaque formation often correlating with the severity of symptoms (Kelly *et al.*, 1994). Colitis can also develop without pseudomembranes with symptoms being less severe than those associated with PMC.

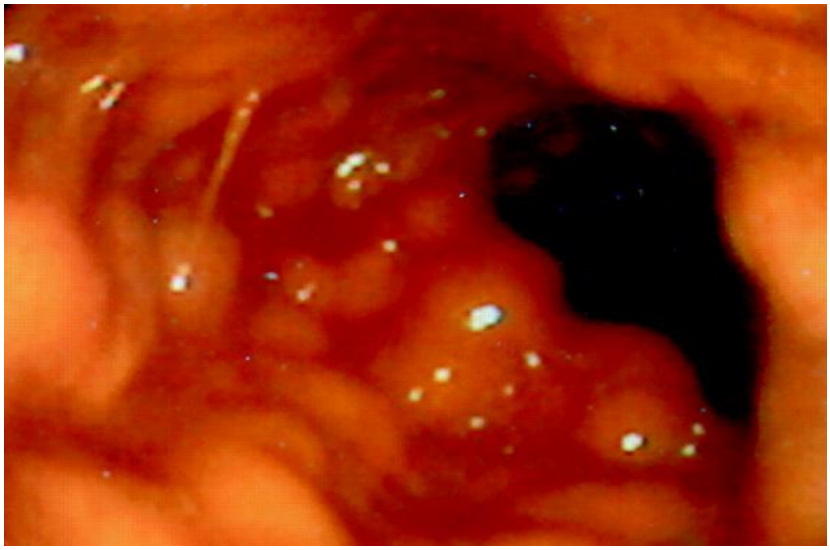


Figure 1.3 Appearance of a colon from a patient suffering from pseudomembranous colitis as a result of *C. difficile* infection. Adapted from (Kawamoto *et al*., 1999).

1.4.4 Fulminant colitis

Fulminant colitis occurs in approximately 1-3% of all cases of CDI (Kelly *et al.*, 1994) and can lead to further complications such as colonic perforation and peritonitis; there is also a high incidence of mortality. Patients are severely ill with abdominal pain and distension, fever and tachycardia; diarrhoea may not be present if toxic megacolon or paralytic ileus has developed resulting in loss of muscle tone. Surgical intervention is often required to prevent further complications and death.

1.4.5 Toxic megacolon

Toxic megacolon is a condition whereby the colon rapidly dilates; this can prevent peristalsis and therefore diarrhoea can be absent in a patient with this condition. The dilation of the colon also causes abdominal distension and tenderness and fever can also be present. Toxic megacolon is a rare but life threatening complication of CDI associated with a high risk of perforation, sepsis and shock. Treatment of toxic megacolon is usually through surgery; either a partial or total colectomy although steroids can also be administered to try to reduce inflammation and dilation.

1.4.6 Colonic perforation and peritonitis

If the colon becomes too inflamed or dilated this can lead to colonic perforation and ultimately peritonitis; a fatal condition if not treated quickly. It is rare that cases of CDI progress to such severity as peritonitis but cases do occur and are associated with high levels of mortality. Antibiotics are often administered for peritonitis in addition to rehydration therapy, surgery is also required.

1.4.7 Relapse and re-infection of *C. difficile* infection

The recurrence rate associated with CDI is high with reported rates of between 7-35% (Barbut *et al.*, 2000, Tabaqchali and Jumaa, 1995, Gerding *et al.*, 1995, Fekety *et al.*, 1997)

however around 20 % is believed to be the mean number (Fekety *et al.*, 1997). It is not clear why recurrence of CDI is so high in comparison to other infections and it is difficult to determine if recurrences are due to relapse or re-infection without the use of molecular typing methods. Relapse is defined as a recurrence of CDI up to twenty eight days since the previous diagnosis, after this period of time it is regarded as re-infection (HPA, 2009a). Relapse is often associated with treatment failure where *C. difficile* has not been successfully eradicated from the gut and following cessation of antibiotic therapy, the patient again becomes symptomatic due to the same strain. It has been suggested that the retention of spores within the gut that are unaffected by antibiotic therapy are likely to be a contributing factor (Kelly *et al.*, 1994, Mcfarland, 2005). Another explanation for the high recurrence rates associated with CDI is that they are not true relapses and are in fact re-infection; studies that have investigated this have reported high levels of re-infection (Wilcox *et al.*, 1998, Barbut *et al.*, 2000, Tang-Feldman *et al.*, 2003, Alonso *et al.*, 2001, O'Neill *et al.*, 1991, Kato *et al.*, 1996, Asha *et al.*, 2006). Re-infection with *C.difficile* is likely to occur while a patient is still recovering from a previous episode of CDI; it can take up to three months for the gut flora to become properly re-established (Mcfarland, 2005) therefore making them vulnerable to infection for prolonged period of time. In order for re-infection to occur a patient not only has be susceptible but also be in an environment where they are likely to ingest spores, unfortunately this is usually a hospital or care home setting.

Lack of typing does make it hard to distinguish between relapse and re-infection however the widespread typing methods used are restrictive. Within the UK where PCR ribotyping is the current method used to type strains, there are a select few ribotypes that predominate. Even if PCR ribotyping were to be carried out on all isolates, the isolation of the same ribotype from a patient on two occasions does not indicate definite relapse; it could be that

a patient has been re-infected with two different strains of the same ribotype. Multilocus variant number tandem repeat analysis (MLVA) is now being offered as a service by the CDRN providing a greater discriminatory power and greater evolutionary information about isolates of *C. difficile* and has the potential to provide useful information about transmission.

1.4.8 Extra-intestinal infections

Cases of *C. difficile* being isolated from infections outside of the intestine are rare although there have been several cases reported (Wolf *et al.*, 1998, Jacobs *et al.*, 2001, Feldman *et al.*, 1995, Rampling *et al.*, 1985, Cid *et al.*, 1998, Chatila and Manthous, 1995, Byl *et al.*, 1996, Gerard *et al.*, 1989, Simpson *et al.*, 1996, Spencer *et al.*, 1984, Saginur *et al.*, 1983, Bhargava *et al.*, 2000, Incavo *et al.*, 1988, Mccarthy and Stingemore, 1999, Brown *et al.*, 2007, Stieglbauer *et al.*, 1995, Studemeister *et al.*, 1987, Pron *et al.*, 1995, Gravisse *et al.*, 2003, Kikkawa *et al.*, 2008, Deptula *et al.*, 2009, Urban *et al.*, 2009, Garcia-Lechuz *et al.*, 2001) . When *C. difficile* is isolated from extra-intestinal sites additional species of bacteria are often present (polymicrobial infection), especially when the infection site is in close proximity to the colon and therefore may be a case of faecal contamination (Garcia-Lechuz *et al.*, 2001). What is often surprising about these cases are that the *C. difficile* strains isolated are often non-toxigenic and there are a large number of cases in children (Garcia-Lechuz *et al.*, 2001).

1.5 Risk factors

1.5.1 Antibiotic therapy

The major risk factor for contracting CDI is recent treatment with antibiotics. Although all antibiotics have been implicated as a cause of CDI; third generation cephalosporins, fluoroquinolones and clindamycin are considered the greatest risk factors (Bartlett,

2006).Several studies have demonstrated that by restricting the use of such antibiotics can significantly reduce CDI rates (Thomas and Riley, 2003, Kallen *et al.*, 2009, Brown *et al.*, 1990, Pear *et al.*, 1994, Gaynes *et al.*, 2004, Mcnulty *et al.*, 1997, Khan and Cheesbrough, 2003, Valiquette *et al.*, 2007, Carling *et al.*, 2003). Often when antibiotics are administered they not only eliminate target bacteria, they also destroy any other species that are susceptible. The commensal bacteria of the gut are particularly vulnerable to such treatments and when eradicated colonization resistance is lost; this allows opportunistic bacteria such as *C. difficile* to colonise the gut and cause infection. Antibiotic therapy is not only a risk factor for the development of CDI but studies have also shown that the presence of antibiotics in the gut can increase toxin production (Honda *et al.*, 1983, Onderdonk *et al.*, 1979, Adams *et al.*, 2007, Nakamura *et al.*, 1982, Pultz and Donskey, 2005, Saxton *et al.*, 2009), germination (Saxton *et al.*, 2009) and the expression of colonization factors (Hennequin *et al.*, 2001a, Deneve *et al.*, 2008) within the gut .

1.5.2 Proton pump inhibitors (PPIs)

It is still not clear whether PPIs are a risk factor for CDI with studies providing conflicting results (Cunningham *et al.*, 2003, Jackson *et al.*, 2006, Dalton *et al.*, 2009, Nerandzic *et al.*, 2009, Ackermann *et al.*, 2003, Al-Tureihi *et al.*, 2005, Dial *et al.*, 2004, Dial *et al.*, 2005, Dial *et al.*, 2006, Kazakova *et al.*, 2006). Proton pump inhibitors reduce gastric acid secretion and are prescribed for a wide range of ailments including acid reflux and peptic ulcers. It is proposed by some that the increased pH in the stomach due to a reduction in acid secretion allows the passage of *C. difficile* into the gut where it can then colonise and cause disease (Jump *et al.*, 2007). Ingestion of *C. difficile* spores is the main route of transmission and considering these are resistant to acid; it is not clear why PPIs may increase the risk of contracting CDI, for example in the medical condition achlorhydria, gastric acid secretions are either absent or very low causing gastric pH to be as high as

seven. If PPIs increased the risk of CDI through the proposed mechanism, it would then be expected that patients suffering from this condition would also be at increased risk (Dalton *et al.*, 2009). It has been proposed that the spores may be stimulated to germinate by bile salts within the stomach and the increase in pH then allows the survival of vegetative cells (Dial *et al.*, 2006); it has also been suggested that the viability of vegetative cells is enhanced on moist surfaces therefore if such cells are ingested, they will survive the passage through the stomach due to the increased pH.

1.5.3 Age

Increasing age is a risk factor for developing CDI with cases being significantly higher in those over sixty five, and when the mandatory reporting scheme was first introduced, only CDI in those over sixty five had to be reported. However, incidences are also increasing in the younger population to the extent that now in England and Wales all cases of CDI have to be reported in those over the age of two. Although age is a risk factor, this is often in combination with other factors such as antibiotic treatment and hospital admission, as those over sixty five are more likely to become ill due to weakened immune responses and are more likely to be prescribed antibiotics and be admitted to hospital.

1.5.4 Hospital admission

Those admitted to hospital have an increased chance of being colonised with *C. difficile*, although colonisation does not mean symptomatic disease. A patient is more likely to either become colonised with *C. difficile* or develop CDI when in hospital with isolation rates of between 10-35% (Barbut and Petit, 2001, Kuijper *et al.*, 2006a) and positive toxin assays in 2-8% reported in hospitalised patients (Barbut and Petit, 2001). In addition to this, isolation of *C. difficile* from stool samples is reported to be proportional to length of

stay in hospital (Kuijper *et al.*, 2006a); this is likely to be due to the greater presence of *C. difficile* spores within the hospital environment (Mcfarland *et al.*, 1989).

1.5.5 Immune response

The symptoms associated with CDI can vary significantly and although this was initially thought to be due to the virulence of different strains of *C. difficile*, it has since been established that a patient's immune response can heavily influence the severity and extent of their symptoms (Kelly, 2007). Those who are immunocompromised are at an increased risk of CDI; however more subtle differences between hosts can also influence the symptoms that a person may experience. Several studies have demonstrated that higher antibody levels towards the toxin levels can provide some form of protection against both symptomatic infection or recurrence of infection against those who have previously suffered from an episode of CDI (Kelly *et al.*, 1992, Kyne *et al.*, 2000, Kyne *et al.*, 2001, Aronsson *et al.*, 1985, Aronsson *et al.*, 1983, Bacon and Fekety, 1994, Leung *et al.*, 1991, Mulligan *et al.*, 1993, Warny *et al.*, 1994) and this has provided the basis for using immunoglobulin therapy as a treatment option (Salcedo *et al.*, 1997). It has also been shown that an alternative genotype in the IL-8 gene may predispose a susceptibility to CDAD (Jiang *et al.*, 2007).

1.6 Detection and diagnosis

1.6.1 Clinical diagnosis

CDI should be suspected in hospitalised or patients in care establishments who present with an unexplained episode of diarrhoea. This alone is often sufficient for a clinician to send a stool sample for testing however this suspicion is likely to be further reinforced if the patient has additional symptoms such as abdominal pain and fever and also if the

patient belongs to a risk group. Diagnosis is confirmed in the laboratory following positive detection of *C. difficile* toxins in a stool sample.

1.6.2 Laboratory diagnosis

1.6.2.1Toxin detection

Within diagnostic laboratories the most common way in which CDI is diagnosed is through the use of enzyme immunoassay (EIA) kits that detect the presence of either Toxin A or both toxins. Lateral flow or membrane bound assay kits are also used to detect the presence of toxin and are generally quicker and easier to perform than EIAs. Both of these methods detect toxin directly from faecal samples and as no culture is required results can be obtained quickly; they also have the advantage of being cheaper and less labour intensive than other diagnostic tests which is why they are so frequently used in the diagnosis of CDI. They are however also associated with poor sensitivity and specificity with both false negative and false positive results being reported (Eltringham, 2009).

As it was previously thought that only *C. difficile* strains producing toxin A were capable of causing disease, diagnostic kits were designed to detect this toxin only. Following the discovery that A-/B+ strains also caused symptomatic disease and the rise in incidence of these strains; it is now recommended that only kits with the capacity to detect both toxins are used. These kits give a positive result if either of the two toxins is present in a sample; allowing for a more accurate diagnosis.

1.6.2.2 Cytotoxicity Assay

The gold standard technique for the diagnosis of CDI is cell cytotoxicity assays however, this method requires cell culture and a 48 hour incubation period. From a centrifuged stool sample, supernatant is removed and exposed to monolayers of cells (cell lines can differ); following incubation cells are observed for cell rounding, indicating cytotoxicity. If these

effects are then neutralised by the addition of anti-*Clostridium sordellii* antiserum, a positive result is recorded.

1.6.2.3 Cytotoxigenic culture

Culturing of stool samples onto selective media followed by cytotoxin assay is another method by which CDI can be diagnosed detecting both the presence of *C. difficile* and its toxins. Faecal samples can be cultured directly onto an agar selective for *C. difficile* such as cycloserine cefoxitin fructose agar (CCFA) which will inhibit the growth of other faecal bacteria and therefore only *C. difficile* should in theory grow. Alternatively, a small amount of the sample can be suspended in absolute ethanol which eliminates any bacterial cells and allows only spores to remain. The suspension is then cultured onto fastidious anaerobe agar supplemented with a known germinant of *C. difficile* spores such as sodium taurocholate; this is again selective for the growth of *C. difficile*. Typical colony morphology of *C. difficile* is greyish, flat colonies with a 'ground glass' appearance; this is also accompanied by a characteristic 'elephant house' odour. Other confirmatory tests such as Gram stains and API testing may also be used alongside culture techniques. Culture of *C. difficile* does however require 48 hours of incubation in anaerobic conditions which is significantly longer than immunoassay based toxin detection tests. Culture alone is not suitable as a diagnostic method due to asymptomatic carriage of *C. difficile*. It has been suggested that both culture and toxin detection is a better technique by which to diagnose CDI and may resolve the issues surrounding misdiagnosis (Delmee *et al.*, 2005) this however has significant cost, labour and turnaround time implications.

1.6.2.4 Glutamate dehydrogenase (GDH) detection

The specific *C. difficile* glutamate dehydrogenase enzyme is a common antigen found on *C. difficile*. Assays which utilise antibodies specific to this antigen can be used to detect

the presence of *C. difficile* however like culture this is not sufficient in the diagnosis of CDI despite accurate results. As GDH is produced by both toxigenic and non-toxigenic strains of *C. difficile*, a positive outcome is not always indicative of CDI. There are however now assays that detect the presence of both GDH and toxin, such tests confirm both the presence of *C. difficile* and the toxigenicity of the strain. These tests provide a two step method for the diagnosis of CDI in addition to providing results quickly.

1.6.2.5 Molecular techniques

Real time PCR techniques have been developed that detect either the genes for either of the toxins of *C. difficile* or the genes for the specific glutamate dehydrogenase. There are now several different published methods on real time PCR for the detection of different *C. difficile* genes including *tcdB* (Van Den Berg *et al.*, 2007, Peterson *et al.*, 2007, Stamper *et al.*, 2009), *tcdA* and *tcdB* (Belanger *et al.*, 2003) and *tcdC* (Sloan *et al.*, 2008) and a multiplex real time PCR assay has also been developed that detects four *C. difficile* genes (*tcdA, tcdB, cdtA* and *cdtB*) (Wroblewski *et al.*, 2009). These methods have demonstrated high levels of sensitivity and specificity in addition to providing results quickly in comparison to the other diagnostic methods (Eastwood *et al.*, 2009, Sloan *et al.*, 2008, Van Den Berg *et al.*, 2007). Due to the success of such diagnostic methods, two PCR assay kits are now available the BD GeneOhm™ Cdiff and ProGastro™ Cd Assay, these assays can give results in under two and three hours respectively. The extraction of genomic *C. difficile* DNA directly from faecal samples significantly enhances the result time as no prior culture of *C. difficile* is required.

1.7 Treatment

1.7.1 Metronidazole

Metronidazole displays bactericidal activity towards both protozoa and many anaerobic bacteria. Metronidazole is a nitroimidazole; a class of antimicrobial pro-drugs that require reduction by low redox conditions in order to demonstrate activity. Metronidazole is now the preferred treatment in the majority of CDI cases as it is more cost effective and selective than vancomycin; the only other widely approved treatment for CDI. The selective activity of metronidazole is attributed to a unique metabolic pathway found only in protozoal and anaerobic cells. When metronidazole diffuses into a cell with a low redox potential, ferredoxin donates electrons to the nitro group present on metronidazole. The reduction of the nitro group allows the drug to take on its active form; generating compounds that interfere with nucleic acid synthesis ultimately leading to cell death.

Metronidazole is most effective when administered orally and is almost completely absorbed. A dosage of 500mg three times a day for ten to fourteen days is usually prescribed in uncomplicated cases however each case is judged individually. It has also been suggested that metronidazole is equally effective when administered intravenously and can even achieve higher therapeutic levels (Bolton and Culshaw, 1986, Dion *et al.*, 1980). Metronidazole is also much cheaper than vancomycin for the treatment of CDI, with metronidazole costing approximately $2 per day in contrast to vancomycin which costs $30 per day (Kyne, 2010). Resistance towards metronidazole has been reported among *C. difficile* isolates by several groups (Wong *et al.*, 1999, Brazier *et al.*, 2001, Pelaez *et al.*, 2002) and in some cases there is no response when a CDI infection when treated with metronidazole (Huang and Nord, 2009).

1.7.2 Vancomycin

Vancomycin is a potent glycopeptide antibiotic used in the treatment of serious Gram positive infections. Vancomycin has a bactericidal effect on cells by inhibiting the synthesis of the peptidoglycan cell wall and is administered intravenously for the majority of infections; this however can lead to side effects and problems relating to toxicity. Vancomycin is a large hydrophilic molecule and does not transfer across the intestinal wall effectively; therefore treatment of CDI requires oral administration in order to establish the high therapeutic concentrations needed in the gut. In recent years the use of vancomycin for the treatment of CDI has declined both due to the cost and also concerns regarding the acquisition of vancomycin resistance by other organisms that reside in the gut such as enterococci. There are cases where vancomycin is still used in the treatment of CDI such as relapse or severe complicated cases also when a patient is pregnant, allergic or unresponsive to metronidazole or suffering from multiple recurrences of CDI (Poutanen and Simor, 2004).

1.7.3 Additional potential antibiotic treatments

1.7.3.1 Rifaximin

Rifaximin is a synthetic antibiotic that inhibits protein synthesis and already approved for the treatment of traveller's diarrhoea in the US. Rifaximin is not absorbed and therefore high concentrations are achieved within the gut (Scarpignato and Pelosini, 2005); it demonstrates excellent *in vitro* activity against *C. difficile* isolates (Marchese *et al.*, 2000) and has also been used *in vivo* with some success particularly as a treatment for multiple recurrences of CDI (Johnson *et al.*, 2009, Hecht *et al.*, 2007). Although a promising treatment option, resistant strains of *C. difficile* have already been recovered from patients

treated with rifaximin (O'connor *et al.*, 2008, Curry *et al.*, 2009, Hecht *et al.*, 2007); it is also very expensive.

1.7.3.2 Nitazoxanide

Nitazoxanide is similar to metronidazole; a pro drug with a similar mechanism of action and primarily used as an anti protozoal agent. Clinical trials have demonstrated that nitazoxanide is as effective as vancomycin and metronidazole in the treatment of CDI (Musher *et al.*, 2006, Mcvay and Rolfe, 2000, Hecht *et al.*, 2007, Musher *et al.*, 2009, Yangco *et al.*, 2009) and is a possible treatment option.

1.7.3.3 Fidaxomicin

Fidaxomicin is a new macrocyclic antibiotic also known by several other names including OPT-80, difimicin, PAR-101 and tiacumicin B (Sullivan and Spooner, 2010); currently undergoing Phase III clinical trials in the United States (Citron *et al.*, 2009). Fidaxomicin works by inhibiting RNA synthesis and has a bactericidal effect on cells and also has a narrower spectrum of activity, having a lesser effect on other flora in the gut (Louie *et al.*, 2009). Studies have reported improved treatment rates and lower recurrence rates with fidaxomicin (Louie *et al.*, 2009) .

1.7.3.4 Linezolid

Linezolid has been shown to have good *in vitro* activity towards isolates of *C. difficile*. Linezolid like vancomycin is only used in the treatment of serious Gram positive infections and is also expensive; it remains one of the few treatment options for bacteria that have developed resistance towards vancomycin and providing other treatments are still available for CDI is unlikely to be adopted as a mainstream treatment.

1.7.3.5 Fusidic acid

Fusidic acid has been shown to demonstrate excellent *in vitro* activity towards *C. difficile* and has also been tested *in vivo* (Wenisch *et al.*, 1996, Wullt and Odenholt, 2004, Noren *et al.*, 2006) Although fusidic acid has proven to be equally as effective as metronidazole for the treatment of CDI, there have been incidences of relapse reported (Wenisch *et al.*, 1996, Wullt and Odenholt, 2004, Noren *et al.*, 2006) . Fusidic acid inhibits protein synthesis in Gram positive cells with a bacteriostatic effect on cells and this may contribute to the high relapse rate. An additional concern about fusidic acid as a treatment option for CDI is resistance as resistant isolates of *C. difficile* have been recovered from patients who have been treated with fusidic acid (Noren *et al.*, 2006). Resistance towards fusidic acid is easily acquired and it is inadvisable for it to be administered alone; because of this fusidic acid has limited potential in the treatment of CDI.

1.7.3.6 Tigecycline

Tigecycline is a bacteriostatic, broad spectrum antibiotic that belongs to the glycylcyclines; a new class of antibiotics of which tigecycline is the first. So far the *in vitro* activity of tigecycline against isolates of *C. difficile* has been found to be excellent (Hecht *et al.*, 2007, Noren *et al.*, 2009, Baines *et al.*, 2006) and it does not appear to effect toxin production or sporulation within the gut (Baines *et al.*, 2006). There is also evidence of successful treatment of CDI using tigecycline however studies have been limited (Herpers *et al.*, 2009).

1.7.4 Probiotics

Probiotics have been suggested as both a prophylactic and treatment in CDI however studies have given variable results into their effectiveness (Mcfarland *et al.*, 1994, Lawrence *et al.*, 2005, Surawicz *et al.*, 2000, Wullt *et al.*, 2003b). The most common

probiotics used are *Saccharomyces boulardii* and *Lactobacillus rhamnosus* with the aim of such therapy to re-populate the gut and prevent CDI through the action of colonisation resistance. Many of the studies that have investigated the effectiveness of probiotics in either the prevention of treatment or CDI have failed to provide any strong evidence for their use (Pillai and Nelson, 2008). Although argued by some that there is no harm in taking probiotics as a preventative measure or as an adjunct to antibiotic therapy; in severely immunocompromised patients probiotics can still cause infection (Munoz *et al.*, 2005).

1.7.5 Immune Therapies

1.7.5.1 *Clostridium difficile* vaccine (ACAM-CDIFF™)

The *C. difficile* vaccine is a toxoid vaccine currently in Phase II clinical trials in the UK (Kyne, 2010); if the vaccine proves effective it is proposed it would be administered to at risk groups. During the current trials at present, the vaccine is being administered to those who are experiencing their first episode of CDI with the hope that the vaccine will prevent relapses. The vaccine contains toxoid A and toxoid B, simulating an immune response to both toxins through the production of serum IgG antitoxin A and serum IgG antitoxin B antibodies (Kotloff *et al.*, 2001).

1.7.5.2 Hyperimmune globulin and intravenous immunoglobulin

Hyperimmune globulin and intravenous immunoglobulin therapies are primarily used to prevent a relapse of CDI infection however such therapies have been used as a treatment in severe cases of CDI when other therapies have proved ineffective or when a patient is suffering from multiple recurrences (Salcedo *et al.*, 1997, Leung *et al.*, 1991, Mcpherson *et al.*, 2006, Murphy *et al.*, 2006, Wilcox, 2004, Beales, 2002, Hassoun and Ibrahim, 2007, Koulaouzidis *et al.*, 2008). In a trial however, intravenous immunoglobulin was found to

be no more effective than other conventional treatments (Juang *et al.*, 2007). The purpose of such therapies is to provide antitoxin antibodies to the immune system of patients who appear to have failed to elicit an effective immune response.

1.7.5.3 Faecal therapy

Faecal transplant therapy is not widely available or an approved therapy but in a review by (Van Nood *et al.*, 2009) has been reported to be extremely effective in both the treatment and the prevention of recurrences of CDI. A faecal donor is usually a relation and preferably a person who shares the same living environment (Van Nood *et al.*, 2009); the faecal transplant is then performed via naso-gastric tube, coloscopy or enema (Van Nood *et al.*, 2009). The aim of faecal transplant therapy is to re-colonise the gut with a population of commensal organisms similar to those present in the patient prior to infection, this then prevents or greatly reduces the risk of further *C. difficile* overgrowth and predomination. Clinical trials using faecal therapy are currently underway in the Netherlands.

1.7.6 Toxin therapies

1.7.6.1 Tolevamer

Tolevamer is a novel non-antimicrobial therapy aimed at inactivation of *C. difficile* toxins, rather than targeting the bacteria, tolevamer binds to the toxins neutralising their activity (Braunlin *et al.*, 2004). A major advantage of this therapy is that as the polymer has no antimicrobial activity there is no disruption to the gut flora. Despite good results in Phase II clinical trials, Phase III trials were ended prematurely as they showed that tolevamer was not as effective as vancomycin in treating CDI; these results were also replicated when using a gut model (Baines *et al.*, 2009).

1.8 The *C. difficile* genome and typing methods

Sequencing of the first *C. difficile* genome was completed and published in 2006. The strain selected was *C. difficile* 630 (PCR ribotype 012); a highly drug resistant strain isolated from a patient with PMC in 1982 (Sebaihia *et al.*, 2006). The chromosome of this strain was found to 4.29mbp in size with nearly 11% of the genome consisting of transposons which it was proposed allowed the potential for the acquisition of genes that could enhance the virulence and pathogenicity of the organism (Sebaihia *et al.*, 2006).

Two other *C. difficile* genomes have since been sequenced and both of these belong to PCR ribotype 027; strains CD196 and R20291. Strain CD196 was isolated from a patient in France in 1985 while the R20291 strain was isolated from patient at Stoke Mandeville hospital, UK in 2006 (Stabler *et al.*, 2009). The investigation into the comparison of these three sequenced genomes has provided insight not only into the variations between two different PCR ribotypes but also differences between strains belonging to the same PCR ribotype and how this cluster of isolates may have evolved.

1.8.1 Immunochemical typing

Immunochemical fingerprinting techniques were some of the first methods used to identify variances between isolates of *C. difficile* (Nakamura *et al.*, 1981, Wust *et al.*, 1982, Poxton *et al.*, 1984), although never used to define distinct types of *C.difficile*; such experiments did however provide the basis for serotyping. Immunochemical techniques investigate the interactions between antigen and antibodies and so in microbiological analysis can be used to determine if particular isolates of bacteria are the same through reactions elicited between the two. Antigens from the cell surface are extracted and reacted with antiserum which has been produced in an animal exposed to whole cell extracts of *C. difficile*; this is often done via crossed immunoelectrophoresis (CIE) and blotting techniques.

Immunochemical methods are now rarely used in the typing of *C. difficile* due to the development of molecular methods.

1.8.2 Serotyping

Serotyping was the first method that was used to discriminate between different types of *C. difficile*, based on variations between strains of the antigenic properties expressed on the cell surface. Serotyping is carried out using slide agglutination or ELISAs using rabbit antisera. Serogroups of *C. difficile* were first defined in 1985 (Delmee *et al.*, 1985) however, such work followed previous work on the immunochemical typing of *C. difficile* (Nakamura *et al.*, 1981, Poxton *et al.*, 1984). Ten defined serogroups have been identified; each group identified using a capital letter, 20 sub-serogroups of group A can be further identified by PAGE. Serotyping has been reported to correlate to some extent with toxinotyping (Rupnik *et al.*, 1998).

1.8.3 Surface layer protein (S-layer) typing

S-layer typing is a phenotypic technique that extracts surface layer proteins from *C. difficile*. The proteins are extracted from the cell surface using EDTA, urea or guanidine hydrochloride before SDS PAGE is performed on the extracted proteins. Most bacteria possess an S-layer that consists of a repeating structure of the same one protein; the S-layer of *C. difficile* however consists of two repeating proteins. The two proteins found in *C. difficile* can vary in their molecular mass; a larger protein of approximately 56-48kDa and a smaller protein in the range of 45-37kDa. The variances observed in these S-layer proteins are encoded by the slpA gene which can also be targeted in a genotypic method. The slight variability in mass that can exist in both proteins allows isolates to be grouped according to their molecular weight. Although S-layer typing does not have the same discriminatory capacity as some molecular techniques it does generally exceed other

phenotypic methods and it has been shown to correlate well with PCR ribotyping (Mccoubrey and Poxton, 2001). The exact properties of the S-layer proteins are unknown but it has been speculated that they could have various adhesive, pathogenic and immunogenic properties (Drudy *et al.*, 2004, O'brien *et al.*, 2005, Ausiello *et al.*, 2006); their role in serotyping is also unclear.

1.8.4 Antibiogram profiling

Antibiogram profiling is the most common phenotypic typing method; it is often used alongside genotypic methods and is frequently used in research studies. Not only do antibiogram profiles provide useful phenotypic information about an isolate but the information is also used in the surveillance of antibiotic resistance in the *C.difficile* population. The emergence of fluoroquinolone resistance amongst isolates of *C. difficile* and high mortality rates associated with such isolates lead to the identification of the hypervirulent PCR ribotype 027 strain (Loo *et al.*, 2005) which is now associated with a particular antibiogram profile (resistance towards fluoroquinolones and erythromycin and sensitivity to clindamycin) (Drudy *et al.*, 2008). Resistance towards antibiotics in *C. difficile* is also highly likely to reflect changes at the genomic level through the uptake of resistance genes into the genome in the form of transposons.

1.8.5 Biochemical typing

Biochemical typing is used to determine the variability in biochemical and metabolic pathways of an organism. The advent of analytical profile index (API) strips has made such typing easier and less labour intensive however biochemical testing is now primarily used as a method of identification for a bacterial species rather than a typing method. Although biochemical typing can provide useful information about a bacterial species; results can be highly variable and hard to quantify. In comparison to more advanced

techniques the discriminatory power of biochemical typing is poor and within many bacterial species unlikely to vary at all.

1.8.6 Toxinotyping

Toxinotyping is used to detect variability in the PaLoc of different isolates of *C. difficile* (Rupnik et al., 1998), although when first developed only genes encoding for toxins A & B were analysed (Rupnik et al., 1997). Toxinotyping combines PCR to initially amplify regions within PaLoc, restriction enzymes are then used within these amplified fragments, from this restriction fragment length polymorphisms (RFLPs) can be identified and the patterns analysed and compared. Restriction patterns from the *C. difficile* reference strain VPI 10463 are used to compare toxinotypes; this strain and all others sharing the same pattern within PaLoc are designated toxinotype 0. So far 24 different toxinotypes have been identified (Kuijper *et al.*, 2006b) with roman numerals assigned to distinguish different toxinotypes. Toxinotyping demonstrates very good correlation with ribotyping (Rupnik *et al.*, 2001), restriction endonuclease analysis (REA) (Geric *et al.*, 2006) and to a lesser extent with serotyping (Rupnik *et al.*, 1998) however, it is not as discriminatory as some other techniques (Martin *et al.*, 2008).

1.8.7 Bacteriophage and bacteriocin typing

There are few published studies that describe the use of bacteriophage and bacteriocin typing for the characterisation of *C. difficile*. As a phenotypic and relatively early typing method, the majority of the studies were performed prior to the widespread use of molecular techniques and therefore no longer used. Despite this it was a useful typing technique and was used for epidemiological typing (Hawkins *et al.*, 1984, Sell *et al.*, 1983, Camorlinga-Ponce *et al.*, 1987, Kaatz *et al.*, 1988). Bacteriophage typing characterises isolates by testing their susceptibility to different bacteriophages; this is based on the

principle that different strains within a species possess different cell surface receptors that can be utilised by different bacteriophages (Pitt and Gaston, 1995b). Bacteriocins are inhibitory products secreted by bacteria that are species specific however susceptibility will vary between strains and it is this variability that is exploited in bacteriocin typing (Pitt and Gaston, 1995a). Both bacteriophage and bacteriocin typing is laborious (Sell *et al.,* 1983) and not as discriminatory as molecular techniques.

1.8.8 Restriction endonuclease analysis

Restriction endonuclease analysis (REA) is a method that allows the analysis of total genomic DNA. Restriction enzymes are used to create REA profiles that characteristically consist of hundreds of DNA fragments. When first used to type *C. difficile* (Devlin *et al.*, 1987), REA was found to be an effective and discriminative method and it is still one of the more discriminative techniques for typing *C. difficile* (Killgore *et al.*, 2008). However, due to the large number of fragments produced by REA, both analysis of the gels and transferability between laboratories is difficult (Marsh *et al.*, 2006).

1.8.9 Multi-locus variable number tandem repeat analysis

Multi-locus variable number tandem repeat analysis (MLVA) is a highly discriminative PCR technique based on variable number tandem repeat (VNTR) methodology. In VNTR the length of a tandem repeat sequence at only one locus is analysed whereas in MLVA several loci are investigated. When using MLVA to genotype *C. difficile* isolates; the length of tandem repeats at seven different loci are used to characterise and determine variability between isolates using primers that flank these variable regions (Marsh *et al.*, 2006). The MLVA method alongside REA has been reported to have the highest level of discriminatory power for the typing of *C. difficile* in a study that compared seven different popular typing methods (Killgore *et al.*, 2008). It has also been reported however that the

high level of discriminatory power also eradicates strain similarities when presented in a dendrogram (Killgore *et al.*, 2008). In 2008, the CDRN announced the availability of MLVA for clinical isolates as part of an enhanced typing service however there are strict criteria that need to be met in order to use this service (HPA, 2009b).

1.8.10 Arbitrarily primed PCR/ Random amplification of Polymorphic DNA

Unlike other PCR based methods random amplification of polymorphic DNA (RAPD)/AP-PCR requires no prior knowledge of the target genome. One short primer (10bp) is used in the reaction to bind to unknown regions within the DNA but in order for regions to be amplified both primers must bind in the right direction and also in close enough proximity to each other for a fragment to be produced. The basis of RAPD/AP-PCR is that polymorphisms within the genome of different isolates will lead to either the absence or addition of amplicon fragments, creating different fingerprint patterns. As it is unknown where primers bind in RAPD/AP-PCR reactions and if in fact they will bind anywhere, initial annealing temperatures are considerably lower than in conventional PCR reactions in order to promote binding. The low temperatures however can also encourage non-specific binding and this then causes issues in the reproducibility and discriminatory capabilities of the method, the use of such short primers can also lead to less specific binding. When RAPD/AP-PCR has been used to type *C. difficile* isolates, reproducibility has often been reported to be an issue (Bidet *et al.*, 2000, Wullt *et al.*, 2003a) and although not as discriminatory as methods such as MLVA, REA and PFGE it is less labour intensive and more cost effective.

1.8.11 Repetitive extragenic palindromic PCR

Repetitive extragenic palindromic PCR (REP-PCR) is a technique that makes use of repetitive sequences, 35-40 bp in length found throughout the genomes of most Gram

negative and several Gram positive bacteria. Complementary primers are used to bind to these sequences leading to the amplification of regions between repetitive sequences. Since first developed (Rademaker et al., 1997), REP-PCR has been used to type many species of bacteria due to high levels of reproducibility and discriminatory power associated with the method. REP-PCR is not frequently used in the typing of *C. difficile*, despite initial studies indicating that REP-PCR is more discriminatory than PCR ribotyping (Spigaglia and Mastrantonio, 2003). Later studies also indicate that REP-PCR has the ability to sub-type within defined PCR ribotypes (Rahmati *et al.*, 2005).

1.8.12 Multi-locus sequence typing

The multi locus sequence typing (MLST) method was one of the first genomic methods with the capability to examine long term bacterial population genetics and epidemiology having been used with several other species of bacteria. The reason why MLST can provide information about the evolution of a species of bacteria is it involves the sequencing of several housekeeping genes (Maiden *et al.*, 1998), and in the case of *C. difficile* typing, only occur once in the genome (Lemee *et al.*, 2004).The sequencing and surveillance of such genes across a range of isolates then allows relationships and genetic lineages to be determined through the use of mathematical analysis programs. When examining *C. difficile* or other bacterial species from an evolutionary perspective MLST is extremely useful however, it is not a method that is suitable for routine typing as the sequencing of seven individual loci make it very labour intensive and although this method does have a good level of discriminatory power a recent study has reported that it does not discriminate as well as MLVA or REA (Killgore *et al.*, 2008). The MLST method can however provide additional lineage information that these other methods do not (Marsh *et al.*, 2009).

1.8.13 Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) is a genotypic technique that was first described in 1993 and has been patented by Keygene (Vos *et al.*, 1995); it uses both restriction enzymes and PCR methodology to detect polymorphisms throughout the entire genomic DNA. Restriction enzymes are first used to digest the entire cellular genomic DNA and adaptors are then ligated to the restriction fragments that have been produced. Two primers are used that are complementary to both the adaptor and the restriction site, and selective nucleotides added to the 3' end of the primers making amplification more specific. Only fragments which posses complementary sequences to the primer-nucleotide complex will be amplified, the results of which are usually visualised by polyacrylamide denaturing gel electrophoresis or capillary electrophoresis. When first used to type *C. difficile*, AFLP was found to be more discriminatory than PFGE (Klaassen *et al.*, 2002) and it has also been reported that it also produces results comparable to PCR ribotyping (Van Den Berg *et al.*, 2004). Although relatively easy to perform and less labour intensive than some other molecular typing methods it is used infrequently as methods such as MLVA, REA and PCR ribotyping are regarded as more discriminatory.

1.8.14 Surface layer protein A gene sequencing (slpAST)

Based on the variation that is seen in the S layer proteins of different isolates of *C. difficile*, surface layer protein A sequencing (*slp*AST) sequences the gene that controls this variation in the S layer proteins and was first used in 2002, originally designed as an alternative to serotyping (Karjalainen *et al.*, 2002). This method combines PCR and subsequent sequencing and therefore is more labour intensive than straight forward PCR techniques; it has however been shown to be more discriminatory than PCR ribotyping and can define types within ribotypes (Killgore *et al.*, 2008, Kato *et al.*, 2005), as a sequencing technique is also more transferable between laboratories (Kato *et al.*, 2005).

1.8.15 Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) was developed in 1984 by Schwartz & Cantor (Schwartz and Cantor, 1984) as a method by which large fragment lengths of DNA (>50 kb) could be separated. The application of periodic changes in the direction of the electrical field enables larger fragments to separate and resolve on the gel. It is frequently used in the characterisation of many bacteria and is often regarded as the gold standard; it is the standard method of typing for *C. difficile* isolates in North America with strains identified with NAP and a preceding number. The discriminatory capacity of PFGE has been known to be of a high standard since it was first used however (Kristjansson *et al.*, 1994), as this and several other investigations discovered, some strains of *C. difficile* could not be typed due to degradation of the DNA. Due to this, other methods had to be developed that would provide reproducible and discriminatory results for the typing of *C. difficile*, it was not until a successful modification of the method that this problem was resolved (Alonso *et al.*, 2005). The discriminatory power of PFGE is very good and similar to that of REA (Kristjansson *et al.*, 1994, Killgore *et al.*, 2008) when typing isolates of *C. difficile*, it is still however a very labour intensive technique (Killgore *et al.*, 2008) in comparison to PCR methods.

1.8.16 PCR ribotyping

PCR ribotyping is currently the gold standard method in the UK for the genotyping of *C. difficile* isolates and so far over one hundred different ribotypes have been defined (Stubbs *et al.,* 1999). Primers are used that flank the 16S-23S intergenic spacer regions of *C. difficile*, although the 16S and 23S genes are highly conserved, the length of the spacer region between them are highly variable; this provides individual fingerprint patterns for the different ribotypes (Brazier *et al.*, 2001). The initial protocol for PCR ribotyping was long and labour intensive however this was later modified to allow for the routine use of

typing clinical isolates (O'Neill *et al*., 1996). PCR ribotypes are identified by the order in which they were discovered and represented by a three digit number. Although PCR ribotyping is very discriminatory, the predominance of particular ribotypes has meant that this method no longer provides sufficient epidemiological information, especially in regards to outbreaks (Fawley *et al.*, 2008).

1.9 Aims and objectives

Clostridium difficile is one of the most common causes of nosocomial infection in the western world and cases have been steadily rising over the past decade. A significant rise in cases over more recent years has been due to the emergence of a hypervirulent strain of *C. difficile* identified as PCR ribotype 027. Toxins A and B are the major virulence factors produced by *C. difficile*; however, information regarding phenotypic characteristics of C. *difficile* isolates and virulence factors such as enzymes is scarce and dated. PCR ribotyping is the gold standard method used to genotypically type *C. difficile* isolates in the UK. The increase in CDI cases in the UK has also seen a predominance of only a few PCR ribotypes and this has restricted the epidemiological use of the PCR ribotyping method. To investigate both phenotypic and genotypic characteristics of two *C. difficile* populations recovered from two separate tertiary referral trusts within the West Midlands, UK, a number of methods were employed.

The aims of this study were to:

- Determine if any of the clinical *C. difficile* isolates recovered from the two tertiary referral trusts produced extracellular enzymes as potential virulence factors.
- Determine the minimum inhibitory concentrations (MICs) of twelve antibiotics in all of the clinical isolates recovered.

- Optimise a random amplification of polymorphic DNA (RAPD) protocol that is reproducible and able to discriminate between different strains of *C. difficile*.
- Characterise clinical isolates using the optimised RAPD protocol and the current gold standard typing method, PCR ribotyping, and compare the discriminatory power of both methods.
- Determine if there are any associations between the phenotypic and genotypic characteristics observed in the clinical isolates.

CHAPTER 2 PHENOTYPIC CHARACTERISATION OF *CLOSTRIDIUM DIFFICILE*

2.1 INTRODUCTION

Phenotypic characteristics are frequently used in the identification of many bacterial species, with colony morphology, odour, Gram stain result and virulence factor production (e.g. haemolysins) often providing enough information to make a preliminary identification in routine clinical microbiology laboratories. In addition to this, antibiotic sensitivity testing is often carried out; this provides additional information including identification of the appropriate treatment option and epidemiological surveillance of antibiotic resistance throughout the species population. Furthermore antibiotic sensitivity testing will generate an antibiogram pattern for each clinical isolate which may be used, albeit with low discriminatory power, to identify strain similarity. However, unlike many other bacterial species, *C. difficile* is not routinely cultured in diagnostic laboratories due to detection and diagnosis being established through direct testing of a faecal sample, this is despite suggestions that culture is required due to constant changes that are occurring within the *C. difficile* population (Wren, 2006, Stabler *et al*., 2009). As a result of this, the phenotypic characteristics of *C. difficile* have not been widely explored.

The lack of recent investigation into the biochemical reactions and enzyme production of *C. difficile* mean that it is not known whether such characteristics have changed within the population; this may be likely as both antibiotic resistance and spore production are known to vary within the species. The detection of previously unseen fluoroquinolone resistance in *C. difficile* isolates highlighted the emergence of the hypervirulent PCR ribotype 027 strain and demonstrates the importance of culture and antibiotic surveillance.

Although vancomycin and metronidazole resistance is not yet a clinical problem there are already isolated reports of metronidazole resistance (Wong *et al.*, 1999, Brazier *et al.*,

2001, Pelaez *et al.*, 2002). Susceptibility testing of other antibiotics, particularly those that are frequently associated with inducing CDI, is important in providing information that can be used locally to influence antibiotic prescribing policies and possibly reduce rates of CDI

The development of molecular techniques over recent years has seen phenotypic characterisation often being overlooked; as a result there has been little research published in recent years that have investigated phenotypic characteristics of *C. difficile*. Although these methods are generally time consuming and slow to produce results in comparison to genotypic methods, the results can often provide considerable insight into an organism.

In this chapter, the phenotypic characteristics of a panel of clinical isolates of *C. difficile* were explored. Firstly the biochemical profile patterns (biotypes) and the ability of the isolates to produce a range of virulence factors was investigated. Secondly the isolates were characterised using S-layer typing using SDS PAGE. Finally, the susceptibility patterns against a panel of twelve antibiotics were determined by minimum inhibitory concentration.

2.2 MATERIALS AND METHODS

2.2.1 Isolates of *C. difficile*

Sixty two clinical isolates of *C. difficile* obtained from two hospital tertiary care Trusts (designated A and B) in the West Midlands, UK were phenotypically characterised: thirty two isolates were recovered from Trust A, and the remaining thirty from Trust B. Isolates were recovered from patients between the time period 2004 -2005. In addition to these clinical isolates two control strains were also included; the reference strain National Collection of Type Cultures (NCTC) 11204 and a reference PCR ribotype 027 strain R20291 were also included in the investigation. A panel of eleven different genetically characterised PCR ribotypes (001, 002, 005, 014, 015, 017,023, 027, 064, 078 and 106) obtained from the North East HPA laboratory, Newcastle-upon-Tyne, UK were also included in MIC determination.

2.2.2 Recovery of *C. difficile* from clinical faecal specimens

Sixty two clinical isolates of *C. difficile* were recovered from *C. difficile* toxin positive faecal specimens obtained from Trusts A and B. Thirty two of the isolates were recovered from Trust A, while the remaining thirty of the isolates were from Trust B. Isolates were stored using a Microbank bacterial preservation system (Pro-Lab Diagnostics, UK) at –70°C until required.

Faecal samples had been stored at -20°C until required, *C. difficile* was recovered from clinical samples from Trust B using alcohol shock methodology. A sterile swab was used to place a pea sized amount of faecal matter into one millilitre of pure ethanol, vortexed and left at room temperature for one hour. One hundred microlitres of suspension was then removed and cultured onto Fastidious Anaerobe Agar (LabM, UK) supplemented with 0.1% ($^{w}/_{v}$) sodium taurocholate (Sigma-Aldrich, UK) and 5% ($^{v}/_{v}$) horse blood (Southern

Group Laboratories, UK); agar plates were then incubated in anaerobic conditions at 37°C for 48 hours.

Following incubation, plates were observed for growth of *C. difficile.* Suspect colonies were identified by typical characteristics including 'fried egg' or 'ground glass' colony morphology and characteristic 'elephant house' odour. Suspected *C. difficile* colonies were then Gram stained to confirm identification and subcultured onto Wilkins-Chalgren anaerobe agar (Oxoid, UK) to ensure pure growth. One colony from a pure culture was then taken and used to inoculate beads which were then stored at -70°C until needed.

2.2.3 API Biotyping

The API (Analytical Profile Index) rapid ID 32A (Biomerieux, France) was used in accordance with the manufacturers recommendations as a confirmatory test for the positive identification of *C. difficile* and also to identify any differences in biotypes between the different isolates.

2.2.4 Detection of extracellular virulence factors

2.2.4.1 Lipase production

Lipase production was detected using olive oil agar (Farrell *et al*., 1993) which comprised of 3% ($^{w}/_{v}$) agar no. 1 (Oxoid, UK), 1% ($^{w}/_{v}$) tryptone (Oxoid, UK), 0.5% ($^{w}/_{v}$) NaCl (Fisher Scientific, UK), 0.001% ($^{w}/_{v}$) rhodamine B (Sigma-Aldrich, UK) and 2.5% ($^{v}/_{v}$) olive oil (Sainsbury's Ltd, UK). Colonies of *C. difficile* from 48 hour culture plates were used to inoculate the surface of the agar which was then incubated in anaerobic conditions at 37°C for a further 48 hours. Lipase production was confirmed through the growth of bright pink colonies that fluoresced orange under UV light. As a positive control *Pseudomonas aeruginosa* American Type Culture Collection (ATCC) 15442 was used and *Streptococcus pyogenes* NCTC 11200 was as a negative control.

2.2.4.2 Non-specific protease production

To detect non specific protease production, 1% ($^{w}/_{v}$) agar no.1 was supplemented with 1 % ($^{w}/_{v}$) skimmed milk powder (Premier Foods, UK). Colonies of *C. difficile* from 48 hour cultures were used to inoculate the surface of the agar and incubated in anaerobic conditions at 37°C for 48 hours; protease production was confirmed through a zone of clearing around the colonies. The positive and negative controls used for this test were *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 29213 respectively.

2.2.4.3 DNase production

DNase production was detected using DNase agar (Oxoid, UK) in accordance with manufacturer's instructions; *S. aureus* ATCC 29213 was used for a positive control and *Staphylococcus epidermidis* RP62A was used as a negative control. Colonies of *C. difficile* from 48 hour cultures were inoculated on to the surface of the agar and incubated in anaerobic conditions at 37°C for a further 48 hours. Following incubation the agar was then flooded with 1M HCl in order to visualise the results; a clearing around the colony indicating DNase production.

2.2.4.4 Haemolysin production

Tests for haemolysis of both horse and sheep blood were investigated using Wilkins-Chalgren anaerobic agar supplemented with 5% ($^{v}/_{v}$) horse blood (Southern Group Laboratories, UK) and 5% ($^{v}/_{v}$) sheep blood (Southern Group Laboratories, UK) respectively. Colonies of *C. difficile* from 48 hour cultures were inoculated on to the surface of the agar and incubated in anaerobic conditions at 37°C for 48 hours. Haemolysin production was confirmed through a clear zone around the colonies. The control strains used for both blood types were *S. pyogenes* NCTC 11200 and *Escherichia coli* ATCC 25922 as positive and negative controls respectively

2.2.4.5 Gelatinase production

Nutrient gelatin (Oxoid, UK) was used to detect the production of gelatinase; *E. coli* ATCC 25922 and *S. epidermidis* RP62A served as positive and negative controls respectively. Nutrient gelatin was poured into test tubes and stab inoculated with one colony of *C. difficile*. Tubes were then incubated for 2 weeks in anaerobic conditions at 37°C. Following incubation tubes were then refrigerated at 4°C for 1 hour. Gelatinase production was indicated when nutrient gelatin remained in liquid form following refrigeration.

2.2.4.6 Urease production

Urease production was detected using urea agar slopes (Oxoid, UK); *Proteus mirabilis* NCTC 8309 and *E. coli* ATCC 25922 were used as positive and negative controls respectively. The surface of urea slopes were inoculated with one colony of *C. difficile* and incubated for 5 hours in anaerobic conditions at 37°C. Urease production was confirmed by a pink discolouration of the urea agar.

2.2.4.7 Toxin production

Toxin A & B production was confirmed using Premier™ Toxins A&B immunoassay kit (Meridian Biosciences, USA) and carried out according to the manufacturer's instructions.

2.2.5 Susceptibility testing

Minimum Inhibitory Concentrations (MICs) of a panel of antibiotics were determined using the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2007) on Wilkins-Chalgren anaerobe agar (Oxoid) and each test was performed in triplicate. Antibiotic solutions were made as described in appendix (1). Ten microlitres of an overnight culture of *C. difficile* grown in Wilkins-Chalgren broth

(Oxoid, UK) was adjusted to 10^7 CFU/ml and inoculated onto agar to give a final concentration of 10^5 CFU per spot. Plates were incubated for 48 hours at 37°C in anaerobic conditions. The MIC was determined as the lowest concentration at which growth was inhibited; growth of a single colony was disregarded. The following antimicrobials were tested: cefotaxime (Melford Laboratories Ltd, UK), chloramphenicol (Sigma-Aldrich), clindamycin (Duchefa, Netherlands), erythromycin (Sigma-Aldrich), fusidic acid (LEO Pharmaceuticals, Buckinghamshire, UK), imipenem (Merck, Sharp & Dohme Ltd, Northumberland, UK), levofloxacin (Hoechst Marion Roussel, Ltd, Middlesex, UK), linezolid (Pfzier Ltd, Kent, UK), metronidazole (Sigma Aldrich), rifampicin (Melford Laboratories Ltd), tetracycline (Sigma Aldrich) and vancomycin (Sigma Aldrich).

Table 2.1 Antibiotics tested and breakpoints used. (Breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)).

Antibiotic	Breakpoint (µg/mL)
Cefotaxime	>2
Chloramphenicol	>8
Clindamycin	>4
Erythromycin	>4
Fusidic Acid	>0.5
Imipenem	>8
Levofloxacin	>2
Linezolid	>4
Metronidazole	>4
Rifampicin	>1
Tetracycline	>2
Vancomycin	>8

2.2.5.1 Calibration curve

Three separate isolates of *C. difficile* were used to produce data for a standard curve; the isolates used were NCTC 11204 and clinical strains numbers B1 and Z1591. Forty eight hour colonies were used to inoculate 10ml of Wilkins-Chalgren anaerobe broth which was then incubated for 24 hours at 37°C in anaerobic conditions.

One hundred microlitres of each culture was used to inoculate a further 20mls of Wilkins Chalgren anaerobe broth and cultures were incubated for 48 hours at 37°C in anaerobic conditions. At intervals within the 48 hour period, one millilitre of each culture was taken and an optical density reading taken at A_{600}. A further 1ml was taken from the culture and serial dilutions were carried out to a concentration of 10^{-6}. Of these dilutions, 100ml of culture at 10^{-2} to 10^{-6} was taken and each spread onto Wilkins-Chalgren Anaerobe Agar with a wedge shaped spreader. Plates were incubated for 48 hours at 37°C in an anaerobic cabinet and average colony counts for each time point and dilution were then used to produce a calibration curve.

2.2.5.2 Chi-square (χ^2) analysis

Chi square analysis was used to determine if there were significant associations between isolate location and antibiotic sensitivity, and also isolate location and resistance towards individual antibiotics. Significant associations were determined using the chi square equation:

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Where *O* is the observed frequency with which resistance occurs within the population and *E* is the expected frequency with which resistance would occur within a population.

2.2.5.3 Simpson's index of diversity

The diversity of antibiogram profiles obtained from the isolates recovered from different trusts was determined using Simpson's index of diversity (D):

$$D = 1 - \frac{\Sigma\, n\,(n-1)}{N\,(N-1)}$$

Where *N* is the total number of strains in the sample population and *n* is the number of strains belonging to the profile type.

2.2.6 S-layer typing

2.2.6.1 Extraction of S-layer proteins with urea to use in SDS-PAGE analysis

Surface layer proteins from *C. difficile* isolates were extracted using a modification of the method of (Cerquetti *et al*, 2000). Colonies of *C. difficile* were taken from 48 hour cultures and used to inoculate 50mls of proteose peptone yeast (PPY) broth (2% ($^{w}/_{v}$) proteose peptone (Difco, USA), 1% ($^{w}/_{v}$) yeast extract (Oxoid, UK) and 0.5% ($^{w}/_{v}$) sodium chloride (Fisher Scientific, UK)), supplemented with 0.04% ($^{w}/_{v}$) sodium carbonate (Fisher Scientific, UK) and 0.075% ($^{w}/_{v}$) cysteine hydrochloride (Sigma-Aldrich, UK). Broths were then incubated for 24 hours and following incubation cells were harvested by centrifugation at 5800 x g for 30 minutes. Pelleted cells were then washed with 1ml of 50mM Tris-HCl pH 7.4 and centrifuged again at 5800 x g for 30 minutes at 4°C and the supernatant was discarded. The resulting pellet was add to 2mls of 8M urea/Tris-HCl pH 8.3 and 500µl of 2mM phenylmethylsulfonylfluoride (PMSF) and vortexed before being incubated for 1hour at 37°C. After incubation the suspension was centrifuged at 19000 x g for 30 minutes and the supernatant containing the extracted surface layer proteins removed and stored at -20°C until required.

2.2.6.2 Preparation of samples for SDS-PAGE analysis

Samples of supernatent containing the extracted S-layer proteins were allowed to thaw at room temperature and vortexed prior to 50µl of sample being added to 50µl of sample denaturing buffer which was prepared as described in appendix (2). Samples were then heated at 94°C for 12 minutes prior to loading into the gel.

2.2.6.3 Preparation and running of gels for SDS-PAGE analysis

Gels and buffers were prepared as outlined in appendix (2) and using the mini PROTEAN 3 cell (Bio Rad, USA).Samples were loaded into the stacking gel using a 30µl Hamilton microlitre syringe. Into the stacking gel, 12µl of sample was loaded into the wells; 8µl of proteins standard was loaded into the wells at the ends of the stacking gel (protein standards are described in appendix 2). Gels were run at 200 volts until samples reached the end of the separating gel.

2.2.6.4 Staining and visualisation of gels

Gels were stained using Coomassie blue, the stain and de-stain were prepared according to the relevant table in appendix (2). Gels were stained by flooding the gel for at least 1 hour in Coomassie blue stain, this was then replaced with Coomassie blue de-stain which was routinely replaced once the de-stain began to change colour. For best results de-stain was replaced once before being left overnight. Once de-stained, gels were photographed using GeneSnap, (Syngene, UK).

2.3 RESULTS

2.3.1 *C. difficile* isolates

All isolates were characteristic of *C. difficile* in colonial morphology; odour and Gram stain although slight variations were also observed among all of these characteristics. The Gram stain and colonial morphology of the *C. difficile* reference strain NCTC 11204 are shown in figures 2.1 and 2.2 respectively and are the standard by which the clinical isolates were compared.

Figure 2.1 Gram stain of *C. difficile* reference strain NCTC 11204 which is representative of the typical appearance of *C. difficile*.

Figure 2.2 Colony morphology of *C. difficile* reference strain NCTC 11204 which is representative of the typical appearance of *C. difficile*.

2.3.2 API Biotyping

All 62 clinical isolates and the two reference strains of *C. difficile* were typed using the Rapid ID 32A API system and from these 6 different biotypes were obtained. Five of the isolates failed to induce reactions in any of the tests and so no profiles were obtained for these isolates. Sixty four percent of isolates produced the same API profile number. All isolates were confirmed to be *C. difficile* despite some the variation observed in some of the tests. The different API profiles produced and the number of isolates that produced each profile is described in table 2.2. The API result for isolate 13 from Trust B is depicted in figure 2.3, displaying positive reactions in proline and leucine arylamidase that are characteristic of *C. difficile*.

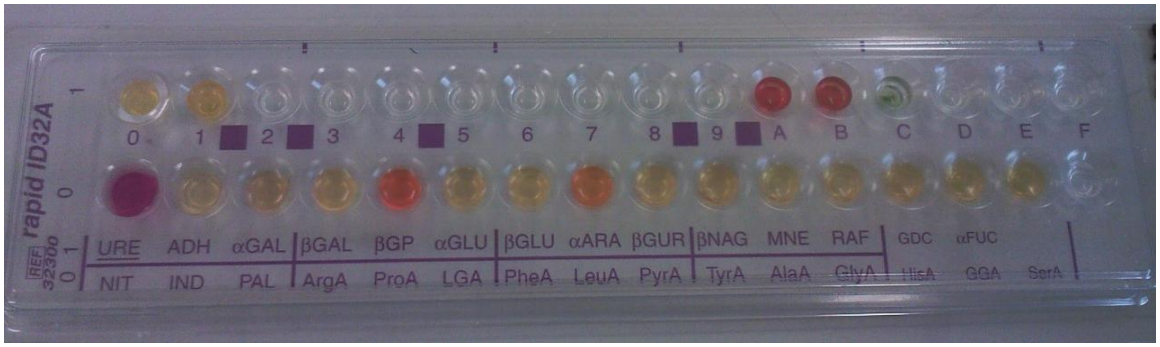


Figure 2.3 API results for isolate B13 recovered from Trust B. Positive reactions in the proline arylamidase (ProA), leucine arylamidase (LeuA) and reduction of nitrates (NIT) were recorded.

Table 2.2 API biotype prevalence and distribution among the 64 isolates of *C. difficile*.

API Profile Number	Number of Isolates	Trust A	Trust B
0000020000	8 (12.5%)	2 (6%)	5(17%)
0000022000	41(64%)	26 (81%)	14(47%)
0000022200	6 (9%)	1 (3%)	5(17%)
0000122000	2 (3%)	0	2(7%)
0002032000	1 (2%)	1(3%)	0
1000020000	1(2%)	1(3%)	0

A positive proline arylamidase reaction test was observed in all isolates that produced a profile result and was overall positive in 92% of the isolates tested. Only isolates that failed to produce a reaction and produced no profile gave negative results for this test. A positive reaction to the leucine arylamidase test was also observed in 80% of the isolates. The frequency with which positive reactions were observed among the tests is depicted in figure 2.4.

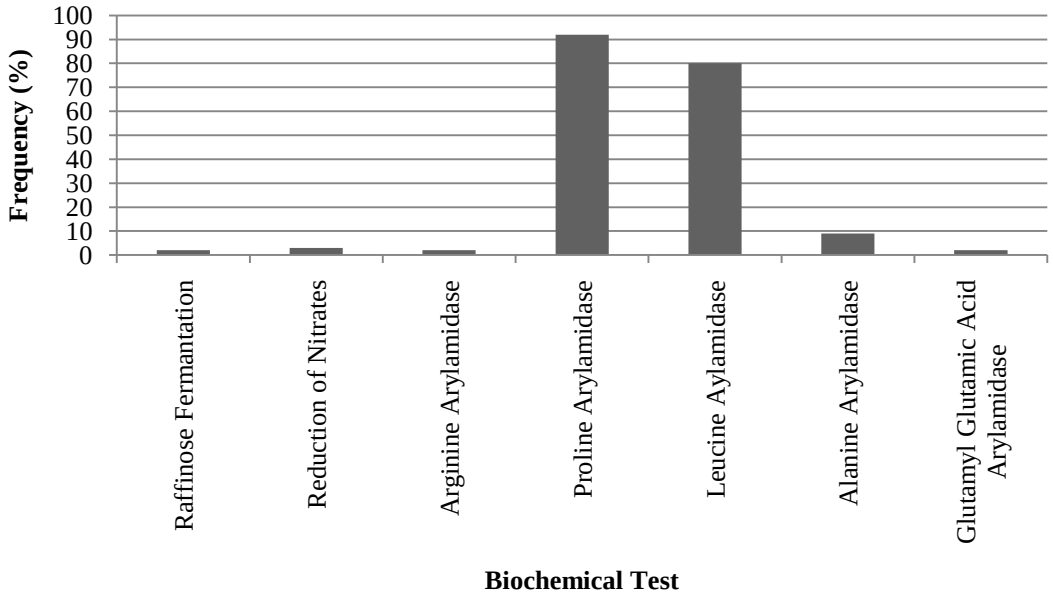


Figure 2.4 The frequency with which positive reactions for the individual biochemical tests in the Rapid ID 32A were recorded.

2.3.3 Extracellular virulence factor production

All clinical isolates tested were negative for lipase, non-specific protease, DNase, haemolysin, urease and gelatinase production; all isolates were however positive for toxin production.

2.3.4 Antimicrobial susceptibility of clinical isolates

All isolates were sensitive to metronidazole and vancomycin as defined by the breakpoints used; varying degrees of resistance was observed towards all of the other antibiotics tested. Among the isolates tested, resistance was observed most frequently towards cefotaxime, clindamycin, fusidic acid and levofloxacin; resistance towards chloramphenicol and linezolid was recorded less frequently in twenty and two percent of isolates respectively. The frequency of resistance towards each antimicrobial between is presented in figure 2.5.

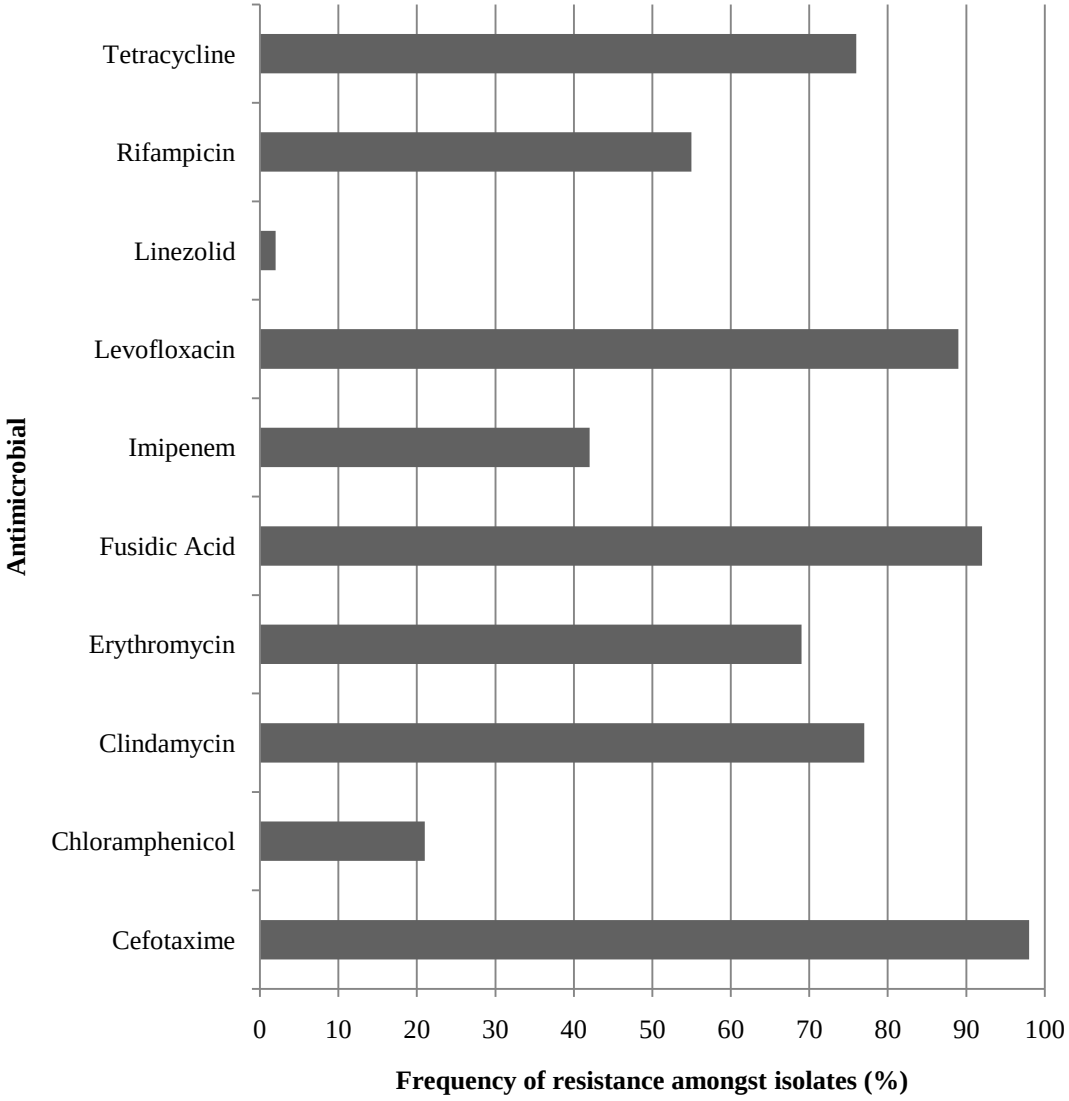


Figure 2.5 The frequency of resistance observed among the clinical isolates against the tested antibiotics.

There were also variances in resistance in the isolates recovered from the two separate locations (Table 2.3). Isolates from Trust A demonstrated increased susceptibility to the panel of antimicrobials tested with isolates displaying resistance to an average of 4 antimicrobials however, isolates from Trust B demonstrated resistance to an average of 8 antimicrobials. The range and modal MICs also varied between the two Trusts, this data is presented in table 2.4. In total 29 different antibiogram profiles were obtained; profiles were exclusive to the location from which the isolate was recovered with no single profile

being observed in both trusts. A more diverse range of antibiogram profiles was observed in the isolates recovered from Trust A; isolates within this population were also resistant to fewer antibiotics in comparison to isolates recovered from Trust B. Isolates recovered from Trust B were resistant to a greater number of the antimicrobials tested and therefore the range of antibiogram profiles in this population was less diverse.

Table 2.3 Frequency of resistance (%) observed in isolates from the two separate Trusts.

Antibiotic	Trust A	Trust B
Cefotaxime	97	100
Chloramphenicol	24	17
Clindamycin	62	97
Erythromycin	50	93
Fusidic Acid	100	83
Imipenem	80	6
Levofloxacin	82	97
Linezolid	0	3
Metronidazole	0	0
Rifampicin	12	100
Tetracycline	50	100
Vancomycin	0	0

Table 2.4 The range and modal MICs recorded for each of the antibiotics tested.

Antimicrobial Agent	Modal MIC (µg/mL)		Range (µg/mL)	
	Trust A	Trust B	Trust A	Trust B
Fusidic Acid	2*	4*	1 - 16	0.125 – 16
Tetracycline	4*	4*	0.5 - 64	4 - 64
Cefotaxime	4*	128*	2 - 128	64 - 128
Levofloxacin	4*	256*	1 - ≥ 256	2 - 256
Clindamycin	4	16*	0.5 - 32	2 - 32
Imipenem	4	16*	0.125 - 16	8 - 16
Rifampicin	0.016	4*	0.016 - 32	4 - 32
Erythromycin	4	512*	0.5 - ≥ 512	2 - ≥ 512
Chloramphenicol	2	4	2 - 32	4 - 64
Linezolid	4	4	1 - 4	0.5 - 8
Metronidazole	0.5	0.5	0.064 - 4	0.25 - 4
Vancomycin	0.5	2	0.5 - 2	0.125 - 4

* Modal MIC value is above resistance breakpoint

2.3.3.1 Calibration curve

A calibration curve for the growth of *C. difficile* was produced by plotting the average OD values of the three isolates used against the average corresponding log cfu/mL results (Figure 2.6). There was a significant correlation observed between OD and cfu/ml.

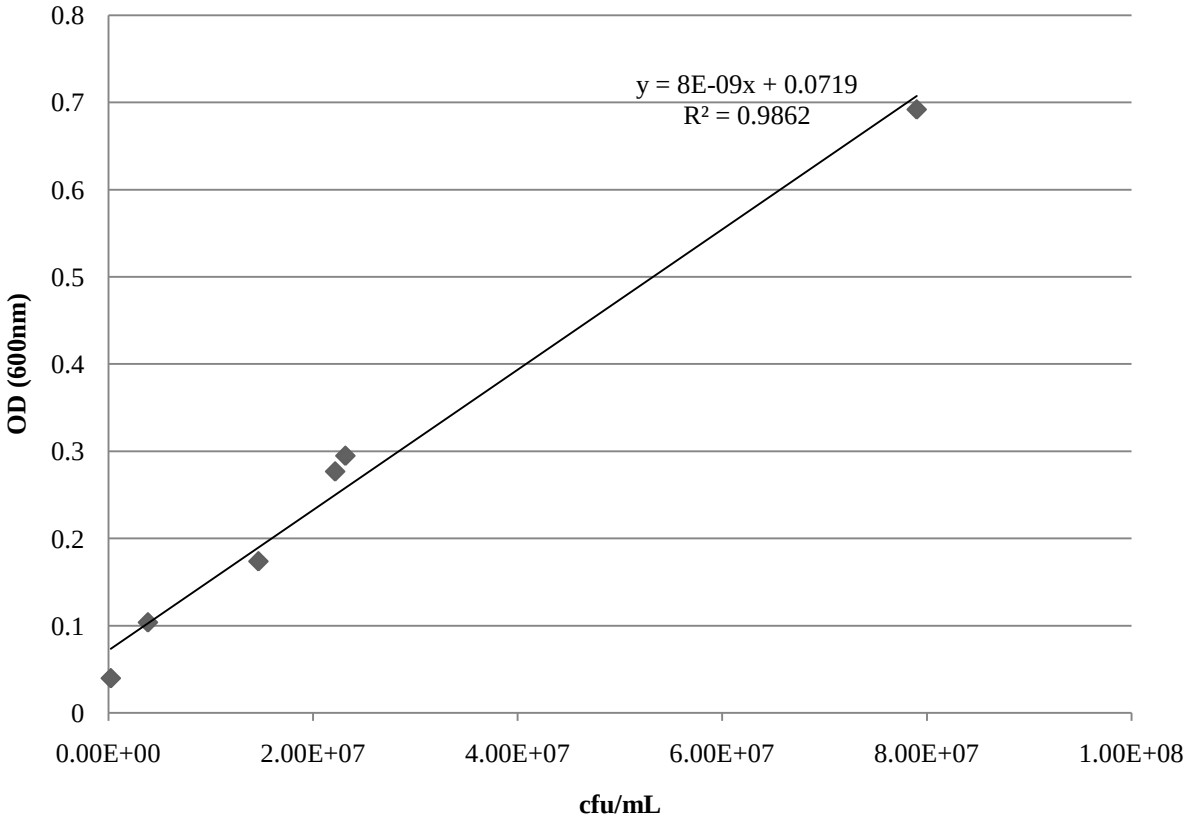


Figure 2.6 Calibration curve for the average growth of three *C. difficile* strains.

2.3.3.2 Chi-square (χ2) analysis

Analysis of the data using Chi-square (χ^2) revealed a significant association (P=0.001) between isolate location and antibiotic susceptibility. When investigated individually significant associations (P=0.001) between location of isolation and susceptibility towards tetracycline, rifampicin, imipenem, clindamycin and erythromycin was observed.

2.3.3.3 Simpson's index of diversity (D)

Simpson's diversity indices of 0.945 and 0.767 were calculated for Trusts A and B respectively, demonstrating a greater diversity of antibiogram profiles amongst the isolates obtained from Trust A.

2.3.5 Antibiotic susceptibility of control ribotypes

No resistance towards metronidazole or vancomycin was observed in this population. The MICs recorded for each antibiotic are presented in Table 2.5. Among the control PCR ribotypes, resistance towards cefotaxime and levofloxacin and sensitivity towards metronidazole, vancomycin, rifampicin and linezolid was uniform throughout the population (Table 2.6). Antibiogram profiles varied between the different PCR ribotypes however for PCR ribotypes 015, 023 and 027 profiles were the same.

Table 2.5 MICs of control PCR ribotype strains against tested antibiotics.

PCR ribotype	[a]MIC (µg/mL)											
	Cef	**Chl**	**Cli**	**Ery**	**Fuc**	**Imi**	**Lev**	**Lin**	**Met**	**Rif**	**Tet**	**Van**
001	16	8	4	1	0.25	4	4	0.25	1	<0.016	4	2
002	>512	8	32	2	2	32	8	0.25	0.25	<0.016	1	1
005	128	16	16	2	1	16	128	0.25	0.25	<0.016	8	1
014	512	16	32	2	0.25	32	8	0.25	0.25	<0.016	0.5	1
015	>512	8	32	2	0.25	32	8	0.25	0.25	<0.016	<0.016	1
017	>512	16	>512	>512	4	32	8	0.25	0.25	<0.016	512	1
023	128	8	8	2	0.5	16	128	0.25	0.5	<0.016	0.5	0.5
027	>512	8	16	>512	1	16	256	0.25	2	<0.016	0.5	1
064	64	8	16	2	0.25	16	128	0.25	0.25	<0.016	4	2
078	128	8	8	2	0.25	16	128	0.25	2	<0.016	0.032	1
106	>512	8	16	512	32	16	>256	0.25	0.25	<0.016	0.064	0.5

[a]Antibiotic abbreviations: Cef (cefotaxime), Chl (chloramphenicol), Cli (clindamycin), Ery (erythromycin), Fuc (fusidic acid), Imi (imipenem), Lev (levofloxacin), Lin (linezolid), Met (metronidazole), Rif (rifampicin), Tet (tetracycline) and Van (vancomycin).

Table 2.6 Resistance/sensitivity profiles for control PCR ribotype strains against tested antibiotics

PCR ribotype	[ab]R/S											
	Cef	**Chl**	**Cli**	**Ery**	**Fuc**	**Imi**	**Lev**	**Lin**	**Met**	**Rif**	**Tet**	**Van**
001	R	S	S	S	S	S	R	S	S	S	R	S
002	R	S	R	S	R	R	R	S	S	S	S	S
005	R	R	R	S	R	R	R	S	S	S	R	S
014	R	R	R	S	S	R	R	S	S	S	S	S
015	R	S	R	S	S	R	R	S	S	S	S	S
017	R	R	R	R	R	R	R	S	S	S	R	S
023	R	S	R	S	S	R	R	S	S	S	S	S
027	R	S	R	R	R	R	R	S	S	S	S	S
064	R	S	R	S	S	R	R	S	S	S	R	S
078	R	S	R	S	S	R	R	S	S	S	S	S
106	R	S	R	R	R	R	R	S	S	S	S	S

[a]Antibiotic abbreviations: Cef (cefotaxime), Chl (chloramphenicol), Cli (clindamycin), Ery (erythromycin), Fuc (fusidic acid), Imi (imipenem), Lev (levofloxacin), Lin (linezolid), Met (metronidazole), Rif (rifampicin), Tet (tetracycline) and Van (vancomycin).
[b]R: resistance, S: sensitive

2.3.6 Surface layer protein typing

There was great variation observed in the S-layer proteins with few isolates possessing both the same high and low molecular mass surface layer proteins. The high molecular mass proteins ranged between 56 – 48 kDa with the low molecular mass proteins ranging between 45 – 36kDa with more variation occurring amongst the low molecular mass proteins. An example of the S-layer proteins extracted is shown in figure 2.7.

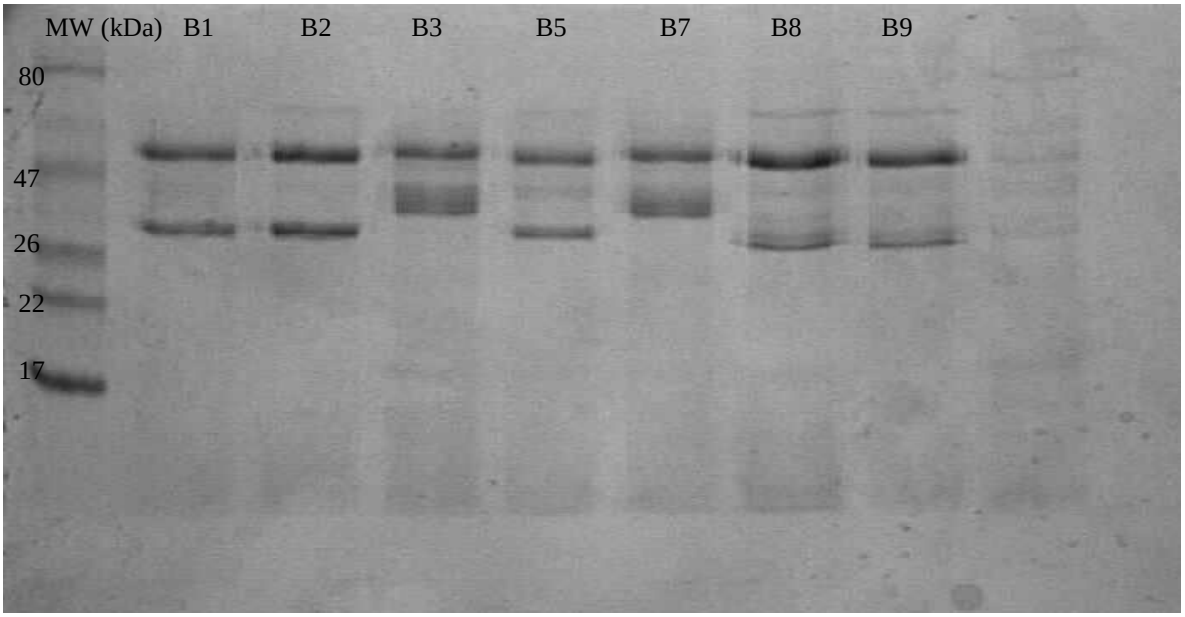


Figure 2.7 Example of gel displaying extracted S-layer proteins from isolates of *C. difficile*. Lanes are labelled according to the appropriate isolate.

2.4 DISCUSSION

Although *C. difficile* has some very distinguishing phenotypic characteristics, slight variations were observed in colony morphology, odour and cell morphology as seen in a Gram stain. Variations in colony and cell morphology have also been reported by others although it is not known why these differences occur. Subtle differences in odour may be due to differences in fermentation and differences in the ratio of volatile fatty acids produced between different strains; this may also be associated with slight differences in their metabolism and biochemical reactions within the cell.

There was little variation in the API profiles of the isolates tested with positive reactions in the proline and leucine arylamidase tests being characteristic of *C. difficile*. The production of such enzymes by most of the *C. difficile* isolates tested here demonstrates that the organism utilises proline and leucine in metabolic reactions. It is known that *C. difficile* requires particular amino acids for growth; utilizing them as a carbon source for fermentation pathways rather than traditional sources such as sugars; this is also clearly apparent in the API tests. Proline, leucine, isoleucine, valine, tryptophan, methionine and cysteine have been reported to be required for the optimal growth of *C. difficile* and it is these unusual fermentation pathways that cause the characteristic odour of *C. difficile*. In six of the isolates tested, positive reactions were also recorded for alanine arylamidase indicating that some isolates may also use alanine in metabolic pathways; a positive result in the nitrate reduction test was also observed in two isolates however this is likely to be a false positive result. Other positive results that were recorded were sporadic and occurred only in individual isolates, this is suggestive of a false positive result however further tests would be required to rule this out.

All sixty two of the clinical isolates were negative for the production of any of the extracellular virulence factors tested with the exception of that for toxin A/B production.

There is limited research available that has investigated this area however the results here concur with work carried out by others (Seddon *et al.*, 1990) with the exception of gelatinase and protease production where activity was reported to vary between the isolates tested (Hafiz and Oakley, 1976, Seddon and Borriello, 1992, Steffen and Hentges, 1981). The observation that the production of additional hydrolytic and proteolytic enzymes (not tested here) varied between isolates in other studies (Seddon and Borriello, 1992, Seddon *et al.*, 1990), indicates that they are unlikely to contribute independently to the pathogenesis of *C. difficile* infection and are more likely to facilitate the survival of the organism within the human gastrointestinal tract (Poxton *et al.*, 2001). This is supported by the observation that strains of *C. difficile* identified as highly virulent are more likely to produce proteolytic enzymes and in higher levels (Seddon and Borriello, 1992). Unlike other organisms, *C. difficile* does not produce a range of virulence factors with its pathogenicity likely to be attributed to toxin production alone and hypervirulence being due only to excessive levels of toxin production. It is known that some strains of *C. difficile* also produces a binary toxin however the role of this in virulence is still unclear. The genome of *C. difficile* is reported to contain a large number of transposons (Sebaihia *et al*., 2006) suggesting the potential for the acquisition of additional virulence factors; there appears to be no evidence however that this occurs and it may be that genes that control such mechanisms are not as easily transferred and acquired as antibiotic resistance genes for example.

There is little current research into the investigation of the production of extracellular virulence factors, earlier and initial research is also limited with sample sizes often being small (Hafiz and Oakley, 1976, Seddon *et al.*, 1990, Steffen and Hentges, 1981, Seddon and Borriello, 1992). Phenotypic changes have been reported in *C. difficile* and it has recently been reported that there are several notable changes in recent hypervirulent PCR

ribotype 027 strains when compared to historic strains, that may be responsible for the phenotypic variations that are being observed (Stabler *et al.*, 2009). Although no changes have yet been reported in the production of extracellular virulence factors by *C. difficile*, such reporting demonstrates that increased surveillance and continued investigation is required.

No resistance was observed in any of the clinical isolates tested towards metronidazole and vancomycin; this was to be expected as vancomycin resistance has yet to be detected and reports of metronidazole resistance are rare and sporadic (Wong *et al.*, 1999, Brazier *et al.*, 2001, Pelaez *et al.*, 2002). The high frequency of resistance towards cefotaxime, clindamycin and levofloxacin among the isolates is a result that correlates well with work by others; these antibiotics are also some of the most frequently implied precipitating antibiotics in CDI. Resistance towards erythromycin, fusidic acid, rifampicin and tetracycline was also relatively widespread throughout this population of *C. difficile* isolates. Both fusidic acid and rifampicin have been reported to show good activity towards *C. difficile in vitro* however the ease with which resistance is acquired and rapid emergence of resistance towards both antibiotics has prevented its use as a viable treatment option. Resistance towards erythromycin and tetracycline is easily acquired by bacteria through the transferring of the *erm* (B) and *tet* genes respectively, both of these genes have been detected in *C. difficile* isolates (Spigaglia *et al.*, 2005a, Spigaglia *et al.*, 2008, Adams *et al.*, 2002, Schmidt *et al.*, 2007, Tang-Feldman *et al.*, 2005, Spigaglia *et al.*, 2005b) and it is therefore likely that these genes are present in the isolates tested.

Antibiograms have been used successfully in the profiling of many species of bacteria, however when used alone they often demonstrate low discriminatory power and fail to differentiate between isolates having limited use in epidemiological studies (Worthington *et al.*, 2000). The use of antibiogram profiling is an invaluable tool in the surveillance of

antibiotic resistance and as demonstrated here can be used to establish associations between sets of isolates and variables such as location. The benefits of antibiogram profiling has already been demonstrated; with previously unseen fluoroquinolone resistance highlighting the emergence of a new strain (Loo *et al.*, 2005) and a particular antibiogram profile also being indicative of the hypervirulent PCR ribotype 027 strain (Drudy *et al.*, 2008). Significant differences were observed in the antibiogram profiles of *C. difficile* isolates recovered from Trusts A & B; similar observations in the antibiogram profiles of *C. difficile* isolates from different locations within the UK have previouslybeen reported (Bendle *et al.*, 2004). Such observations highlight differences in the susceptibility of *C. difficile* isolates to particular antibiotics and are likely to be a response to differences in local selective pressures such as prescribing policies and antibiotic pressures (Taori *et al.*, 2010). The differences observed in isolates from the two separate locations indicate that *C. difficile* has the ability to successfully adapt to a local environment despite multiple selective pressures being exerted. The adaptability of *C. difficile* to its environment is likely to be due to the presence of a high number of transposons within the genome (11%) (Sebaihia *et al*., 2006), allowing genes to be acquired that will ultimately enhance the survival of the organism particularly within the gut. In this instance it is proposed that *C. difficile* may acquire genes that confer resistance to antibiotics that are often prescribed as a prophylactic measure for other infections in contrast to those used in the treatment of CDI. Treatment of CDI is standardised between hospital Trusts (mainly due to the lack of treatment options) however prophylactic prescribing policies are not, thus potentially explaining the antibiotic resistance variability that has been observed in this study. Despite the observation of such differences, there has also been widespread increase in MICs towards metronidazole, moxifloxacin and clindamycin amongst *C. difficile* isolates in England and Wales (HPA, 2008), most notably among the most predominant ribotypes.

This suggests that prolonged exposure to these antibiotics has led to an increase MICs which could then lead to resistance in isolates of *C. difficile*. Although the development of resistance in bacteria is a natural phenomenon, excessive antibiotic use within an environment can also exert a selective pressure (Cohen, 2000), leading to reduced susceptibility and ultimately antimicrobial resistance (Austin *et al.*, 1999). The differences observed in this investigation are likely to have been influenced by non-standardised prescribing policies within the Trusts from which they were obtained. This same reasoning however also suggests that uniformity and standardisation in prescribing policies would lead to high levels of widespread resistance towards selected antibiotics amongst *C. difficile* isolates. Therefore restrictions in antibiotic administration and/or uniform rotation of antibiotics across Trusts may be more appropriate in reducing the spread of resistance in *C. difficile* isolates.

The antibiogram profiles for PCR ribotypes 015, 023 and 078 were the same although the individual MICs did vary. Resistance was observed towards clindamycin in PCR ribotype 027 and although sensitivity to clindamycin was previously observed to be a characteristic of PCR ribotype 027 strains, resistance in now being observed in these strains (Drudy *et al.*, 2008). A high level of resistance (256 µg/ml) was observed towards levofloxacin in PCR ribotype 027 strain which is frequently observed in high levels in these strains (Deneve *et al.*, 2009, Razavi *et al.*, 2007, Mcdonald *et al.*, 2005). Due to the small sample size used here it is not possible to determine if the antibiogram profiles or any of the susceptibility traits observed here are characteristic of a particular ribotype.

A great deal of variation was observed among the isolates tested. This method of typing has been reported to correlate well with PCR ribotyping (Mccoubrey and Poxton, 2001) however it would seem that with the variation observed this is unlikely in this group of isolates. It has been proposed that the S-layer proteins are involved in cell attachment and

immune responses and therefore the variances that occur may be responsible for differences that have been observed in CDI and symptoms reported.

2.5 CONCLUSION

Clinical isolates of *C. difficile* were indistinguishable when compared on the results from extracellular virulence factor production indicating that toxin production alone is responsible for the virulence of the organism. This suggests that the virulence mechanisms that are already possessed by *C. difficile* are sufficient for pathogenesis and survival and therefore the acquisition of further virulence factors are unlikely to be of benefit to the organism. Antibiogram profiles were highly variable between isolates as a whole population and also between the two trusts from which they were recovered; however, discriminatory power was limited by the level of resistance in Trust endemic strains which is likely to be influenced by prescribing policy. These results highlight how local selective pressures influence isolates of *C. difficile* and demonstrate a need for restriction and or rotation in antibiotic usage policies between Trusts in contrast to uniform standardisation.

CHAPTER 3 OPTIMISATION OF A RANDOM AMPLIFIED POLYMORPHIC DNA PCR PROTOCOL FOR THE GENOTYPIC CHARACTERISATION OF *CLOSTRIDIUM DIFFICILE*

3.1 INTRODUCTION

Prior to the routine use of molecular techniques, phenotypic and immunochemical methods were adopted to identify and characterise bacteria. Phenotypic characteristics of microorganisms including colonial morphology, Gram stain reaction and odour can often identify bacteria to at least the genus level however additional tests are usually required for species identification. Phenotypic characterisation of microorganisms often lacks discriminatory power and rarely detects variances between strains that belong to the same species. However, genotypic characterisation of microorganisms can provide a much greater discriminatory capacity and can generate much more information about an organism. Despite providing greater discriminatory capabilities, many molecular techniques are time consuming and often require specialist equipment which can render them unsuitable for routine clinical purposes; they also require a great deal of development and optimisation in order to produce desired results.

Random amplification of polymorphic DNA (RAPD PCR) (also referred to as arbitrary primed PCR) has been applied to genetically type many different species of bacteria and is characterised by its use of short primers, low annealing temperatures and unique to other PCR methods, no prior knowledge of the genome being required. RAPD PCR is a rapid and cost effective method and its user friendly application makes it attractive for genetic characterisation of microorganisms in routine laboratories. However, RAPD PCR is often criticised for its reduced discriminatory power and lack of reproducibility in comparison to other methods. The gold standard typing method for *C. difficile* isolates in the UK is currently PCR ribotyping which is documented to be both highly reproducible and

discriminatory. However, there are limitations to the discriminatory power of PCR ribotyping and therefore other methods which are more discriminative are now being employed. RAPD PCR has previously been used to characterise *C. difficile* using a variety of different primers (Barbut *et al.*, 1993, Barbut *et al.*, 1994, Chachaty *et al.*, 1994, Van Dijck *et al.*, 1996, Wilcox *et al.*, 1998, Pituch *et al.*, 2001, Silva *et al.*, 1994, Killgore and Kato, 1994, Wilks and Tabaqchali, 1994, Martirosian *et al.*, 1995, Collier *et al.*, 1996, Lemann *et al.*, 1997, Cohen *et al.*, 1997, Samore *et al.*, 1997, Rafferty *et al.*, 1998, Wullt and Laurell, 1999, Titov *et al.*, 2000, Bidet *et al.*, 2000, Martirosian *et al.*, 2005), and often compared with other methods such as pulsed field gel electrophoresis (PFGE), PCR ribotyping and REP PCR (Chachaty *et al.*, 1994, Van Dijck *et al.*, 1996, Killgore and Kato, 1994, Martirosian *et al.*, 1995, Tang *et al.*, 1995, Collier *et al.*, 1996, Bidet *et al.*, 2000, Wada *et al.*, 1980, Wullt *et al.*, 2003a, Fawley *et al.*, 2005). Whilst these studies generated promising data with RAPD PCR, problems associated with reproducibility and discriminatory power were documented when compared to other methods. Published RAPD PCR methodologies that have been used to genetically characterise isolates of *C. difficile* show little evidence of the optimisation of the methods which may influence the results.

To improve the reproducibility of RAPD PCR, the components of the reaction need careful titration to maximise and ensure the efficiency of the primer used (Hilton *et al.*, 1997, Perry, 2004). Furthermore, optimisation of the concentration of all components in a PCR reaction is crucial and is largely influenced by the primers used; therefore RAPD protocols cannot always be transferred between species of bacteria, especially if different areas of the genome are being targeted.

In this chapter the optimisation of a RAPD protocol that is suitable for the typing of *C. difficile* is described.

3.2 MATERIALS AND METHODS

3.2.1 DNA extraction

3.2.1.1 Method 1 (DNA extraction using phenol extraction and ethanol precipitation)

Colonies of *C. difficile* were used to inoculate 10mls of Wilkins-Chalgren anaerobe broth (Oxoid, UK). Following incubation at 37°C in anaerobic conditions for 24 hours, 1.5mls of culture was removed and centrifuged at 15000 x g for 4 minutes. The supernatant was discarded and pellet re-suspended in 300µl of TE buffer (10mM Tris, pH8, 10mM EDTA, pH8) before being added to 500µl of lysozyme (8 mg/ml) (Sigma Aldrich, UK) and incubated for 15 minutes at 37°C and then for a further 10 minutes at 75°C. After these initial stages of incubation, 100µl 10% ($^{w}/_{v}$) SDS (Fisher Scientific, UK) and 5µl DNase (10mg/ml) (Sigma Aldrich, UK) were added to samples before being incubated for a further 10 minutes at 75°C. Before a final incubation period at 65°C for 2 hours, 1µl proteinase K (20mg/ml) (Sigma Aldrich, UK) was added to the sample.

Following incubation 200µl of sample was removed and added to an equal volume of phenol (Sigma Aldrich, UK), before being vortexed to form an emulsion and then centrifuged for 1 minute at 15000 x g. The top aqueous layer containing the DNA was then removed and dispensed into a separate centrifuge tube; to the bottom remaining layer a volume of SDW equal to half the volume of this layer was added before being vortexed briefly and centrifuged at 15000 x g for 1 minute. The upper phase resulting from the second centrifugation was added to the upper aqueous phase that had been removed previously and the tube containing the remaining lower phenol layer discarded. To the centrifuge tube containing both upper aqueous layers an equal volume of phenol: chloroform (50:50) was added; again the mixture vortexed to an emulsion and centrifuged for 1 minute at 15000 x g. The top phase was again removed and placed in a separate

centrifuge tube; to the remaining lower layer a volume of SDW equal to half the volume of this remaining layer added as previous and the mixture again vortexed, centrifuged and the upper layer added to the previous extract taken. To the extracted upper layers, an equal volume of chloroform was added, vortexed and centrifuged. The top layer was removed to a new tube before another volume of chloroform was added before being vortexed and centrifuged again. The resulting upper layer was again removed to the tube containing the first extract and the tube containing the chloroform discarded.

To the extracted DNA solution, a volume of 3M sodium acetate (Fisher Scientific, UK) equal to 1/10 of the final sample was added and vortexed. Absolute ethanol that had previously been stored at -70°C was then used to fill the centrifuge tube before being stored at -20°Cfor 15-18 hours. Samples were then centrifuged at 15000 x g for 20 minutes and the resulting pellet left to air dry for 1 hour. The pellet was then suspended in 200µl of SDW and stored at -20°C until required.

3.2.1.2 Method 2 (DNA extraction through boiling of whole cells)

C. difficile was cultured onto Wilkins-Chalgren anaerobe agar (Oxoid, UK) and incubated for 24 hours at 37°C in anaerobic conditions. All colonies were then taken and suspended in 1 ml of SDW and centrifuged for 4 minutes at 15000 x g, the supernatant was discarded and pellet washed once with 1 ml of SDW. The resulting pellet was re-suspended in 100µl of SDW in a PCR tube and incubated for 12 minutes at 94°C before being centrifuged for 4 minutes at 1500 x g. The supernatant containing the crude DNA extract was then used in PCR reaction.

3.2.1.3 Method 3 (DNA extraction through boiling of whole cells with Chelex® 100 resin)

C. difficile isolates were cultured onto Wilkins-Chalgren anaerobe agar and incubated for 24 hours at 37°C in an anaerobic cabinet (Don Whitley, UK) for 24 hours. All colonies were then suspended in a 0.5% ($^{w}/_{v}$) Chelex® 100 resin (Bio-Rad, UK) suspension which was briefly vortexed before being incubated in a water bath at 94°C for 12 minutes. Samples were then centrifuged for 10 minutes at 15000 x g and the supernatant containing the crude DNA extract used in the RAPD reaction.

3.2.2 DNA analysis

3.2.2.1 Analysis of DNA quality

The quality of extracted DNA by each of the methods described in 3.2.1 was determined by gel electrophoresis to ensure that the DNA had remained intact. Ten microlitres of the DNA suspension was mixed with 2µl of loading buffer (0.25% ($^{w}/_{v}$) bromophenol blue (Sigma Aldrich, UK), and 30% ($^{v}/_{v}$) glycerol (Fisher Scientific, UK)) and loaded into a 2% ($^{w}/_{v}$) agarose gel (Geneflow, UK) containing 1µg/ml ethidium bromide (Sigma Aldrich, UK); 5 microlitres of a DNA ladder 3000-300bp (Geneflow, UK) was also loaded as a standard. Gel electrophoresis was performed in 1 x TAE (40mM Tris-HCl, 1mM EDTA and 0.1% ($^{v}/_{v}$) glacial acetic acid, pH 8) for 1 hour at 100V. Gels were visualised under UV light using the GBOX-EF Gel Documentation System (Syngene, UK) and images captured using Genesnap software (Syngene, UK).

3.2.2.2 Analysis of DNA quantity and purity

A UV spectrophotometer (Jenway, UK) was used to determine both DNA quantity and purity. Ten microlitres of DNA sample was mixed with 990µl of SDW in a UV compatible cuvette and the contents measured at wavelengths of both A_{260} and A_{280}. The analysis of

samples at A_{260} allowed the quantity of extracted DNA to be measured; values expressed as a ratio of values obtained from both measurements determines the purity of the DNA that has been recovered. Ratios of between 1.7 and 1.9 indicate good quality DNA and reading between these two values are the ideal for PCR reactions.

3.2.3 RAPD protocol

As a template for further development and characterisation of *C. difficile* isolates a previously published RAPD protocol for the genetic characterisation of MRSA was adopted (J. Caddick, PhD Thesis, Aston University, 2005). This initial RAPD reaction contained 2µl of 5ng/µl DNA template, 2.5µl 10x PCR Buffer (100mM Tris-HCl pH 8.8, 250 mM KCl, 35mM $MgCl_2$), 0.6µl 100µM primer (Eurofins MWG biotech, Germany), 0.5µl 10mM dNTPs (Promega, UK), 19.15 µl SDW and 0.25µl of 5 units/µl Taq DNA polymerase (Promega, UK), making a final reaction volume of 25µl. Amplification cycles were carried out on a Peltier Thermal Cycler-200 (MJ Research, USA) using the cycles as follows: an initial cycle at 94°C for four minutes and thirty seconds; five cycles of thirty seconds at 94°C, two minutes at 20°C and one minute at 72°C followed by thirty five cycles of thirty seconds at 94°C, thirty seconds at 30°C and one minute at 72°C and finally a extension of five minutes at 72°C. Samples were then stored at 4°C until analysis by gel electrophoresis.

3.2.4 RAPD reaction optimisation

3.2.4.1 Isolate selection

Three *C. difficile* isolates were selected for use in the optimisation of the RAPD protocol: *C. difficile* NCTC 11204 and two clinical isolates designated AZ169 and B17.

3.2.4.2 Primer selection

Four primers were initially selected based on work published by other authors (Table 3.1) and are shown in table 3.1. All primers were initially tested with the RAPD protocol described in section 3.2.3 using DNA extracted as described in section 3.2.1.1. Primers producing clear amplicon patterns that demonstrated the potential for discrimination between different strains were selected to continue with the optimisation process.

Table 3.1 RAPD Primers

Primer	Sequence (5'-3')	Reference
AP1	TCA CGA TGC CA	(Wullt and Laurell, 1999)
AP2	CTA GGA CCG C	(Killgore and Kato, 1994)
AP3	TCA CGA TGC A	(Martirosian *et al.*, 1995)
AP4	TCA CGC TGC A	(Barbut *et al.*, 1993)

3.2.4.3 Buffer Selection

Buffers were made up according to the composition of the buffers described in the Opti-prime™ Optimisation Kit (Stratagene, USA). The compositions of all the buffers tested are described in table 3.2. Each buffer was tested in combination with all five primers outlined

in table 3.1 and with phenol extracted DNA (section 3.2.1.1) from the strains described in section 3.2.3.2. The optimal buffer was selected where clear distinct amplicons were produced and adopted for use in future RAPD reactions.

Table 3.2 Buffer composition (Opti-prime™ Optimisation Kit, Stratagene, USA)

Buffer	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
$MgCl_2$ mM	1.5	1.5	3.5	3.5	1.5	1.5	3.5	3.5	1.5	1.5	3.5	3.5
KCl mM	25	75	25	75	25	75	25	75	25	75	25	75
pH	8.3	8.3	8.3	8.3	8.8	8.8	8.8	8.8	9.2	9.2	9.2	9.2
Tris-HCl mM	10	10	10	10	10	10	10	10	10	10	10	10

3.2.4.4 Primer concentration

Optimal concentration of the selected primers was determined using phenol extracted DNA (section 3.2.1.1) from reference strain NCTC 11204 and clinical isolates AZ169 and B17. Using the RAPD protocol in section 3.2.3 duplicate reactions were performed using primer concentrations of 50, 100, 200 and 300µM. The primer concentration that produced clear distinct amplicon patterns was selected and adopted for use in future RAPD reactions.

3.2.4.5 dNTP concentration

Optimal dNTP concentration was determined using phenol extracted DNA (section 3.2.1.1) from reference strain NCTC 11204 and clinical isolates AZ16 and B17. Using the RAPD protocol in section 3.2.2 duplicate reactions were performed at dNTP concentrations of 5, 10, 20 and 30 mM. The dNTP concentration that produced clear distinct amplicon patterns was selected and adopted for use in future RAPD reactions.

3.2.4.6 Template DNA extractions

The modified RAPD reaction was performed using DNA from reference strain NCTC 11204 and clinical isolates AZ169 and B17 using three methods described in sections 3.2.1.1-3.2.1.3. Profiles were compared to determine if the crude extraction methods produced results comparable to that of high quality phenol extracted DNA. Comparisons of the two crude extraction methods were also made to determine which was superior for use in RAPD reactions.

3.2.4.7 Template DNA concentration

The effect of DNA template concentration on the modified RAPD reaction was investigated using phenol extracted DNA (section 3.2.1.1) from reference strain NCTC 11204 and clinical isolates AZ169 and B17. Using the optimised RAPD protocol, reactions were performed using DNA template at concentrations of 5, 10, 15, 20 and 25 ng/µl. This was to determine if the RAPD reaction was affected by the quantity of DNA template used; this is a factor when using crude extractions of DNA where the quantity is not measured and calibrated. Profiles were compared to determine if results appeared impaired through the presence of additional DNA in the reaction.

3.2.4.8 Transferability between thermocyclers

The modified RAPD protocol was used in reactions that were then placed on a different thermocycler.

3.2.4.9 Discrimination of PCR ribotypes using optimised RAPD protocol

Eleven known PCR ribotypes (001, 002, 005, 014, 015, 017, 023, 027, 064, 078 and 106) were characterised using the modified RAPD protocol to determine if this method had a similar discriminatory capacity to PCR ribotyping. This protocol was also used to type

isolates known to be of the same PCR ribotype to determine if this method also identified them as the same.

3.2.4.10 RAPD reproducibility

DNA extractions were performed over different periods of times, using different subcultures of cells to ensure that the RAPD protocol produced sable reproducible profiles.

3.3 RESULTS

3.3.1 Effects of primer and reaction buffer on RAPD reaction

Four primers were initially selected for the optimisation process; the template DNA from the three different strains (NCTC 11204, 027 and AZ 172) was tested alongside all four primers (AP1, AP2, AP3, AP4) and all twelve buffers; these profiles are shown in figures 3.1-3.12.

The primers found to have the best discriminatory capacity were AP3 and AP4 (in reactions independent of each other); these primers produced clear patterns with a sufficient number of amplicons demonstrating greater potential to be able to discriminate between different PCR ribotypes. The patterns produced by these primers were also more consistent across the three DNA templates; providing discriminate results for each. The amplicon patterns produced by primers AP1 and AP2 did not discriminate between different PCR ribotypes.

Buffer composition significantly influences amplification results and for primers AP3 and AP4 a KCl concentration of 25mM was required for successful amplification (lanes 1, 3, 5, 7, 9 & 11) although amplification could sometimes be seen at a 75mM concentration of KCl. This result suggests that these primers therefore remain stable over a range of pH values and $MgCl_2$ concentrations and are KCl concentration dependent. Amplification appeared most severely inhibited by both a low $MgCl_2$ concentration (1.5mM) and high KCl (75mM) concentration within the same reaction (lanes 2, 6 and 10).

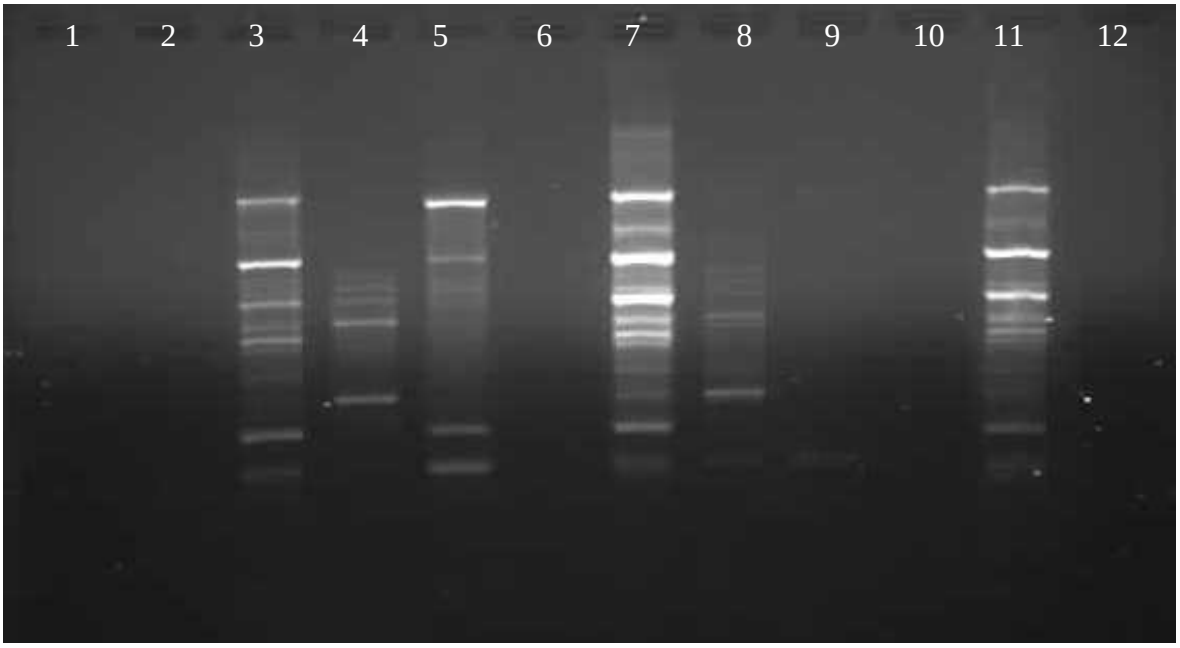


Figure 3.1 RAPD profiles of strain NCTC 11204 using primer AP1 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

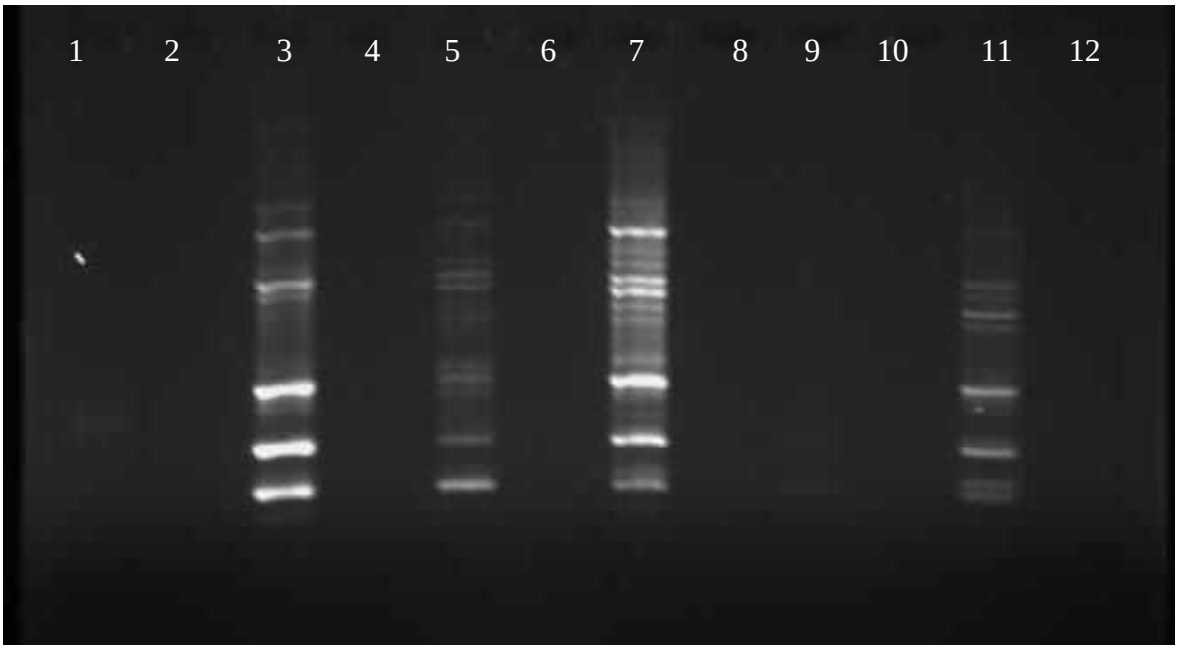


Figure 3.2 RAPD profiles of strain NCTC 11204 using primer AP2 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

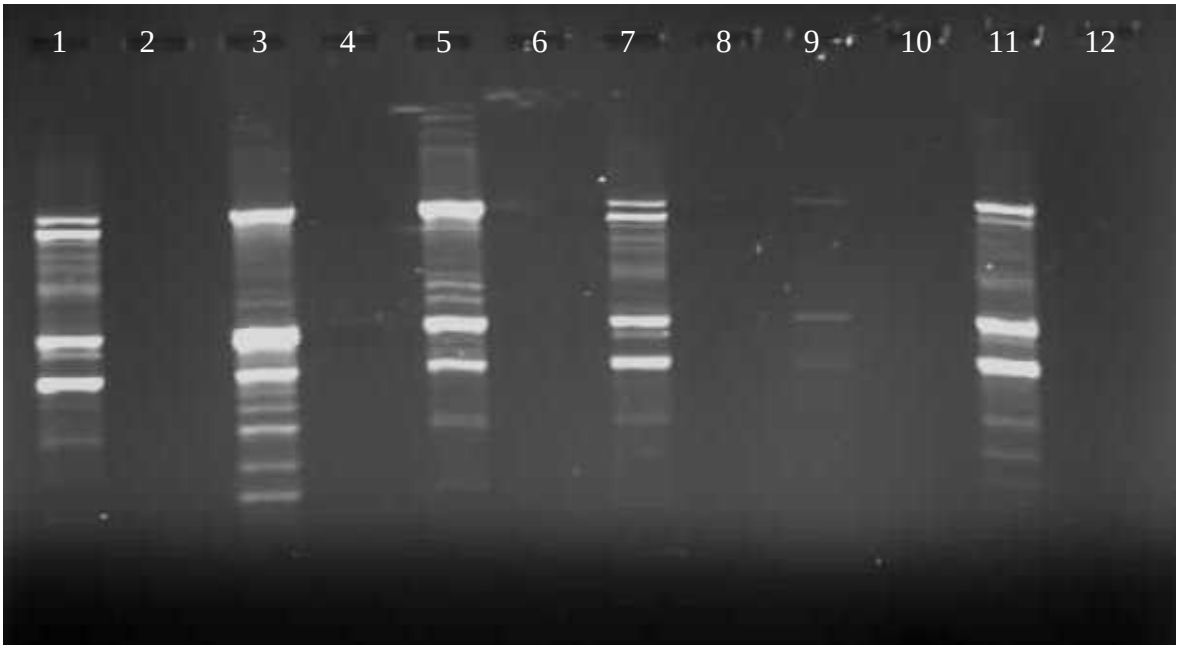


Figure 3.3 RAPD profiles of strain NCTC 11204 using primer AP3 using reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

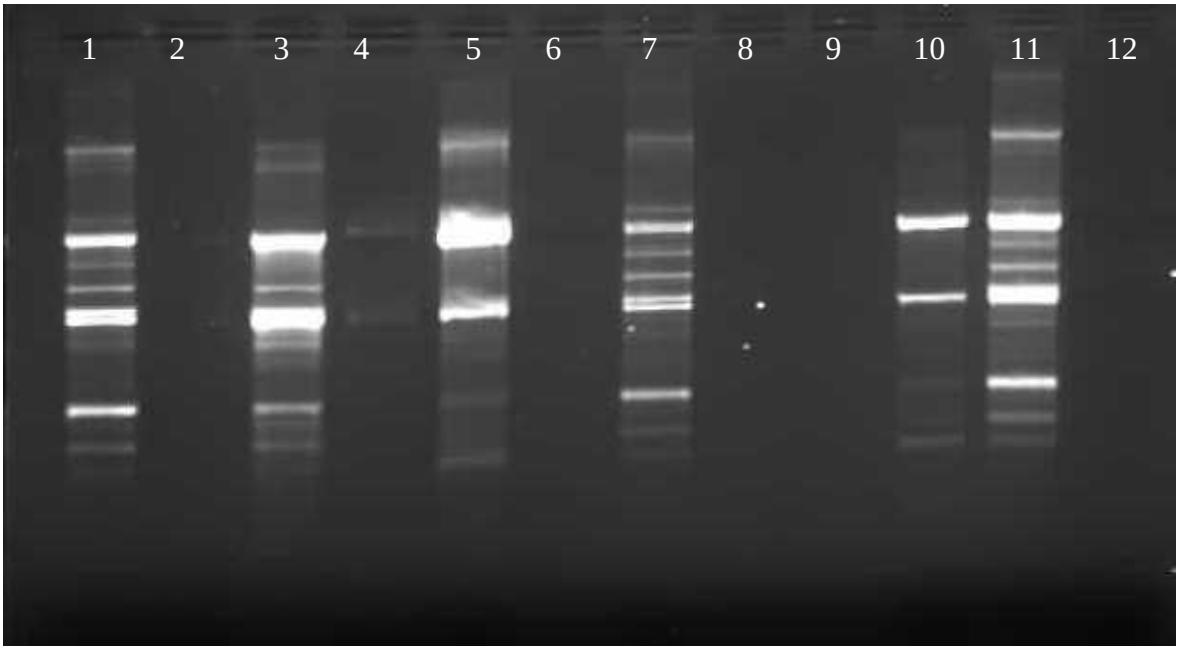


Figure 3.4 RAPD profiles of strain NCTC 11202 using primer AP4 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

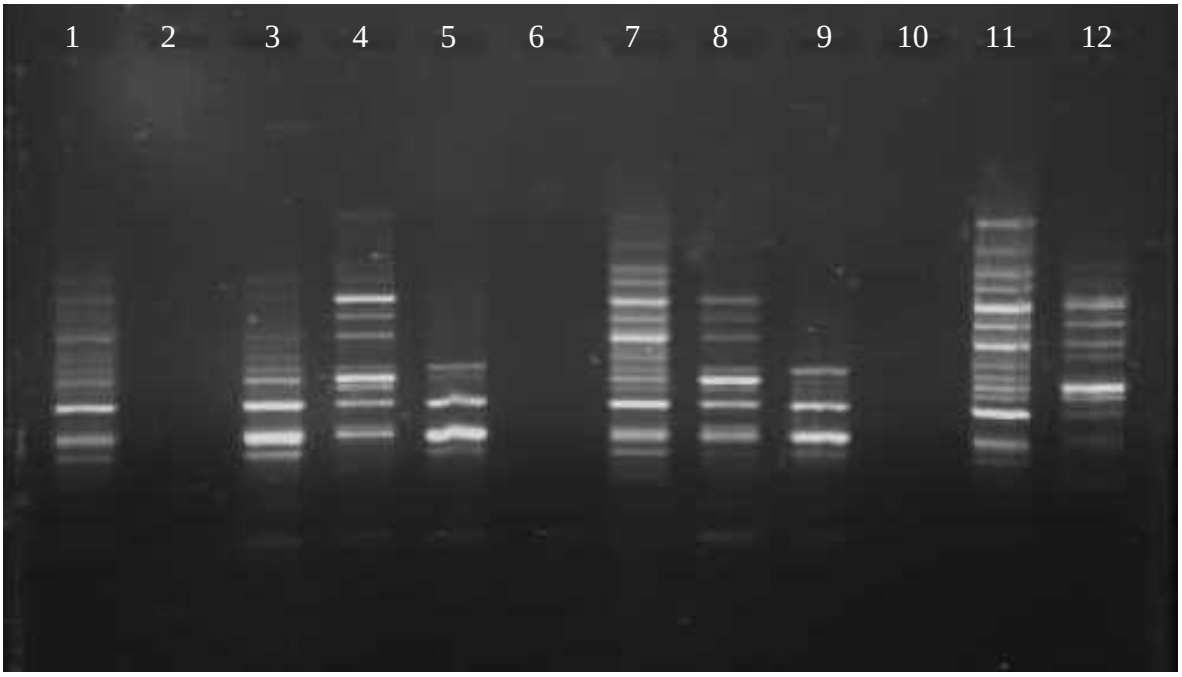


Figure 3.5 RAPD profiles of strain 027 using primer AP1 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

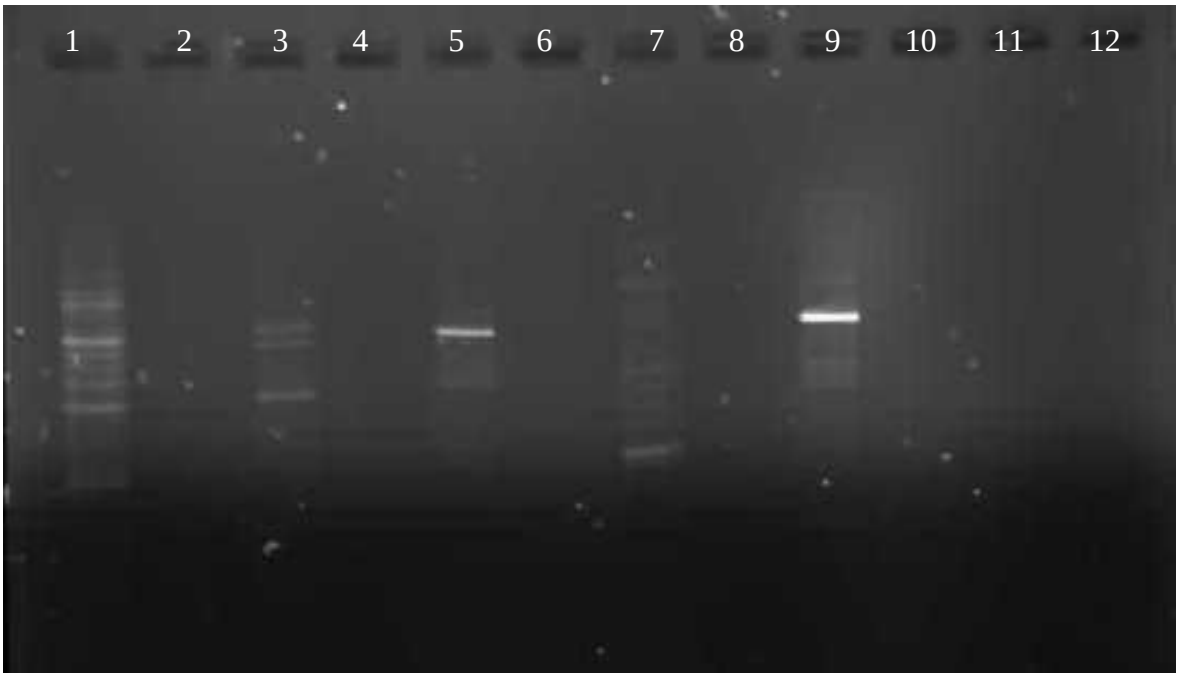


Figure 3.6 RAPD profiles of strain 027 using primer AP2 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

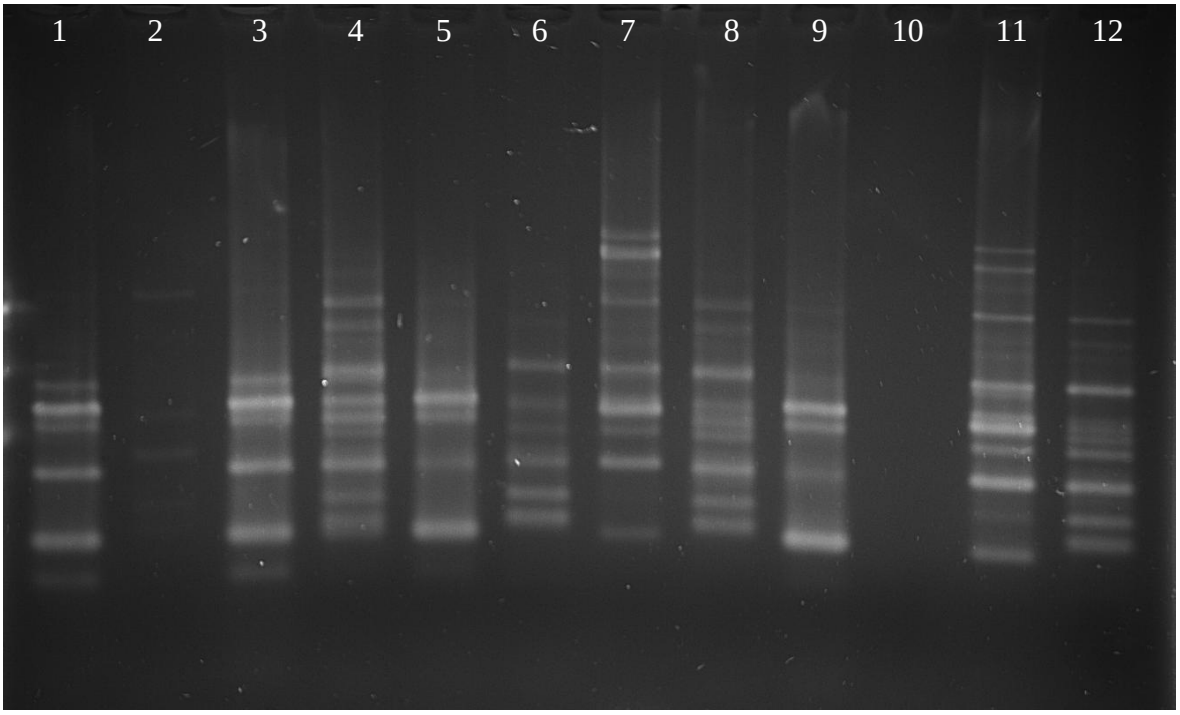


Figure 3.7 RAPD profiles of strain 027 using primer AP3 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

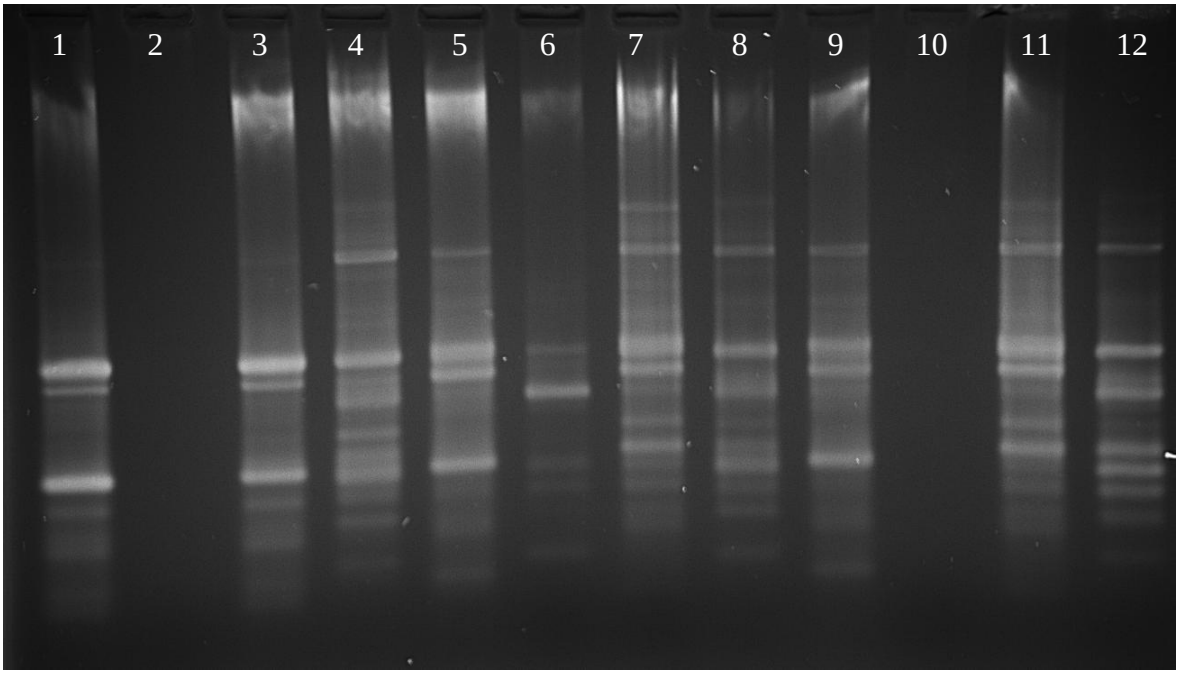


Figure 3.8 RAPD profiles of strain 027 using primer AP4 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

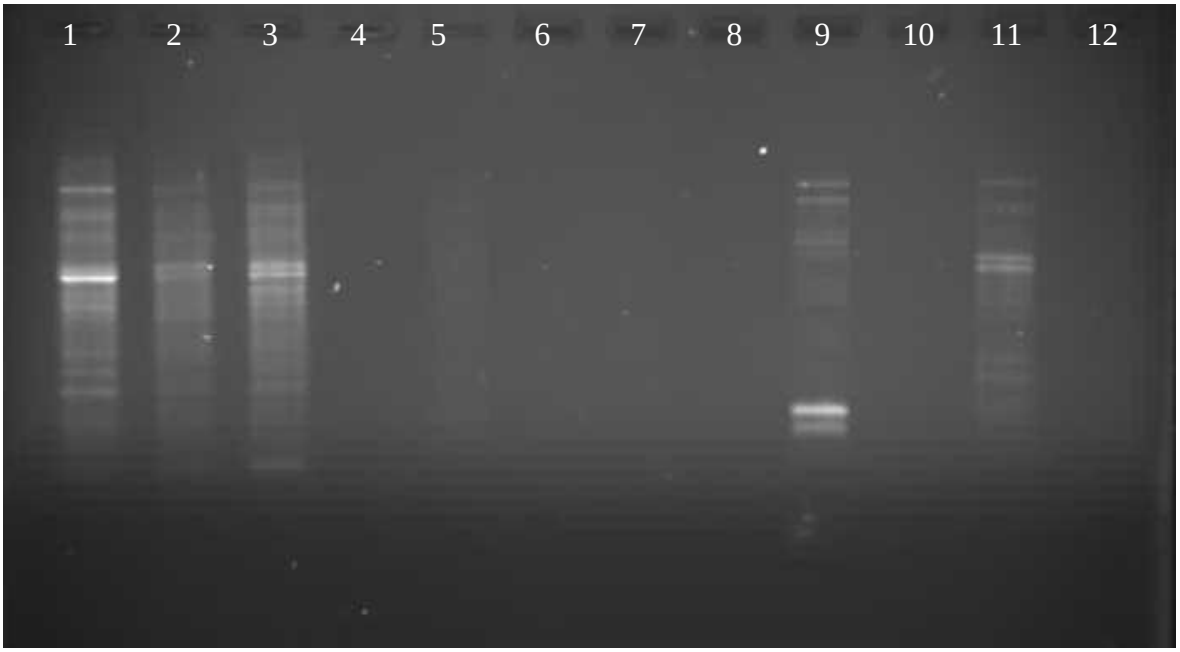


Figure 3.9 RAPD profiles of strain AZ172 using primer AP1 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

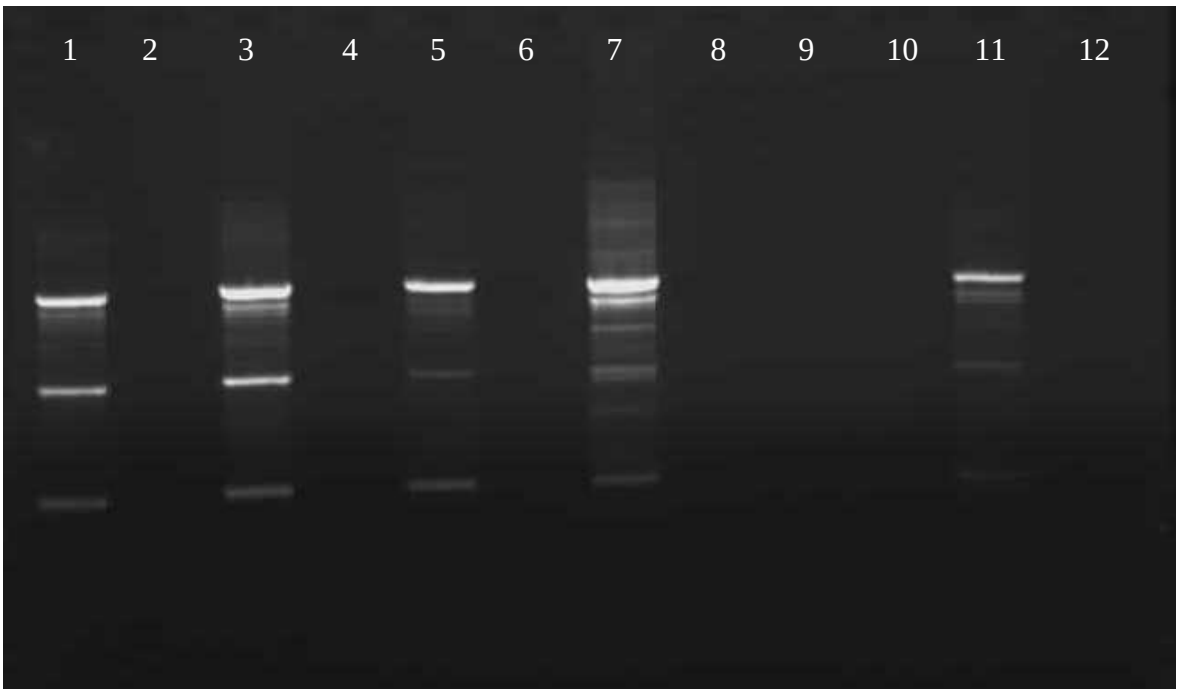


Figure 3.10 RAPD profiles of strain AZ172 using primer AP2 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

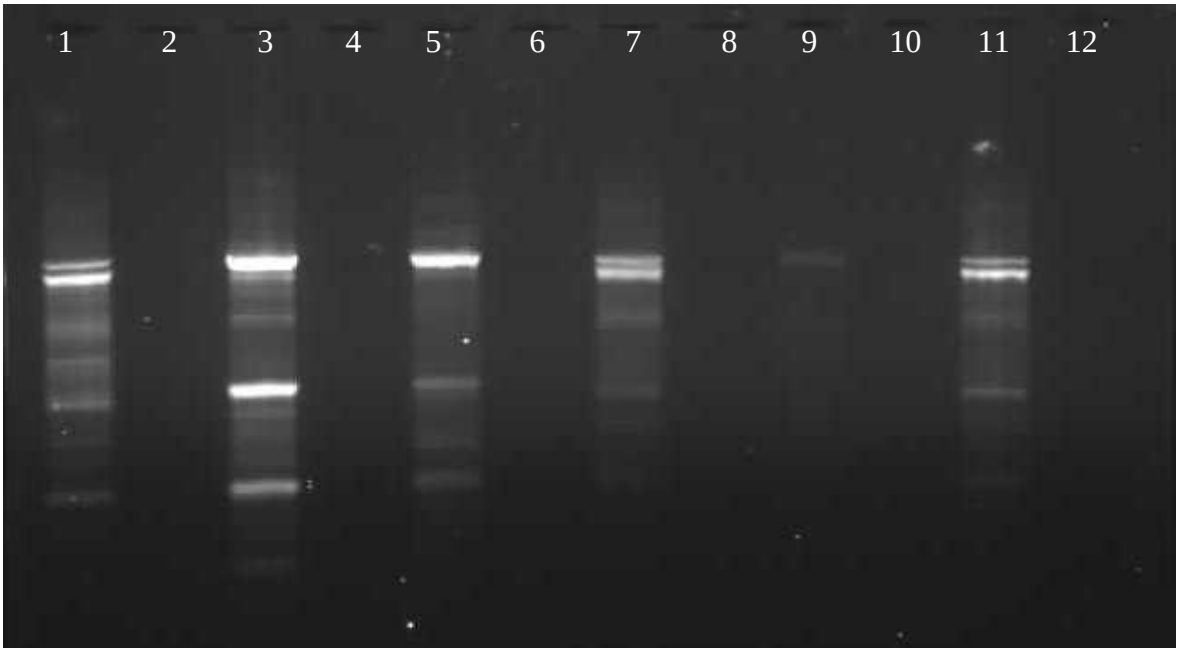


Figure 3.11RAPD profiles of strain AZ172 using primer AP3 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

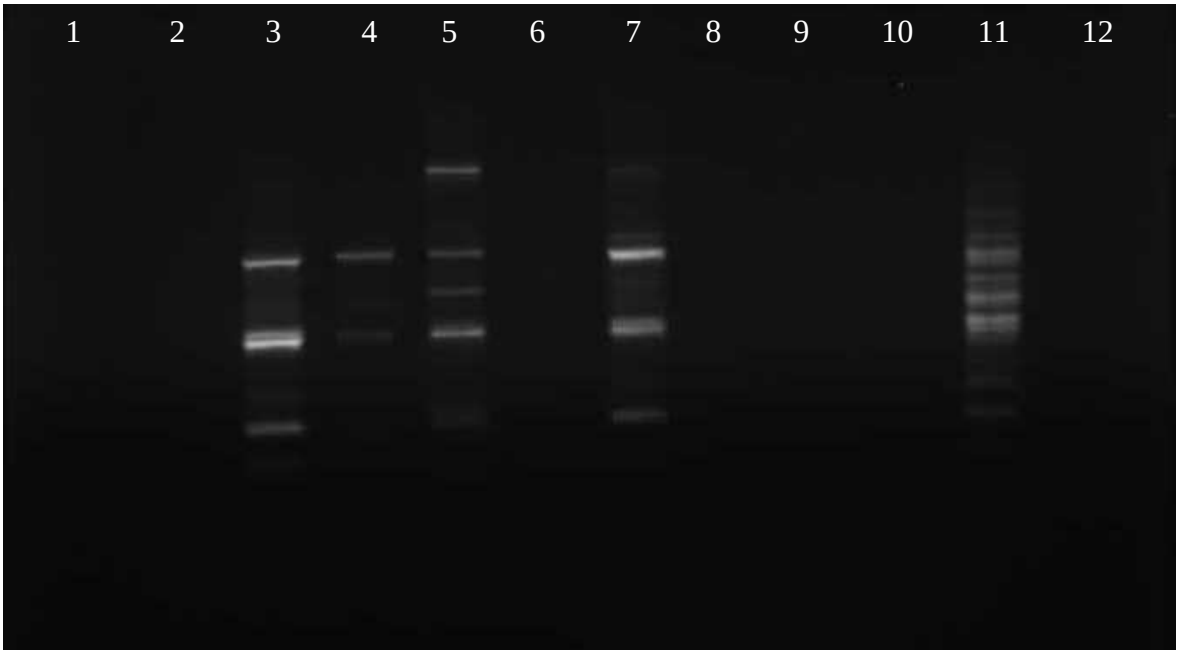


Figure 3.12 RAPD profiles of strain AZ172 using primer AP4 and reaction buffers 1-12 detailed in table 3.2. Lanes are labelled according to the appropriate buffer.

3.3.2 Effect of primer concentration

Primers AP3 and AP4 were used in concentrations of 50, 100, 200 and 300µM alongside buffer 7 in RAPD reactions. Primer AP3 produced amplicon patterns at both 50µM and 100µM concentrations, above these concentrations however amplification was inhibited. When using primer AP3 in RAPD reactions, there was little difference in the quality of profiles produced using primer concentrations of 50µM and 100µM and therefore the lower of the two was selected for future experiments. In contrast to primer AP3, primer AP4 produced amplicon patterns at all of the primer concentrations tested. When using primer AP4 in RAPD reactions, the profiles produced at primer concentrations of 100µM and 200µM were regarded as being better quality and producing more consistent profiles across the three stains of DNA. As a result a primer concentration of 100µM was selected for used in RAPD reactions using primer AP4.

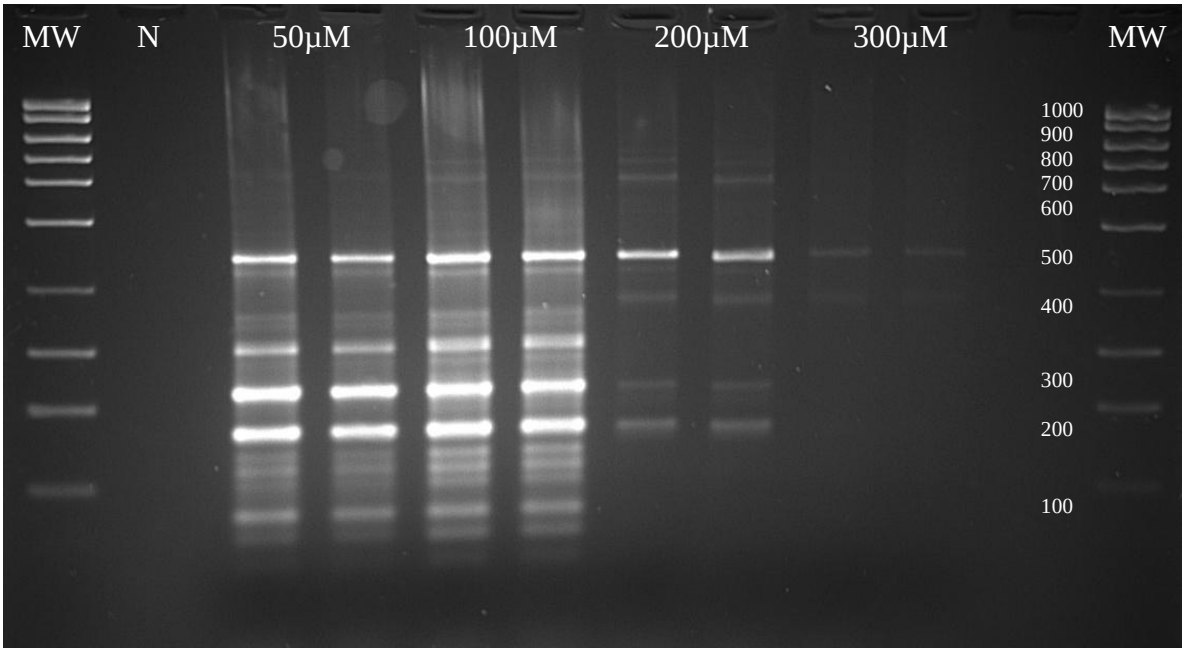


Figure 3.13 RAPD profiles of strain NCTC 11204 using primer AP3. Lanes are labelled according to the appropriate primer concentration. N: negative control; MW: molecular weight ladder (bp)

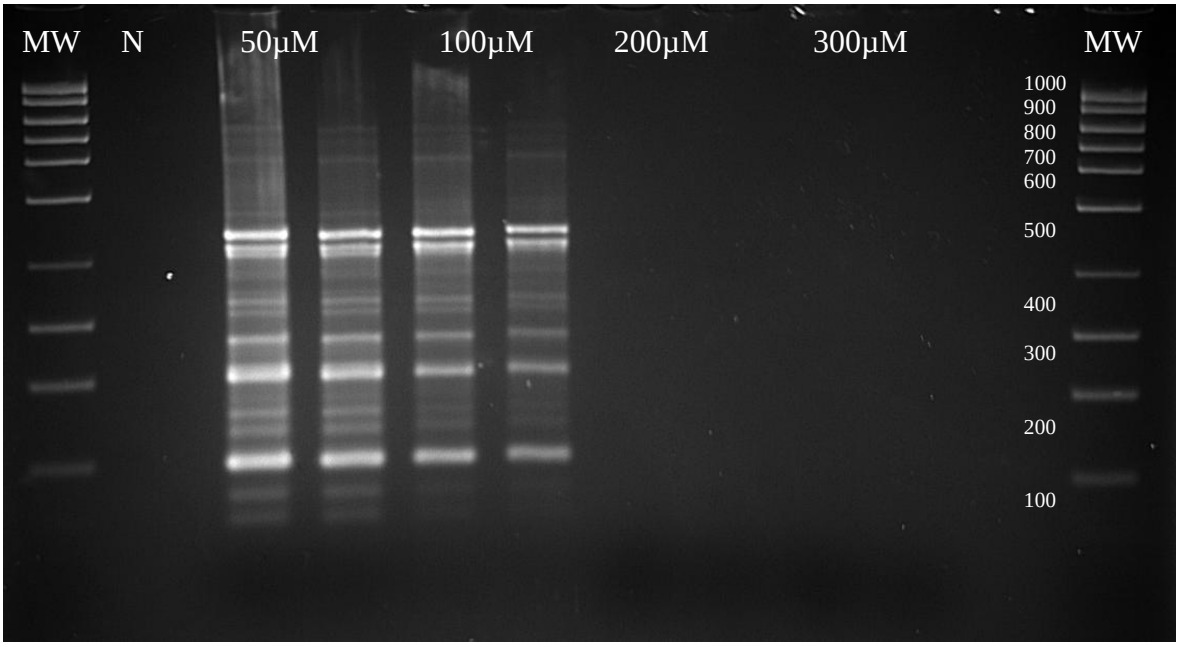


Figure 3.14 RAPD profiles of strain 027 using primer AP3. Lanes are labelled according to the appropriate primer concentration. N: negative control; MW: molecular weight ladder (bp).

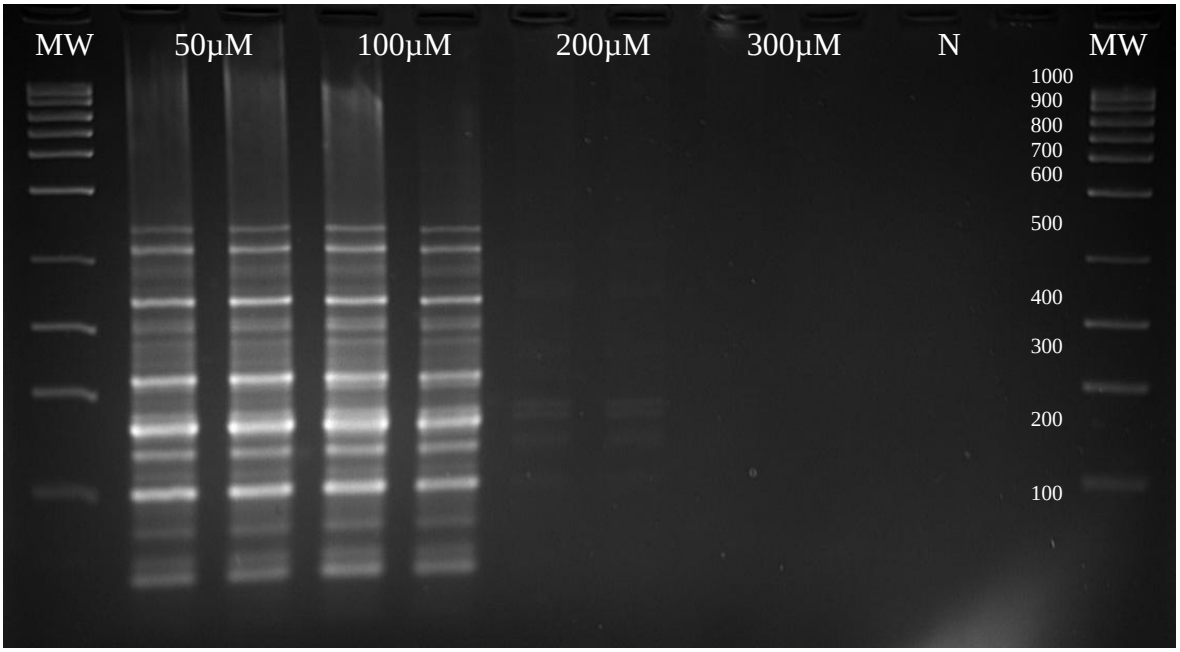


Figure 3.15 RAPD profiles of strain AZ172 using primer AP3. Lanes are labelled according to the appropriate primer concentration. N: negative control; MW: molecular weight ladder (bp).

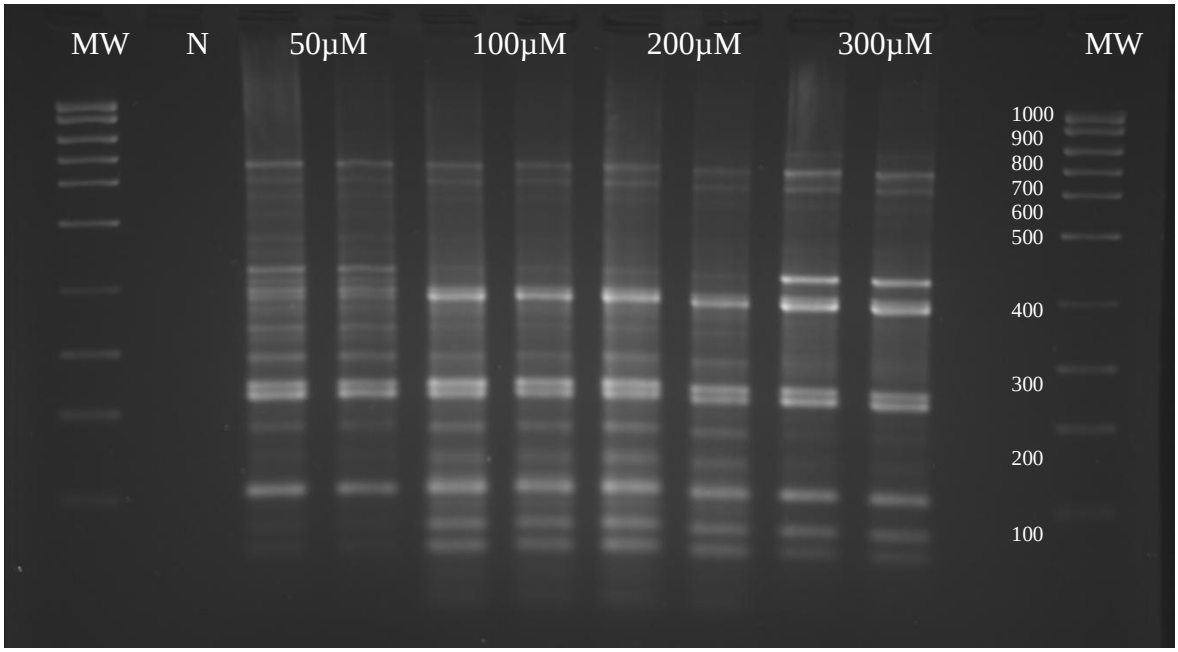


Figure 3.16 RAPD profiles of strain NCTC 11204 using primer AP4. Lanes are labelled according to the appropriate primer concentration. N: negative control; MW: molecular weight ladder (bp).

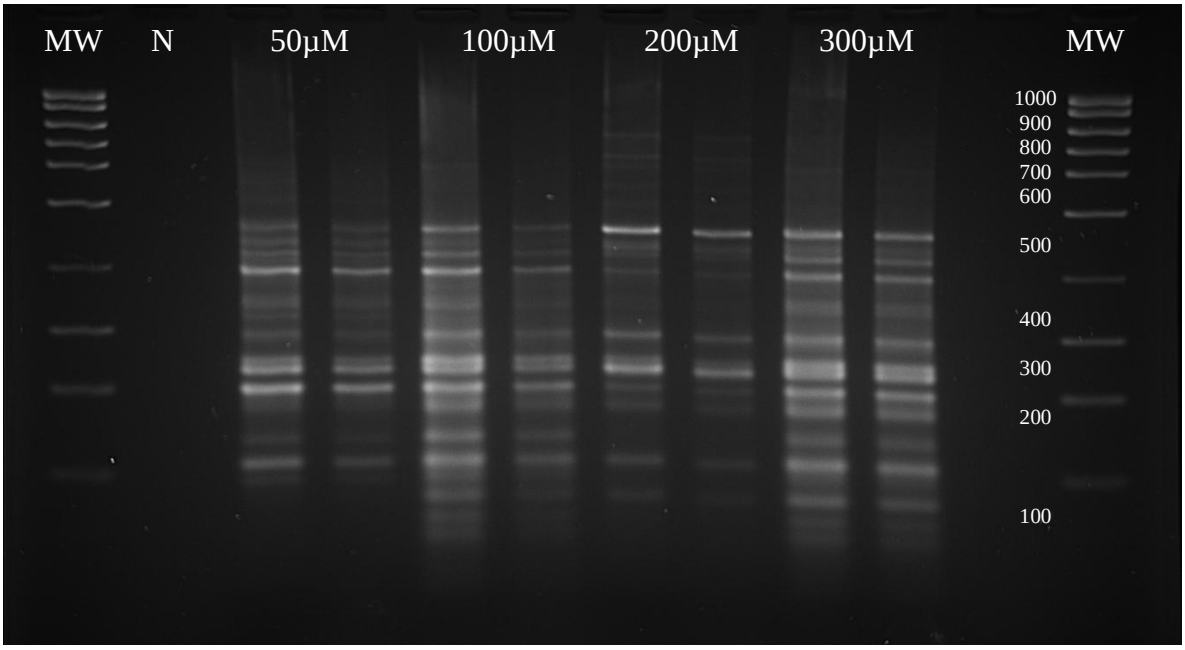


Figure 3.17 RAPD profiles of strain 027 using primer AP4. Lanes are labelled according to the appropriate primer concentration. N: negative control; MW: molecular weight ladder (bp).

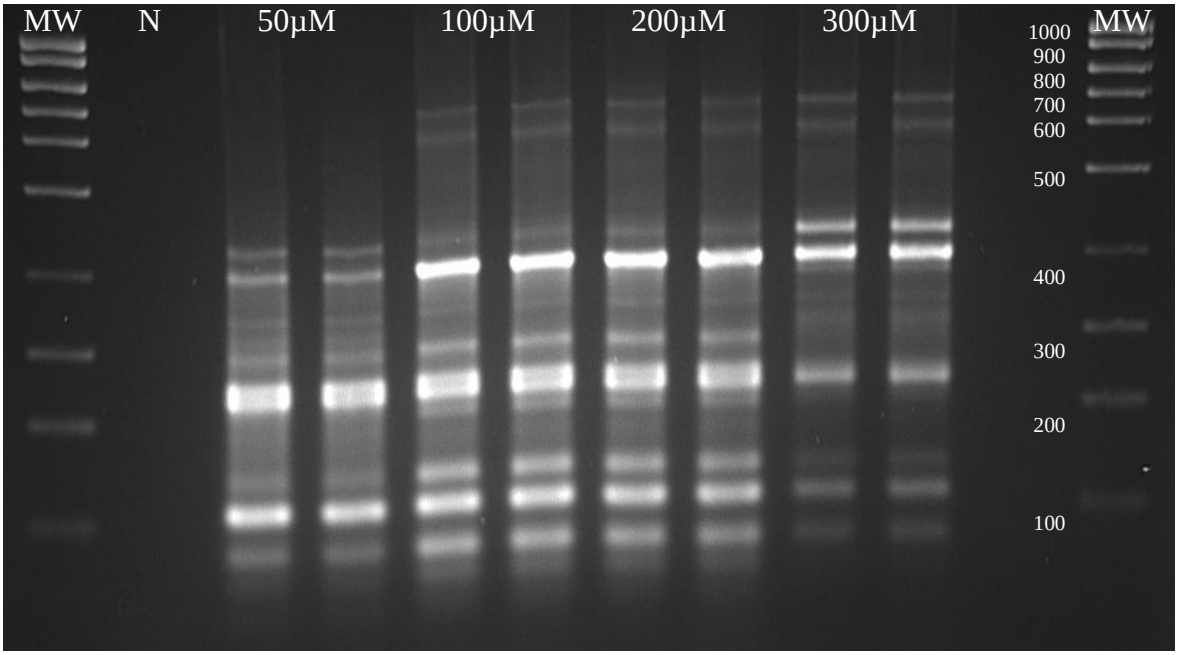


Figure 3.18 RAPD profiles of strain AZ172 using primer AP4. Lanes are labelled according to the appropriate primer concentration. N: negative control; MW: molecular weight ladder (bp).

3.3.3 Effect of dNTP concentration

Primers AP3 and AP4 were used in separate reactions alongside buffer 7 and 5mM, 10mM, 20mM and 30mM concentrations of dNTPs. Within the RAPD reaction using primer AP3, 5mM and 10mM concentrations of dNTPs both supported amplification to a similar extent; producing profiles of a similar quality. Concentrations of 20mM and 30mM inhibited the reaction with no profiles being produced at a concentration of 30mM. A concentration of 10mM was selected for use in future reactions with primer AP3. When optimising dNTP concentrations for RAPD reactions using primer AP4, amplification was also more successfully supported at the lower concentrations of 5mM and 10mM, the optimal concentration of 10mM was therefore also selected for RAPD reactions using primer AP4.

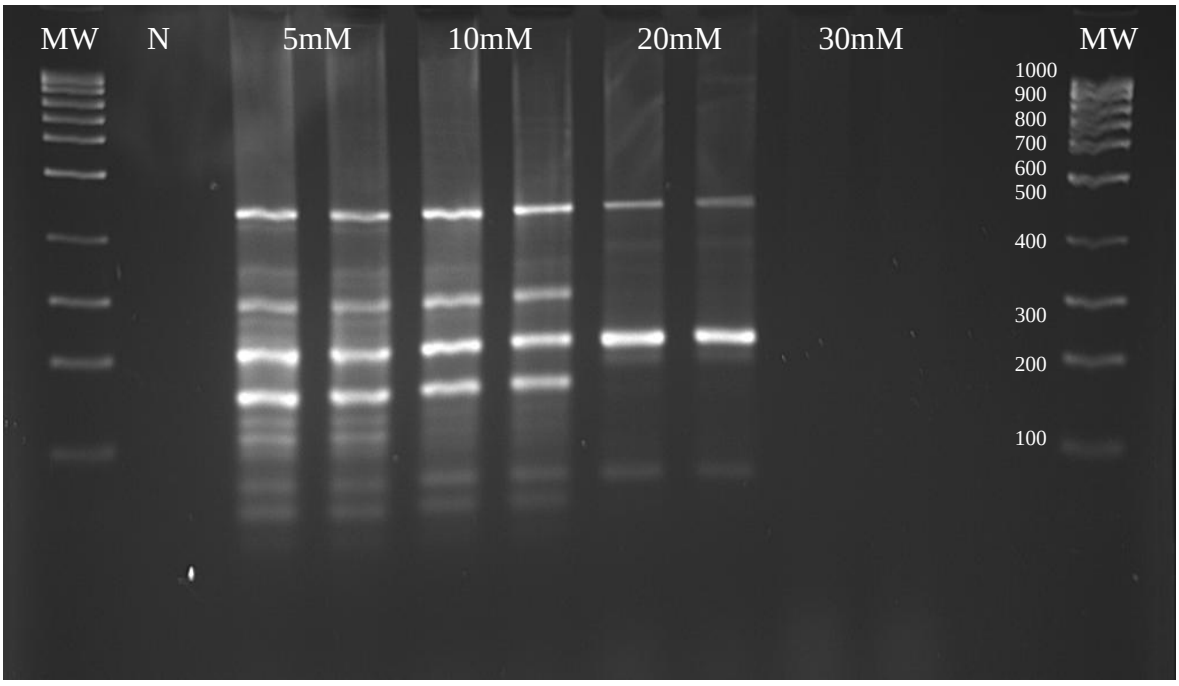


Figure 3.19 RAPD profiles of strain NCTC 11204 using primer AP3. Lanes are labelled according to the appropriate dNTP concentration. N: negative control; MW: molecular weight ladder (bp).

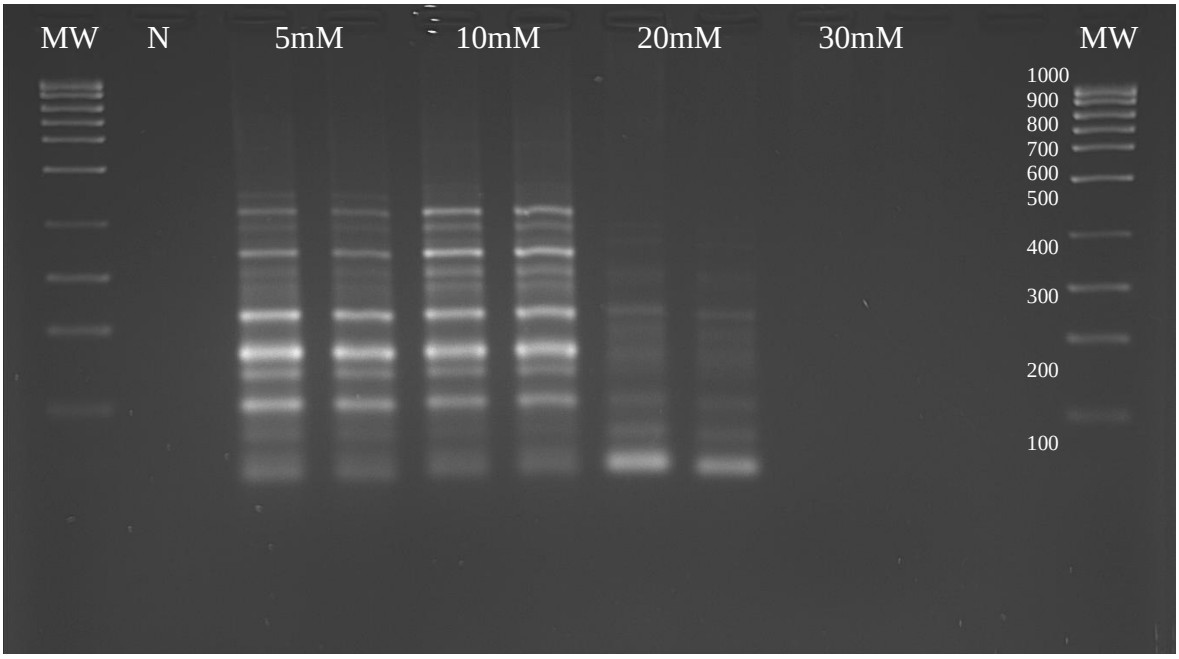


Figure 3.20 RAPD profiles of strain 027 using primer AP3. Lanes are labelled according to the appropriate dNTP concentration. N: negative control; MW: molecular weight ladder (bp).

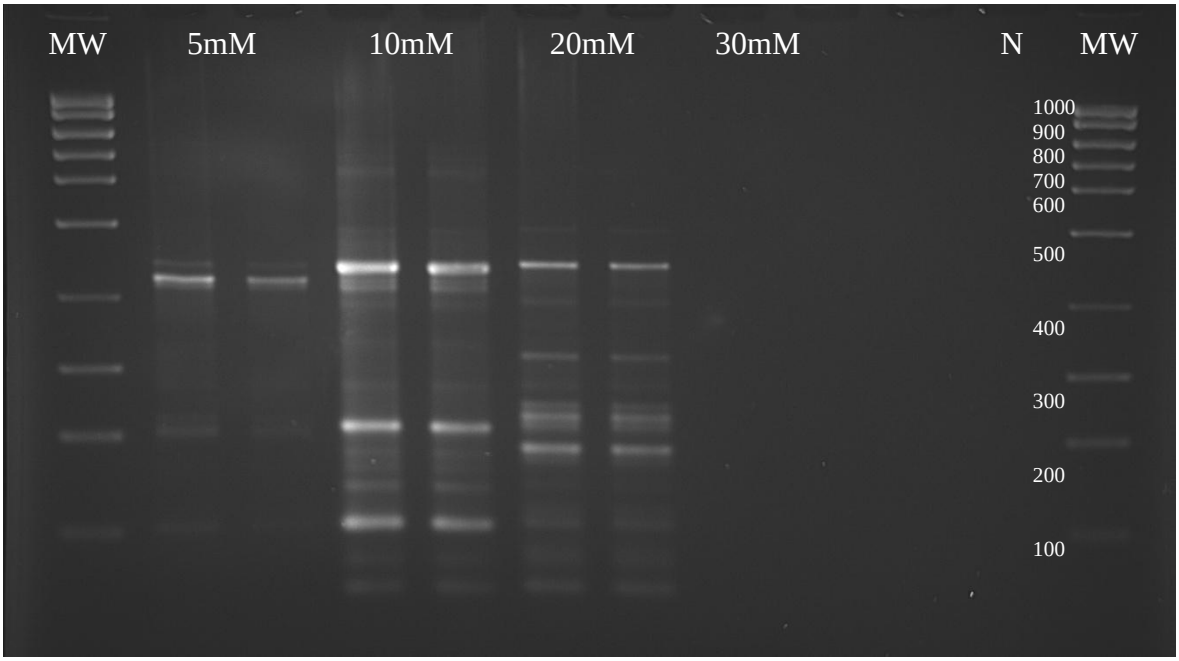


Figure 3.21 RAPD profiles of strain AZ172 using primer AP3. Lanes are labelled according to the appropriate dNTP concentration, N: negative concentration; MW: molecular weight ladder (bp).

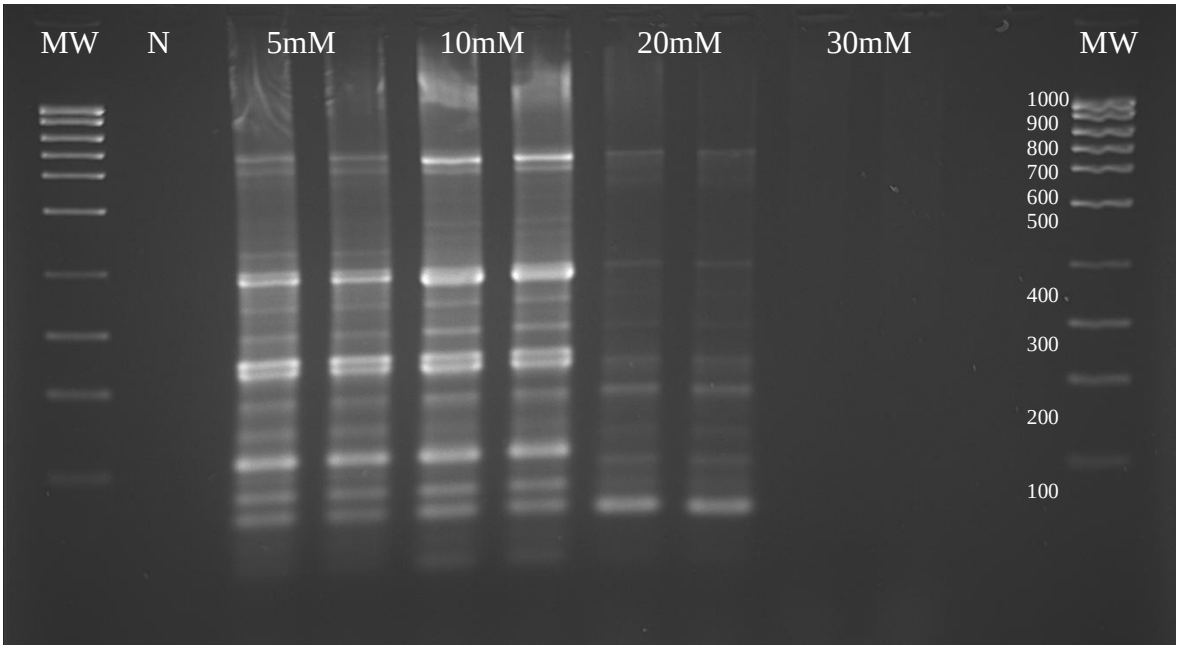


Figure 3.22 RAPD profiles of strain NCTC 11204 using primer AP4. Lanes are labelled according to the appropriate dNTP concentration. N: negative control; MW: molecular weight ladder (bp).

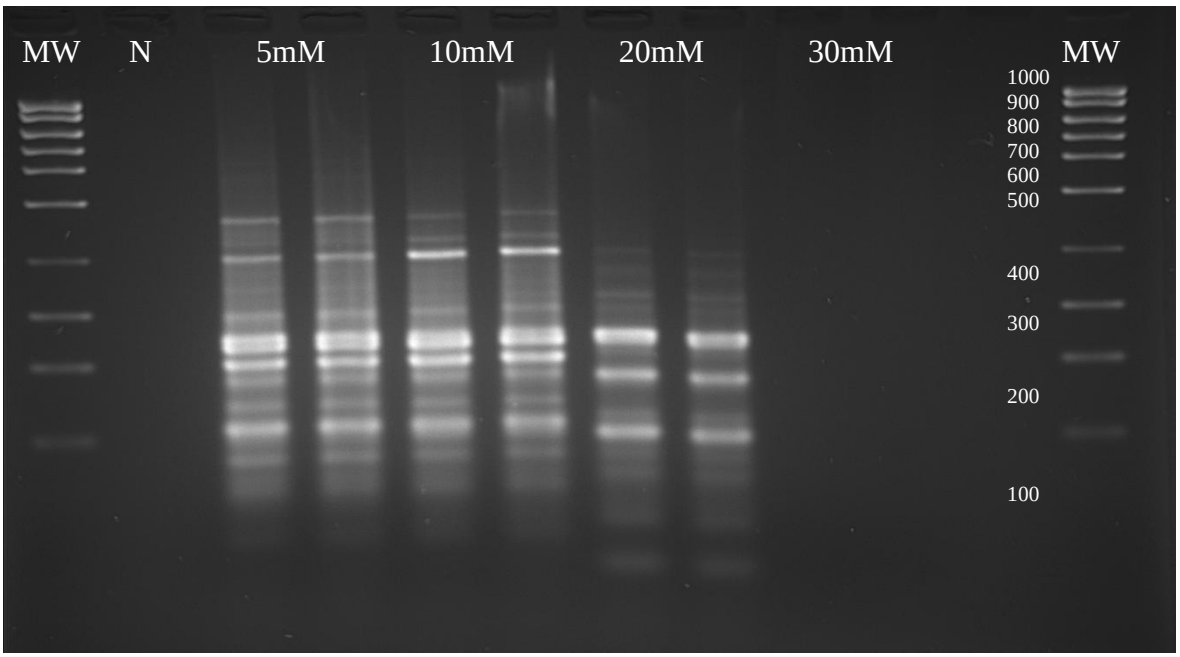


Figure 3.23 RAPD profiles of strain 027 using primer AP4. Lanes are labelled according to the appropriate dNTP concentration. N: negative control; MW: molecular weight ladder (bp).

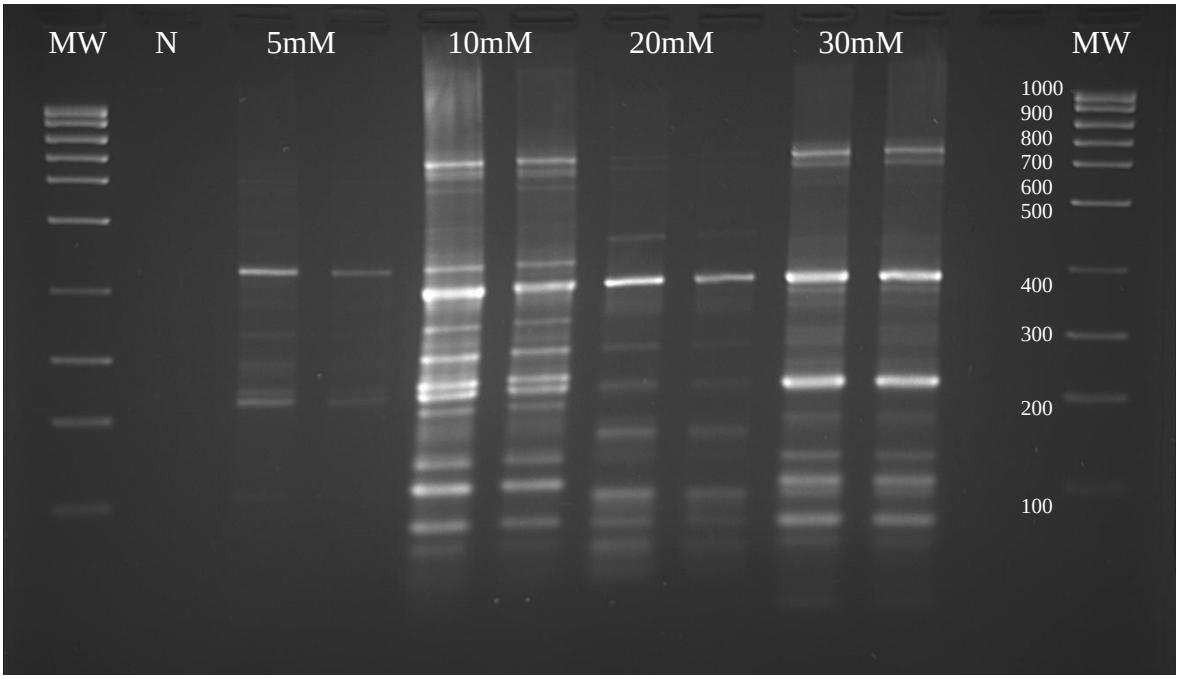


Figure 3.24 RAPD profiles of strain AZ172 using primer AP4. Lanes are labelled according to the appropriate dNTP concentrations. N: negative control; MW: molecular weight ladder (bp).

3.3.4 Comparison of DNA extraction methods

Phenol extracted template DNA (section 3.2.1.1) was used throughout optimisation and this was then compared with two DNA boil extraction methods (section 3.2.1.2-3.2.1.3). DNA from three strains (NCTC 11204, 027, Z172) were used in optimised RAPD protocols using AP3 and the profiles obtained compared and shown in figure 3.25. The RAPD profiles obtained using phenol extracted DNA (method 1) were superior to those obtained using DNA from boil extraction methods (method 2 & 3). Template DNA extracted using method one produced more amplicons above three hundred base pairs which were largely absent in the reactions using DNA from boil extraction methods; below this profiles produced were very similar and therefore discrimination between the strains was still possible. Profiles were stable and reproducible for all extraction methods when repeated on separate occasions. Template DNA obtained from boiling cells with chelex-

100 resin (method 3) produced clearer profiles than from boiling cells alone and this extraction method was selected for future RAPD reactions.

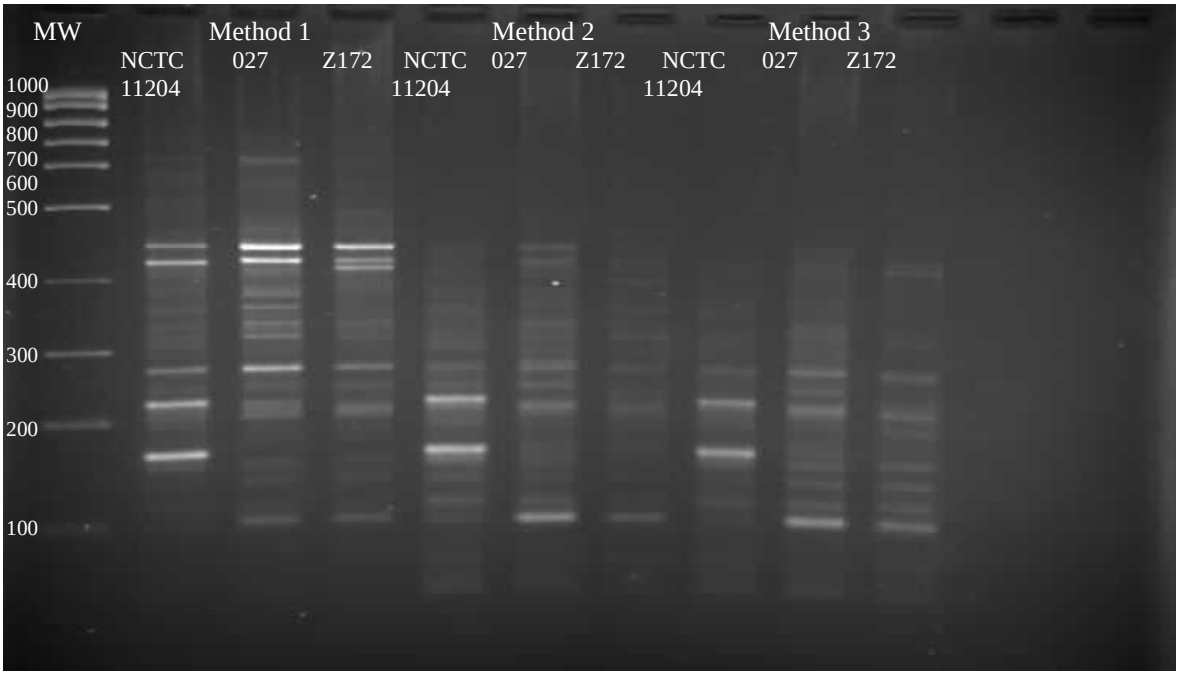


Figure 3.25 RAPD profiles of strains NCTC 11204, 027 and AZ172 using DNA obtained from three different extraction methods as described in section 3.2.1 and amplified using primer AP3.

3.3.5 Template DNA concentration

Known concentrations of phenol extracted DNA were used to determine the effect of template DNA concentration on the optimised RAPD reactions. The effect of DNA template concentration was tested with strain NCTC 11204 using primer AP3 shown in figure 3.26; and primer AP4 shown in figure 3.27. Using the concentrations of template DNA 5, 10, 20, 30, 40 and 50 ng per reaction; for both primers none of the tested concentrations appeared to be inhibitory or optimal to the RAPD reaction with the same profiles of the same quality produced for each concentration. The lack of effect this range of DNA concentrations had on the modified RAPD reaction demonstrates that a crude boil extract is suitable for use with such reactions where DNA concentration is not measured and likely to vary.

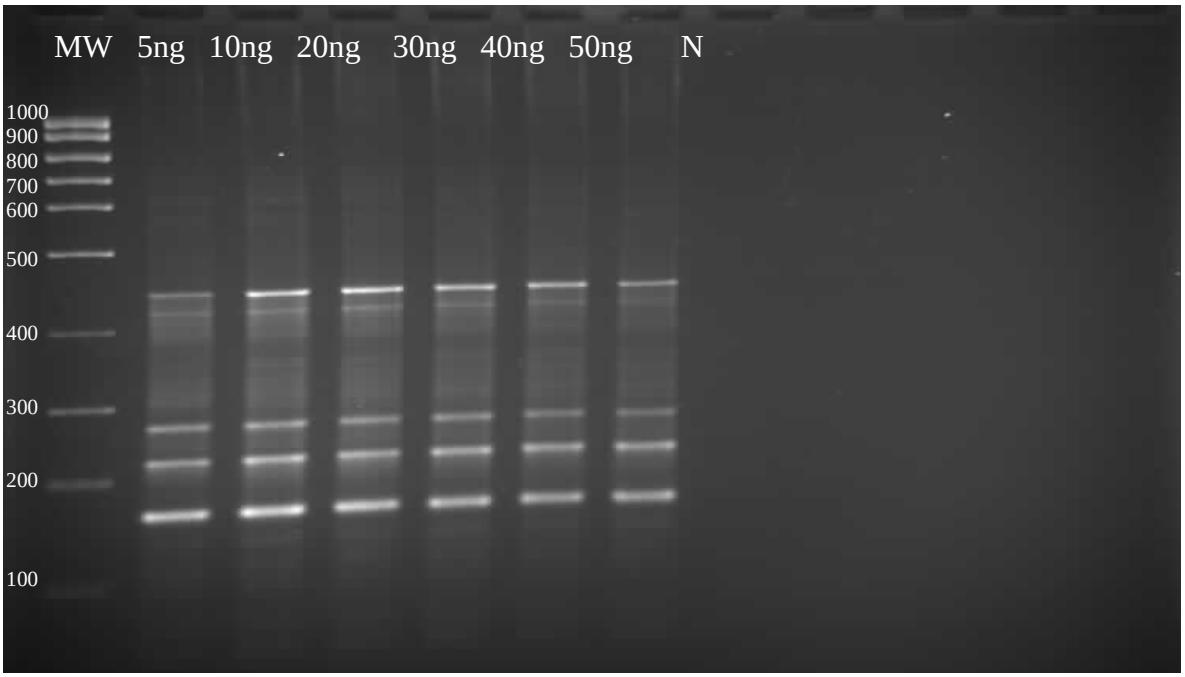


Figure 3.26 RAPD profiles of strain NCTC 11204 using primer AP3 with different concentrations of template DNA per reaction. Lanes are labelled according to DNA template concentration. N: negative control; MW: molecular weight ladder (bp).

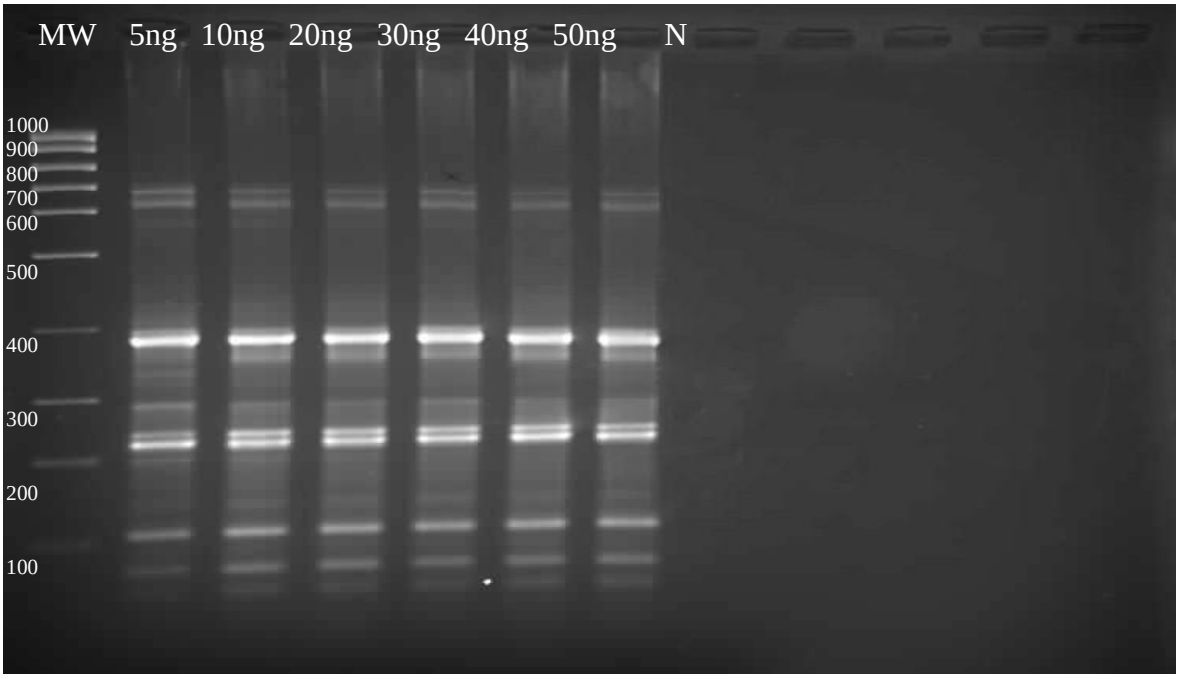


Figure 3.27 RAPD profiles of strain NCTC 11204 using primer AP4 with different concentrations of template DNA per reaction. Lanes are labelled according to DNA template concentration. N: negative control; MW: molecular weight ladder (bp).

3.3.6 Transferability between PCR thermocyclers

Throughout optimisation, the same PCR thermocycler was used, when reactions were performed on a different thermocycler, little variation was observed between the profiles produced.

3.3.7 Discrimination of PCR ribotypes

Eleven different PCR ribotypes were analysed using the optimised RAPD protocol using primers AP3 and AP4 in independent reactions; DNA for all reactions was extracted using method 3 (section 3.2.1.3). Both primers when used in independent reactions produced discriminate RAPD profiles for each of the PCR ribotypes tested as shown in figure 3.28 and figure 3.29. Isolates known to be the same PCR ribotype were also analysed by this RAPD protocol to determine if they were identified as the same. Reactions using primers AP3 and AP4 in independent tests were used to characterise eight clinical isolates of PCR ribotype 106, five clinical isolates of PCR ribotype 001 and five clinical isolates of PCR ribotype 027. The primers used in both RAPD reactions grouped isolates the same as PCR ribotyping shown in figures 3.30-3.33.

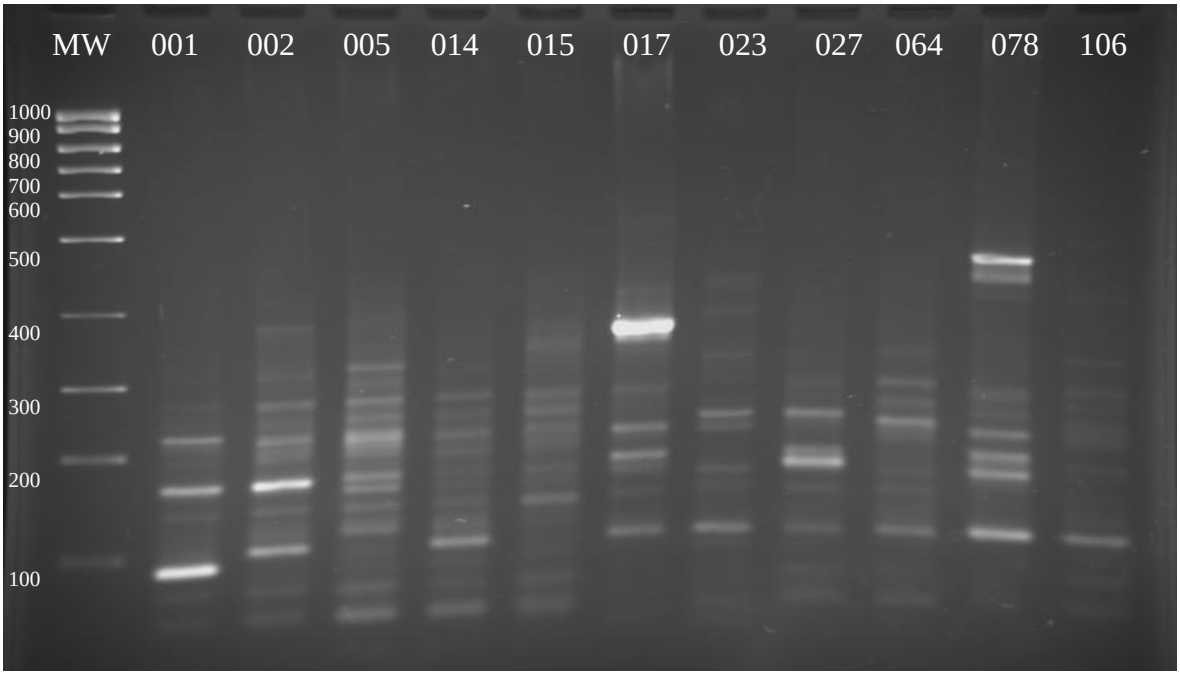


Figure 3.28 RAPD profiles of eleven known PCR ribotypes using primer AP3. Lanes are labelled according to PCR ribotype. MW: molecular weight ladder (bp).

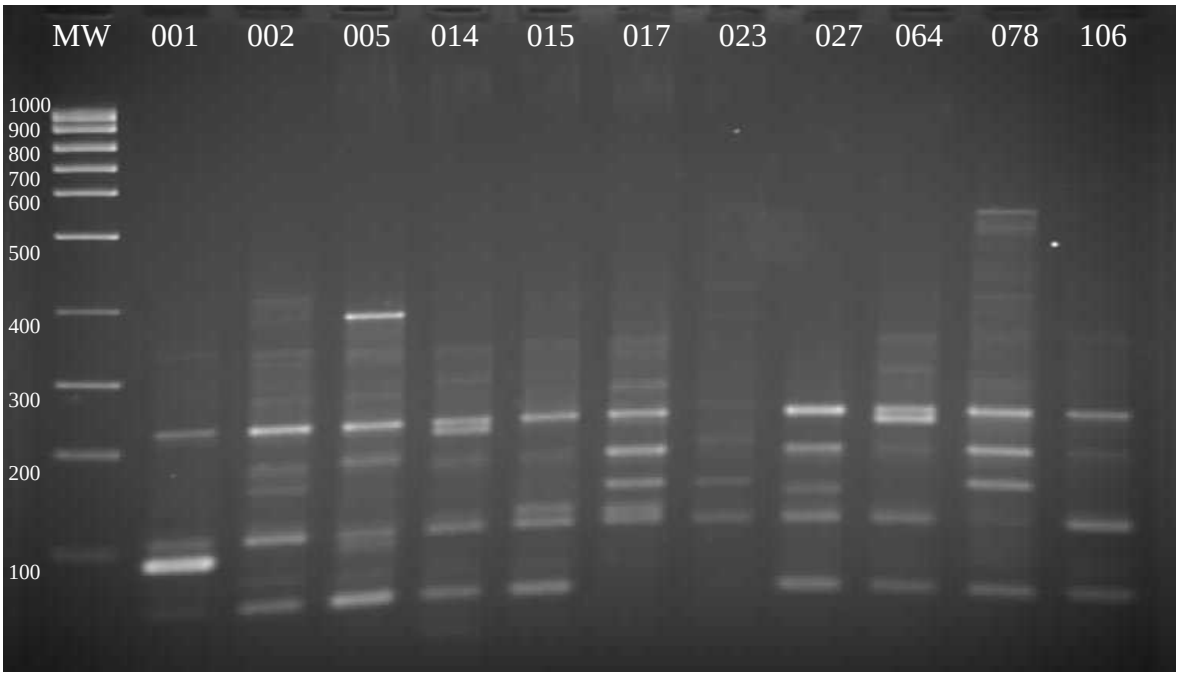


Figure 3.29 RAPD profiles of eleven known PCR ribotypes using primer AP4. Lanes are labelled according to PCR ribotype. MW: molecular weight ladder (bp).

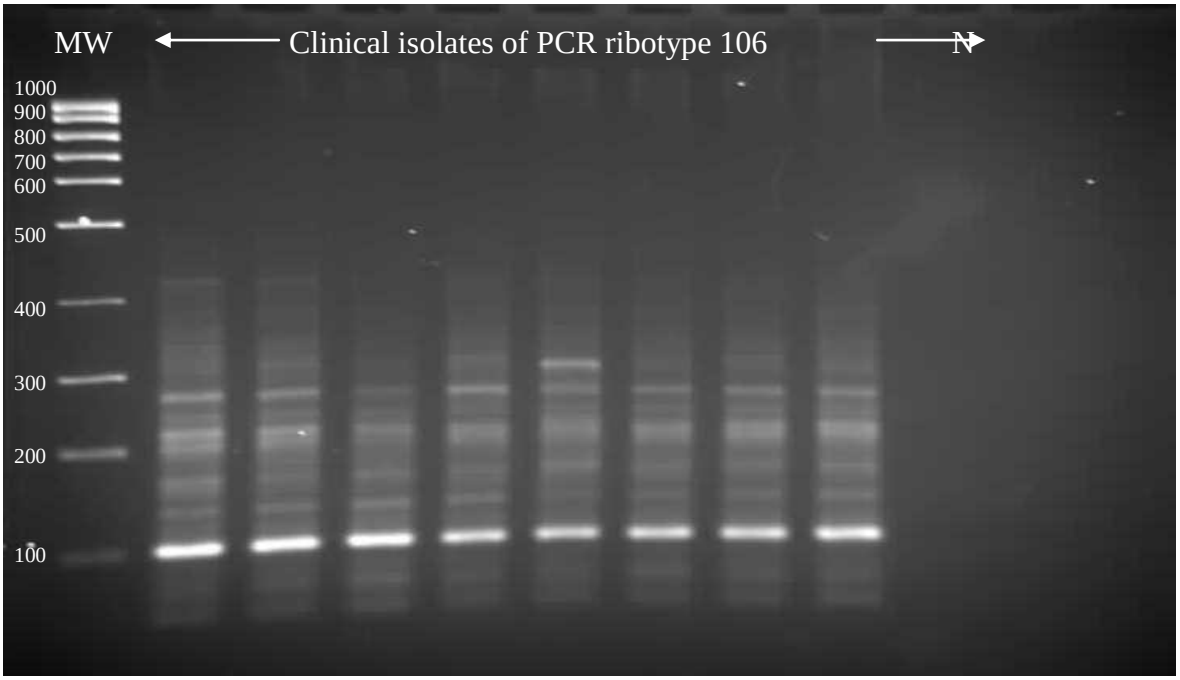


Figure 3.30 RAPD profiles of eight clinical isolates of PCR ribotype 106, types using primer AP3. N: negative control; MW: molecular weight ladder (bp)

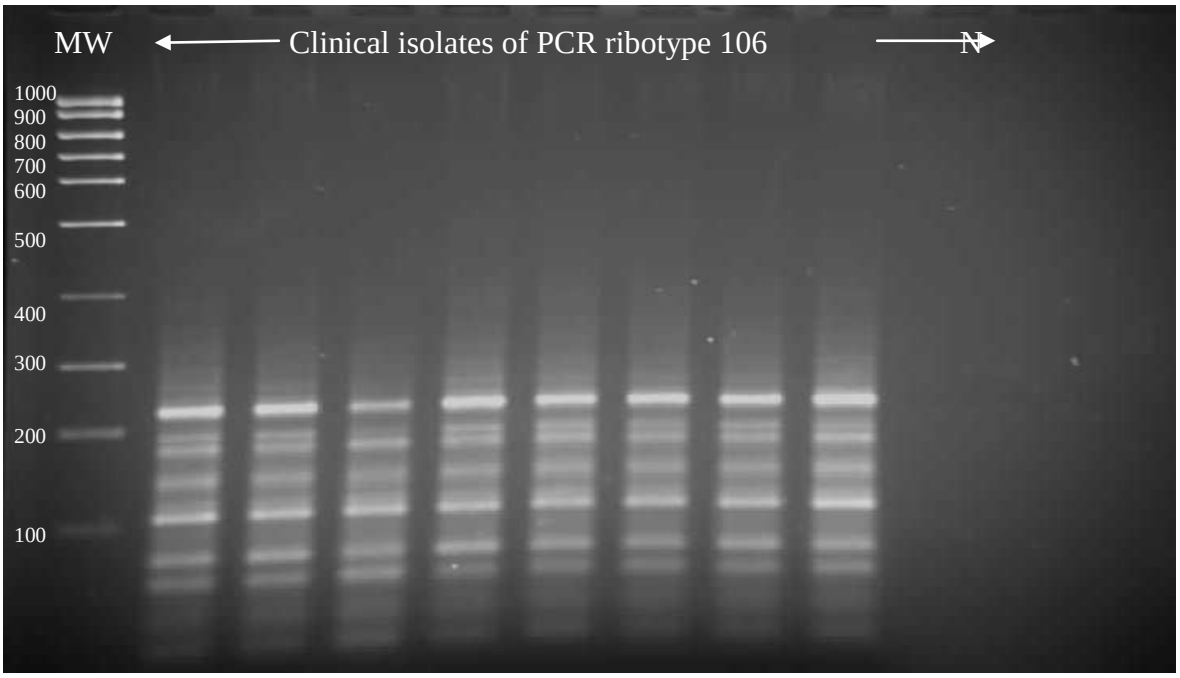


Figure 3.31 RAPD profiles of eight clinical isolates of PCR ribotype 106, typed using primer AP4. N: negative control; MW: molecular weight ladder (bp).

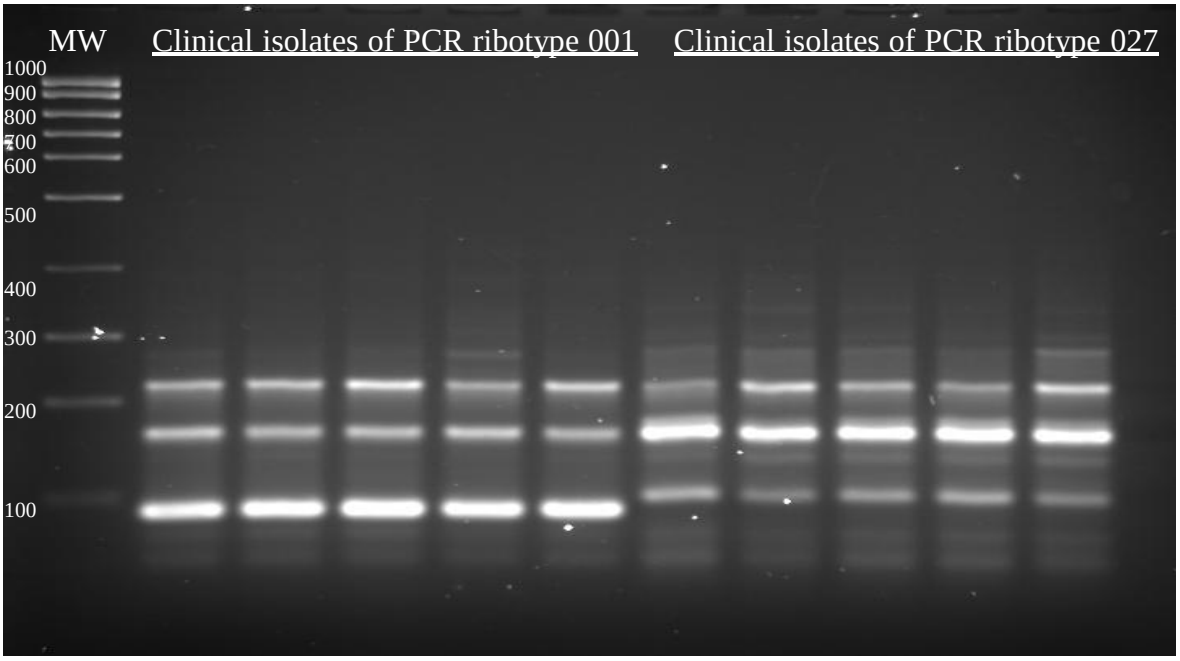


Figure 3.32 RAPD profiles of five clinical isolates of PCR ribotype 001 and five clinical isolates of PCR ribotype 027, typed using primer AP3. N: negative control; MW: molecular weight ladder (bp).

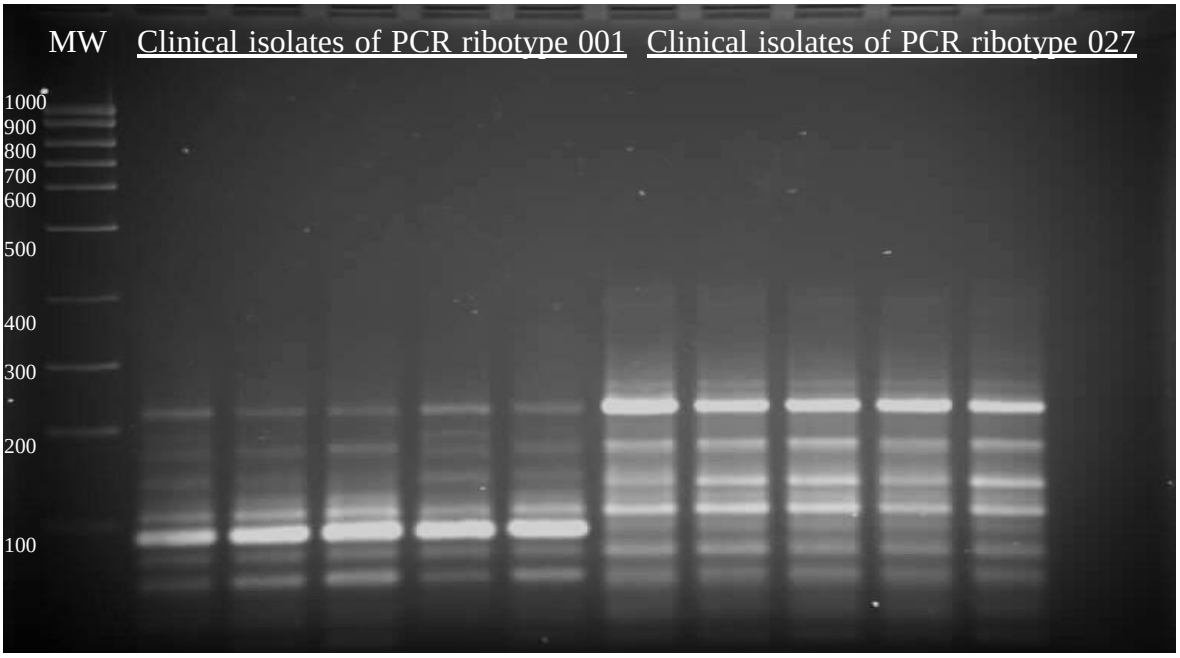


Figure 3.33 RAPD profiles of five clinical isolates of PCR ribotype 001 and five clinical isolates of PCR ribotype 027, typed using primer AP4. N: negative control; MW: molecular weight ladder (bp).

3.4 DISCUSSION

The aim of this chapter was to develop and optimise two independent RAPD protocols for the genetic characterisation of *C. difficile* isolates to a standard of discriminatory power and reproducibility comparable to PCR ribotyping. Both optimised protocols were then used to characterise eleven control isolates of *C. difficile* known to belong to eleven different PCR ribotypes to determine if the optimised protocols could discriminate between them. Identical PCR ribotypes of *C. difficile* were also analysed to determine if the RAPD protocol would identify these as the same type.

Despite being a relatively quick and cost effective method of genetic characterisation, RAPD is often criticised on grounds of reproducibility and discriminatory power. As a result RAPD has been used infrequently in the typing of *C. difficile* isolates when compared to other methods such as PCR ribotyping and PFGE which are the gold standard methods used in the UK and North America respectively. The PCR methodology is very specific and RAPD is no exception to this; as a result protocols are not always readily transferable between laboratories and if not properly optimised can produce sub-standard results (Cobb and Clarkson, 1994), it is therefore recommended that RAPD protocols are thoroughly optimised in order to provide optimal amplification (Tyler *et al.*, 1997). Both PCR ribotyping and PFGE are regarded as much more robust and by some have been reported to be more discriminative than RAPD for the typing of *C. difficile*; however for both of these methods results are time consuming and PFGE is renowned for being a labour intensive technique. Although over one hundred different PCR ribotypes have been identified, very often only limited PCR ribotypes will predominate within a region or area causing limitations in the PCR ribotyping technique as although isolates may belong to the same ribotype, they may still be different strains. This lack of further discrimination with

PCR ribotyping hinders epidemiological mapping as further strain variability cannot be detected.

As the target of amplification is unknown in RAPD, ideally more than one primer should be selected for initial testing as amplicon patterns will differ depending on the primer sequence. The four primers initially selected here were chosen as they had been used with some success by others in earlier work using RAPD to type *C. difficile* (Barbut *et al.*, 1993, Killgore and Kato, 1994, Martirosian *et al.*, 1995, Wullt and Laurell, 1999) and therefore it had been demonstrated that these primers had the potential to produce amplicons in an optimised PCR reaction. In this study, primers AP3 and AP4 were selected for optimisation due to ability to potentially provide an adequate number of amplicons to allow for discrimination between strains but not so many amplicons that discrimination would be difficult. Although some of the patterns produced by AP1 and AP2 also showed potential, results were less consistent across the different template DNA.

Optimisation was carried out according using a systematic methodology similar to that described by (Hopkins and Hilton, 2001) using a buffer matrix design (Optiprime, Stratagene) that allows optimal concentrations of $MgCl_2$ and KCl and the pH of the reaction to be determined simultaneously. The pH and concentrations of $MgCl_2$ and KCl are crucial to a PCR reaction and if too high or low can inhibit amplification completely or cause non specific priming and inaccurate results. PCR reactions are often reported to be $MgCl_2$ dependent with this variable being regarded more important than others due to the range of effect the ions have on the reaction. During the optimisation process here amplification was supported at both $MgCl_2$ concentrations tested (1.5mM and 3.5mM). Within the PCR reaction $MgCl_2$ has multiple roles; it acts as a cofactor to *Taq* polymerase in addition to promoting primer template binding and forming complexes with dNTPs allowing them to be utilised by *Taq* polymerase (Kolmodin and Birch, 2002). If

concentrations of $MgCl_2$ are too low this is likely to inhibit amplification with insufficient amplicons being produced (Williams *et al.*, 1993) as primers may not bind efficiently to the template, dNTPs may not be utilised and *Taq* polymerase may not function. High concentrations of $MgCl_2$ can also have a detrimental effect on PCR reactions causing non specific binding (Williams *et al.*, 1993) likely due to enhanced activity of *Taq* polymerase that allows non specific binding to both the DNA and dNTPs and can also inhibit denaturation of template DNA (Perry *et al.*, 2003).

Salt concentration is also important in PCR reactions and like $MgCl_2$ influences primer binding and the activity of *Taq* polymerase with incorrect concentrations causing similar problems associated with $MgCl_2$ variation. The primers selected here for optimisation appear to be dependent on KCl concentration for successful amplification. Like $MgCl_2$, KCl also influences primer binding and *Taq* polymerase activity (Hilton *et al.*, 1997) and therefore concentrations that are too high or low can also cause the problems that are observed with inaccurate $MgCl_2$ concentrations.

The influence of pH on PCR reactions is not well documented and the primers selected for use in this investigation were stable across the pH range tested; despite this optimal pH in PCR reactions should not be overlooked especially as enzymes such as *Taq* polymerase can be very sensitive to such changes. High KCl concentration in most reactions appeared to be inhibitory with no amplicons being produced although there were some exceptions. Amplification appeared most severely inhibited in the presence of higher KCl concentration (75mM) and a low $MgCl_2$ concentration (1.5mM) although amplification has been seen to occur in the presence of one of these variables. Results such as this indicate that the RAPD reaction is not only influenced by individual components but also by the interaction of these with each other. Buffer 7 (3.5mM $MgCl_2$, 25mM KCl, 10mM Tris-Hcl,

pH 8.8) was selected as it consistently produced higher quality amplicon patterns across the three strains used.

Optimal primer concentration varied for both of the primers thus demonstrating the importance of optimisation. Amplification was inhibited when primer AP3 was used at higher concentrations (200µM and 300µM) possibly due to insufficient concentrations of other components in the reaction to support adequate amplification throughout all of the cycles. Amplification was supported across all primer concentrations when using primer AP4 although the optimal concentration was at 100µM. If primer concentration is too low then a lack of product will often result producing fewer or no bands at all; if primer concentration is too high then this can cause non specific binding or completely inhibit the reaction (Muralidharan and Wakeland, 1993). It has also been reported that products of RAPD reactions are influenced by different batches of primer (Tyler *et al.*, 1997) and as a result different batches were used throughout optimisation to ensure that this had no influence and therefore unlikely to effect future reactions. Like primer stocks, variability between different stocks of dNTPs have also been reported to influence RAPD reactions (Tyler *et al.*, 1997) however this was not found to be the case here. Reactions were inhibited at the highest dNTP concentration with lower concentrations producing better results, because dNTPs also utilise $MgCl_2$, the ratio between these two components is also very important (Blanchard *et al.*, 1993). As this was the final stage in the optimisation process it is likely that the lower concentrations of dNTPs here were optimal for the concentration of $MgCl_2$ present, with there being insufficient levels of $MgCl_2$ to support higher concentrations of dNTPs.

Although template DNA concentration was kept at a constant concentration through the optimisation process, the use of phenol extracted DNA is not practical for routine testing of isolates both due to the time taken to extract the DNA but also the hazards associated with

using phenol and chloroform. Cruder extraction techniques whereby whole cells are boiled have been used by others with results for both methods being reported to be of similar quality (Mazurier and Wernars, 1992, Lawrence *et al.*, 1993, Howell *et al.*, 1996, Hilton *et al.*, 1997). An additional extraction method that boiled cells with Chelex-100 resin was also tested based on DNA extraction methods used for PCR ribotyping of *C. difficile* (Stubbs *et al.*, 1999). As would be expected the reactions that contained phenol extracted DNA produced the clearest profiles; despite this there was good similarity between the profiles obtained for the three methods tested therefore demonstrating that boiling of whole cells is a suitable extraction method for RAPD analysis. Using a crude DNA extraction method means that the quantity of DNA within a reaction will vary and this is known to influence RAPD reactions. Due to the optimisation process, DNA concentration had to remain constant and this is not a true reflection of a RAPD reaction using a crude DNA extract. Using the optimised RAPD reaction mixture, the concentration of the DNA template was varied to determine if this would influence the results. As the concentration of DNA template did not affect results, this confirmed that a crude but less labour intensive method of DNA extraction may be incorporated within the RAPD protocol. Transferability is often reported to be an inherent problem with molecular protocols, in particular the thermocycler used (Tyler *et al*., 1997) however this was not found in this study.

Following optimisation it was important to determine if the protocol could discriminate between different PCR ribotypes and also group isolates of the same known PCR ribotype together using crude extracted template DNA. If the protocols had failed to do this, the optimisation process would have had to been repeated using different primers. Both primers were able to discriminate between the PCR ribotypes of *C. difficile*, genetic characterisation of further ribotypes is required to fully assess the value of the method. When DNA was extracted from the defined PCR ribotypes using the phenol extraction

method however, some strains appeared very similar in the amplicon patterns that were produced. This may be due to the DNA being better preserved when extracted using phenol allowing more amplicons to be produced; it therefore appears that the use of boil extracted DNA allows for discrimination between these profiles as there is likely to be some damage to the DNA. Testing was performed on several different occasions using different subcultures of bacterial strains in order to assess the reproducibility of the protocol and reproducibility was excellent. Sometimes when experiments were repeated, some of the fainter bands varied between repetitions however the stronger clearer amplicons remained constant and it these amplicons on which discrimination was based.

3.5 CONCLUSION

Optimisation is very important when a new primer-template DNA combination is being tested and this has been demonstrated here. Although phenol extracted DNA produced clearer amplicon patterns, not only is this not a practical extraction method for RAPD analysis, its use compromises the discriminatory power of the protocol. The interaction of all variable components of the reaction appears to be very influential on the results obtained for this protocol with KCl appearing to be the most crucial factor. Based on the eleven defined PCR ribotypes used here, RAPD appears to be equally as discriminative as PCR ribotyping, producing reproducible profiles and grouping known PCR ribotypes together.

It may be possible that RAPD could be used as a suitable typing method for *C. difficile* isolates due to its reproducibility; simplicity and relatively quick results. Further PCR ribotypes would need to be tested in addition to other factors such as transferability of the method between laboratories.

CHAPTER 4 GENOTYPIC CHARATERISATION OF *CLOSTRIDIUM DIFFICILE* USING PCR RIBOTYPING AND A RANDOM AMPLIFIED POLYMORPHIC DNA PCR PROTOCOL

4.1 INTRODUCTION

Many different methods have been used in the genotypic characterisation of *C. difficile* since its emergence as a serious hospital associated infection. In the genotypic characterisation of other species of bacteria, pulsed field gel electrophoresis (PFGE) is often regarded as the gold standard by which to type isolates. In North America, PFGE is widely used to characterise isolates of *C. difficile* for epidemiological purposes. When PFGE was first used to characterise isolates of *C. difficile*, some isolates could not be typed due to apparent degradation of the genomic DNA prior to the addition of restriction enzymes. This has since been resolved and PFGE can now type all strains of *C. difficile* (Alonso *et al.*, 2005). Prior to this however, other suitable methods had to be investigated leading to the development of PCR ribotyping for the characterisation of *C. difficile* isolates. The PCR ribotyping method has gained worldwide acceptance as a suitable and reproducible method by which to type isolates of *C. difficile*, and is used in many countries as the gold standard method including the UK. Although PCR ribotyping can provide effective discrimination, the predominance of selected ribotypes within different geographical areas has meant that this method now provides limited information on the transmission and epidemiology of *C. difficile* strains and possible subtypes (HPA, 2009b).

In contrast to PCR ribotyping, RAPD when previously used to characterise *C. difficile* has often been disregarded in favour of other methods on the grounds of reproducibility and discriminatory power (Brazier, 1998). Most studies that have used RAPD have done so in order to compare different typing methods (Chachaty *et al.*, 1994, Van Dijck *et al.*, 1996) however, it has been used in various studies to genotypically characterise bacterial isolates

(Barbut *et al.*, 1994, Van Dijck *et al.*, 1996, Wilcox *et al.*, 1998, Fawley and Wilcox, 2001, Pituch *et al.*, 2001, Baker *et al.*, 2010). Although issues regarding reproducibility and discrimination are often reported with the RAPD method, it has been demonstrated that effective optimisation when used to characterise other species of bacteria can greatly enhance results (Hilton *et al.*, 1997, Perry *et al.*, 2003). As a genotypic method, RAPD is cost effective and can provide results in a relatively short space of time. The use of random primers also lends adaptability to the methodology allowing different sequences to be targeted within the bacterial genome.

The aim of this chapter was to determine the PCR ribotypes and RAPD profiles of the sixty two clinical isolates of *C. difficile* (from Trust A and B) using a standard protocol and the optimised protocols outlined in chapter 3 and compare the discriminatory power of both methods.

4.2 MATERIALS AND METHODS

4.2.1 PCR ribotyping

Isolates of *C. difficile* were cultured onto Wilkins-Chalgren agar and incubated overnight at 37°C in an anaerobic cabinet. Colonies were then suspended in 100µl of a 0.5% ($^{w}/_{v}$) Chelex® 100 resin solution and vortexed briefly before being incubated in a boiling water bath for 12 minutes. A negative control containing only a suspension of Chelex® 100 resin was also used for each experiment. Following incubation, samples were then centrifuged at 15000 x g for 10 minutes and the supernatant removed and stored at 4°C for no longer then 24 hours until required. The PCR reaction mixture was prepared as follows: 5µl DNA sample, 0.5µl 50µM P3 primer (5' CTG GGG TGA AGT CGT AAC AAG G 3'), 0.5µl 50µM P5 primer (5' GCG CCC TTT GTA GCT TGA CC 3'), 10µl (4mM total concentration) dNTPs, 1.5µl 1.5mM $MgCl_2$, 0.25µl (5U/µl) *Taq* polymerase and 32.25µl sterile distilled water to give a total volume of 50µl per reaction. Amplification cycles were then carried out using a Peltier Thermal Cycler-200 (MJ Research, USA) as follows: thirty cycles of 95°C for two minutes, 92°C for one minute and 55°C for one minute followed by a final cycle of 72°C for ninety seconds, 95°C for one minute, 55°C for forty five seconds and a final extension stage of 72°C for five minutes. Following amplification, tubes were then removed from the cycler and heated at 75°C on a heating block for 45 minutes with the lids open in order to concentrate the PCR products. Samples were stored at 4°C if not immediately required. To the PCR product 5µl of loading buffer was added ((0.25% ($^{w}/_{v}$) bromophenol blue (Sigma Aldrich, UK), and 30% ($^{v}/_{v}$) glycerol (Fisher Scientific, UK)) and 5µl of sample was loaded into wells of a 3% agarose gel. In addition 5µl of DNA ladder (Super ladder Low 100bp marker, AB gene, UK) was also added to both end wells and between every 5-6 sample lanes. Gel electrophoresis was performed in 1 x TAE

(40mM Tris-HCl, 1mM EDTA and 0.1% ($^{v}/_{v}$) glacial acetic acid, pH 8) and gels ran for 3 hours at 200 volts.

To enable gels to be visualised, gels were stained by soaking in 0.005% ($^{w}/_{v}$) ethidium bromide solution (Sigma Aldrich, UK) for 20 minutes and then rinsing in sterile distilled water. Gels were visualised under UV light using the GBOX-EF Gel Documentation System (Syngene, UK) and images captured using Genesnap software (Syngene, UK).

4.2.2 RAPD protocol

Isolates were cultured onto Wilkins-Chalgren anaerobe agar and incubated for 24 hours at 37°C in an anaerobic cabinet (Don Whitley, UK). Colonies were then suspended in a 0.5% ($^{w}/_{v}$) Chelex® 100 resin (Bio-Rad, UK) suspension which was briefly vortexed before being incubated in a water bath at 94°C for 12 minutes; a negative control containing only a suspension of Chelex® 100 resin was also used for each experiment. Samples were then centrifuged for ten minutes at 15000 x g and the supernatant containing the crude DNA extract used in the RAPD reaction, samples were stored at 4°C until required. The relevant PCR reaction mixtures used are outlined in sections 4.2.2.1 and 4.2.2.2. Amplification cycles were then carried out using a Peltier Thermal Cycler-200 (MJ Research, USA) using cycles as follows: five cycles of 94°C for four minutes and thirty seconds, 94°C for thirty seconds, 24°C for two minutes and 72°C for one minute followed by thirty cycles of 94°C for thirty seconds, 30°C for thirty seconds and 72°C for one minute. Following amplification, 5µl of loading buffer was added to the PCR samples which were stored at 4°C if not used immediately. Gel electrophoresis was carried out using a 2% ($^{w}/_{v}$) agarose gel and performed in 1 x TAE; 5µl of DNA ladder were added to the centre and end wells and 15µl of PCR product was loaded into the remaining wells. Gels were run at 80 volts for 1 hour and 24 minutes until samples reached the end of the gel. To enable gels to be

visualised, gels were stained by soaking in 0.005% ($^{w}/_{v}$) ethidium bromide solution (Sigma Aldrich, UK) for twenty minutes and then rinsing in sterile distilled water. Gels were visualised under UV light using the GBOX-EF Gel Documentation System (Syngene, UK) and images captured using Genesnap software (Syngene, UK).

4.2.2.1 Protocol using primer AP3

The PCR reaction mixture when characterising with primer AP3 was follows: 2µl DNA sample, 0.3µl 100µM primer (5' TCA CGA TGC A 3'), 1µl 10mM dNTPs, 2.5µl buffer 7 (3.5mM $MgCl_2$, 25mM KCl, 10mM Tris-HCl, pH 8.8), 0.25µl (5U/µl) *Taq* polymerase and 18.95µl sterile distilled water to give a total volume of 25µl per reaction.

4.2.2.2 Protocol using primer AP4

The PCR reaction mixture used when characterising with primer AP4 was as follows: 2µl DNA sample, 0.6µl 100µM primer (5' TCA CGC TGC A 3'), 1µl 10mM DNTPs, 2.5µl buffer 7 (3.5mM MgCl2, 25mM KCl, 10mM Tris-HCl, pH 8.8), 0.25 (5U/µL) *Taq* polymerase and 18.65µl sterile distilled water to give a total volume of 25µl per reaction.

4.2.2.3 Typeability and discriminatory power of PCR ribotyping and the optimised RAPD protocol

The typeability and discriminatory power of PCR ribotyping and both primers used in the optimised RAPD protocol was calculated using Simpson's index of diversity (D) (given in section 2.2.5.3).

4.3 RESULTS

4.3.1 PCR ribotyping of reference strains

Amplicon profiles of reference PCR ribotype isolates produced at Aston University, and reference profiles provided by the HPA North East Laboratory were compared for similarity to determine the validity of the method used. Dendrograms produced by the two separate institutions were identical (Figure 4.1 and 4.2). The amplicon profiles produced using the modified PCR ribotyping protocol were comparable to the reference profiles indicating reproducibility of the method employed. Four distinct clones were produced at the 70% similarity level; the largest cluster formed contained four isolates (PCR ribotypes 001, 014, 015 and 064) at the 88% similarity level; PCR ribotype 017 demonstrated no similarity to the other PCR ribotypes at the 70% similarity level.

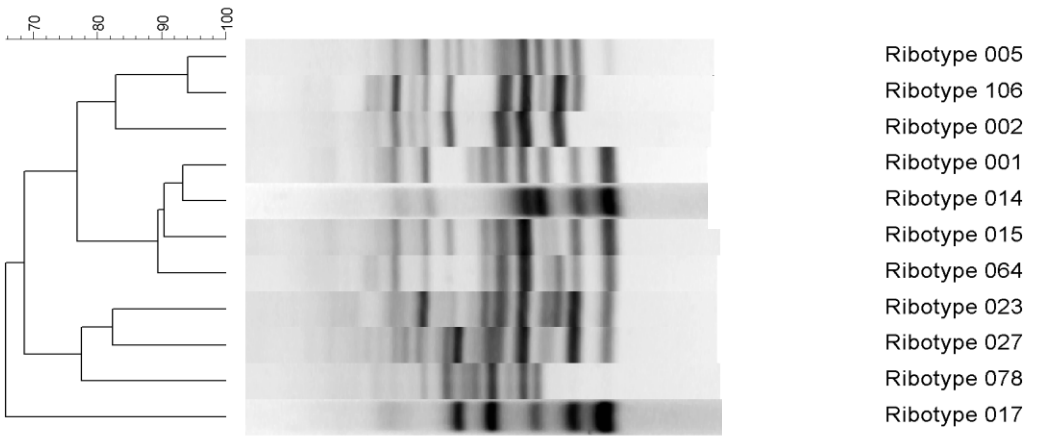


Figure 4.1 Dendrogramatic representation of the eleven PCR ribotype profiles. Similarity was calculated using Dice coefficient and represented by UPGMA clustering. Profiles were provided by Andrew Sails, North East HPA laboratory, Newcastle-upon-Tyne, UK.

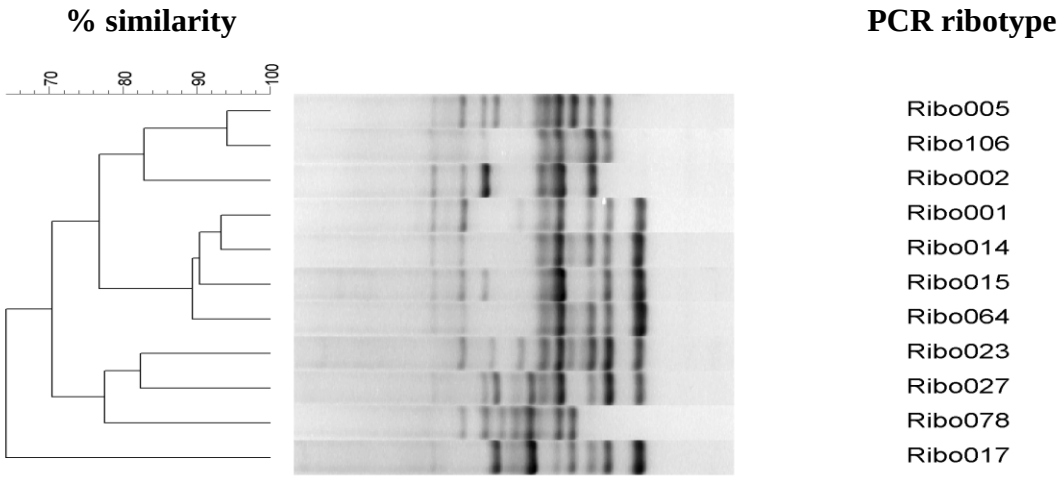


Figure 4.2 Dendrogramatic representation of the eleven PCR ribotypes. The profiles here were produced using reference PCR ribotypes at Aston University. Similarity was calculated using Dice co-efficient and represented by UPGMA clustering.

4.3.2 PCR ribotyping of clinical isolates of *C. difficile*

The sixty two clinical isolates of *C. difficile* were characterised by the PCR ribotyping protocol outlined in section 4.2.1. Using the reference profiles isolates were identified as belonging to PCR ribotypes 001, 002, 014, 027, 064 and 106 (Figure 4.3). The PCR ribotype of fourteen of the sixty two isolates (23%) could not be identified as amplicon profiles did not match any of the reference profiles available. The thirty two isolates recovered from Trust A formed ten separate clones at a 68% similarity level when analysed with GelCompar (Figure 4.4); clones consisted of between one and five isolates. Among the isolates recovered from Trust A, six different PCR ribotypes were identified with PCR ribotypes 001 and 014 were most frequently observed in this population of isolates (Table 4.1).Eleven of the isolates recovered from Trust A formed four clones that could not be

assigned to a PCR ribotype using the reference profiles available; two of these clones each comprised of four isolates with the two further clones consisting of two and a single isolate. The thirty isolates recovered from Trust B formed six distinct clones at the 74% similarity level when analysed with GelCompar (Figure 4.5); twenty six of the isolates (87%) were identified as belonging to either PCR ribotype 027 and 106. One isolate was assigned to PCR ribotype 001 with the PCR ribotypes of the remaining three isolates being unidentifiable based on the reference profiles available; these isolates formed three separate clones consisting of one isolate each. When all sixty two isolates were compiled together on the same dendrogram (Figure 4.3), six clones were produced where isolates could not be assigned to a PCR ribotype based on the reference profiles available. Clones were exclusive to the location from which they were recovered with the exception of one (four isolates from Trust A and one isolates from Trust B).

Table 4.1 Frequency with which PCR ribotypes occurred amongst the isolates (percentage frequency indicated in parentheses).

PCR Ribotype	Trust A	Trust B	Total
001	5 (16%)	0	5 (8%)
002	4 (13%)	0	4 (6%)
014	5 (16%)	0	5 (8%)
027	3 (9%)	14 (47%)	17 (27%)
064	2 (6%)	1 (3%).	3 (5%)
106	2 (6%)	12 (40%)	14 (23%)
Undefined	11 (34%)	3 (10%)	14 (23%)

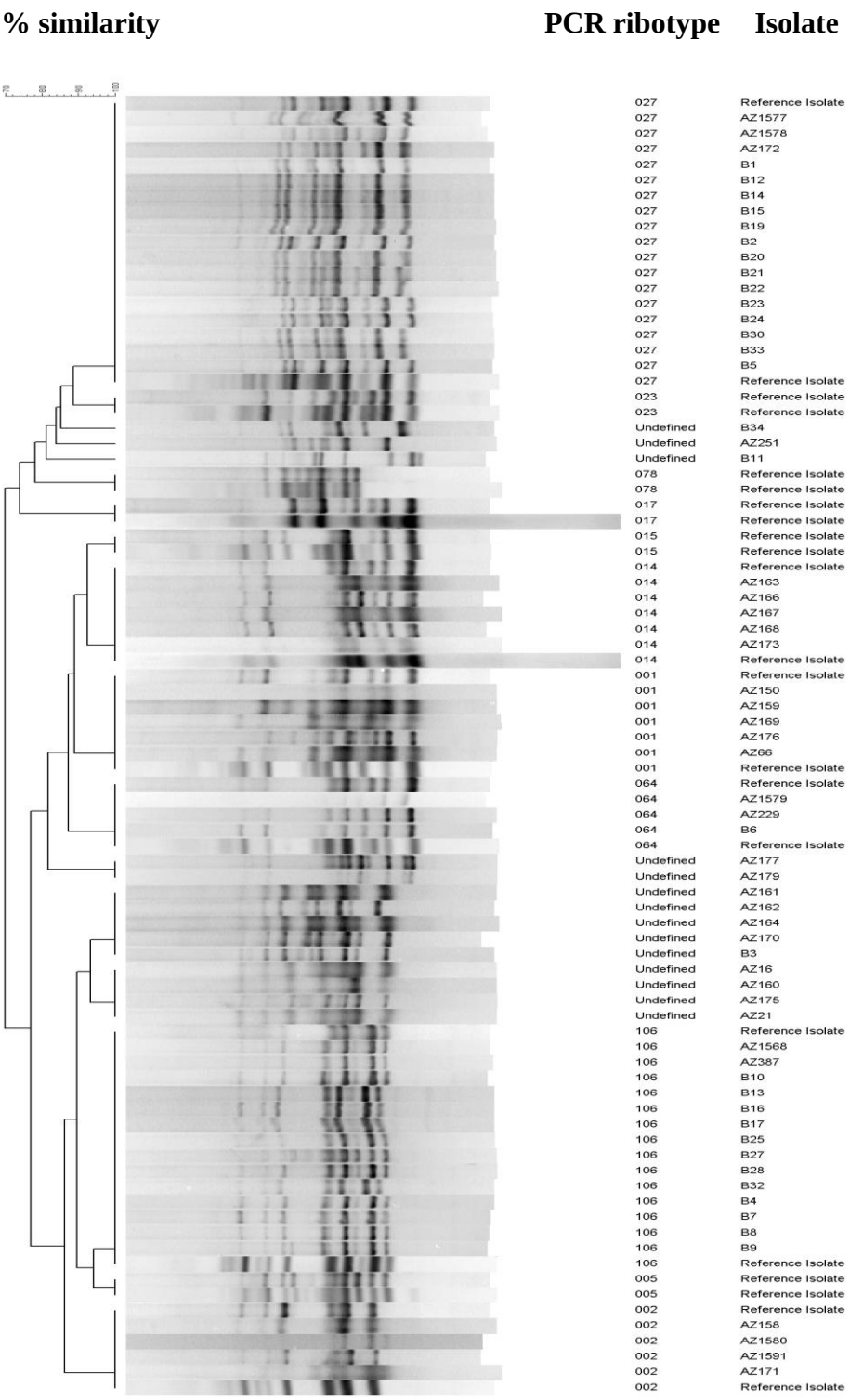


Figure 4.3 Dendrogramatic representation of all sixty two clinical isolates of *C. difficile* and reference PCR ribotypes characterised by PCR ribotyping. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

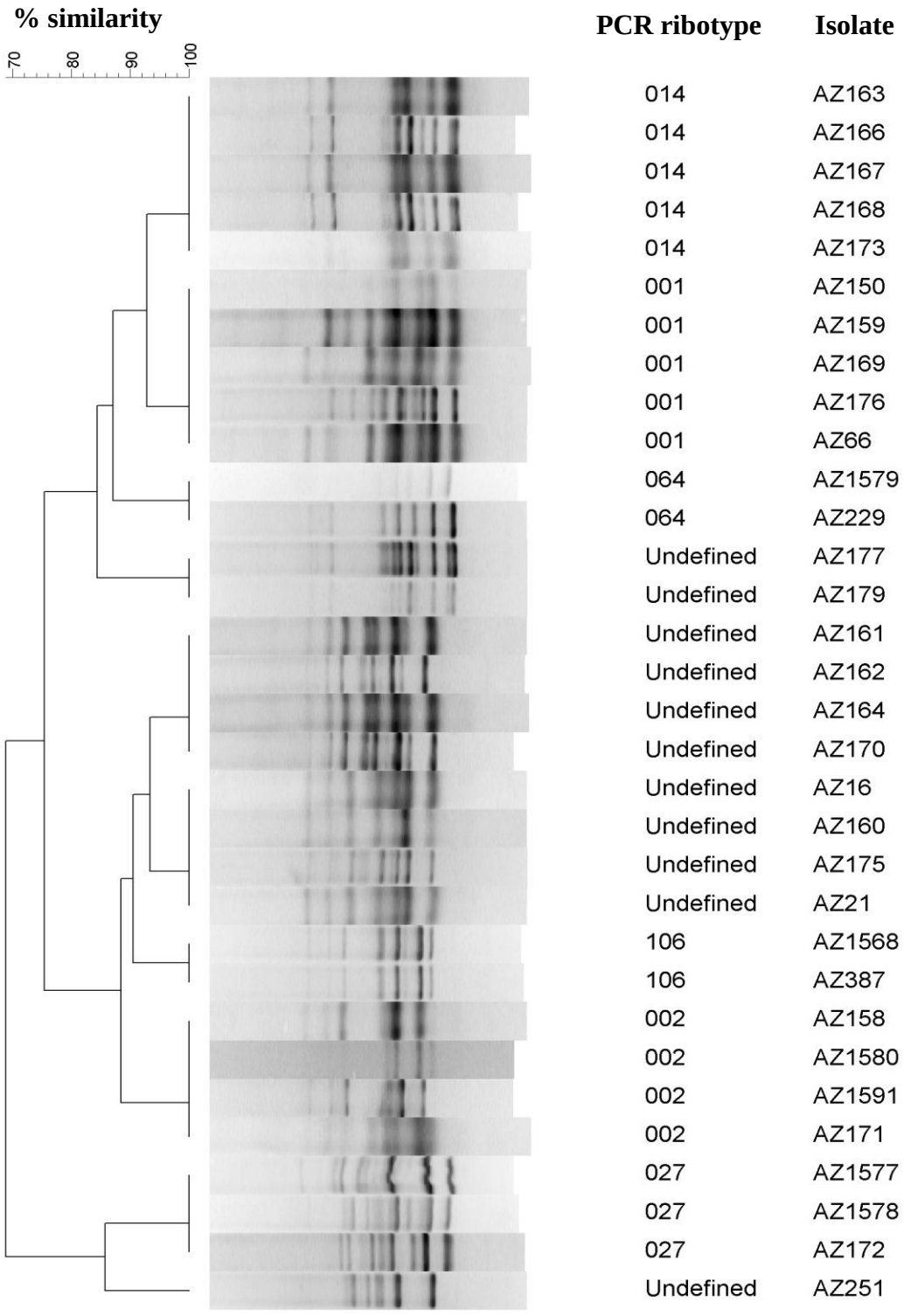


Figure 4.4 Dendrogramatic representation of the thirty two clinical isolates of *C. difficile* recovered from Trust A characterised by PCR ribotyping. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

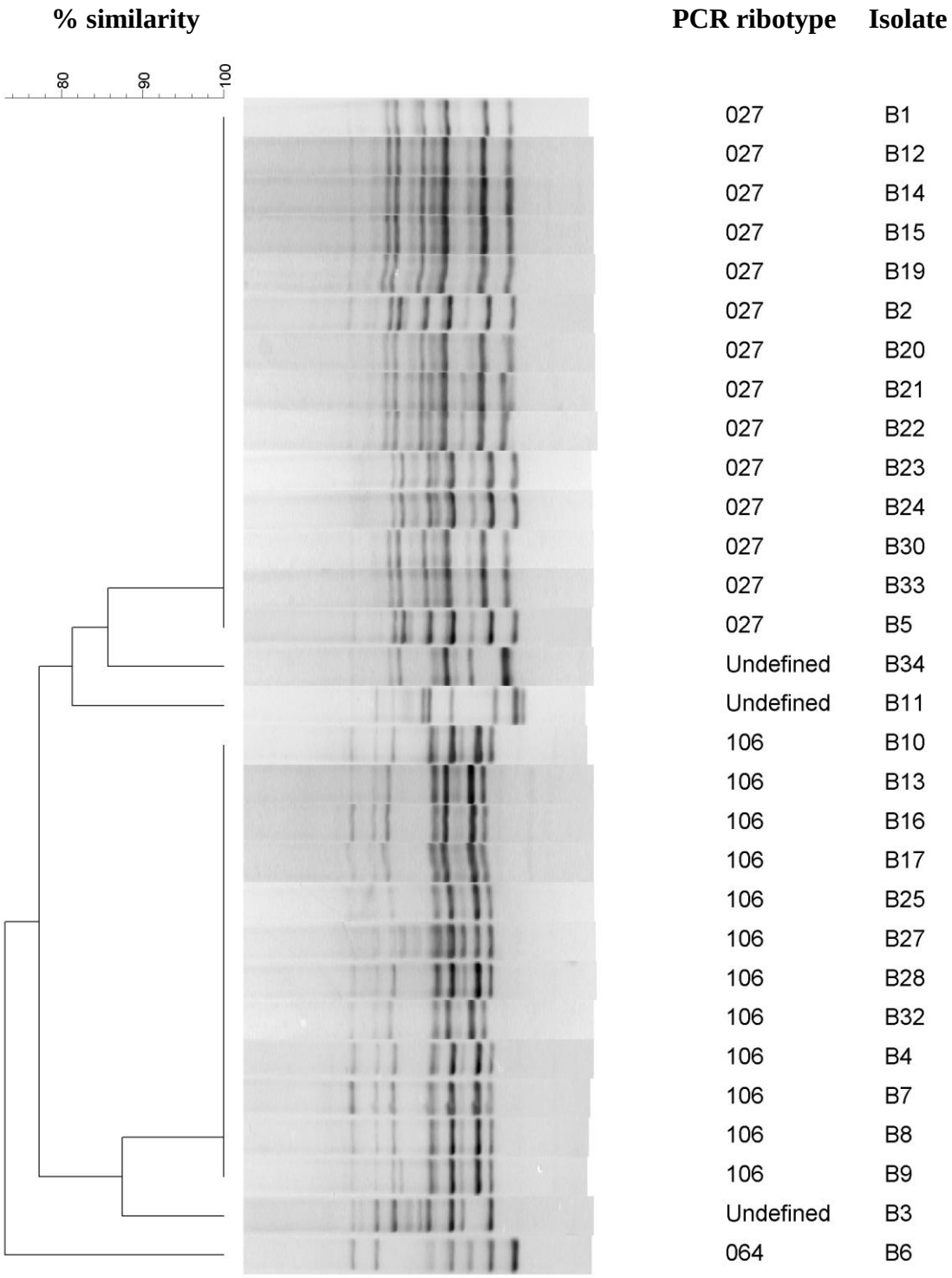


Figure 4.5 Dendrogramatic representation of the thirty clinical isolates of *C. difficile* recovered from Trust B characterised by PCR ribotyping. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

4.3.3 RAPD of reference isolates

The amplicon patterns produced from RAPD reactions using both primers AP3 and AP4 were found to discriminate between the eleven PCR ribotype reference strains used (Figure 4.6 and 4.7). The RAPD method employed was also deemed reproducible following triplicate application of the method to any one reference isolate (Figure 4.8 and 4.9). When analysed using GelCompar, the RAPD amplification profiles of the PCR ribotype control strains produced two distinct clusters at the 40% similarity when primer AP3 was used (Figure 4.6); the two clusters were formed from nine of the eleven isolates with the final cluster consisting of two isolates. When primer AP4 was used in the RAPD reaction, the clones produced were much more distinct (Figure 4.7); three clones were produced consisting of one, four and six isolates. Two clones were present at the 60% similarity level that consisted of ten isolates; at this level of similarity the remaining isolate was clustered separately.

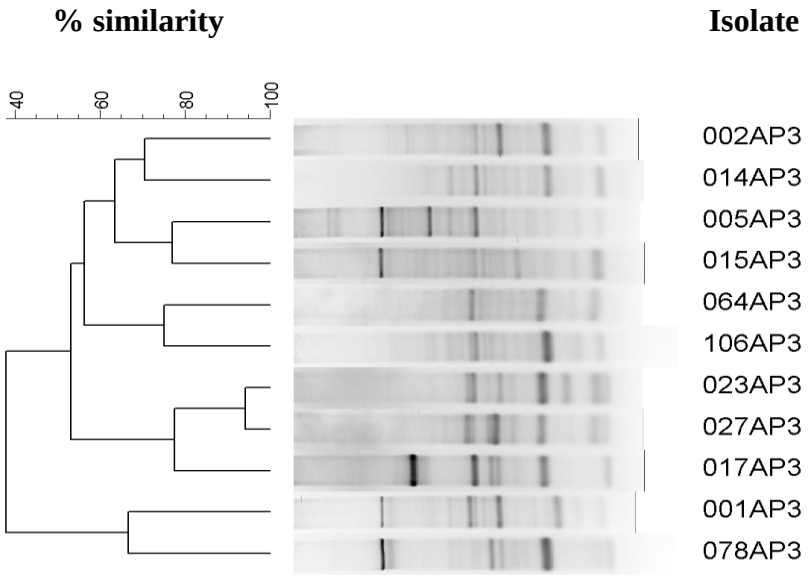


Figure 4.6 Dendrogramatic representation of the eleven reference PCR ribotype isolates characterised using RAPD primer AP3. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

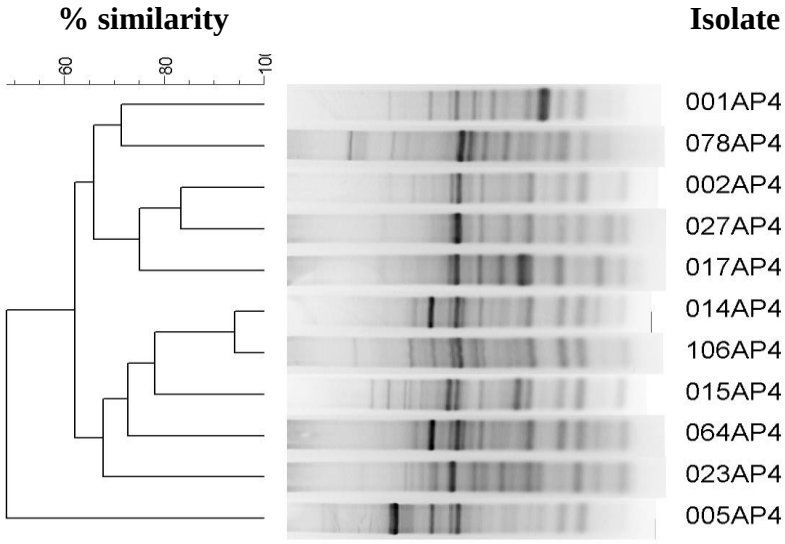


Figure 4.7 Dendrogramatic representation of the eleven reference PCR ribotype isolates characterised using RAPD primer AP4. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

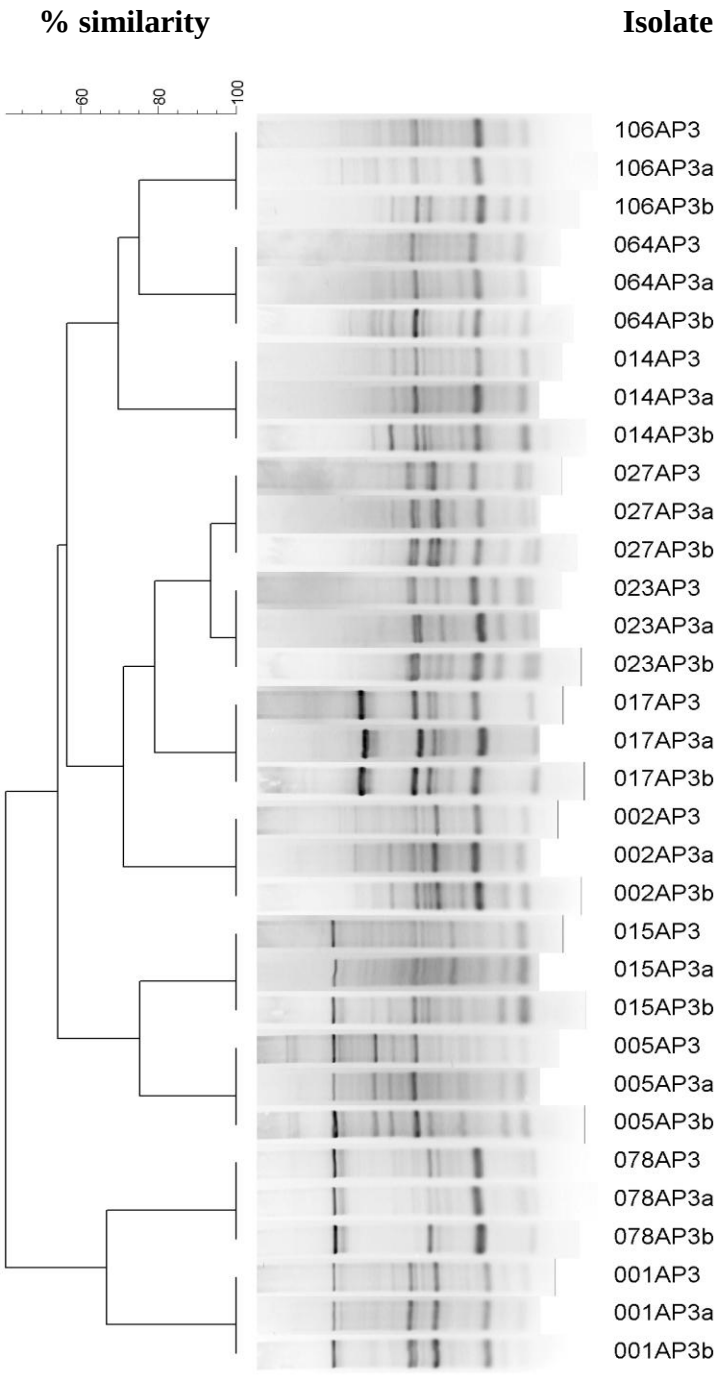


Figure 4.8 Dendrogramatic representation of amplicon profiles obtained from multiple RAPD reactions using primer AP3 and DNA extracted from reference PCR ribotypes at different times. Similarity was calculated using Dice coefficient and UPGMA clustering.

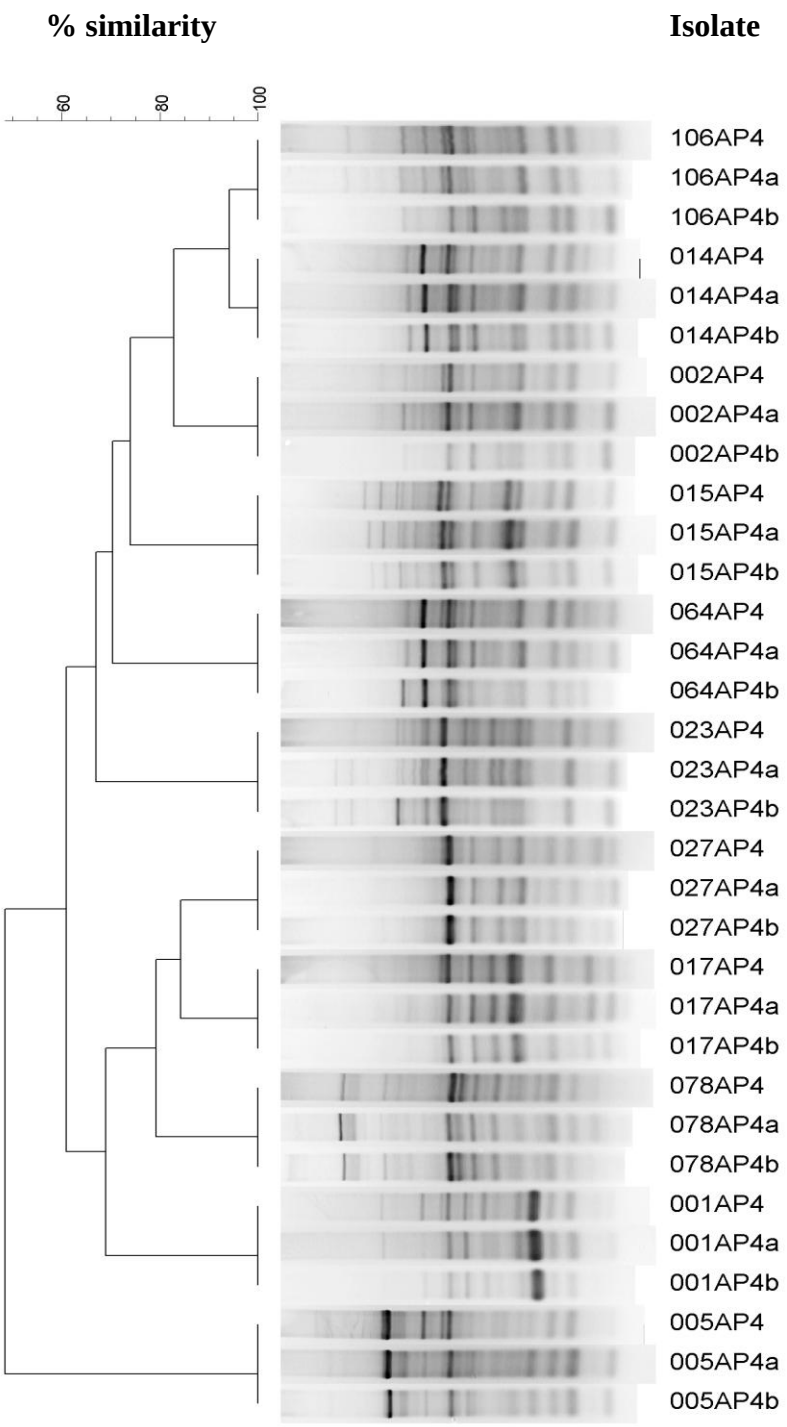


Figure 4.9 Dendrogramatic representation of amplicon profiles obtained from multiple RAPD reactions using primer AP4 and DNA extracted from reference PCR ribotypes at different times. Similarity was calculated using Dice coefficient and UPGMA clustering.

4.3.4 RAPD of clinical isolates of *C. difficile*

When the clinical isolates recovered from Trust A were characterised using primer AP3 in the RAPD reaction, thirteen clusters were formed at the 60% similarity level (Figure 4.10), however five of these clusters were individual isolates. The remaining isolates were grouped into eight clusters comprising of between two and seven isolates. When the isolates recovered from Trust A were characterised using primer AP4 (Figure 4.11), seven clusters were formed at the 58% similarity level. Four of these were individual isolates with twenty eight of the isolates being grouped into three clusters comprising of five, nine and fourteen isolates. The three major clusters within this population of isolates were formed at the 76% similarity level. The clustering observed amongst the isolates recovered from Trust A differed greatly for both primers.

When the clinical isolates recovered from Trust B were characterised using primer AP3, six clusters were formed at the 66% similarity level (Figure 4.12). Twenty six isolates were assigned to two major clusters of fifteen and eleven isolates; the remaining four isolates formed four individual and distinct clones. The same pattern of clustering was also evident when the clinical isolates recovered from Trust B were characterised using primer AP4 although the six clones were formed at a similarity level of 70% and the relationships and similarity between the isolates did differ (Figure 4.13). Both primers did however designate isolates B3, B6, B11 and B27 as isolated clones.

The profiles produced using RAPD primer AP3 were unique to the Trust from which they were recovered however this was not evident when isolates were characterised using primer AP4. When all sixty two isolates characterised with the same primer were compiled on the same dendrogram, isolates were very clearly segregated. When isolates were characterised using primer AP3, two very distinct clusters were formed, each comprising

of isolates recovered from the corresponding trust (Figure 4.14). There was one exception to this however with isolate B6 which demonstrated a greater level of similarity to isolates from Trust A than those recovered from Trust B. The cluster mainly consisting of isolates from Trust A was split into two further clusters at the 70% similarity level, each of these comprised of smaller distinct clones. When isolates were characterised using primer AP4 again two distinct clusters were formed; each representing isolates from the two respective trusts (Figure 4.15). There were also two exceptions to this, isolates B3 and B11 demonstrated a greater degree of similarity to the isolates recovered from Trust A than those recovered from Trust B. In contrast to the multiple clones formed among isolates recovered from Trust A; isolates from Trust B demonstrated greater similarity to each other both with larger clusters being formed and also greater similarity being observed between the clusters produced.

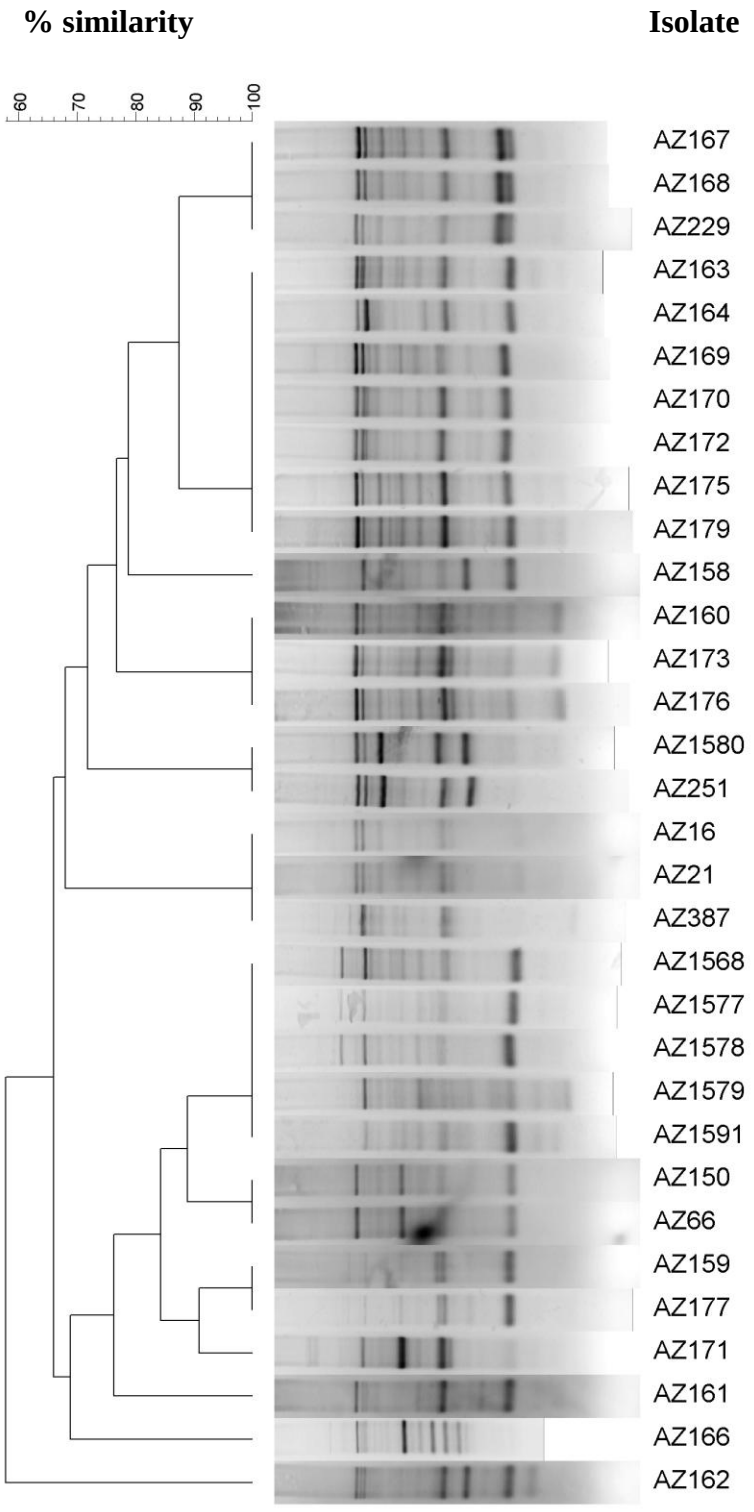


Figure 4.10 Dendrogramatic representation of the thirty two clinical isolates of *C. difficile* recovered from Trust A and characterised using RAPD primer AP3. Similarity was calculated using Dice coefficient and UPGMA clustering.

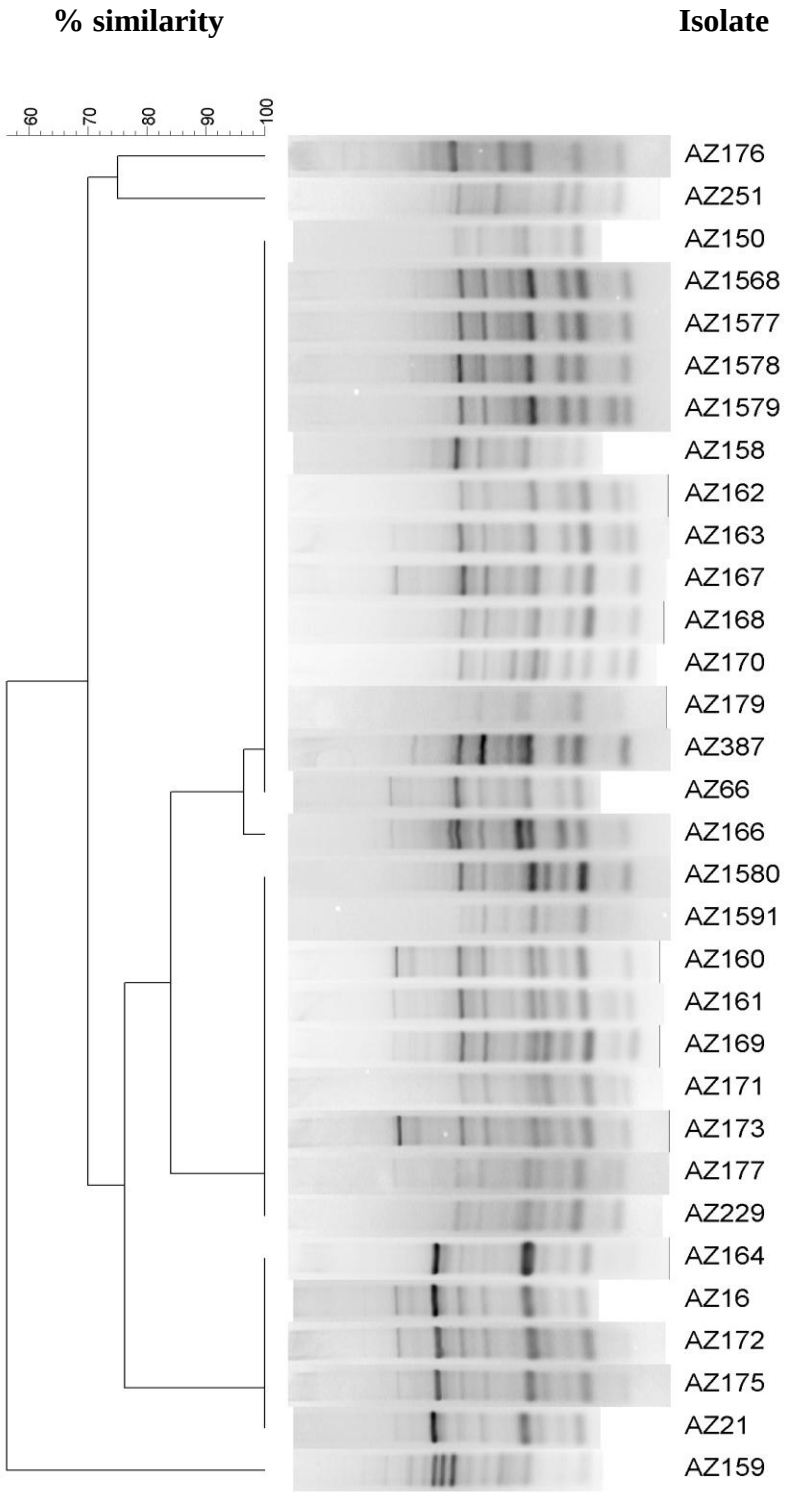


Figure 4.11 Dendrogramatic representation of the thirty two clinical isolates of *C. difficile* recovered from Trust A and characterised using RAPD primer AP4. Similarity was calculated using Dice coefficient and UPGMA clustering.

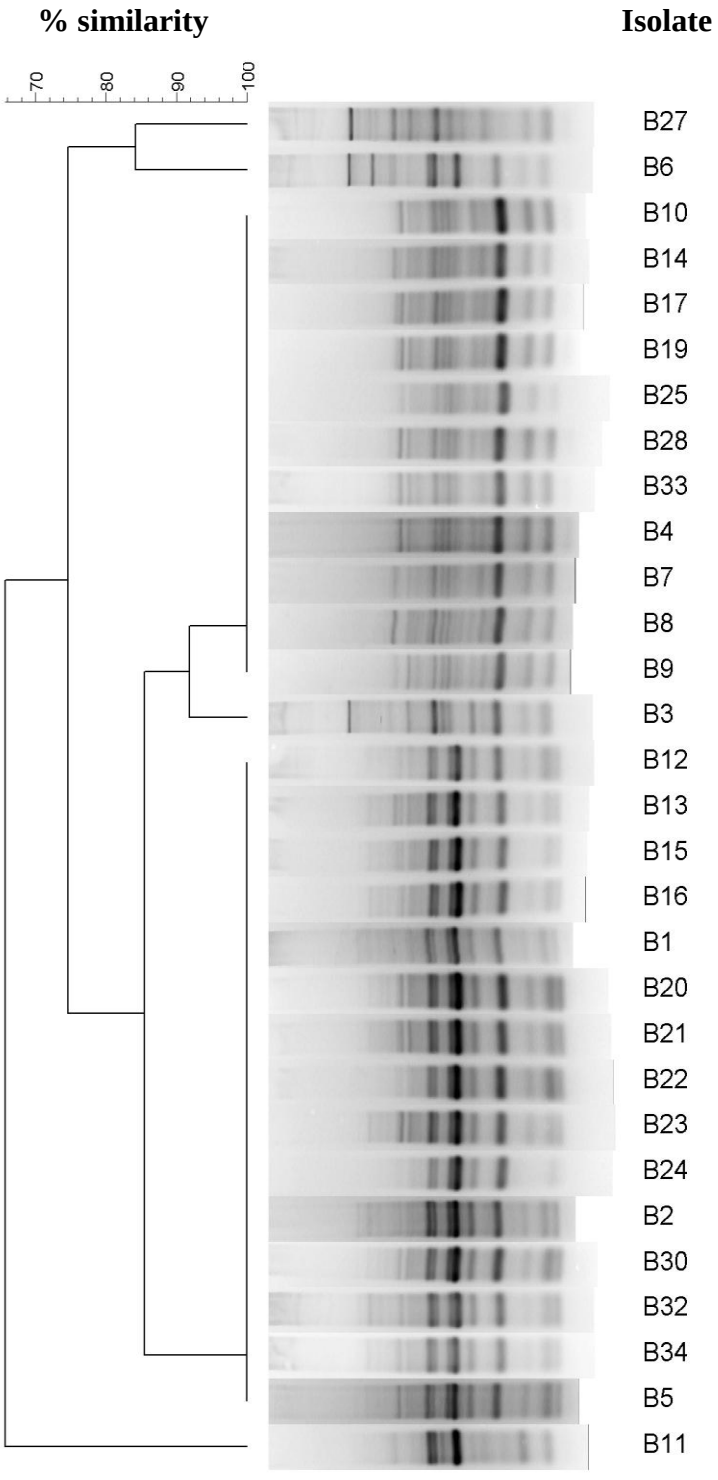


Figure 4.12 Dendrogramatic representation of the thirty clinical isolates of *C. difficile* recovered from Trust B and characterised using RAPD primer AP3. Similarity was calculated using Dice coefficient and UPGMA clustering.

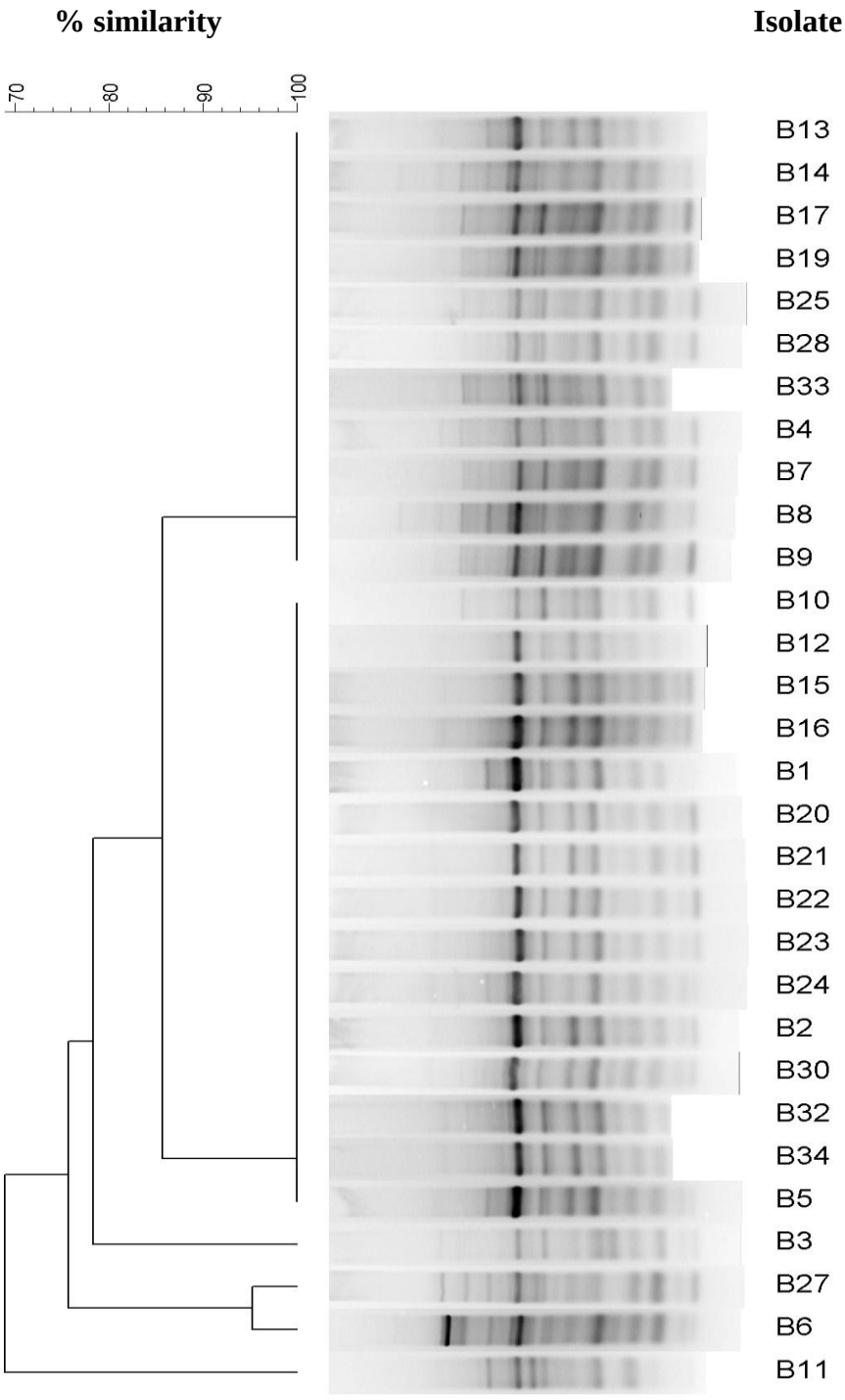


Figure 4.13 Dendrogramatic representation of the thirty clinical isolates of *C. difficile* recovered from Trust B and characterised using RAPD primer AP4. Similarity was calculated using Dice coefficient and UPGMA clustering.

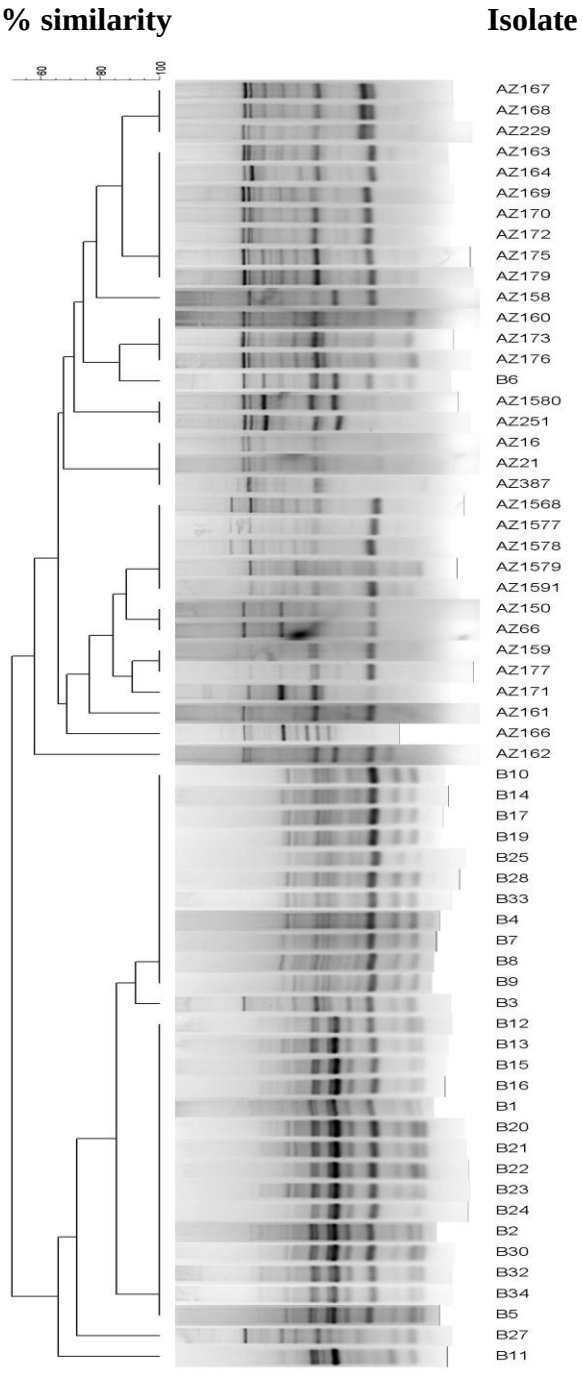


Figure 4.14 Dendrogramatic representation of all sixty two clinical isolates of *C. difficile* characterised by RAPD using primer AP3. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

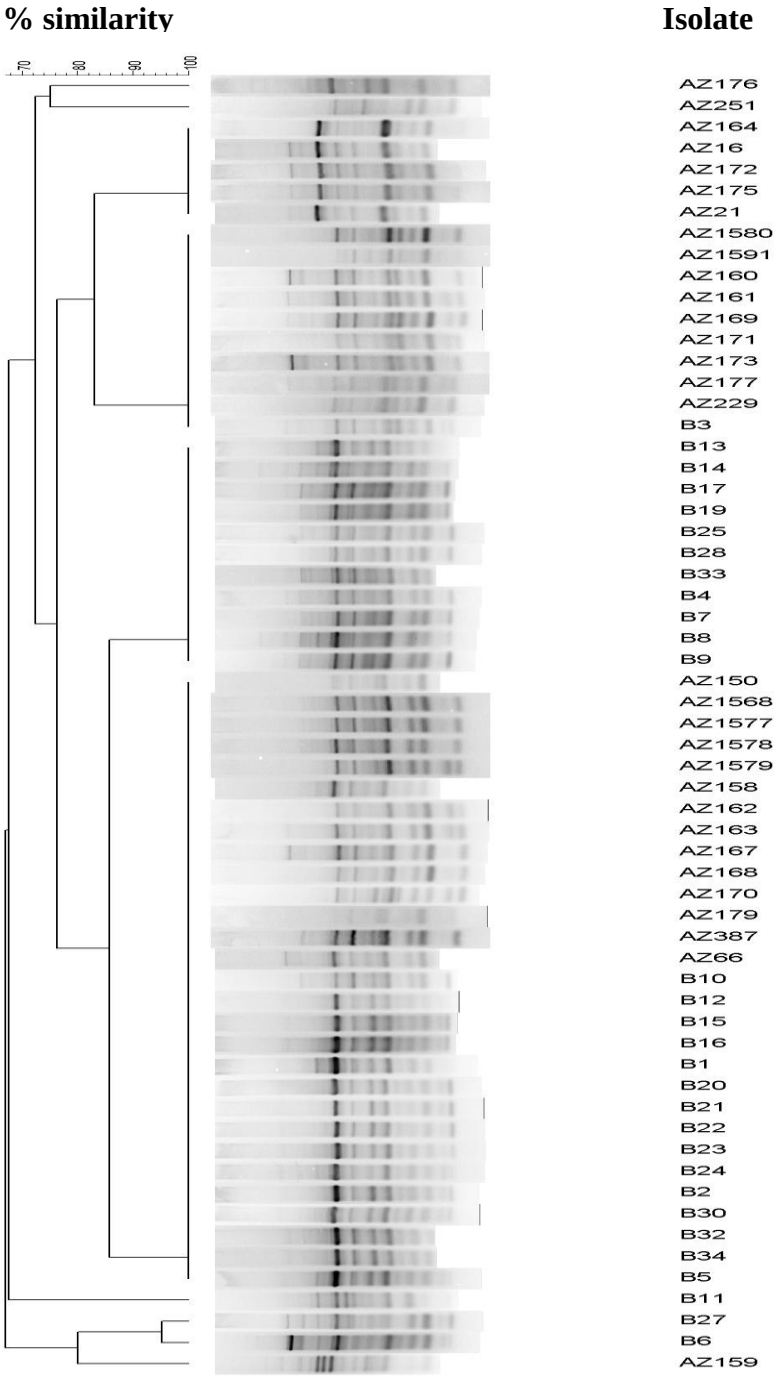


Figure 4.15 Dendrogramatic representation of all sixty two clinical isolates of *C. difficile* characterised by RAPD using primer AP4. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

4.3.5 Typeability and discriminatory power of PCR ribotyping and the optimised RAPD protocol

All isolates were typeable by both PCR ribotyping and the optimised RAPD protocol. The discriminatory powers of both methods are outlined in table 4.2. Characterisation of isolates using RAPD and primer AP3 demonstrated the greatest discriminatory power (0.89) followed by PCR ribotyping (0.86) and then RAPD using primer AP4 (0.58).

Table 4.2 Discrimination indices of PCR ribotyping and RAPD calculated from typing of all sixty two clinical *C. difficile* isolates.

Typing Method	Number of types	Type	Number of isolates	Discriminatory index (D)
PCR Ribotyping	**12**	**001**	5 **(5)**	**0.86**
		002	4 **(4)**	
		014	5 **(5)**	
		027	17 **(3, *14*)**	
		064	3 **(2,*1*)**	
		106	14 **(2, *12*)**	
		U(a)	1 **(*1*)**	
		U(b)	1 **(1)**	
		U(c)	1 **(*1*)**	
		U(d)	2 **(2)**	
		U(e)	5 **(4, *1*)**	
		U(f)	4 **(4)**	
RAPD (AP3)	**18**	**A**	3 **(3)**	**0.89**
		B	7 **(7)**	
		C	1 **(1)**	
		D	3 **(3)**	
		E	1 **(*1*)**	
		F	2 **(2)**	
		G	3 **(3)**	
		H	5 **(5)**	
		I	2 **(2)**	
		J	2 **(2)**	
		K	1 **(1)**	
		L	1 **(1)**	
		M	1 **(1)**	
		N	11 **(11)**	
		O	1 **(1)**	
		P	15 **(*15*)**	
		Q	1 **(*1*)**	
		R	1 **(*1*)**	
RAPD (AP4)	**10**	**a**	5 **(5)**	**0.58**
		b	1 **(*1*)**	
		c	1 **(1)**	
		d	1 **(1)**	
		e	8 **(7, *1*)**	
		f	4 **(4)**	
		g	39 **(13, *26*)**	
		h	1 **(*1*)**	
		i	1 **(*1*)**	
		j	1 **(1)**	

[a]Total number of strains (Bold text indicates isolates recovered from Trust A and bold italicised text indicates isolates recovered from Trust B).

4.3.6 Comparison of PCR ribotyping and the optimised RAPD protocol for the characterisation of *C. difficile* isolates

When isolates were characterised by RAPD, eighteen and ten different types were defined using primers AP3 and AP4 respectively (Figure 4.16 & Figure 4.17). When isolates were characterised by PCR ribotyping, twelve different types were defined. The types defined by both RAPD primers therefore did not map directly to the types defined by PCR ribotyping. Characterisation and the types defined by both primers using the optimised RAPD protocol showed little resemblance to those types defined by PCR ribotyping. Although typing of the clinical isolates using RAPD did not discriminate between each individual ribotype and cluster isolates of the same PCR ribotype together, isolates were clearly characterised and grouped together based on other polymorphisms. The more common PCR ribotypes (in particular PCR ribotypes 027 and 106) when characterised by RAPD, produced profiles that were often clustered together on a dendrogram, however this did not occur in all cases.

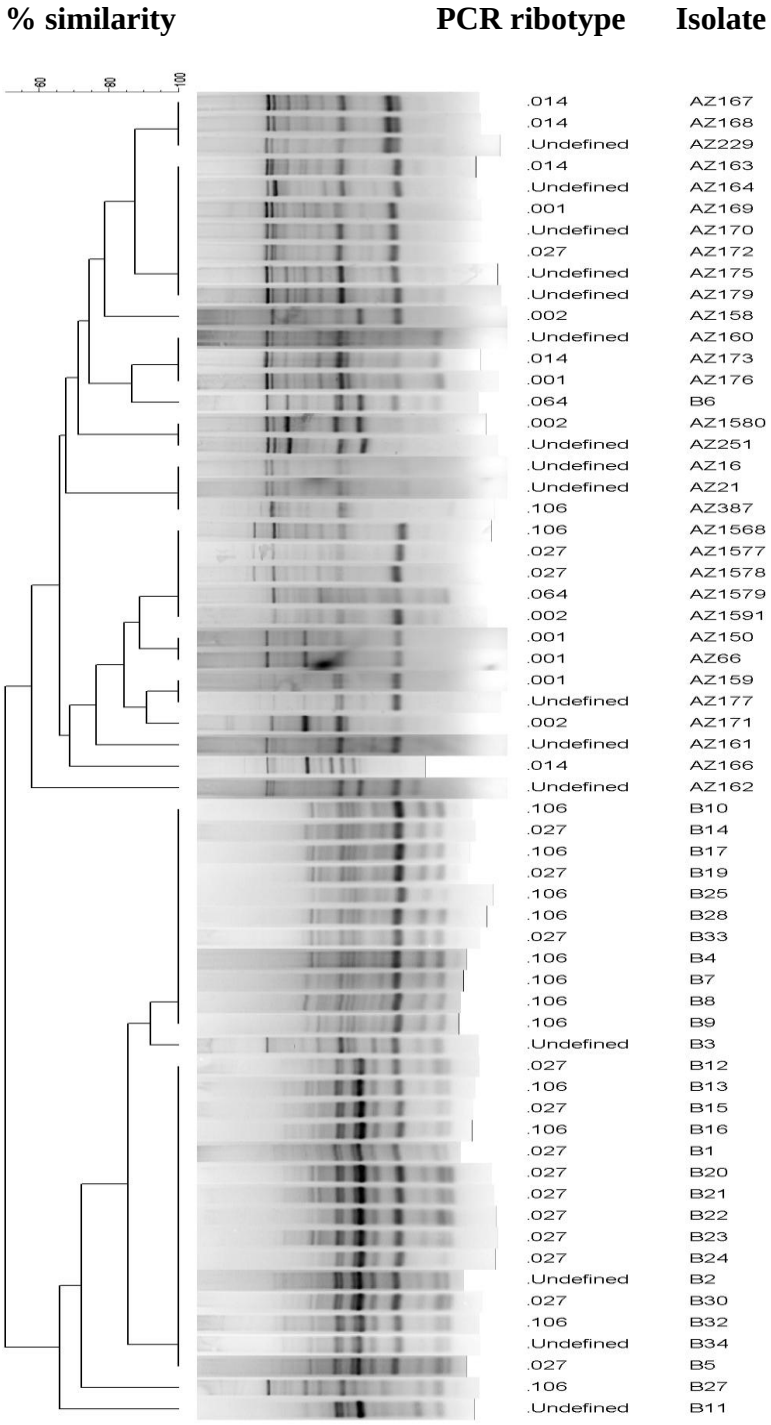


Figure 4.16 Dendrogramatic representation of all sixty two clinical isolates of *C. difficile* and corresponding PCR ribotypes characterised by RAPD using primer AP3. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

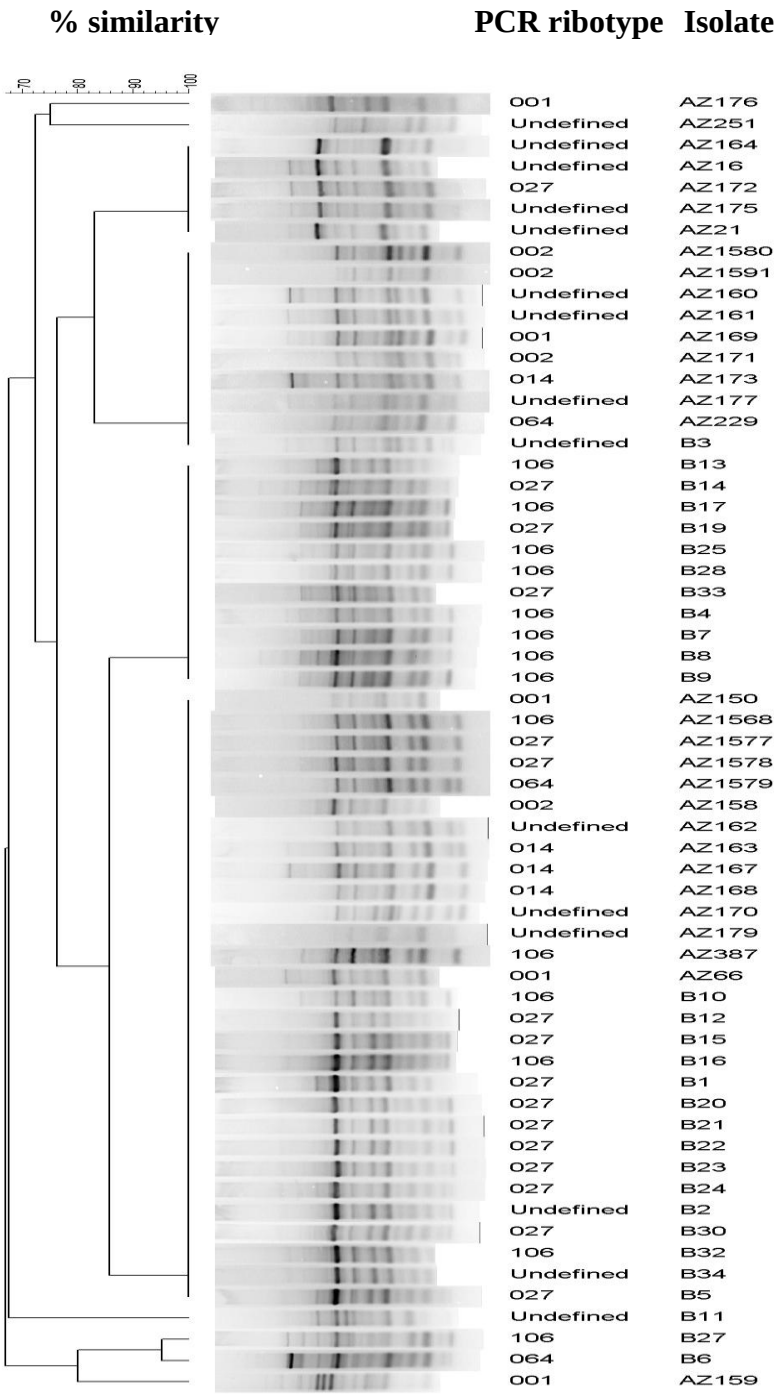


Figure 4.17 Dendrogramatic representation of all sixty two clinical isolates of *C. difficile* and corresponding PCR ribotypes characterised by RAPD using primer AP4. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

4.3.7 RAPD Types present within PCR ribotypes

The different RAPD types defined for each PCR ribotype are shown in table 4.3. The use of both PCR ribotype and RAPD type has allowed possible subtypes to be identified within PCR ribotypes. This has then been used to determine if isolates belonging to the same PCR ribotype are in fact the same or variants within the PCR ribotype. The use of primer AP3 in the RAPD reaction identified a greater number of subtypes within a defined PCR ribotype when compared to primer AP4. The only exception to this was PCR ribotype 001, where only one PCR ribotype was identified. When using RAPD, all isolates belonging to a defined PCR ribotype (where there was more than one isolate) could be further subtyped.

Table 4.3 The RAPD types defined within each PCR ribotype.

PCR Ribotype	No. of RAPD Types Defined (AP3)	Isolates	No. of RAPD Types Defined (AP4)	Isolates
001	3	(AZ66, AZ150, AZ159) (AZ169) (AZ176)	4	(AZ66, AZ150) (AZ159) (AZ169) (AZ176)
002	4	(AZ158) (AZ171) (AZ1580) (AZ1591)	2	(AZ158) (AZ171,AZ1580, AZ1591)
014	4	(AZ163) (AZ166) (AZ167, AZ168) (AZ173)	3	(AZ166) (AZ163,AZ167, AZ168) (AZ173)
027	4	(B1, B5, B12, B15, B20, B21, B22, B23, B24, B30) (B14, B19, B33) (AZ172) (AZ1577, AZ1578)	2	(B1, B5, B12, B14, B15, B19, B20, B21, B22, B23, B24, B30, B33, AZ1577, AZ1578) (AZ172)
064	2	(B6) (AZ229, AZ1579)	2	(B6) (AZ229, AZ1579)
106	4	(B4, B7, B8, B9, B10, B17, B25, B28) (B13, B16, B32) (AZ387, AZ1568) (B27)	2	(B4, B7, B8, B9, B10, B13, B16, B17, B25, B28, B32, AZ387, AZ1568) (B27)
U(a)	1	B34	1	B34
U(b)	1	AZ251	1	AZ251
U(c)	1	B11	1	B11
U(d)	2	(AZ177) (AZ179)	2	(AZ177) (AZ179)
U(e)	4	(B3) (AZ161) (AZ162) (AZ164, AZ170)	3	(B3, AZ161) (AZ162, AZ170) (AZ164)
U(f)	4	(AZ16) (AZ21) (AZ160) (AZ175)	2	(AZ16, AZ21, AZ175) (AZ160)
U(g)	1	(B2)	1	(B2)

4.4 DISCUSSION

The distribution of PCR ribotypes varied greatly between the two locations. A greater diversity of PCR ribotypes was observed among the isolates recovered from Trust A compared to the isolates recovered from Trust B which predominantly belonged to PCR ribotypes 027 and 106. Differences in the PCR ribotype epidemiology of *C. difficile* isolates recovered from different locations have been previously reported with the distribution of PCR ribotypes being known to vary significantly between countries (Kuijper et al., 2008). In this study the significant variation in PCR ribotypes observed here between two locations in relatively close proximity appears to be a unique and previously unreported occurrence. All of the clinical isolates were recovered from their respective Trusts in 2004-2005; prior to well documented outbreaks of CDI in UK hospital trusts such as Stoke Mandeville and the significant increase in CDI cases in the years that followed. This increase in CDI cases often due to outbreak situations saw not only the emergence of PCR ribotype 027 but also predominance of particular ribotypes (001, 027 and 106). The predominance of PCR ribotypes 027 and 106 observed amongst the isolates recovered from Trust B is now a frequent occurrence in healthcare settings across the UK, however this observation is unexpected amongst a population of historical isolates from this time period. Although the isolates from both trusts are likely to have been acquired within the trust environment, the variability in the PCR ribotypes observed amongst isolates recovered from Trust A suggest that transmission may have been more restricted and less widespread between patients; possibly due to better infection control procedures. The predominance of only two PCR ribotypes amongst the isolates from Trust B indicate that isolates may have been acquired and continually spread within the trust environment; either through direct contact with other infected patients or transmission via contaminated surfaces. However, it has now been established that PCR ribotyping as a genotyping

method for the characterisation of *C. difficile* no longer provides enough discrimination to accurately determine the spread and epidemiology of CDI as sub types within PCR ribotypes are known to exist (Rhamati *et al*., 2005, Northey *et al*., 2005). As a result more discriminatory genotyping methods such as MLVA are now being employed to characterise isolates.

The PCR ribotype of fifteen of the clinical isolates (representing six different PCR ribotypes) were indefinable based on the eleven reference amplicon profiles available and therefore inhibited the PCR ribotype identification of some of the isolates. The remaining reference isolates and associated profiles obtained were eleven of the most common PCR ribotypes in the UK at present. The predominance and prevalence of PCR ribotypes are known to change over time (Brazier, 1998) and considering that these isolates were recovered from the respective trusts over five years ago indicates a possible reason why the PCR ribotype of some of the isolates could not be defined.

Although both primers in the RAPD protocol were found to discriminate between the eleven reference PCR ribotype isolates; the same pattern of results were not replicated when applied to the population of clinical isolates. Both primers had the capacity to type and discriminate between different isolates of *C. difficile* when the clinical isolates were characterised using RAPD. Eighteen and ten different RAPD types were defined using primers AP3 and AP4 respectively; this was in comparison to the twelve different types identified with PCR ribotyping. Discriminate RAPD profiles were produced for different PCR ribotypes however, this result was not always consistent and also isolates known to belong to different PCR ribotypes were found to produce the same RAPD profile type. The variability observed explains why distinct profiles were produced for each of the different PCR ribotype isolates; there appeared to be no association between the profiles produced for each typing method and therefore is likely that the initial results from the

characterisation of the reference strains were due to chance. The lack of association between the profiles produced for the two typing methods and also the way different PCR ribotypes were clustered together on a dendrogram, demonstrates that although the RAPD primers used were not capable of distinguishing between different PCR ribotypes they clearly had the capacity to discriminate between different strains of *C. difficile* by using different genotypic markers. This is further supported by the comparison of RAPD profiles produced from clinical isolates with those of the reference isolates. Profiles from clinical isolates could not be effectively matched to reference profiles and results did not always correlate with that of the ribotype.

When the typeability and discriminatory power of both methods was investigated, the discriminatory index of PCR ribotyping and RAPD using primer AP3 were similar with RAPD typing demonstrating a slightly higher index. Typing by RAPD using primer AP4 however had a significantly lower discriminatory index. When primer AP3 was used to characterise isolates, the amplicon profiles were more varied in the number and position of the amplicons produced when compared to the patterns produced with primer AP4. This is likely to contribute to the why the discriminatory index of primer AP3 is so much better. This indicates that the regions where polymorphisms were being detected within the genome with primer AP3 were likely to vary to a greater extent than those being detected with primer AP4. Notably however, primer AP4 does not type isolates of *C. difficile* to the same standard as AP3 and therefore is not an ideal primer for the characterisation of isolates.

Although the types defined using primers AP3 and AP4 with the RAPD protocol clearly did not map onto the PCR ribotypes, there were similarities observed between PCR ribotyping and RAPD. The variability in the number of PCR ribotypes identified between the two trusts was also observed with RAPD typing; a greater number of different types

were consistently defined amongst isolates recovered from Trust A in comparison to those from Trust B. Such similarities between the different methods lends support to the typeability and discriminatory capacity of RAPD as a genotypic method for the typing of *C. difficile* isolates as a similar level of discrimination is evident. Isolates belonging to PCR ribotype 027 and 106 would often produce the same RAPD profile with the two PCR ribotypes often being indistinguishable from each other. In addition to this, when RAPD profiles were complied on a dendrogram it was apparent that isolates belonging to PCR ribotypes 027 and 106 were more frequently clustered together to form several clones of distinct RAPD types. The observation that isolates belonging to more frequently encountered PCR ribotypes appear to posses the same polymorphisms indicates that such changes may be associated with the frequency with which they are encountered within a population. Such an example may be the acquisition of a gene that promotes or enhances the likelihood of survival within an environment; this has already been seen in isolates of *C. difficile* where resistance and reduced susceptibility towards some antibiotics is more prevalent amongst isolates belonging to more common PCR ribotypes (HPA, 2008). What is also apparent from the results here is that RAPD has identified variability in isolates between two locations. This therefore suggests that RAPD may also be useful in providing epidemiological information for populations of *C. difficile* isolates, both within local environments and also in the identification of endemic clones.

By combining the information obtained from characterising the clinical isolates by both PCR ribotyping and RAPD, this has established that polymorphisms exist between isolates belonging to the same ribotype. This therefore indicates that different isolates may have been responsible for infection despite the ribotypes being the same. Another explanation however could also be that subtle polymorphisms may have occurred between the same strain in different patients. Due to the nature of RAPD it is not known what

polymorphisms are being detected within the genome and therefore it can be difficult to determine where variability is occurring; the polymorphisms being detected here could be the result of gene loss or acquisition, or single nucleotide polymorphisms (SNPs).

4.5 CONCLUSION

Significant differences were observed in both PCR ribotype and RAPD type epidemiology amongst the isolates recovered from the two different trusts; both the number of types and the predominance of types varied between the two locations. Although RAPD typing does not map directly onto PCR ribotyping, the optimised protocol can discriminate between different isolates and notably also identify subtypes within PCR ribotypes. The two RAPD primers produced very different discriminatory indices and if future work were to be done, primer AP4 would not be used. The results here indicate that not only has RAPD the potential to discriminate between different strains of *C. difficile*, this methodology could be further developed and used to genotype isolates of *C. difficile* as both an independent method but also in combination with other genotypic methods such as PCR ribotyping.

CHAPTER 5 GENOTYPIC CHARACTERISTICS OF ISOLATES OF *CLOSTRIDIUM DIFFICILE* AND ASSOCIATIONS BETWEEN GENOTYPIC AND PHENOTYPIC CHARACTERISTICS

5.1 INTRODUCTION

Alterations in both the phenotype and genotype of a bacterial cell can have a significant effect on bacterial pathogenicity. A change or polymorphism within the genome does not always influence phenotypic characteristics and this is frequently the case; with changes often undetected and insignificant. In a small proportion of cases however, variations often in the form of mutations or through the acquisition of genes, are detected through the development of resistance to antibiotics or the production of additional or enhanced levels of extracellular virulence factors. Within many species of pathogenic bacteria, strains are often present within the population that have the capacity to induce either a more virulent state of disease or that are more difficult to treat due to increased resistance towards antibiotics (e.g. MRSA, Panton-Valentine Leukocidin (PVL) MRSA and extended spectrum beta lactamase (ESBL) producing *E.coli*). The acquisition or development of characteristics that are directly related to the virulence of the organism often promote its survival and are generally regarded with more interest than other phenotypic characteristics that are not directly associated to virulence or pathogenicity. As has been demonstrated with the PCR ribotype 027 strain of *C. difficile*, a change in one phenotypic characteristic can also be associated with other phenotypic changes such as a altered antibiotic resistance profiles and sporulation rate (Loo *et al*., 2005, Ackerlund *et al*., 2008), in addition to the acquisition of other genes (Huang *et al*., 2009). It is not known why so many changes appear to have occurred within the PCR ribotype 027genome and if these are all the result of one significant alteration or several. It is however possible that some of the phenotypes may have been present prior to the change that resulted in excess toxin production and

were only identified following this observation. Such variability and the known mobility of the *C. difficile* genome (Sebaihia *et al*., 2006) demonstrate a need for further investigations into both phenotypic and genotypic traits of *C. difficile* isolates. Associations between genotypic and phenotypic characteristics can initially be made from observing particular phenotypes and seeing if these can be associated with any genotypic traits; RAPD provides an ideal tool for such detection as a large population of isolates can be investigated relatively quickly and cost effectively with the potential of identifying several associations. As a genotypic method, RAPD is often rejected in favour of other methods; however, due to no specific targets being used and the application of the primer to the whole of the genome, RAPD has the potential to provide much information about the genome of the organism. Although RAPD detects polymorphisms within isolates which can be used to detect variability, this same methodology can also be used to define characteristics of a species.

In this chapter the phenotypic and genotypic characteristics observed amongst isolates of *C. difficile* were analysed to determine if any associations between particular phenotypic and genotypic traits exist.

5.2 MATERIALS AND METHODS

5.2.1 PCR ribotyping

PCR ribotyping was performed as described in section 4.2.1.PCR ribotyping was performed on both reference and clinical isolates described in section 2.2.1.

5.2.2 RAPD

RAPD was performed as described in section 4.2.2. RAPD was performed on both the reference and clinical isolates described in section 2.2.1.

5.2.3 API Biotyping

API Biotyping was carried out as described in section 2.2.3. Biotyping was performed on only the clinical isolates described in section 2.2.1

5.2.4 MIC determination

MIC values were determined as described in section 2.2.5. MIC determination was performed on both the reference and clinical isolates described in section 2.2.1.

5.2.5 S-layer typing

S-layer typing was performed as described in 2.2.6. S-layer typing was performed on the clinical isolate described in section 2.2.1.

5.3 RESULTS

5.3.1 PCR ribotypes and antibiogram profiles

There were no antibiogram profiles that appeared to be exclusive or characteristic of any of the PCR ribotypes identified. The antibiogram profiles of PCR ribotype reference isolates 015, 023 and 078 were identical, but varied for each of the other PCR ribotypes. Resistance towards chloramphenicol, erythromycin, fusidic acid and tetracycline varied most frequently between the eleven reference strains. Resistance towards cefotaxime and levofloxacin was consistent amongst the reference isolates alongside susceptibility towards linezolid, metronidazole, rifampicin and vancomycin. The antibiogram profiles for each of the reference PCR ribotype isolates is shown in table 5.1.

When the clinical isolates were defined by PCR ribotype and antibiogram profiles compared, none of the isolates defined as belonging to the same ribotype displayed the same antibiogram profile; this included both the defined and indefinable PCR ribotypes. There was however some trends identified where resistance or sensitivity was constant throughout a particular PCR ribotype. Levofloxacin resistance was observed amongst all clinical isolates that were identified as belonging to PCR ribotype 001, 027 and 064; this was also observed in the corresponding reference isolates. Fusidic acid resistance was observed amongst all isolates that were identified as belonging to PCR ribotypes 002 and 014 and although resistance was detected towards fusidic acid in the PCR ribotype 002 reference isolate, the PCR ribotype 014 reference isolate was susceptible. All isolates identified as belonging to PCR ribotypes 001, 002, 014 and 064 were sensitive to linezolid and again this was mirrored in the reference isolates. Isolates belonging to PCR ribotype 001 and 002 all displayed susceptibility to imipenem with those that belonged to PCR ribotype 002 all also displaying susceptibility to rifampicin. Although PCR ribotype

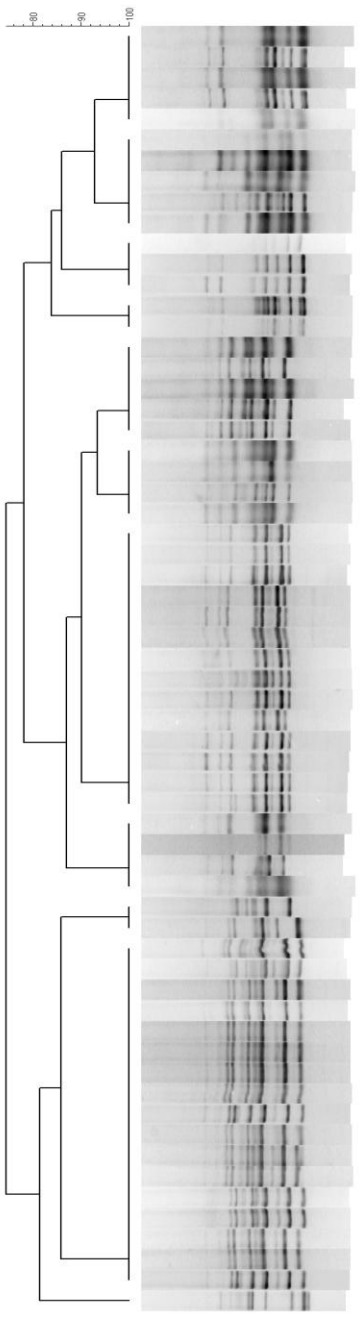
reference isolates 001 and 002 were susceptible to imipenem and rifampicin respectively, resistance towards imipenem was detected in PCR ribotype reference strain 002. Amongst all of the isolates where PCR ribotype was indefinable, all of the isolates displayed resistance to cefotaxime and fusidic acid and sensitivity towards chloramphenicol and linezolid. When these isolates were further divided into the six distinct types that were defined, the only consistency observed was imipenem sensitivity in the undefined group comprising of isolates AZ16, AZ21, AZ160 and AZ175. The distribution of antibiogram profiles amongst the different PCR ribotypes is shown in figure 5.1.

Resistance towards both clindamycin and erythromycin is known to be encoded on the *erm*B gene and resistance to both of these antibiotics was detected in thirty six (58%) of the clinical isolates. Of these isolates, thirteen were identified as belonging to PCR ribotype 106 (93% of isolates identified as belonging to PCR ribotype 106 in this population) and twelve were identified as belonging to PCR ribotype 027 (75% of isolates identified as belonging to PCR ribotype 027 in this population). The antibiogram profiles for the PCR ribotype reference strains 027 and 106 also displayed resistance to both of these antibiotics in addition to PCR ribotype 017. Amongst isolates that displayed resistance to clindamycin and erythromycin, tetracycline and rifampicin resistance was also frequently observed in these isolates. Additional tetracycline resistance was observed in thirty four of the thirty six isolates and rifampicin resistance in twenty nine of these isolates. With the exception of one isolate from Trust A, resistance towards rifampicin and tetracycline together was only observed in isolates recovered from Trust B.

Table 5.1 Antibiogram profiles of reference PCR ribotype isolates.

PCR ribotype	Antibiotic											
	Cef	**Chl**	**Cli**	**Ery**	**Fuc**	**Imi**	**Lev**	**Lin**	**Met**	**Rif**	**Tet**	**Van**
001	R	S	S	S	S	S	R	S	S	S	R	S
002	R	S	R	S	R	R	R	S	S	S	S	S
005	R	R	R	S	R	R	R	S	S	S	R	S
014	R	R	R	S	S	R	R	S	S	S	S	S
015	R	S	R	S	S	R	R	S	S	S	S	S
017	R	R	R	R	R	R	R	S	S	S	R	S
023	R	S	R	S	S	R	R	S	S	S	S	S
027	R	S	R	R	R	R	R	S	S	S	S	S
064	R	S	R	S	S	R	R	S	S	S	R	S
078	R	S	R	S	S	R	R	S	S	S	S	S
106	R	S	R	R	R	R	R	S	S	S	S	S

S: sensitive; R: resistant; cef: cefotaxime; chl: chloramphenicol; ery: erythromycin; fuc: fusidic acid; imi: imipenem; lev: levofloxacin; lin: linezolid; met: metronidazole; rif: rifampicin; tet: tetracycline; van: vancomycin



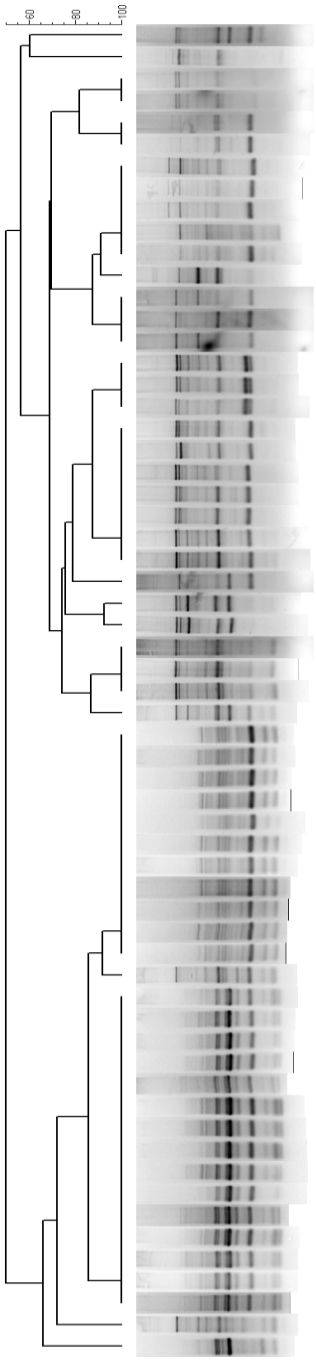
cef	chl	cli	ery	fuc	imi	lev	lin	met	rif	tet	van	PCR ribotype	Isolate
S	S	S	S	R	S	S	S	S	S	R	S	014	AZ163
R	S	R	S	R	S	R	S	S	S	R	S	014	AZ166
R	S	S	R	R	S	R	S	S	S	S	S	014	AZ167
R	S	R	S	R	S	R	S	S	S	R	S	014	AZ168
R	R	R	R	R	S	R	S	S	R	S	S	014	AZ173
R	R	S	S	R	S	R	S	S	R	S	S	001	AZ150
R	R	S	S	R	S	R	S	S	S	S	S	001	AZ159
R	S	S	S	R	S	R	S	S	S	S	S	001	AZ169
R	R	R	R	R	S	R	S	S	S	R	S	001	AZ176
R	S	R	S	R	S	R	S	S	S	S	S	001	AZ66
R	S	R	S	R	S	R	S	S	S	R	S	064	AZ1579
R	S	R	S	R	S	R	S	S	S	R	S	064	AZ229
R	S	R	R	R	R	R	S	S	R	R	S	064	B6
R	S	R	S	R	S	R	S	S	S	R	S	Undefined	AZ177
R	S	S	R	R	S	R	S	S	S	R	S	Undefined	AZ179
R	S	R	R	R	S	R	S	S	S	R	S	Undefined	AZ161
R	S	S	R	R	S	S	S	S	S	R	S	Undefined	AZ162
R	S	S	S	R	S	S	S	S	S	S	S	Undefined	AZ164
R	S	R	R	R	S	S	S	S	R	R	S	Undefined	AZ170
R	S	R	R	R	S	R	S	S	R	R	S	Undefined	B3
R	S	R	R	R	S	S	S	S	R	R	S	Undefined	AZ16
R	S	R	R	R	S	R	S	S	S	R	S	Undefined	AZ160
R	S	S	R	R	S	R	S	S	S	S	S	Undefined	AZ175
R	S	R	S	R	S	R	S	S	S	R	S	Undefined	AZ21
R	R	R	R	R	R	R	S	S	S	S	S	106	AZ1568
R	S	R	R	R	S	S	S	S	S	S	S	106	AZ387
R	R	R	R	R	R	R	R	S	R	R	S	106	B10
R	S	R	R	R	R	R	S	S	R	R	S	106	B13
R	S	R	R	S	R	R	S	S	R	R	S	106	B16
R	S	R	R	S	R	R	S	S	R	R	S	106	B17
R	S	R	R	R	R	R	S	S	R	R	S	106	B25
R	S	R	S	S	S	R	S	S	R	R	S	106	B27
R	R	R	R	R	R	R	S	S	R	R	S	106	B28
R	S	R	R	R	R	R	S	S	R	R	S	106	B32
R	S	R	R	R	R	R	S	S	R	R	S	106	B4
R	R	R	R	R	R	R	S	S	R	R	S	106	B7
R	S	R	R	R	S	R	S	S	R	R	S	106	B8
R	S	R	R	R	R	R	S	S	R	R	S	106	B9
R	R	S	S	R	S	S	S	S	S	S	S	002	AZ158
R	R	R	S	R	S	R	S	S	S	R	S	002	AZ1580
R	R	S	R	R	S	R	S	S	S	S	S	002	AZ1591
R	S	S	S	R	S	R	S	S	S	R	S	002	AZ171
R	S	R	R	R	S	R	S	S	S	S	S	Undefined	AZ251
R	S	R	R	R	R	R	S	S	R	R	S	Undefined	B34
R	S	S	S	R	S	R	S	S	S	S	S	027	AZ1577
R	S	R	S	R	S	R	S	S	S	S	S	027	AZ1578
R	S	R	R	R	S	R	S	S	S	R	S	027	AZ172
R	S	R	R	R	R	R	R	S	R	R	S	027	B1
R	S	R	R	R	R	R	S	S	R	R	S	027	B12
R	S	R	R	R	R	R	S	S	R	R	S	027	B14
R	S	R	R	R	R	R	S	S	R	R	S	027	B15
R	S	R	R	R	S	R	S	S	R	R	S	027	B19
R	S	R	R	R	R	R	S	S	R	R	S	027	B2
R	S	R	R	R	R	R	S	S	R	R	S	027	B20
R	S	R	R	R	R	R	S	S	R	R	S	027	B21
R	S	R	R	R	R	R	S	S	R	R	S	027	B22
R	R	R	R	R	R	R	S	S	R	R	S	027	B23
R	R	S	R	R	R	R	S	S	R	R	S	027	B24
R	S	R	R	S	R	R	S	S	R	R	S	027	B30
R	R	R	R	S	R	R	S	S	R	R	S	027	B33
R	S	R	R	R	S	R	S	S	R	R	S	027	B5
R	S	R	S	R	S	S	S	S	R	R	S	Undefined	B11

R: resistant; S: sensitive; cef: cefotaxime; chl: chloramphenicol; cli: clindamycin; ery: erythromycin; fuc: fusidic acid; imi: imipenem; lev: levofloxacin; lin: linezolid; met: metronidazole; rif: rifampicin; tet: tetracycline; van: vancomycin

Figure 5.1 Dendrogramatic representation of profiles generated from PCR ribotyping of all clinical isolates and associated antibiogram profiles. Similarity was calculated using Dice coefficient and represented by UPGMA clustering

5.3.2 RAPD types and antibiogram profiles

The antibiogram profiles defined for each RAPD type appeared completely random with no associations being made between a particular antibiotic resistance profile or trait. Consistent with PCR ribotyping, the isolates identified as belonging to a particular RAPD type did not demonstrate a single or predominant antibiogram profile. Resistance towards rifampicin and tetracycline was predominant in isolates that were defined as belonging to the two largest RAPD clusters when characterised using primer AP3. Although trends such as these did appear amongst isolates belonging to certain types, they were neither consistent nor exclusive, therefore could not be defined as a characteristic of that genotype. When isolates were characterised using primer AP4, all isolates that were defined as belonging to one cluster displayed resistance to both clindamycin and tetracycline but there was no resistance towards either erythromycin or rifampicin as seen in other genotypes.



cef	chl	cli	ery	fuc	imi	lev	lin	met	rif	tet	van	PCR ribotype	Isolate
R	S	S	R	R	S	S	S	S	S	R	S	Undefined	AZ162
R	S	R	R	R	S	S	S	S	S	S	S	106	AZ387
R	S	R	R	R	S	S	S	S	R	R	S	Undefined	AZ16
R	S	R	S	R	S	R	S	S	S	R	S	Undefined	AZ21
R	R	S	S	R	S	R	S	S	S	S	S	001	AZ159
R	S	R	S	R	S	R	S	S	S	R	S	Undefined	AZ177
R	R	R	R	R	R	R	S	S	S	R	S	106	AZ1568
R	S	S	S	R	S	R	S	S	S	S	S	027	AZ1577
R	S	R	S	R	S	R	S	S	S	S	S	027	AZ1578
R	S	R	S	R	S	R	S	S	S	R	S	064	AZ1579
R	R	S	R	R	S	R	S	S	S	S	S	002	AZ1591
R	S	S	S	R	S	R	S	S	S	R	S	002	AZ171
R	R	S	S	R	S	R	S	S	R	S	S	001	AZ150
R	S	R	R	R	S	R	S	S	S	R	S	Undefined	AZ161
R	S	R	S	R	S	R	S	S	S	S	S	001	AZ66
R	S	S	R	R	S	R	S	S	S	S	S	014	AZ167
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R	S	R	S	R	S	R	S	S	S	R	S	Undefined	AZ229
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R	S	S	S	R	S	S	S	S	S	S	S	Undefined	AZ164
R	S	S	S	R	S	R	S	S	S	S	S	001	AZ169
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R	S	R	R	R	S	R	S	S	S	R	S	027	AZ172
R	S	S	R	R	S	R	S	S	S	S	S	Undefined	AZ175
S	S	S	R	R	S	R	S	S	S	R	S	Undefined	AZ179
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R	R	R	R	R	R	R	S	S	R	R	S	106	B28
R	R	R	R	S	R	R	S	S	R	R	S	027	B33
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R	S	R	R	R	S	R	S	S	R	R	S	Undefined	B3
R	S	R	R	R	R	R	S	S	R	R	S	027	B12
R	S	R	R	R	R	R	S	S	R	R	S	106	B13
R	S	R	R	R	R	R	S	S	R	R	S	027	B15
R	S	R	R	S	R	R	S	S	R	R	S	106	B16
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R	S	R	R	R	R	R	S	S	R	R	S	027	B20
R	S	R	R	R	R	R	S	S	R	R	S	027	B21
R	S	R	R	R	R	R	S	S	R	R	S	027	B22
R	R	R	R	R	R	R	S	S	R	R	S	027	B23
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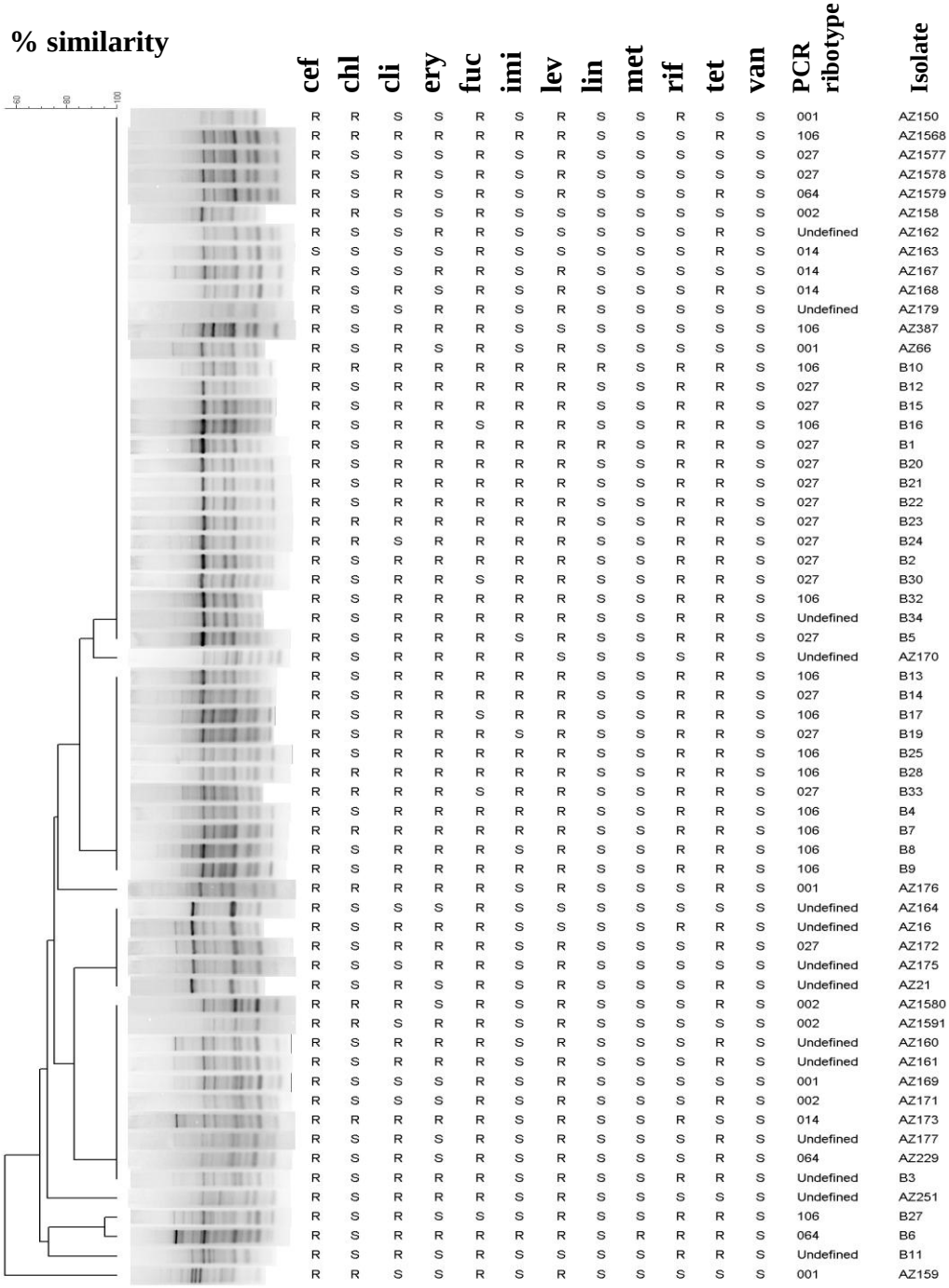
R	S	R	R	S	R	R	S	S	R	R	S	027	B30
R	S	R	R	R	R	R	S	S	R	R	S	106	B32
R	S	R	R	R	R	R	S	S	R	R	S	Undefined	B34
R	S	R	R	R	S	R	S	S	R	R	S	027	B5
R	S	R	S	S	S	R	S	S	R	R	S	106	B27
R	S	R	S	R	S	S	S	S	R	R	S	Undefined	B11

R: resistant; S: sensitive; cef: cefotaxime; chlor; chloramphenicol; cli: clindamycin; ery: erythromycin; fuc: fusidic acid; imi: imipenem; lev: levofloxacin; lin: linezolid; met: metronidazole; rif: rifampicin; tet: tetracycline; van: vancomycin

Figure 5.2 Dendrogramatic representation of profiles generated from RAPD using AP3 and associated antibiogram profiles. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.



cef	chl	cli	ery	fuc	imi	lev	lin	met	rif	tet	van	PCR ribotype	Isolate
R	R	S	S	R	S	R	S	S	R	S	S	001	AZ150
R	R	R	R	R	R	R	S	S	S	R	S	106	AZ1568
R	S	S	S	R	S	R	S	S	S	S	S	027	AZ1577
R	S	R	S	R	S	R	S	S	S	S	S	027	AZ1578
R	S	R	S	R	S	R	S	S	S	R	S	064	AZ1579
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R	S	S	R	R	S	S	S	S	S	R	S	Undefined	AZ162
S	S	S	S	R	S	S	S	S	S	R	S	014	AZ163
R	S	S	R	R	S	R	S	S	S	S	S	014	AZ167
R	S	R	S	R	S	R	S	S	S	R	S	014	AZ168
R	S	S	R	R	S	R	S	S	S	S	S	Undefined	AZ179
R	S	R	R	R	S	S	S	S	S	S	S	106	AZ387
R	S	R	S	R	S	R	S	S	S	S	S	001	AZ66
R	R	R	R	R	R	R	R	S	R	R	S	106	B10
R	S	R	R	R	R	R	S	S	R	R	S	027	B12
R	S	R	R	R	R	R	S	S	R	R	S	027	B15
R	S	R	R	S	R	R	S	S	R	R	S	106	B16
R	S	R	R	R	R	R	R	S	R	R	S	027	B1
R	S	R	R	R	R	R	S	S	R	R	S	027	B20
R	S	R	R	R	R	R	S	S	R	R	S	027	B21
R	S	R	R	R	R	R	S	S	R	R	S	027	B22
R	R	R	R	R	R	R	S	S	R	R	S	027	B23
R	R	S	R	R	R	R	S	S	R	R	S	027	B24
R	S	R	R	R	R	R	S	S	R	R	S	027	B2
R	S	R	R	S	R	R	S	S	R	R	S	027	B30
R	S	R	R	R	R	R	S	S	R	R	S	106	B32
R	S	R	R	R	R	R	S	S	R	R	S	Undefined	B34
R	S	R	R	R	S	R	S	S	R	R	S	027	B5
R	S	R	R	R	R	S	S	S	S	R	S	Undefined	AZ170
R	S	R	R	R	R	R	S	S	R	R	S	106	B13
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R	S	R	R	S	R	R	S	S	R	R	S	106	B17
R	S	R	R	R	S	R	S	S	R	R	S	027	B19
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R	R	R	R	R	R	R	S	S	R	R	S	106	B28
R	R	R	R	S	R	R	S	S	R	R	S	027	B33
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R	S	R	R	R	S	R	S	S	R	R	S	106	B8
R	S	R	R	R	R	R	S	S	R	R	S	106	B9
R	R	R	R	R	S	R	S	S	S	R	S	001	AZ176
R	S	S	S	R	S	S	S	S	S	S	S	Undefined	AZ164
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R	S	R	R	R	S	R	S	S	S	R	S	027	AZ172
R	S	S	R	R	S	R	S	S	S	S	S	Undefined	AZ175
R	S	R	S	R	S	R	S	S	S	R	S	Undefined	AZ21
R	R	R	S	R	S	R	S	S	S	R	S	002	AZ1580
R	R	S	R	R	S	R	S	S	S	S	S	002	AZ1591
R	S	R	R	R	S	R	S	S	S	R	S	Undefined	AZ160
R	S	R	R	R	S	R	S	S	S	R	S	Undefined	AZ161
R	S	S	S	R	S	R	S	S	S	S	S	001	AZ169
R	S	S	S	R	S	R	S	S	S	R	S	002	AZ171
R	R	R	R	R	S	R	S	S	R	S	S	014	AZ173
R	S	R	S	R	S	R	S	S	S	R	S	Undefined	AZ177
R	S	R	S	R	S	R	S	S	S	R	S	064	AZ229
R	S	R	R	R	S	R	S	S	R	R	S	Undefined	B3
R	S	R	R	R	S	R	S	S	S	S	S	Undefined	AZ251
R	S	R	S	S	S	R	S	S	R	R	S	106	B27
R	S	R	R	R	R	R	S	R	R	R	S	064	B6
R	S	R	S	R	S	S	S	S	R	R	S	Undefined	B11
R	R	S	S	R	S	R	S	S	S	S	S	001	AZ159

R: resistant; S: sensitive; cef: cefotaxime; chl: chloramphenicol; cli: clindamycin; ery: erythromycin; fuc: fusidic acid; imi: imipenem; lev: levofloxacin; lin: linezolid; met: metronidazole; rif: rifampicin; tet: tetracycline; van: vancomycin

Figure 5.3 Dendrogramatic representation of profiles generated using RAPD and primer AP4 and associated antibiogram profiles. Similarity was calculated using Dice coefficient and represented by UPGMA clustering.

5.3.3 Genotypic characteristics of PCR ribotypes

Amplicons that appear characteristic of many isolates of *C. difficile* were produced in the majority of the isolates when typed using PCR ribotyping. An amplicon of 299bp was evident in nine of (82%) the eleven reference profiles produced, absent in the isolates 002 and 078. This amplicon was also absent from eight of the profiles generated from the clinical isolates; these isolates belonged to PCR ribotype 002 with the remaining isolates being of undefined PCR ribotype (Figure 5.4). Among the profiles generated from the reference isolates, an amplicon of 362bp was present in all isolates (Figure 5.4).In contrast to this, an amplicon of 584bp was present in a large majority of both clinical and reference isolates.

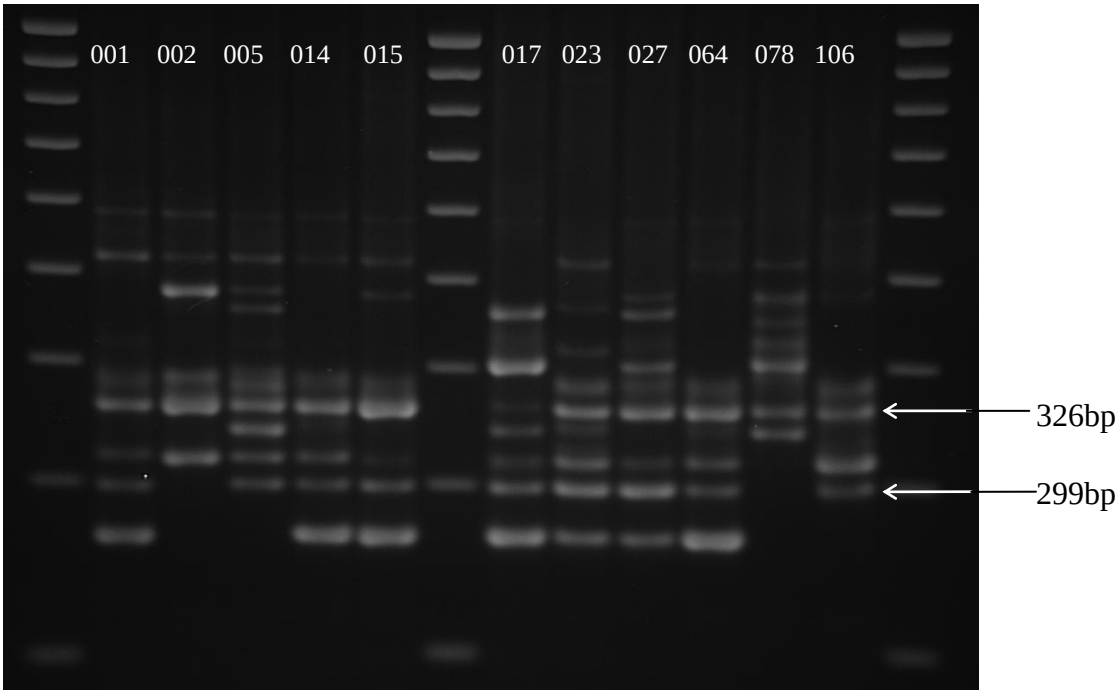

Figure 5.4 Image of PCR ribotype reference isolates and identification of 299bp amplicon. Lanes are labelled according to the relevant PCR ribotype isolate.

5.3.4 Genotypic characteristics of defined RAPD types

Consistent with PCR ribotyping, characteristic amplicons were evident when all isolates were typed using both AP3 and AP4 RAPD primers. Both primers also generated amplicons that were only present in isolates recovered from one location; there were however no amplicons that were consistently produced in all of the isolates recovered from one location. When typed using primer AP3, amplicons of 928bp, 1038bp and 1099bp were only seen in isolates recovered from Trust A, however each of these amplicons was not present in all of these isolates (Figure 5.5) The 1038bp amplicon was present in all isolates identified as belonging to one particular RAPD type when characterised using AP3 and was only seen in one other isolate (AZ387). An amplicon of 697bp was also present in all isolates belonging to a different RAPD type (Figure 5.6). This amplicon was also only generated in isolates recovered from Trust B. Two amplicons were generated in a high frequency amongst isolates regardless of the location from which they were recovered; these were identified as being 520bp and 307bp in size (Figure 5.7). The amplicon of 520bp was generated in all isolates whereas the amplicon of 307bp was only present in fifty six of the clinical isolates.

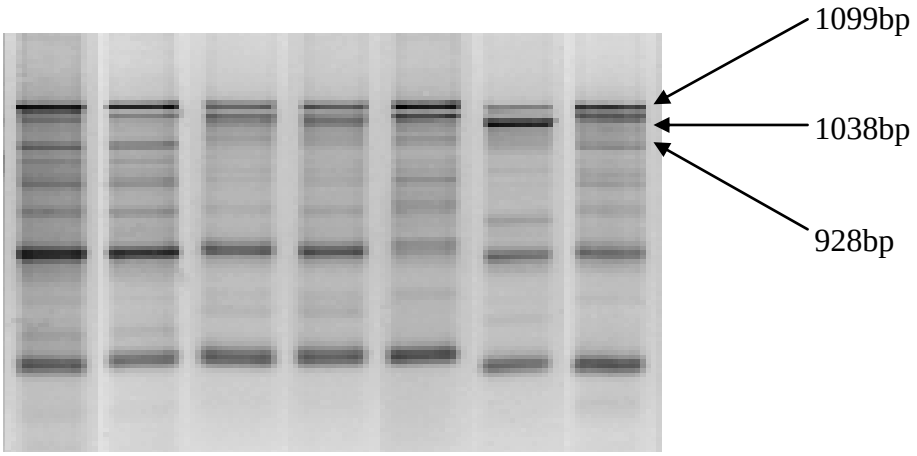

Figure 5.5 Image of clone of isolates highlighting amplicons of 1099bp, 1038bp and 928bp. These amplicons were only observed in isolates recovered from Trust A.

When isolates were characterised using primer AP4, an amplicon of 214bp was present in all of the isolates with the exception of one (isolate B11); amplicons of 542bp and 452bp were also present in sixty one (98%) and fifty nine (95%) of the isolates respectively (Figure 5.6). While the majority of isolates generated profiles that contained each of these amplicons, at least one of each of these three amplicons was present in each isolate. An amplicon of 663bp in length was present in isolates belonging to three different RAPD types when characterised using primer AP4. In isolates belonging one other RAPD type a pattern of four amplicons were present (567bp, 542bp, 474bp and 452bp), this characteristic was not observed in any of the other isolates.

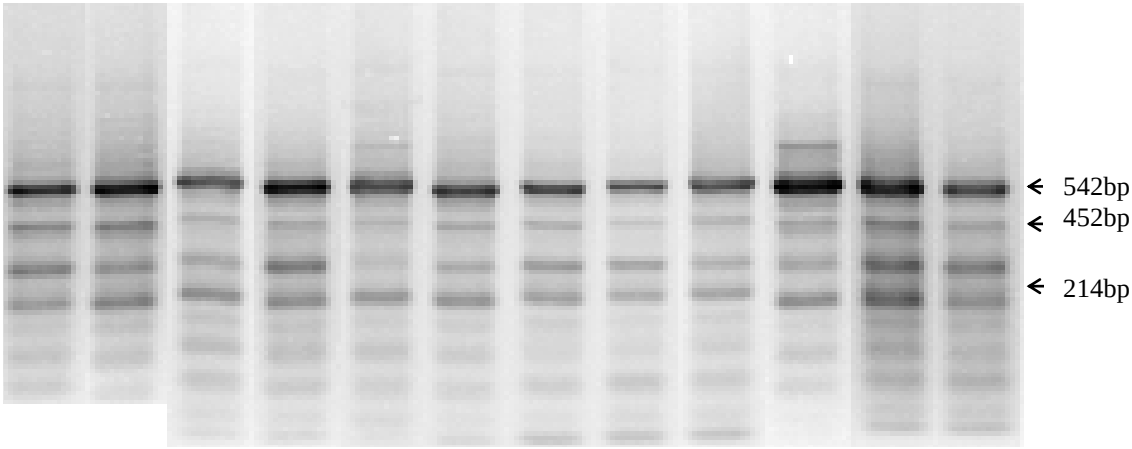

Figure 5.6 Image of profiles generated using RAPD and primer AP4, indicating amplicons of 214bp, 452bp and 542bp.

Amongst the six isolates that generated a positive reaction for the alanine arylamidase biochemical test in the API, four of these isolates (66%) belonged to PCR ribotype 027; the remaining two isolates belonged to PCR ribotype 106 and an undefined PCR ribotype. No other associations were evident between genotype and the restricted biotype profiles produced.

5.3.5 Associations between S-layer proteins and genotypic characteristics

No significant associations between isolates of a particular genotype defined by either RAPD, PCR ribotyping, or S layer protein type could be made. The S-layer types defined for the isolates here appeared to vary randomly with no particular S-layer type appearing to be unique to a specific genotype. However, an association could be made between PCR ribotype and S-layer; PCR ribotypes 014 and 106 were S layer types 5236 and 5436 and were present in 88% and 80% of isolates respectively. When isolates were typed using RAPD and primer AP3, a clone of eleven isolates all possessed the S-layer type 5236, this was also observed in a clone of isolates characterised by primer AP4. The isolates that formed each of these clones were almost exactly the same with the exception of one isolate.

5.4 DISCUSSION

When characterising the *C. difficile* isolates using both PCR ribotyping and RAPD, it was apparent that there were amplicons produced that both varied between isolates as would be expected for any typing method but also those that appeared to be characteristic and consistent between the isolates. Such amplicons were produced in all isolates when typed using RAPD and in a large proportion of the isolates tested with PCR ribotyping. For both of the typing methods, the amplicons produced that were present in all or a large proportion of the isolates were generally of smaller size. When characterised using RAPD, the amplicons of a more variable nature were typically around 500bp-1000bp; the amplicons produced by PCR ribotyping were no greater than 600bp with variable amplicons of between 300bp and 500bp. This observation was consistent across all of the isolates tested; probably due to smaller amplicons being easier to generate in comparison to those which are larger. The DNA extraction protocol for each of these typing methods is relatively crude which although quickly extracts DNA from the cell also compromises the integrity of the DNA, making it more vulnerable to shearing and therefore prevents amplification. This is likely to have occurred during DNA extraction of both of these typing methods and is therefore why larger amplicons were less frequently observed and not regarded as robust enough amplicons for use in differentiation.

Although it is not known what regions of the genome the consistent amplicons represent, they suggest that these are regions that are highly conserved within the genome. It may be possible that these regions are highly conserved amongst several species of bacteria however it may also be possible that these amplicons may represent regions that are characteristic of the Clostridium genus or *C. difficile* as a species. Whether these amplicons are species specific or found in a greater number of bacteria, the high frequency of

occurrence suggests that these regions may represent genes that are essential to the survival of the bacteria. Further investigations involving the typing of other species of bacteria and Clostridia to determine if similar amplicons are also produced, and sequencing experiments to identify the amplified region would be beneficial in identifying possible targets that could be used to differentiate between strains. Although RAPD has been shown to be a discriminatory and robust technique by which to characterise isolates of *C. difficile*, it is unlikely that it would ever be adopted as a widespread method to genotype *C. difficile* isolates. This is in part due to previous criticisms of the RAPD method and also the need for typing techniques such as MLVA, which demonstrates a much greater discriminatory power than either PCR ribotyping or RAPD, can offer. The profiles produced here however may indicate that RAPD could be used to identify possible targets for more specific characterisation and discrimination.

As described in Chapter 4, a greater variability was observed in the types defined by both genotypic methods amongst isolates recovered from Trust A compared to Trust B; this pattern of results was also observed in the antibiogram profiles of the isolates. Although the level of diversity in both the genotypes defined and antibiogram profiles were similar for isolates from each of the trusts, there was no clear mapping or association with one particular antibiogram type and a corresponding genotype. No significant associations could be made between PCR ribotype and antibiogram profile, with no resistance towards a particular antibiotic being exclusive to one ribotype. Associations have been made with fluoroquinolone resistance and clindamycin susceptibility amongst isolates belonging to PCR ribotype 027; there are also associations amongst isolates belonging to more common PCR ribotypes in the UK and an increase in MICs towards metronidazole. Although isolates belonging to PCR ribotype 027 were initially associated with susceptibility to

clindamycin, this is no longer the case and additionally, clindamycin resistance was observed amongst the majority of PCR ribotype 027 isolates in this study. Although there was little cross over between the PCR ribotypes observed between the two trusts, isolates belonging to PCR ribotypes 027, 064 and 106 were recovered from both locations with quite significant differences observed depending on recovery location. Isolates identified as belonging to PCR ribotype 027 recovered from Trust B generally displayed resistance to a greater number of antibiotics in comparison to those recovered from Trust A, this was also found with isolates belonging to both PCR ribotype 064 and 106. Results such as this suggest that the PCR ribotype of an isolate is unlikely to be an indicator of antibiotic resistance in *C. difficile*, probably due to the ease with which antibiotic resistance can spread through a population and the predominance of few PCR ribotypes. These results suggest that the location from which an isolate is recovered is more likely to be an indicator of antibiotic resistance and although antibiotic prescribing polices are highly likely to be a factor in this, other pressures such as infection control procedures and the prevalence of other bacteria in the environment may also be of influence. Biocide resistance has been reported in other species of bacteria; and although the spores of *C. difficile* are known to be difficult to eradicate, vegetative cells of *C. difficile* are generally quite susceptible. However, just as antibiotic resistance has developed in bacteria through exposure to sub-inhibitory levels of antimicrobials, this same reasoning can be applied to biocide resistance and there are also reports of cross resistance towards both antibiotics and biocides (Russell, 1998, Braoudaki & Hilton, 2004). The cleaning products used in hospitals vary between trusts and therefore different populations of bacteria may be exposed to different pressures based on the cleaning product used. In addition to this and harder to control is the population of bacteria that are present within a clinical setting. One reason why vancomycin has been restricted for the treatment of CDI is to try to prevent the

further development of VRE as both organisms are found in the gut. The ability of *C. difficile* to acquire resistance genes and the presence of the many other species of bacteria that are found in the gut provides many opportunities for genetic information to be exchanged; this can be problematic if resistance within a bacterial population in a hospital is widespread. The different environmental conditions as described above are likely to have influenced the isolates investigated here. A high proportion of both PCR ribotype 027 and 106 isolates, both where clindamycin and erythromycin resistance was observed were recorded. Although it is not known whether the resistance observed in the isolates here has been encoded by the *erm*B gene it is likely. The prevalence of such a resistance pattern in these two predominant PCR ribotypes suggests that such resistance has spread among a population of isolates that are more frequently encountered and therefore have more exposure to antimicrobials. This again lends support to the suggestion that location and prevalence may be a greater indicator of antibiotic resistance in *C. difficile* as a greater proportion of isolates recovered from Trust B displayed this resistance pattern. Also, this profile was not observed in all isolates belonging to PCR ribotype 027 and, isolates that did not belong to PCR ribotypes 027 or 106 also displayed this antibiotic resistance profile.

No associations could be made between biotype and genotype this however is likely to be due to the restricted biotype profiles that were produced. The isolated positive results for the alanine arylamidase test were most frequently recorded in isolates belonging to PCR ribotype 027; this could therefore be linked to the virulence of this strain. The PCR ribotype 027 strain is regarded as more virulent due to its enhanced toxin production; this however does not explain other aspects of the strains virulence such as enhanced resistance and sporulation rate. Although the ability to produce alanine arylamidase does not directly link to the virulence of the strain either, this could provide nutritional or other advantages.

There was no association between the defined genotypes and S-layer types of the clinical isolates of *C. difficile* investigated here. There have been reports that S-layer typing correlates well with ribotyping (McCoubrey *et al*., 2001). Whilst a small association could be made between S-layer and PCR ribotype in this study, in general, S-layer type varied both between PCR ribotype and RAPD type. It has been reported by others (McCoubrey & Poxton , 2001) that the most common S-layer type observed (5236) here correlates with PCR ribotype 001. Although this S-layer type was found frequently in this population of *C. difficile* isolates, the isolates were found to belong to a variety of different ribotypes. Two clusters produced from RAPD typing, one from each of the RAPD primers, which contained only isolates that had S-layer types 5263. This however was not exclusive to the clusters formed.

5.5 CONCLUSION

The phenotypic characteristics displayed by *C. difficile* isolates appeared to be influenced to a greater extent by location and prevalence within a population rather than the PCR ribotype to which they belonged suggesting that maybe too much emphasis is placed on the PCR ribotype of an isolate. No significant associations could be made between the phenotypic and genotypic traits observed here. The variability that was observed both in genotypic profiles and antibiotic resistance suggest that in order for such associations to be made further investigation of both phenotypic traits and genotypic analysis of *C. difficile* is required.

CHAPTER 6 GENERAL DISCUSSION

This research aimed to identify the phenotypic and genotypic characteristics of two populations of *C. difficile* isolates that had been recovered from two separate tertiary referral trusts. Isolates were then characterised by both PCR ribotyping and RAPD and the discriminatory power of both methods compared. Finally, the phenotypic characteristics and amplicon profiles produced by both genotypic methods were analysed to determine if any associations between the phenotype and genotype of the isolates could be determined.

There is a lack of current research into the phenotypic characteristics of *C. difficile* and there is a need for this to be addressed in order to gain further insight into the microorganism. Although Toxins A and B are the major virulence factors of *C. difficile*, there may be other factors present in some strains which enhance virulence and promote further pathogenicity. If other virulence factors were present and characteristic of some strains, not only would a greater understanding of the microorganism be gained but also could potentially be incorporated into a diagnostic test or typing method. As highlighted in this current study, the phenotypic characteristics of *C. difficile* are likely to be influenced by many factors and therefore it is possible that such influences have led to changes within some strains which are at present unknown as they have not been investigated. Historical phenotypic data such as that reported by (Seddon & Boriello, 1978, Hafiz, 1980) is still relied upon when reporting the phenotypic characteristics of *C. difficile*; however, evolutionary changes may have occurred within the species that have altered this. This is supported by work undertaken by (Stabler *et al*., 2099), where despite a large proportion of conserved genes within both current and historical isolates of *C. difficile* PCR ribotype 027, there were also regions within the genome where significant differences were observed. It is thought that these alterations are responsible for the hypervirulent state observed in current PCR ribotype 027 isolates. Although many of the phenotypic

characteristics observed in this study provided little data to allow discrimination between *C. difficile* isolates (with the exception of antibiogram profiles), there are other traits which could be investigated such as the sporulation rate of different isolates and biocide resistance. The antibiogram profiles observed in the current investigation, and the variation observed between the two different trusts is likely to be the result of differences in the antibiotic prescribing policies of the two different trusts. This observation has been suggested by others who have undertaken similar studies (Taori et al., 2009). Although the results are suggestive that a difference in antibiotic prescribing policies influenced the differences observed in the antibiogram profiles, the prescribing policies for the two Trusts were not available and therefore no further information can be gained. Historic antibiotic prescribing policies from when the isolates were recovered from the respective Trusts would be needed in order to confirm that antibiotic prescribing policies were responsible for the differences observed in the antibiogram profiles between the two Trusts. In addition to this, further studies using current *C. difficile* isolates and antibiotic prescribing policies from the same two Trusts would be needed. Such work may then be able to determine if antibiotic resistance had been influenced over time and exposure to particular antibiotics. There is a lack of treatment options for CDI and therefore treatment is generally quite uniform between different trusts. This therefore indicates that antibiotics prescribed for prophylaxis prior to high risk surgical procedures and for the treatment of other infections such as MRSA are more likely to be causing the selective pressure that is driving the antibiotic resistance that was observed in this study Although these antibiotics are not being used in the treatment of CDI, it is still likely that the microorganism is being exposed to sub-inhibitory concentrations within the gut of patients which can allow resistance to develop. It has been demonstrated that *C. difficile* spores are prevalent in many clinical settings, and the ingestion of these spores is known to be the main route of transmission for

CDI. Spores are also ingested by patients who do not have a compromised gut flora and therefore cause no symptomatic disease; however, *C.difficile* spores will still germinate into vegetative cells within a healthy human gut and therefore can still be exposed to other antimicrobials. Although it is the vegetative cells of *C. difficile* that initially acquire such resistance, if the cell then sporulates, genomic information is passed on to the spore. Although the development of resistance to some of the antibiotics tested in this study has no effect on the treatment of CDI, this allows isolates of *C. difficile* to become reservoirs of antibiotic resistance which can then be transferred to other species of bacteria. It is therefore possible that other bacteria encountered by *C. difficile* in different healthcare settings can also influence the characteristics of different isolates. As the results in this study indicate; *C. difficile* does appear to adapt to the selective pressures of the local environment. There are other pressures within a healthcare setting that *C. difficile* is likely to encounter such as biocides and hand hygiene products and these again could also be responsible for phenotypic and genotypic changes within the bacteria. At present there is no published work that has examined resistance or the development of resistance to such agents in *C. difficile* vegetative cells; this again highlights the need for further investigation into phenotypic characteristics of *C. difficile*.

The acceptance of RAPD as a widespread typing method for *C. difficile* isolates is unlikely. This is in part due to criticisms regarding discriminatory power and reproducibility (Brazier, 1998) and although it has been shown that the thorough optimisation of RAPD can improve results (Hilton *et al*., 1997, Perry *et al*., 2003) it is still not a favoured technique. In addition to this, the development of other typing methods that are highly discriminatory such as MLVA and MLST are now being favoured over even standard methods such as PCR ribotyping. Although RAPD may not be adapted as a widespread method for the typing of *C. difficile* isolates, what has been established in this

study is that RAPD could be useful in local epidemiological typing. As has also been identified in this study, RAPD identified different types in a similar pattern to both PCR ribotyping and antibiogram profiling, providing support to the RAPD method. Within each of the various PCR ribotypes defined, varying RAPD types were identified, thus identifying further 'subtypes' within PCR ribotypes; subtypes have also been defined by REP-PCR and PFGE (Rahmati *et al*., 2005, Northey *et al*., 2005). Such typing is very useful as an epidemiological tool as it demonstrates that although isolates are identified as the same PCR ribotype, this does not necessarily mean that they are the same strain. The use of RAPD within a clinical setting could therefore potentially identify whether patients have been infected with the same isolate of *C. difficile*. The PCR ribotyping method and RAPD protocol used here both target different areas of the genome; PCR ribotyping targets the intergenic spacer regions while RAPD is applied to the whole genome. As a result the two methods are not strictly comparable due to different targets however; the results here do suggest that they may be compatible. Initially a PCR ribotype could be defined, followed by a RAPD type to determine if infecting isolates are the same. The results here suggest that either RAPD alone or RAPD in combination with PCR ribotyping could be used to map the local epidemiology of CDI. As has been demonstrated here, this appears to work well however; testing of a greater number of isolates from more Trusts would be required in addition to possible sequencing of the genome.

Another potential use for RAPD would be to use the protocol to characterise a variety of different *C. difficile* isolates and identify the regions of DNA that were being targeted by the primers used. This would involve sequencing amplicons generated and identifying the amplified regions of DNA and the genes which they were part of. If these targets were found to be suitable for the characterisation of *C. difficile* isolates, these could be developed into a more specific PCR protocol and if multiple targets were identified this

could be used in a multiplex PCR protocol. If both species specific targets and strain variable targets were identified then this could be further developed into a protocol that is used in the identification of *C. difficile*. Although sequencing DNA is ultimately the best way to both characterise isolates and identify potential targets to be used in a typing or identification protocol, this is costly and labour intensive. In addition to this, sequencing would need to be carried out on a variety of isolates which again would increase both costs and labour. In contrast to this, RAPD is a cost effective and quick technique, allowing a lot of isolates to be analysed over a shorter period of time and could potentially identify targets more quickly.

The potential influence of environmental pressures on *C. difficile* may demonstrate why few apparent associations could be made between the phenotypic and genotypic traits investigated here. There did however appear to be some association between the traits due to the patterns that were observed between the two trusts for both genotypic methods and antibiogram profiling. Phenotypic characteristics of *C. difficile* isolates appeared to be associated with location or prevalence within an environment rather than any particular genotype. Associations between PCR ribotyping and particular phenotypic characteristics are often made; however, such characteristics may actually be due to the prevalence within an environment rather than being due to the PCR ribotype of an isolate. An example of this is the increase in MICs towards clindamycin, metronidazole and moxifloxacin (HPA, 2008) that has been reported among the more predominant PCR ribotypes. If other PCR ribotypes were observed to be predominant, then it is likely that the same patterns in antibiotic resistance would be seen in these.

From the results presented in this study it appears that both genotypic and phenotypic characteristics of *C. difficile* isolates are influenced by the local environment; most likely through local selective pressures in the form of antibiotic prescribing and infection control

policies. The variability that was observed using both genotypic methods and also the antibiogram profiles suggests that *C. difficile* is a highly adaptive microorganism with the potential to acquire mechanisms that are specific to the promotion of its survival in a given environment. The differences that were observed using antibiogram profiling, PCR ribotyping and RAPD indicate that changes may be occurring in several different areas of the genome. The use of these three methods alone to characterise *C. difficile* has provided insight into the organism and demonstrates the need for further investigations into the characteristics and local epidemiology of the bacteria.

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APPENDICES

Appendix (1)

Preparation of antibiotic solutions

Antibiotics were in powder or crystalline form except levofloxacin and linezolid which were in solution at concentrations of 5mg/ml and 2 mg/ml respectively. Antibiotics were soluble in sterile distilled water with the exception of erythromycin and metronidazole which were dissolved in 20% ethanol and rifampicin which was dissolved in a weak acetic acid solution before being further diluted. As a control, both ethanol and the weak acetic acid were incorporated into agar without any antimicrobial to determine if these solutions were having an inhibitory effect rather than the antibiotic tested. Antibiotics were made up to a concentration twenty times greater than the highest concentration required and dilutions made from this where required. Antibiotic solutions were freshly prepared each day with the exception of levofloxacin and linezolid which were already in solution.

Appendix (2)

Composition of the gels, buffers and stains used in S-layer protein analysis.

Sample Denaturing Buffer	
10% ($^{w}/_{v}$) SDS	5mls
0.5M Tris HCl (pH 6.8)	2.5ml
Sterile double distilled water	5mls
Glycerol	2.5ml
2-mercaptoethanol	250µl
5% (w/v) Bromophenol Blue	200µl

Protein Standard Markers	
Bovine Albumin	66 (kDa)
Ovalbumin	45 (kDa)
Glyceraldehyde 3-phosphate Dehydrogenase	36 (kDa)
Carbonic anhydrase	29 (kDa)
Trypsinogen	24 (kDa)

Separating Gel (12%)	
Stock 1 (40% (w/v) acyrlamide 0.8% (w/v) bis (N,N'-methylene bisacrylamide)	6mls
10% (w/v) SDS	0.5ml
1.5M Tris HCl pH 8.8	6.2ml
Sterile double distilled water	7.8mls
TEMED	50µl
10% (w/v) AMPS	70µl

Stacking Gel	
Stock 2 (30% (w/v) acrylamide 0.8% (w/v) bis (N-N'-methylene bisacrylamide)	2.5ml
10% SDS	150µl
0.5M Tris-HCl buffer (pH 6.8)	3.8ml
Sterile Double Distilled Water	8mls
TEMED	40µl
10% AMPS	50µl

Running Buffer	
10% (w/v) SDS	20mls
Tris	6g
Glycine	28.8g
Make up to 2 litres with double distilled water	

CONFERENCES ATTENDED

April 2010	European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 20th Meeting Vienna, Austria.
May 2009	European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 19th Meeting Helsinki, Finland.
December 2008	Society for Anaerobic Microbiology (SAM) December 2008 Scientific Meeting London, UK.
April 2008	European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 18th Meeting Barcelona, Spain.

LIST OF PUBLICATIONS

Peer reviewed publications

Phenotypic characterisation of *Clostridium difficile* reveals lack of extracellular virulence factor production and significant differences in antibiogram profiles.

L. M. Green, T. Worthington, A.C. Hilton and P. A. Lambert.

International Journal of Antmicrobial Agents, Volume 35, Issue 3, March 2010, Pages 305-306

Poster Presentations

Antimicrobial susceptibility patterns of *Clostridium difficile* from two tertiary referral trusts within Birmingham, UK.

L.M. Green, T. Worthington, A.C. Hilton, P.A. Lambert.

The Eighteenth European Congress of Clinical Microbiology and Infectious Disease

An optimised random amplification of polymorphic DNA (RAPD) protocol for rapid genotyping and local mapping of *Clostridium difficile*.

L.M. Green, J. Rollason, T. Worthington, A.C. Hilton, P.A. Lambert.

The Nineteenth European Congress of Clinical Microbiology and Infectious Disease.

Comparison of PCR ribotyping and an optimised random amplification of polymorphic DNA (RAPD) protocol for genotyping of Clostridium difficile produces profiles similar in discriminatory power and reproducibility.

L.M. Green, T. Worthington, A.C. Hilton, P.A.Lambert

The Twentieth European Congress of Clinical Microbiology and Infectious Disease.