Investigation into the dominant strains of Clostridium difficile within hospitals and strategic cleaning regimes

Investigation into the dominant strains of *Clostridium difficile* within hospitals and strategic cleaning regimes

by

Krusha Vasanti Patel, BSc.

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Abstract

Clostridium difficile is a common and potentially fatal cause of antibiotic-associated diarrhoea and pseudomembranous colitis worldwide. It has been isolated from patients and their surroundings, in healthcare facilities and from the community. *C. difficile* is able to survive for many months on inanimate surfaces in the form of spores. PCR ribotyping is used in the UK to characterise and identify strain diversity. Investigating how the most problematic strains respond to cleaning regimes may influence the control of disease. This work used the University Hospitals of Leicester Trust as a case study for this purpose of understanding the epidemiology of this pathogen within healthcare facilities. Five individual agar media were compared based on their abilities to recover and resuscitate damaged ribotype 027 spores, a strain associated with disease outbreaks and increased severity. Controlled laboratory experiments with a sub-lethal dose of a germicide were conducted before *C. difficile* recovery from hospital wards. An additional two sampling campaigns acquired environmental strains. *C. difficile* isolation after routine cleaning demonstrated the inefficiency of the current recovery regime as *C. difficile* spores were recovered using direct contact plates, enrichment broths, and resuscitation media. This study used layering of non-selective agar over selective agar, identifying a potential link in the proportions of media following the use of sponges in environmental sampling. All strains were characterised by ribotyping; ribotype 027 was isolated from all sampling cohorts. A four-month epidemiological study was conducted into the ribotype prevalence and distribution from *C. difficile*-positive faecal specimens. A second survey investigated these effects with a modification of *C. difficile* detection from faecal samples. Hydrogen peroxide vapour is currently being explored as a means of decontamination of healthcare-associated infections. Inactivation kinetics of ribotype 027 spores were analysed in response to vapour and liquid exposure of hydrogen peroxide. No reports thus far have explored such kinetics and controlled decontamination with both clinical and non-clinical strains. Evidence strongly suggests spores can be inactivated with its application. Furthermore, this study revealed there appears to be significant differences in susceptibility and inactivation of different *C. difficile* ribotypes.

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Abbreviations

ABHR	Alcohol-based hand rubs
BHI	Brain-Heart Infusion
bp(s)	base pairs
CCAB	Cycloserine-cefoxitin-amphotericin B agar
CCEY	Cycloserine-cefoxitin egg yolk agar
CCFA broth	Cycloserine-cefoxitin fastidious anaerobe broth
CDAD	*Clostridium difficile*-associated disease
CDMN	*Clostridium difficile* moxalactam norfloxacin
CDRN	*Clostridium difficile* Ribotyping Network
CFU	colony-forming units
DH	Department of Health
DNA	Deoxyribose nucleic acid
dNTPs	Deoxyribose triphosphates
ELISA	Enzyme-linked immunosorbent assay
FAA	Fastidious anaerobe agar
GDH	Glutamate dehydrogenase
GGH	Glenfield General Hospital
GS-BHI	Glucose-supplemented BHI agar
HCAI	Healthcare-associated infections
HCl	Hydrochloric acid
HPA	Health Protection Agency
hr	hour(s)
IMS	Industrial Methylated Spirits
kV	kilo Volts
LGH	Leicester General Hospital
LRI	Leicester Royal Infirmary
min	minute(s)
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaDCC	Sodium dichloroisocyanurate
NCTC	National Collection of Type Cultures
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
ppm	parts per million
RODAC	Replicate Organism Detecting and Counting
rpm	revolutions per minute
rRNA	Ribosomal ribonucleic acid
sec	second(s)
SEM	Scanning Electron Microscopy
ST	Sodium taurocholate
TAE	Tris-acetate and ethylenediaminetetraacetic acid buffer
TCCFA	Taurocholate cycloserine-cefoxitin fructose agar
UHL	University of Leicester
UV	Ultra-violet
v/v	volume/volume
VRE	Vancomycin-resistant *Enterococcus*

Chapter 1. Introduction and Literature Review

1.1 Healthcare-associated infections

Healthcare-associated infections (HCAI) can be distinguished as those not present or developing during admission, but occurring in a patient throughout the duration of stay and whilst in the care of a hospital or other healthcare facility (World Health Organization 2002). This also accounts for infections acquired in the hospital but not manifesting prior to discharge, as well as occupational infections possessed by members of staff (Allegranzi et al. 2007). HCAI have been reported to account for 300,000 infections in UK hospitals every year, with 1 in 10 patients contracting an infection during their stay; the estimated annual cost of treating HCAI in the UK is £1 billion (HCAI Research Network & Department of Health). In 70% of nosocomial infections where HCAI are the problem, the pathogen in question was resistant to at least one anti-microbial drug. This is interesting as the now resistant drug was previously effective. Those infections, caused by a range of microbial agents, included: *Acinetobacter* spp., *Staphylococcus aureus*, *Enterococcus* spp., and *Clostridium difficile* (Carmeli 2008).

1.1.1 Transmission of HCAI

Studies have been previously conducted on how HCAI establish infection and how they are spread in healthcare facilities, as well as into the external environment (White et al. 2008). It is known that contamination of a pathogenic organism can occur from a patient to the hospital surroundings, and these are able to thrive and survive on surfaces in the environment for many months (Boyce et al. 1997; Kramer et al. 2006). From these locations, others are able to acquire these pathogens; these include members of staff in the healthcare facility in addition to visitors and potentially, other patients. Verity et al. (2001) also alluded to the containment of bacteria through isolation areas, thereby reducing the possibility of transmission between affected and non-affected patients. Reports have shown levels of contamination exist on the surrounding surfaces and sites around patients, indicating sources of infection (Cotterill et al. 1996). This is not to suggest that the only means for transfer is through physical contact of contaminated surfaces; it has been shown that aerial dissemination of pathogens can also result in these infections (Beggs et al. 2008). Further to this, increased numbers of bed occupancy have also been linked to increased numbers of

contamination of these bacteria, as there is subsequently a higher chance of prevalence and spread of infection (White et al. 2008).

1.1.2 UK surveillance of HCAI

Mandatory surveillance helps to ensure proficient and accurate systems are implemented to manage the spread of infections efficiently. The Department of Health (DH) and Health Protection Agency (HPA) have enforced compulsory surveillance of a number of HCAI in order to focus on those that are currently posing the most problems in hospitals, such as *Enterococcus* spp., *Staphylococcus aureus*, and *C. difficile*. These three infections are particularly important, as their prevalence is higher than most underlying pathogens due to antibiotic resistance.

Monitoring was first enforced in the UK for *Staphylococcus aureus*, as well as methicillin-resistant *Staphylococcus aureus* (MRSA), after a bacteraemia out-break in April 2001 (Johnson et al. 2005). This allows the pooling of data from different locations and NHS Trusts, allowing a clearer overall geographical conclusion. This was then followed by the surveillance of the vancomycin-resistant enterococci (VRE) in October 2003 (Brown et al. 2006). VRE infections and mortalities were recorded following outbreaks in the UK (Kuriyama 2003; Kawalec et al. 2007). In January 2004, monitoring of *C. difficile* was introduced following the growing trend of associated infections and mortalities. This is particularly problematic as *C. difficile* produces spores, increasing the ease with which the infection can be transmitted. The HPA has since implemented an informative scheme investigating which strains of *C. difficile* are causing disease within the NHS (Health Protection Agency 2012). This is explained further in Section 1.3.2.2.

1.1.3 Preventing the spread of HCAI

In 1999, a plan was devised where 'visual cleanliness' was the aim to effectively reduce the numbers of infections in hospitals and successfully monitor the hygiene procedures as stated in the guidelines of "Standards for Environmental Cleanliness in Hospitals" (NHS Estates 2000). This was based on the surroundings being 'visually' clean. However, Griffith et al. (2000) and Malik et al. (2003) proved that this 'visual cleanliness' is not a successful means of

eliminating contamination from the environment. Both groups concluded that surfaces thought to be free from contamination were not, using adenosine triphosphate (ATP) bioluminescence techniques and microbiological sampling, detecting the presence of pathogens.

Nevertheless, it has been shown that reducing the environmental contamination has the ability to reduce the transmission, and therefore infection of these bacteria in hospitalised patients (Cotterill et al. 1996). Increasing the time spent cleaning, with reagents currently used, can lead to a decrease in the infection rate, as found by Rampling et al. (2001) when investigating an outbreak of MRSA.

1.1.3.1 Enforcing hand washing and use of alcohol-based hand rubs

Clearly, poor environmental hygiene can lead to the transmission of these healthcare infections (Wilcox 1996). Through the implementation of thorough hand washing with soap and water, or with alcohol-based hand rubs (ABHR), the spread of some nosocomial infections has been controlled (Curtis 2008). This called for the need to minimise the spread and infection of HCAI through improvements to hand hygiene.

ABHR are frequently utilised in hospitals due to the ease in which it can be used compared to hand washing with soap and water. In addition, the application of ABHR reportedly irritates the hands less and has the benefit of accessibility in that it can be made available in multiple sites within a facility (Ellingson & McDonald 2010). Despite its activity against the spread of MRSA and VRE, a number of studies have suggested that ABHR are less effective against *C. difficile* (Oughton et al. 2009; Ellingson & McDonald 2010; Jabbar et al. 2010). Furthermore, these studies also acknowledge that the application of ABHR does not produce statistically significant $\log_{10}$ reductions in *C. difficile* spore concentrations. These authors all report that hand washing with soap and water is superior in effectiveness in reducing the concentration of *C. difficile* spores present on hands when compared with ABHR. Interestingly, Jabbar et al. (2010) reported that *C. difficile* spores may be transferred from person to person via a handshake, with a transmission rate of 30% immediately after the use of an ABHR. Therefore,

in spite of the increased use and availability of ABHR in healthcare facilities, it may well be counter-intuitive in the control of *C. difficile* infection.

1.1.3.2 Use of cleaning agents and disinfectants

Altering the procedures and materials which are used to clean wards and bays can also have an influence on the recovery of bacteria (Eckstein et al. 2007). This was shown in a study conducted by Fawley et al. (2007). This group investigated different detergents and disinfectants on vegetative cells of *C. difficile* and their effects on inhibiting germination of the bacterial spores, including ChlorClean, a product standardly used in UK hospitals at 1000 ppm (mg/m^3). Effectiveness was recorded when a chlorine-based product was added to the cells and significant reductions in the spore germination were found. Numbers of recovered *C. difficile* spores also decreased when chlorine-cleaning agents were used in conjunction with a hydrogen peroxide pre-clean. However, the study concluded that the use of hydrogen peroxide with a neutral detergent was unable to reduce the numbers of spores germinating as successfully. Mayfield et al. (2000) recorded a mean decrease in incidence from 8.6 to 3.3 cases per 1000 patient-days in *C. difficile* rates following the application of hypochlorite solution in comparison to a quaternary ammonium disinfectant. This ammonium-based agent also failed to be as effective as aldehydes and peroxides against *Staphylococcus aureus* (Exner et al. 2004).

Irrespective of these findings, studies with *C. difficile* have found that cleaning agents can instead have an adverse effect. Wilcox et al. (2000) found that the application of a disinfectant or cleaning products to specific clinical strains could lead to the increase in the numbers of spores germinated. This was increased further with the use of particular chlorine-free cleaning agents. A suggestion for this could be due to a response to the exerted environmental stress in the form of a virulence factor. Other groups have also eluded to the routine use of disinfectants not being efficient enough to reduce the infection rates alone (Danforth et al. 1987; Dettenkofer et al. 2004).

1.2 Clostridium difficile

First identified over seventy years ago from a collection of healthy neonatal faecal samples, *C. difficile* is an anaerobic, Gram-positive, spore-forming bacillus (Hall & O'Toole 1935). Arguably, it is one of the most important causes of infectious diarrhoea, pseudomembranous colitis and toxic mega colon (Sorg & Sonenshein 2009). There were 34,268 reports of *Clostridium difficile*-associated disease (CDAD) in people aged 65 years and over, in the UK from April 2008 to April 2009 (Health Protection Agency 2010). A recent report into the financial burden of *C. difficile* infections identified an average of £7,000 is spent per case (Department of Health 2012). Although often present in the gut, when patients are immunocompromised, disruption to the normal colonic flora can cause CDAD which manifests usually after administration of broad spectrum antibiotics (Eggertson & Sibbald 2004; Eckert & Barbut 2010). The result of this is either an overgrowth of *C. difficile* or colonisation with environmental clostridia that are often present in healthcare environments (Dubberke et al. 2007).

1.2.1 Bacteriology

C. difficile infection has been linked to the administration of the drug clindamycin by Tedesco et al. (1974) and Cleary (1998). Often occurring in the elderly or immunocompromised patients following the use of broad-spectrum antibiotics as well as antineoplastic drugs that possess antibiotic properties have led to an increase in the incidence of the toxigenic antibiotic-associated colitis (Saxton et al. 2009).

There appears to be a general decrease in recorded *C. difficile* infections in those over the age of 65 over an annual period; there is a rise in the first quarter which falls over time, only to increase again over the winter months (Polgreen et al. 2010). This could be attributed to immune systems being less tolerant of the cold during this period and there being an increased probability of their admittance to a healthcare facility. Further risk factors are: infections to the enteric system that result in alterations in the colonic microflora, being in intensive care and high-dependence units, undergoing mechanical bowel cleansing, being over 60 years old, and conditions that affect the host-immune systems and defences such as

chemotherapy, human immunodeficiency virus (HIV) and malnutrition. A combination, or indeed any of these factors alone, is able to lead to a rise in the incidence of the *C. difficile*-associated colitis (Cleary 1998).

Through the production of spores that are passed out with faeces, *C. difficile* is able to persist in the environment. These allow the persistence on a number of surfaces in healthcare facilities such as floors, tables, chairs, bedding, and clothes for up to five months, increasing the chance of further infection, and spreading to others (Kim et al. 1981; Mayfield et al. 2000).

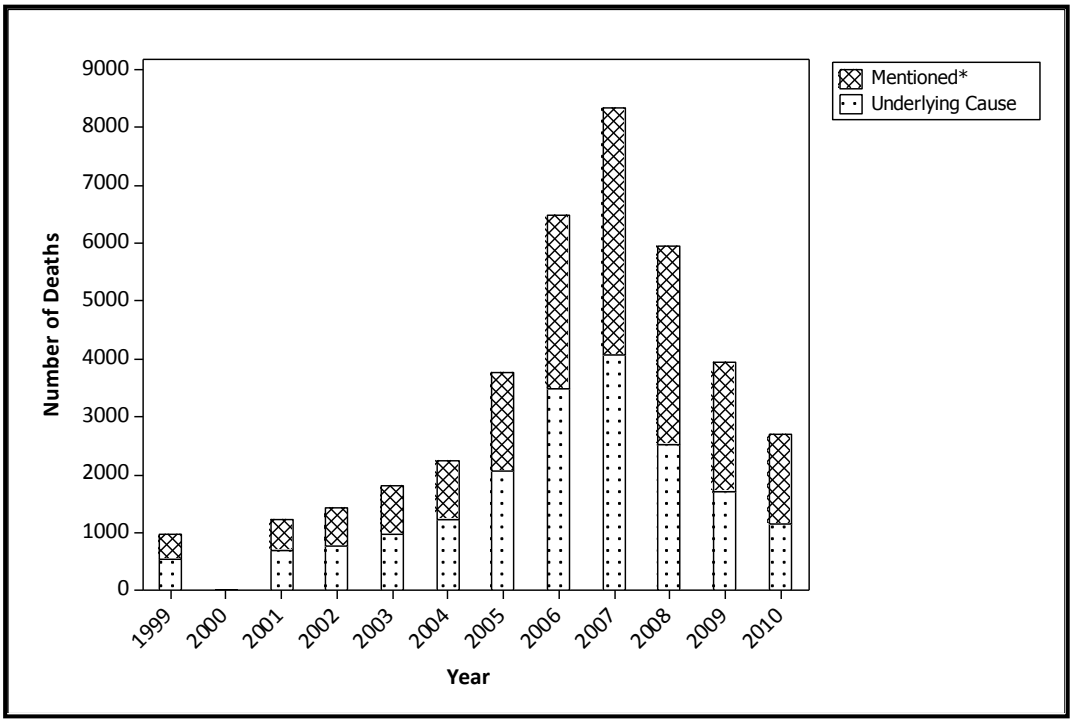

Figure 1.1: Number of death certificates reporting *C. difficile* infection (hatched) in comparison with infection as the underlying cause (dotted) in England and Wales between 1999 and 2010.

Data was not provided for the year 2000. Data was obtained from the Office of National Statistics (http://www.statistics.gov.uk). '*' - refers to *C. difficile* infection mentioned as a contributory factor on a death certificate.

Figure 1.1 shows considerable rises in the numbers of incidence of CDAD in the early 2000s, and Figure 1.2 illustrates rates of *C. difficile*-positive faecal specimens, which had the effect of increasing the duration of stay in hospitals, in addition to mortality rates (Aslam et al. 2005). There has been a decrease in the latter part of this decade due to the introduction of procedures and practices to limit the spread of infection. Controlling the administration of broad-spectrum antibiotics, careful patient management and monitoring CDAD has also

contributed to this decline (Healthcare Commission 2005). Mandatory surveillance of *C. difficile* allows the monitoring of whether the implemented control methods have an effect on the infection rates.

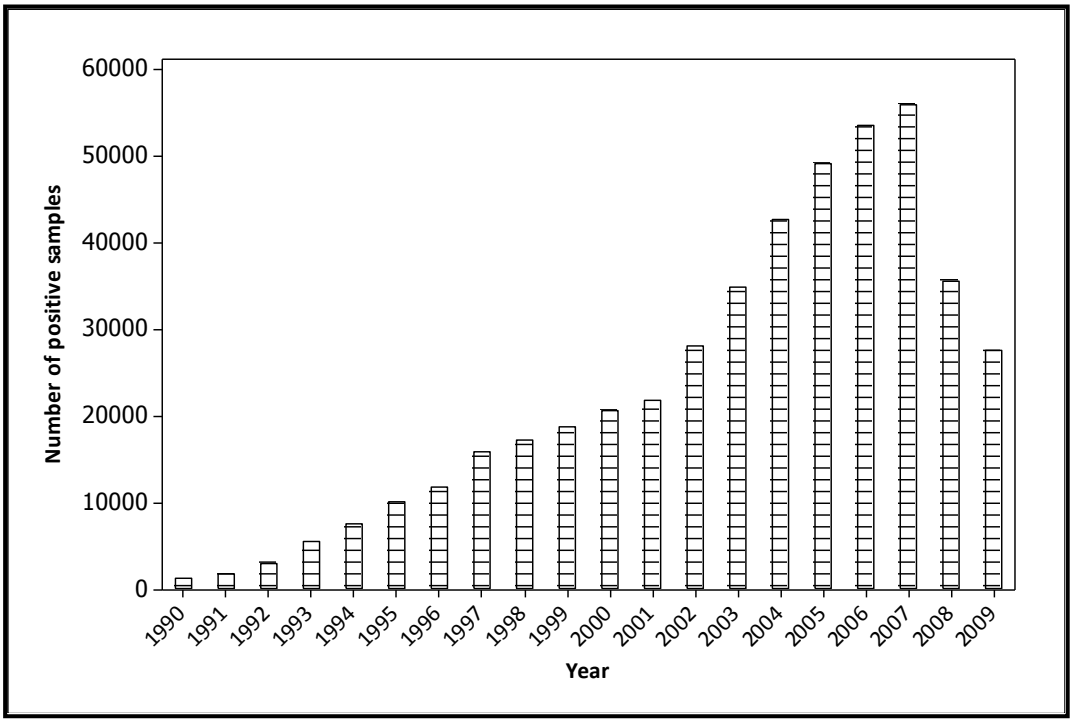

Figure 1.2: Number of *C. difficile*-positive faecal specimens obtained through a voluntary surveillance in England and Wales between 1990 and 2009.

From 2004, surveillance was made mandatory to monitor *C. difficile* infection in hospitals in the UK. Data was obtained from the Health Protection Agency (http://www.hpa.org.uk).

1.2.2 Pathogenesis

C. difficile can exist endogenously unable to colonise in healthy individuals until the normal gut flora is altered. This is inhibited through patients receiving antibiotics, thus resulting in over-growth of toxigenic strains in the gut and disease. Ingested *C. difficile* spores that colonise and proliferate in the human colonic flora produce toxins A and B; CDAD can only be caused by toxinogenic strains of *C. difficile*. These toxins produced following spore germination result in fluid secretion, inflammation and mucosal damage (Berrington 2004). This is what leads to the symptoms of diarrhoea and pseudomembranous colitis, although the process in which the intestinal microbiota is reduced is not fully understood. The two afore-mentioned toxins are considered to be the major virulence factors in CDAD (Barbut & Petit 2001).

Toxin A is a potent enterotoxin, which causes necrosis, increases intestinal permeability, through the inhibition of protein synthesis that results in complete erosion of the colonic mucosa. This then damages the intestinal villi and erodes the brush border membranes (Poxton et al. 2001). Toxin B is thought to enhance the activity of toxin A as well as playing a role in virulence (Berrington 2004; Kuehne et al. 2010). Most clinical strains of *C. difficile* are capable of producing both toxins A and B, while some only produce toxin B. It is rare to identify strains which are positive for toxin A alone (Cohen et al. 1998; van den Berg et al. 2004).

1.2.3 Methods of detection

There are several methods for the detection of *C. difficile* from faecal specimens used in a microbiology laboratory. Methods such as direct culturing and the glutamate dehydrogenase (GDH) antigen assay target *C. difficile* itself; whereas others detect the presence of the *C. difficile* toxins from the specimen, such as the enzyme-linked immunosorbent assay (ELISA). A third group of tests detect the presence of the toxin genes via molecular methods, such as nucleic acid amplification tests (NAATs) (Kufelnicka & Kirn 2011).

The most accurate method for *C. difficile* detection from faecal specimens remains laboratory culturing on agar plates. Samples are shocked with either alcohol or industrial methylated spirits (IMS). This eliminates contamination of cells which are not *C. difficile*; in addition, it encourages the sporulation of *C. difficile* (Clabots et al. 1989). This faecal-alcohol mixture is then sub-cultured onto a cycloserine-cefoxitin egg yolk (CCEY) agar plate. Within 48 hr, positive *C. difficile* colonies should appear. These can then be used in sub-typing of strains, as described in Section 1.3.1. However, this method has many inherent problems. Culturing individual samples of *C. difficile* is time-consuming, costly, and labour-intensive. In addition to this, it is dependent on the presence of spores or vegetative cells. The turn-around time for identification is also slow, and therefore would not be ideal in outbreaks and for diagnostic purposes (Vaishnavi 2009).

The most common technique is the use of commercial kits for ELISA that detect either toxin A or both toxins A and B, even when present in low levels (0.8 ng/ml and 2.5 ng/ml, respectively) (Techlab Enteric Diagnostics n.d.). The results

are obtained relatively quickly, boding well and aiding in the diagnosis of infection. The sensitivity of ELISA ranges from 50-90% and specificity from 70-95% (Vaishnavi 2009). Recently, the detection of the bacterial antigen GDH has also been targeted. These assays are most sensitive but less specific due to GDH being expressed by toxigenic and non-toxigenic *C. difficile* strains. Positive samples are often further tested for the presence of toxins or their genes (Kufelnicka & Kirn 2011).

The use of polymerase chain reaction (PCR) primers and NAATs have also been utilised in the detection of *C. difficile* from faecal specimens, targeting the enterotoxin gene or toxin B (Vaishnavi 2009). Following the use of PCR, analysis of the results can be time-consuming, requiring technical skills. Commercial kits have become available including GeneOhm Cdiff [BD Diagnostics, USA], *illumi*gene *C. difficile* [Meridian Biosciences, USA], and GeneXpert *C. difficile* [Cepheid, USA] (Kufelnicka & Kirn 2011). The use of NAATs is often carried out following initial ELISA, for example with GDH-detection. This entails the cheaper, quicker, and more sensitive test to be conducted first and subsequently the specific molecular method (Kawada et al. 2011). Kufelnicka & Kirn (2011) suggest such combinations increase the sensitivity of tests to 75-100%, with high specificity.

1.2.4 Transmission and patient susceptibility

Patients, both asymptomatic carriers as well as those infected, are crucial in the spreading of the disease to those susceptible to infections (Clabots et al. 1989). The transmission and maintenance of spores, passed out with faeces, can occur unknowingly as *C. difficile* is capable of surviving in a dormant phase for a number of weeks or months, remaining on surfaces such as chairs, tables, floors, even linens despite washing (Kim et al. 1981). These hardy spores can reside on inanimate objects which means there is a high chance of the organism transferring from patient to patient within bays and wards, in addition to infection spread via the hands, jewellery and stethoscopes of clinical staff (Bradbury & Barrett 1997; Alleyne et al. 2009).

Cephalosporins, penicillins, and clindamycin are known to increase the incidence of hospital-acquired infections and are the three most common

antibiotics linked to *C. difficile* colitis. Those less likely to cause colitis are trimethoprim, quinolones, ureidopenicillins and aminoglycosides (Cleary 1998). A study was conducted where 17% of patients who received clindamycin were subsequently diagnosed with associated colitis over two months (Kabins & Spira 1975). Due to increased administration of cephalosporins, there appeared to be an increase in antibiotic-associated diarrhoea incidences, with Keighley & Matheson (1980) and Talbot et al. (1986) reporting 5-38% of patients under treatment with antibiotics as sufferers. The duration and route of antibiotic administration has also been shown to affect the development of CDAD; oral treatment is thought to increase the risk greater than intravenous (Bignardi 1998; Barbut & Petit 2001). Furthermore, McFarland et al. (1989) reported that of 428 patients tested at admission, 7% were positive for antibiotic-associated diarrhoea; throughout the course of hospitalisation, this rose to 21% as culture-positive. Fekety (1997) stated that in 15-20% of antibiotic-associated diarrhoea cases, *C. difficile* is responsible, and of the remaining instances, symptoms are settled when the administration of antibiotics is terminated. In addition to this, the chances of contracting CDAD have been shown to increase in relation to the length of the course of antibiotic use as well as the number of antibiotics prescribed (Hensgens et al. 2012).

1.2.5 Current treatment

This association to antibiotics means that the treatment plan for *C. difficile* can be quite problematic. Two antibiotics have been routinely used over the past thirty years in treating *C. difficile* infection: vancomycin and metronidazole (Gerding 2000). The use of these drugs is often sparing to avoid the development of antibiotic-resistant strains, as seen with VRE and MRSA. Nevertheless, susceptibility to metronidazole has declined although the reasons why have yet to be determined (Kuijper & Wilcox 2008). Additionally, there has been the recent introduction of fidaxomicin, the first in a new group of narrow spectrum macrocyclic antibiotics, as a replacement for the use of vancomycin (Louie et al. 2011; Lancaster & Matthews 2012). Reports have suggested fidaxomicin disrupts the microbiota less when treating *C. difficile* than vancomycin, as well as acting against many VRE (Nerandzic et al. 2012). There have also been fewer cases of *C.*

difficile infection recurrence with the administration of fidaxomicin than vancomycin and a reduction in the number of cases of ribotype 027, a problematic *C. difficile* strain (Louie et al. 2011; Mullane et al. 2011).

1.3 Monitoring and characterisation of C. difficile strains

Monitoring which strains of *C. difficile* are present in healthcare facilities is important because it allows the identification of the strains responsible for disease outbreaks. Furthermore, it provides a discriminatory data set to be collected that can provide the framework required to understand the epidemiology of the disease. Therefore, correct characterisation of strains is highly important to aid in the recognition of new emerging ribotypes.

1.3.1 Strain differentiation with PCR ribotyping

Some *C. difficile* strains are more problematic in causing disease or infection than others, with a continuous rise in CDAD reports worldwide. Therefore, several typing methods have been used to study the epidemiology of *C. difficile*. This allows the identification of which strains are responsible for outbreaks of disease, and which may be presently underlying. Several methods have been developed to classify the sub-types of *C. difficile* including restriction endonuclease analysis, pulsed-field gel electrophoresis, multi-locus sequence typing, multi-locus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing (Killgore et al. 2008).

However, the most widely used technique in the UK and throughout Europe, is polymerase chain reaction (PCR) ribotyping which targets the 16S and 23S rRNA interspacer regions of the bacterial gene with specific primers (Brazier 2001). Gurtler (1993) showed the *C. difficile* genome to be heterogeneous, as it possesses many copies of these rRNA genes. These were found to differ in number between strains as well as the size between different copies on the same genome. It was then demonstrated that these fragments can be amplified from 250 to 600 base pairs (bp); following separation using the techniques described and modified primers (O'Neill et al. 1996). This is then used as a signature for the strains and referred to as a ribotype (Stubbs et al. 1999). Different ribotypes of *C. difficile* are then subject to differentiation due to the number and the size of the spacer gene present within the bacterial genome (Brazier 2001). This is why PCR ribotyping is thought to be extremely useful in determining the genetic variation of *C. difficile* (Terhes et al. 2006). This methodology is also highly discriminative, reproducible

and as it is easier and quicker than other molecular typing methods (Stubbs et al. 1999).

As PCR ribotyping uses universal primers, the *C. difficile* strain has to be isolated from patient faecal samples and then cultured to ensure that only DNA from this species is present as a template for PCR. Traditionally, the PCR products from ribotyping are separated on a high-resolution agarose gel, the sizes of the bands are then estimated using gel analysis software; size standards are used as markers. There are inherent problems with this as DNA can separate differently within gels, and this creates issues regarding comparability; in addition to this analysis of the bands is notoriously subjective (Zaiss et al. 2009).

An alternative approach to identifying the ribotyping profiles is to size the fragments on a capillary-based system, having first incorporated a fluorescent primer into the PCR mixture. A fluorescent standard marker is also added to each sample before the fragments are analysed on a DNA sequencing machine. The output is a series of peaks, which are constructed on a chromatogram, where each peak corresponds to a particular fragment size. Several authors have suggested that using capillary electrophoresis to identify genotype profiles allows greater precision than gel-based systems as it can resolve the size of DNA fragments to within ±4 bp (Indra et al. 2008; Xiao et al. 2012). This allows samples to be standardised both within and between laboratories as it is far less prone to individual bias that occurs using gel-based systems (Indra et al. 2008).

The use of PCR ribotyping has been further developed since 2008 to produce a more reliable system of strain characterisation (Indra et al. 2008). The capillary-based method makes use of the already successful PCR ribotyping technique, but overcomes the emerging problem hampering European reference laboratories: exchanging data between laboratories. This results in differing nomenclatures between groups, resulting in confusion as to which strain has been identified and investigated (Kato et al. 2001; Spigaglia & Mastrantonio 2004; Aspevall et al. 2006). The HPA also uses this method of identifying PCR ribotypes for its annual reports of ribotype dominance in the UK (Health Protection Agency 2011).

1.3.1.1 C. difficile PCR ribotype 027

The first recorded case of a *C. difficile* ribotype 027 outbreak occurred in Québec in 2003, where over 1,400 cases of infection were recorded in six Montreal hospitals, over an eighteen month period (Eggertson & Sibbald 2004). In 2005, a subsequent outbreak of *C. difficile* occurred in the Netherlands, and therefore, in the following year, a surveillance programme was initiated to investigate the strain differences. Fourteen Dutch hospitals took part in a three-year long surveillance project, which investigated the incidence and distribution of different strains of *C. difficile* based on their PCR ribotypes (Hensgens et al. 2009).

Arguably, one of the worst series of *C. difficile* outbreaks was within the United Kingdom at Stoke Mandeville Hospital, Aylesbury. According to the findings by the Healthcare Commission following two outbreaks in quick succession, there were 174 cases of *C. difficile* infection from 2003 to 2004, of which nineteen were fatal. Furthermore, in the following year, a further 160 patients were confirmed with *C. difficile* infection and another nineteen deaths were reported (Healthcare Commission 2006). Interestingly, the strain that caused these large outbreaks was the same.

Kuijper et al. (2006) reported that in 2003, ribotype 027 was one of the rarest strains in the UK; by 2004, it was recognised as the strain causing the most severe outbreaks in hospitals (McDonald et al. 2005; Arvand et al. 2009). This strain has since been identified as prevalent in hospitals worldwide and is also known as North American pulsed-field gel electrophoresis type 1 (NAP1), restriction endonuclease analysis group BI and toxinotype III (O'Connor et al. 2009).

This strain is often referred to as a 'hyper-virulent variant', although this term is somewhat controversial (Stabler et al. 2006; Hubert et al. 2007; Morgan et al. 2008). Investigations into the reasons for the prevalence of this strain have had conflicting results. A frame-shift mutation and 18 bp deletion in the tcdC gene of ribotype 027 pathogenicity locus is thought to be responsible for a consequent increase in the production of toxin A and B, by 16 and 23 times, respectively (McDonald et al. 2005; McFarland et al. 2007). Studies have suggested however

that this increase of toxins has no bearing on an increase in disease severity (Walk et al. 2012).

Kuijper & Wilcox (2008) reported that these virulence factors are not exclusive to ribotype 027. They have also been identified in other ribotypes, such as ribotype 078, which was initially isolated from bovine and porcine origins (Goorhuis et al. 2008). The spread of this strain is still debated, with a European-based survey by Barbut et al. (2007) reporting the presence of ribotype 078 in Greece only, whereas Bauer et al. (2011) identified it as the third most prevalent strain in their subsequent continent-based study. Interestingly, recent reports of this strain were made from the Middle East, where reports on *C. difficile* infection are highly infrequent (Jalali et al. 2012). Nevertheless, these virulence markers are not found within all problematic strains. In 2008, one of the most frequently found strains in the UK was ribotype 106, and this strain does not possess any of the deletions identified in ribotype 027 and 078 (Kuijper & Wilcox 2008).

Despite the increasing emergence of new ribotypes of *C. difficile*, PCR ribotype 027 is still one of the most prevalent strains of *C. difficile* in the UK, across Europe and North America.

1.3.2 C. difficile surveillance

1.3.2.1 Monitoring of C. difficile in Europe

Since 2006, samples from any patient presenting with *C. difficile* in the Netherlands are submitted to Leiden University Medical Centre (LUMC), for culture isolation, propagation and PCR ribotyping (Hensgens et al. 2009). Therefore, the Netherlands has a significant and comprehensive set of data describing the emergence and predominance of different strains of *C. difficile.* This shows that, although still currently dominant, the 027 strain is actually in decline and has revealed the emergence of ribotype 078 (Goorhuis et al. 2008; Hensgens et al. 2009). However, this degree of surveillance is unique and the high costs associated with it have precluded its implementation in the UK.

A survey was carried out in Hungary where 105 clinical isolates were investigated over two years from three geographical regions of Hungary, of which

65 were from inpatients and the remaining 40 from outpatients (Terhes et al. 2006). This study found five distinct PCR ribotypes which failed to map any which had been previously determined, identifying the prevalence of types 014 and 002 (24.8% and 13.3%, respectively) (Terhes et al. 2006).

There have been two European-wide studies conducted by the European *Clostridium difficile* infection study (ECDIS) that investigated the prevalence of ribotypes between different countries. These surveys aim to identify the key strains causing infections and therefore potential geographical networks of strains (Barbut et al. 2007; Bauer et al. 2011). Collaborations between fourteen European countries permitted the identification of sixty-six ribotypes from 411 isolates (Barbut et al. 2007). This group reported the most commonly isolated strains at the time of the study were ribotypes 001 (13%), 014 (9%) and 027 (6.2%). It was surprising to find that despite its predominance in particular regions, such as the Netherlands (40% of isolates) and Belgium (31.4%), ribotype 027 was not as prevalent across Europe. It was not identified in the UK at all, although the authors concede this may be due to only one UK hospital participating in the study. Ribotype 001 accounted for 73% of the isolates from three Spanish hospitals; ribotype 017 was identified in 80% of the toxigenic samples from Irish and Polish hospitals (Barbut et al. 2007).

Bauer et al. (2011) isolated 389 samples from thirty-four countries, comprising sixty-five PCR ribotypes. Contrasting with the findings four years prior, the ribotype cluster 014/020, 001 and 078 were the most commonly identified, at 16%, 10%, and 8%, respectively. Barbut et al. (2007) interestingly attributed this strain to predominantly causing *C. difficile* infection in younger patients. The overall proportion of ribotype 027 was 5%, demonstrating a further decline in European prevalence. Nevertheless, it was still one of the most identified strains from UK and Ireland. These data demonstrate the variation of ribotypes between countries, irrespective of geographical distance, indicating patterns of strain diversity exist. Particular strains were isolated exclusively within countries, such as ribotype 017 in three Spanish hospitals within the study, suggesting the localisation of ribotypes may also exist (Barbut et al. 2007). The comparison of

ribotypes between the two studies has also shown the conformation of which strains are dominant can change over time (Barbut et al. 2007; Bauer et al. 2011; Kachrimanidou & Malisiovas 2011).

1.3.2.2 Monitoring of C. difficile in the UK

The HPA in the UK has a programme where it conducts mandatory surveillance of *C. difficile* infection rates, and routinely releases documents within the NHS Trusts (both Acute Trusts and Primary Care Organisations). Every three months, hospitals are required to report these rates, however, it is not mandatory to submit all samples for culturing. This is largely due to cost and the labour intensive nature of the procedures.

In the UK, the *C. difficile* Ribotyping Network (CDRN) has been operating under the HPA since 2007, conducting mandatory weekly surveillance of *C. difficile* infection rates from all NHS Trusts. The CDRN ribotypes samples using the capillary-based system, analysing isolates where there are increases in incidence, mortality, and recurrence rates, severity of cases, or failure to meet *C. difficile* infection targets. This provides an invaluable resource for understanding national *C. difficile* distribution, and consequently a framework in which to contextualise studies based on a smaller spatial scale (Health Protection Agency 2012).

Although ribotyping in UK hospitals is confined to a subset of strains, it has allowed the recent emergence of new dominant ribotypes to be closely followed. For example, ribotypes 002, 015, and 078 have become more abundant over the periods 2007/8. They then gained even greater prominence in 2008/9 (Health Protection Agency 2009). This information is critical in order to monitor which strains of *C. difficile* are problematic, related to outbreaks and potentially to then investigate why they are so successful in the hospital environment. Patterns have also been identified in terms of where strains are found; particular strains are found more commonly within communities rather than within healthcare facilities, in addition to the ability of different strains to produce different amounts of the *C. difficile* toxins (McDonald et al. 2005; Warny et al. 2005; Bauer et al. 2011). This suggests there is variation within the virulence of strains. These findings therefore demonstrate the need for constant monitoring and surveillance programmes for *C.*

difficile infection, within not only continents but also countries, to identify the epidemiological changes exhibited by strains which could in turn aid in the strategic therapeutic responses (Kuijper & Wilcox 2008).

1.4 Isolation of C. difficile from the environment

In addition to identifying which strains exist within patient specimens, it is also important to determine the presence of *C. difficile* within the environment. Knowledge of which strains are capable of residing in healthcare facilities despite the cleaning regimes enforced by healthcare professionals and governing bodies provides an insight into the problematic types. There have been many studies carried out whereby environmental sampling has been conducted and *C. difficile* is recovered from healthcare facilities. Different techniques for recovering *C. difficile* spores and vegetative cells have been implemented.

C. difficile has not only been isolated from healthcare facilities and patient specimens, but other sources (al Saif & Brazier 1996; Rodriguez-Palacios et al. 2007; Bakri et al. 2009; Songer et al. 2009; Weese et al. 2009). Its presence has been detected in pre-packaged salad bags as well as meat products. Bakri et al. (2009) reported the detection of *C. difficile* from 7.5% of ready-to-eat salad bags tested. Worryingly, as these salads are served uncooked, there is the concern that ingestion may result in the colonisation of *C. difficile*. The authors suggested that this could increase the asymptomatic carriage and risk of transferring *C. difficile* among humans. Rodriguez-Palacios et al. (2007) isolated *C. difficile* from 20% (*n*=60) of retail beef and veal as ground-meat samples from Canada. Of these, eleven samples were toxigenic, and eight were ribotype 027. A further Canadian study identified 12% of samples (*n*=115) of ground-beef and 12% (*n*=115) ground-pork as *C. difficile*-positive (Weese et al. 2009). Ribotypes 078 (73%) and 027 (27%) were identified from the 42% (*n*=88) *C. difficile*-positive samples isolated from cooked and raw beef, pork and turkey products, in North America (Songer et al. 2009).

Contrastingly, studies conducted in Sweden found 2.4% (*n*=82) and the Netherlands only 0.8% (*n*=500) of meat product samples as *C. difficile*-positive, despite testing chickens, turkeys, sheep, calves and pigs (Von Abercron et al. 2009; Freeman et al. 2010). Despite these findings, there has yet to be any evidence that definitively suggests *C. difficile* contamination in food can result in *C. difficile* infection in humans.

1.4.1 C. difficile agar media

Many comparative *C. difficile* medium studies have been designed for the isolation of *C. difficile* from faecal specimens. The composition of agar media and optimised conditions may not be ideal in environmental recovery, although this is seldom explored. Media are often formulated as an 'agar base' requiring additional supplements, each with a specific objective, such as an antibiotic mixture hindering growth of any bacteria, except for *C. difficile*. Other supplements can be added in order to enhance spore germination or to improve *C. difficile* colony growth.

Fastidious anaerobe agar (FAA) and brain heart infusion (BHI) agar are used in selectivity for *C. difficile* in conjunction with different additional components. However, alone they both have been found to enhance the colony fluorescence of *C. difficile* when performing one of the characteristics tests for identification: viewing under an ultra-violet (UV) light (Levett 1985; Tabaqchali & Jumaa 1995). *C. difficile* is also thought to utilise fructose in base agars as a nutrient, and so there is a visible colour change in the medium upon acid production and metabolism (Buggy et al. 1983). These two agar bases are often utilised in laboratory culturing of *C. difficile*.

1.4.1.1 Use of supplements in C. difficile media

There are antibiotics used in addition to the agar base when selecting for *C. difficile*. The purpose of antibiotics in selective media is to inhibit most microbial flora growth, except that of *C. difficile*.

Norfloxacin, a broad-spectrum antibiotic that interferes with bacterial DNA replication, is commonly used in addition to moxalactam, another broad-spectrum antibiotic that acts by interrupting synthesis of peptidoglycan. This then inhibits the growth of various bacteria and other clostridia. A study found the use of norfloxacin inhibited strains of Enterobacteriaceae and faecal streptococci when tested at a concentration of 16 mg/l (Aspinall & Hutchinson 1992). When used in combination with moxalactam (32 mg/l), *C. difficile* moxalactam-norfloxacin (CDMN) also successfully inhibited the growth of all the clostridia, without affecting the growth of *C. difficile*. Upon comparison with cycloserine-cefoxitin

fructose agar (CCFA), CDMN gave a 20% higher isolate rate and a 33% reduction in growth of contaminants.

D-cycloserine (commonly known as cycloserine) is a third broad-spectrum antibiotic, which inhibits two enzymes of the murein production cycle. This is frequently used in conjunction with cefoxitin, also a broad-range antibiotic that interacts with cell wall synthesis. This antibiotic combination is a common mixture that inhibits a host of bacteria found in high proportions within faecal samples, when used at 250 mg/l and 8 mg/l. These concentrations have been reported to be more successful at growing a diverse range of *C. difficile* strains than 500 mg/l and 16 mg/l, which were initially utilised in the production of selective media (Levett 1985). It is also the most commonly utilised selective medium in the isolation of *C. difficile* from faecal specimens.

An antifungal antibiotic administered in hospitals that has been associated with an increase of CDAD is amphotericin B. Although not well documented in literature, because of its recorded effect on *C. difficile*-infected patients, is thought to aid in the germination of spores and maintain *C. difficile* growth. Therefore, there is an instance of its use in the preparation of media prior to environmental sampling (Gerding et al. 1995).

Egg yolk emulsion is used in the preparation of clostridial media for the detection of enzymatic activity of lecithinase and lipase. *C. difficile* produces neither, and so the need for egg yolk in a selective medium has not been identified. Although, based on the dull, grey colony morphology produced, the addition of egg yolk proves useful with respect to confirmation and identification (Wilson et al. 1982).

George et al. (1979) found that with direct application of faecal samples to a medium containing horse blood, larger colonies were produced than with egg yolk emulsion. It has also been shown that 7% (v/v) defibrinated horse blood with BHI agar (BHI blood) is beneficial in culturing *C. difficile* due to its nutrient composition, increasing sporulation rates and for identification purposes in the production of a greenish-yellow fluorescence of colonies when exposed to long-wave UV light.

Cysteine hydrochloride is another agent which speeds up the growth of *C. difficile* following application of the bacterium to the media (Aspinall & Hutchinson 1992). The addition of lysozyme into a medium has also been found to increase the recovery of *C. difficile*. It acts through the stimulation of its spore germination where it often replaces the egg yolk emulsion in the cycloserine-cefoxitin supplemented agar (Verity et al. 2001; Wilcox & Fawley 2000; Dubberke et al. 2007).

Some cholate derivatives that are normal components of bile can act with glycine in inducing the germination of *C. difficile* spores. Sorg & Sonenshein (2008) produced evidence to show that one of these, chenodeoxycholate, was responsible in inhibiting growth and germination of spores. Sodium taurocholate, however, is the most common in *C. difficile* selective media, as it enhances spore recovery. Buggy et al. (1983) reported a six-fold increase in the recovery of *C. difficile* spores following the addition of taurocholate to cycloserine-cefoxitin fructose agar (TCCFA). This group identified an increase in spore density, and subsequently an increase in the colonies formed following standard *C. difficile* anaerobic incubation for 48 hr at 37°C. The success of this media was supported by Kaatz et al. (1988), who reported an increase in *C. difficile* spore recovery from the environment, with 31.4% of cultures testing positive. Upon administration of 0.1% sodium taurocholate in the place of 2.5% egg yolk in CCFA, the spore recovery increased twenty-fold (Buggy et al. 1983; Buggy et al. 1985). The purity of the bile salt is also thought to be linked to the recovery, with a higher grade being more successful, as well as unrefined deoxycholate salts potentially inhibiting cell multiplication (Brazier 1998). Using more than 0.1% sodium taurocholate appears to have no significant increase in recovery (Buggy et al. 1983; Wilson 1983).

Despite the number of studies that have recovered *C. difficile* from the environments, and reported on hospital contamination rates, there has been little found in literature to suggest an overview into the most successful media, and/or its selective agents when conducting environmental sampling. Research groups predominantly utilise a particular growth medium for the isolation and recovery of *C. difficile* with little to no justification as to its choice. A comparative table into

the success of different *C. difficile* media and their incorporation in environmental sampling has been presented (Table 1.1).

Media	Group	Journal	Success
C. difficile brucella agar with 0.05% ST	(Nerandzic & Donskey 2009)	*Journal of Clinical Microbiology*	100% (direct application)
C. difficile moxalactam-norfloxacin*	(Alfa et al. 2008)	*BMC Infectious Diseases*	33%
	(Bakri et al. 2009)	*Emerging Infectious Diseases*	3/40 (7.5%) – from salad → enrichment broth
Cycloserine-cefoxitin (Brazier's) with egg yolk*	(McCoubrey et al. 2003)	*Journal of Medical Microbiology*	185/1348 (14%)
	(Shapey et al. 2008)	*Journal of Hospital Infection*	48/203 (24%)
Cycloserine-cefoxitin (Brazier's) with 5 mg/l lysozyme	(Roberts et al. 2008)	*BMC Infectious Diseases*	23/32 (72%) – from air samples → recovery solution
	(Verity et al. 2001)	*Journal of Hospital Infection*	16-35% - from swabs
Cycloserine-cefoxitin (Brazier's) without egg yolk with 5 mg/l lysozyme and 1% lysed blood	(Wilcox et al. 2000)	*Journal of Hospital Infection*	24% - from swabs (11% without lysozyme)
Cycloserine-cefoxitin-amphotericin B with 0.1% ST*	(Martirosian et al. 2005)	*Diagnostic Microbiology and Infectious Disease*	22/180 (12.2%)
	(Martirosian et al. 2005)	*Anaerobe*	5/14 (36%)
Cycloserine-cefoxitin-fructose	(Clabots et al. 1991)	*Journal of Clinical Microbiology*	4-17%
	(Kim et al. 1981)	*Journal of Infectious Diseases*	85/910 (9%)
	(Mundy et al. 1995)	*American Journal of Clinical Pathology*	100% (direct application)
Cycloserine-cefoxitin-fructose with 0.1% ST	(Weese et al. 2000)	*Journal of Veterinary Diagnostic Investigation*	24/381 (6%)
	(Buggy et al. 1983)	*Journal of Clinical Microbiology*	14.5-45.8%
	(Cohen et al. 1997)	*Clinical Infectious Disease*	9.1%
	(Niyogi & Pal 1992)	*Indian Journal of Medical Research*	73% (direct application)
Cycloserine-cefoxitin-fructose with 5 mg/l lysozyme and 0.1% ST	(Sethi et al. 2010)	*Infection Control and Hospital Epidemiology*	60% and 37%, 32% and 14%, 58% and 50% - from gauze pads and swabs
Cycloserine-cefoxitin-fructose with 5 mg/l lysozyme	(Dubberke et al. 2007)	*American Journal of Infection Control*	13/48 (27%) – from sponges
Cycloserine-cefoxitin-fructose with egg yolk	(Fekety et al. 1981)	*American Journal of Medicine*	110/1086 (10%) in areas with known *C. difficile*
Cycloserine-mannitol blood agar	(Mundy et al. 1995)	*American Journal of Clinical Pathology*	43/64 (67%) (direct application)
Fastidious anaerobe agar with 0.1% ST*	(Wheeldon et al. 2008)	*Journal of Applied Microbiology*	No data, reported as 'success'
GS-BHI agar with 0.1% ST*	(Kamiya et al. 1989)	*Journal of Medical Microbiology*	After heat-treatment: <1%; after alkali treatment: <6%
GS-BHI agar with 10 mg/l lysozyme*	(Kamiya et al. 1989)	*Journal of Medical Microbiology*	(Same as above): 10-47%; >90%

Table 1.1: Comparison of environmental studies that utilise *C. difficile* selective media.

Summary of different media used in the environmental recovery of *C. difficile*. ST: sodium taurocholate. '*' - denotes media (or a variation) has been used in this thesis.

1.4.2 Direct contact plates in environmental sampling

RODAC (Replicate Organism Detection And Counting), or direct contact plates have been used in the recovery of environmental samples of *C. difficile* (Buggy et al. 1983; Kaatz et al. 1988). These plates have a raised agar surface that is exposed to environmental sites and through direct contact with these areas, are able to collect bacteria. The presence of a grid outline on the base of the plate also facilitates the counting of bacterial colonies. Preparing the RODAC plates with *C. difficile*-selective media acts to eliminate the requirement for a recovery medium or enrichment broth as growth of the bacteria occurs directly on the surface of the agar. The use of RODAC plates can be used as a direct method in the investigation into media selectivity and recovery success.

The aforementioned study by Buggy et al. (1983) utilised TCCFA in RODAC plates. Kaatz et al. (1988) also used TCCFA to increase spore recovery of *C. difficile* from the environment, finding 31.4% of cultures testing positive after direct contact.

Alfa et al. (2008) used RODAC plates prepared with CDMN agar in conjunction with UV markers to detect the presence of *C. difficile* contamination of toilets and commodes within a hospital ward, stating all the sites sampled should be routinely cleaned. Their investigation identified 33% of samples were toxigenic *C. difficile*. A follow-up study identified 86% recovery of *C. difficile* spores from contaminated toilet surfaces using the same techniques (Alfa et al. 2010). The authors conclude that the use of RODAC plates allows direct comparisons as to the influence a disinfectant has on the inactivation of spores.

However, a potential disadvantage in the use of RODAC plates, is the difficulties that arise in the process of environmental sampling are irregular areas, such as door handles and taps, both of which are considered 'high' contact sites. Recovery of *C. difficile* from these sites would prove difficult with the flat surface of the agar.

1.4.3 Pre-moistened sponge-sticks and swabs in environmental sampling

A final method in the recovery of *C. difficile* from environmental sampling is with the use of sponges and swabs. The pre-moistened swabs and sponge-sticks enable the recovery of microorganisms with ease when collecting samples at multiple sites. The larger surface area, which can be covered with their use, also reduces the time taken to sample within healthcare facilities. A further benefit of this method is the ability to sample 'high' contact sites that are not flat surfaces, for example, sink taps, and hand rails. This suggests the use of this technique may provide a detection method that is more sensitive for *C. difficile* environmental contamination.

Dubberke et al. (2007) showed recovery of *C. difficile* using pre-moistened cellulose sponges with neutralising broth following spreading over an area of 1 m^2. The presence of the broth encourages the microbes to attach to the surface of the sponge, increasing the potential recovery. This group found 27% samples (n=48) collected from these sponges were positive for *C. difficile* from the six healthcare facilities they investigated. They concluded an increase in contamination was observed in those rooms with a *C. difficile*-infected patient present, in addition to the prevalence of ribotype 027.

Otter et al. (2009) compared the use of these sponges against swabs with broth enrichment following the wards in question being exposed to hydrogen peroxide vapour decontamination; they found the percentage recovery for each was 28 and 1.5, respectively. This study also showed there was a greater recovery in terms of *C. difficile* being able to be cultured with the sponges. There was no information as to the strains identified from this sampling campaign.

A disadvantage to using the pre-moistened sponges is despite the ability to sample multiple sites, it is not possible to identify the exact location of the source of contamination. Therefore, it is also difficult to determine the significance of the contamination, which would be possible with direct contact sampling (Otter et al. 2009).

1.4.4 Recovery of damaged cells from the environment

Subsequent to environmental sampling, samples from sponges and swabs are often immersed into enrichment broths or plated onto the surface of agar media. Enrichment broths frequently contain supplements, such as sodium taurocholate or antibiotics, to enable damaged spores to recover from the stresses induced and encourage spore germination (Arroyo et al. 2005; Bakri et al. 2009; Rodriguez-Palacios 2009; Jalali et al. 2012). These can then be cultured further, for example for strain typing. The second methodology used is the application of the sample onto a *C. difficile*-selective agar medium to encourage proliferation (Clabots et al. 1992; Dubberke et al. 2007; Roberts et al. 2008). These two techniques can be used in conjunction to initially enrich the sample and then isolate *C. difficile* following growth on a selective medium (Rodriguez-Palacios 2009).

Kang & Fung (2000) have investigated how the preparation of media can have an effect on its ability to resuscitate bacteria following environmental sampling. This group have examined the recovery of *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Salmonella typhimurium*, employing a method of layering a thin non-selective media onto antibiotic-containing media. This practice allows the development and nurturing of the damaged spores and cells onto the non-selective media. Following the initial growth period, the role of the antibiotics is to inhibit the formation of other species and microorganisms, selecting primarily for the desired bacteria. The results produced by this group with the application of heat-treated and damaged cells onto the double-layered agar media demonstrate a higher success rate than the bacterial resuscitation of single-layered agar media. No studies have utilised this method to-date for the recovery of environmentally collected and damaged *C. difficile* spores.

1.5 Hydrogen peroxide vapour decontamination

A common method for the deep cleaning of hospitals that is currently becoming incorporated into cleaning regimes is the use of hydrogen peroxide vapour decontamination. Based on the number of HCAI in hospitals, it is clear that a means to control the spread of contamination needs to be developed. There are surfaces and medical equipment in the healthcare facilities that can act as reservoirs for *C. difficile*. The resistance of *C. difficile* to cleaning methods has been established, and this is attributed to its presence in the environment in spore form (Sorg & Sonenshein 2008). This increases the chances of cross-contamination through contact by healthcare staff, contributing to the spread of infection rather than reducing it. Few studies have optimised the hydrogen peroxide exposure with respect to time and concentration. Therefore, there is a need for data on the effects hydrogen peroxide can have on the inactivation kinetics of *C. difficile* spores.

Of the many decontamination methods that could be implemented in the cleaning of hospitals, hydrogen peroxide could potentially be the most advantageous. Originally designed for the decontamination of rooms in the pharmaceutical industry, it has been developed for use in healthcare facilities. Any variability in manually cleaning would also be reduced with its use, although it is often recommended that a pre-clean be conducted prior to exposure of hydrogen peroxide to remove any organic material (Pottage et al. 2010).

Catalase is an enzyme some bacteria naturally produce which breaks down hydrogen peroxide into its constituent components: water and oxygen. Hydrogen peroxide is an oxidising agent, and it is known that bacterial catalase-peroxidase systems exist to combat against such oxidative stress (Otter & French 2009). Problematic pathogens responsible for many HCAI are highly catalase-positive and therefore this catalase presence could account for difficulties arising in the inactivation of these bacteria. Interestingly, *C. difficile* does not produce catalase (Fu et al. 2012).

Contrasting reports have also been found that demonstrate that re-colonisation of certain nosocomial pathogens can occur within a week in some cases, as well as within a day after hydrogen peroxide decontamination (Hardy et

al. 2007; Otter et al. 2007). This is not to suggest the use of hydrogen peroxide would bear no effects if implemented in healthcare facilities as a means for cleaning. Dependent on the surface exposed to the vapour, there are data to suggest varying efficacy in decontamination. Rogers et al. (2005) have shown significant differences on porous and non-porous surfaces with *Bacillus subtilis* spores. A 1.2-$\log_{10}$ CFU reduction was reported with exposure on carpet and 2.2-$\log_{10}$ CFU reduction on bare pine wood (J. V Rogers et al. 2005). However, when recovering the spores from paper wallboard, formica laminate and glass, this group reported a $\log_{10}$ CFU reduction of greater than 7.5 (J. V Rogers et al. 2005).

Application of hydrogen peroxide also has repercussions in healthcare facilities if this is to be used as a viable option for cleaning. Inevitable drawbacks of this technique are the requirement of removing patients from rooms, the relatively high costs associated in comparison to the traditional method of manual cleaning, and the requirement for specialised equipment and personnel to operate the decontamination systems. Although for safety reasons these cannot be changed, a disadvantage can be investigated: the times and concentrations required for decontamination. Operating the decontamination systems at concentrations such as these would prolong the re-admission of patients. An average of 4.5 hr is required following a hydrogen peroxide vapour decontamination session, whereas it is only 67 min after a manual bleach clean (Davies et al. 2011). This longer wait time subsequently has an impact on the turnaround time for hospitals, limiting the number of patients that the hospital can be newly admitted into vacant rooms (Boyce et al. 2008; Otter et al. 2009; Davies et al. 2011).

Nevertheless, hydrogen peroxide vapour has also been shown to reduce the proportion of *Staphylococcus aureus*, *Acinetobacter baumannii*, and VRE in hospital environments within other studies (French et al. 2004; Otter et al. 2007; Ray et al. 2010; Chan et al. 2011). The application in its vaporised form does not appear to cause any damage to, or affect the functionality of, medical equipment that suggests it could be used as an alternate tool for cleaning. It is also a substitute to

the potentially hazardous sterilisation of heat-labile materials (Andersen et al. 2006).

1.5.1 Deep Clean Project

In order to combat HCAI, a key component is introducing and maintaining cleanliness in and around hospitals. In September 2007, the British government declared a change in the cleaning procedures and an increased level of hygiene was set. This announcement forced NHS Trusts to assess the existing states of the hospital environments, as well as encouraging them to recognise which particular areas would require refurbishment or a comprehensive deep clean, allowing a baseline of cleanliness to be established. With a budget set of £57 million by the Government, the Deep Clean Project aimed to target different NHS Trusts on an individual basis, dependent on where they deem the most action is required (Department of Health 2008).

There was some success reported soon after the instigation and initiation of the Deep Clean Project. The Weston Area Health NHS Trust implemented a pilot scheme whereby bed areas were steam cleaned via aerosols by staff, following the discharge of a patient. This technique was used in addition to the Trust purchasing new commodes and bedpan washers, increasing the number of hand-wash basins within wards and replacing one hundred bedside lockers. There was also the introduction of a 'bare below the elbows' initiative enforced by all clinical members of staff to eliminate bacterial contamination from contact with clothing (Department of Health 2008). A 25% reduction in the numbers of *C. difficile* infection was recorded.

In order to ensure certain levels of cleanliness were maintained, wards were potentially subject to being fined and, if problems persisted, closed down. The DH stated specific attention was to be paid to Patient Environment Action Team (PEAT) scores that focus on the cleanliness in and around hospitals, national specification scores, and any complaints and incidents relating to the environment (Department of Health 2008). Other criteria were in-patient surveys as well as local patient and staff satisfaction surveys, and trends in infection rates, in particular, with regards to *C. difficile* and MRSA (Department of Health 2008).

1.5.2 Hydrogen peroxide decontamination systems

There are two different hydrogen peroxide systems predominantly used in cleaning: vapour decontamination and in the form of a dry-mist.

The vapour decontamination system uses 30-35% (w/w) hydrogen peroxide solution, with an air stream to maximise the coverage of exposure throughout a room (Otter et al. 2007; Boyce et al. 2008). In order to operate the system, rooms must be entirely enclosed, including sealing of air-conditioning ducts and any sources of ventilation. A uniform layer of 2-6 μm of hydrogen peroxide is applied onto the surfaces following air saturation, before the catalysis to water vapour and oxygen is conducted with an active aeration system. The use of activated charcoal has also been shown to reduce the concentration of hydrogen peroxide (Aguinaco et al. 2011). Utilising this subsequent to hydrogen peroxide exposure would decrease the waiting time for a safe level prior to the re-admission of patients.

Otter & French (2009) investigated the resistance of a range of microorganisms to hydrogen peroxide. This group successfully inactivated all tested organisms within 90 min: *Acinetobacter baumannii* was the most resistant to the hydrogen peroxide vapour, followed by MRSA and *Klebsiella pneumoniae*, *C. difficile* and finally, VRE. The reports suggest only 10 min was required to inactivate more than 6-$\log_{10}$ CFU of VRE, a catalase-negative organism, and 30 min for *C. difficile*. The authors contribute the presence of catalase in the other microorganisms tested to their comparative resistance to hydrogen peroxide.

The hydrogen peroxide dry-mist systems aerosolise a solution of 3-10% (w/w) hydrogen peroxide, (<50 ppm) silver ions, and (<50 ppm) orthophosphoric acid (Shapey et al. 2008; Barbut et al. 2009; Boyce 2009). The dry-mist produces droplets between 8-12 μm in diameter, which can then be directed onto surfaces with nozzles. The mist naturally decomposes into water vapour and oxygen following its application. Some of these systems reportedly do not measure the concentration of hydrogen peroxide in the air at the time of exposure, which could be potentially dangerous.

Andersen et al. (2006) observed complete inactivation of *Bacillus atrophaeus* spores (6-$\log_{10}$ CFU reduction) with the use of a dry-mist hydrogen peroxide system (Sterinis) [Gloster Sante Europe, France]. This group tested the decontamination of spore strips in hospital rooms and ambulances. In closed test rooms, 87% (*n*=146), and in a surgical department, 100% (*n*=48), of samples were decontaminated. The authors also report 100% (*n*=60) success in ambulances, with the dry-mist fumigating glove compartments, under mattresses, equipment and the drivers' cabins. They concluded these findings were subsequent to three cycles of the system, with each taking about 3 hr; one or two cycles were not deemed as successful.

1.5.3 Hydrogen peroxide inactivation of C. difficile strains

Boyce et al. (2008) investigated the effects of hydrogen peroxide decontamination with a vaporised system [Bioquell Ltd., UK], in a university-affiliated hospital known to be affected with *C. difficile*, in particular the infection of ribotype 027. There were control measures implemented prior to the study: increasing *C. difficile* toxin testing and isolation of infected patients, encouraging hand hygiene with washing using soap and water, and reducing the administration of broad-spectrum antibiotics. These changes were reported to have little impact on the proportion of patients with CDAD. This group sampled wards pre-intervention as well as during the intervention, for two ten month periods to allow for any seasonal fluctuations in infection. Each patient room was reported to have taken 3-4 hr, and 12 hr per ward for complete decontamination. The use of hydrogen peroxide vapour significantly reduced *C. difficile* infection in all five of the tested wards, with a 53% reduction in those months when ribotype 027 was identified.

Shapey et al. (2008) investigated *C. difficile* contamination in three wards inhabited by elderly patients with the Sterinis system. The wards were sampled both before and after the usage of the hydrogen peroxide exposure. Prior to its application, 24% samples were *C. difficile*-positive. With only one cycle of its application, 3% (P<0.001) of samples recovered were *C. difficile*-positive. These were also characterised by ribotyping, identifying the strains as ribotypes 001,

027, and 106. The counts recorded were similar to those from isolation rooms which were deemed low risk (elective orthopaedic, obstetrics and paediatric wards) (Shapey et al. 2008). This group reports that the effects of hydrogen peroxide exposure to these sites could have been bactericidal and/or sporicidal. Sampling was conducted again three to six weeks following the intervention and there was no increase in the number of *C. difficile*-positive samples.

Another report of the application of hydrogen peroxide and different strains of *C. difficile* was conducted by Barbut et al. (2009). An *in vitro* study was conducted with hydrogen peroxide dry-mist exposure, with application to three different *C. difficile* strains (toxinotype 0, and a historical ribotype 027 and an epidemic ribotype 027), using the aforementioned Sterinis system (Barbut et al. 2009). Following a single cycle, this group identified a mean 4.18-$\log_{10}$ CFU reduction on coupons infected with spores.

1.5.4 Kinetic inactivation of C. difficile

Currently, groups often utilise the aforementioned hydrogen peroxide decontamination equipment for large-scale operations in healthcare facilities, employing vapour concentrations within the range of 500-2000 ppm (Hall et al. 2007; Hardy et al. 2007; Otter et al. 2007). It has been suggested that hydrogen peroxide vapour concentrations exceeding 75 ppm are hazardous to human health (Krishnan et al. 2006). There has been little investigation into the kinetic inactivation of *C. difficile*, focusing on the combination of time of exposure against the active concentration of hydrogen peroxide.

Lawley et al. (2010) investigated the effects of adding 1 and 10% hydrogen peroxide solutions to pure *C. difficile* ribotype 017 spores, a non-clinically relevant strain. This group identified with the lower concentration that 75% of spores are inactivated after 1 min of exposure; there is no change in the subsequent 19 min of exposure. With 10% hydrogen peroxide, >99% inactivation was observed within 1 min, and complete kill of all spores occurred by the end of the 20 min experiment. Ribotype 017 spore strips prepared on sterile filter paper, concentration of 6-$\log_{10}$, were exposed to 400 ppm hydrogen peroxide vapour for 1, 5, 20 and 60 min. The group studied the efficiency of the hydrogen peroxide decontamination,

identifying over 75% inactivation after 5 min with *C. difficile* and 100% after 20 min. These findings contrast with the data from the *Geobacillus stearothermophilus*, which identified 60 min was required for complete inactivation, demonstrating the sensitivity of *C. difficile* spores to hydrogen peroxide in comparison.

Many studies utilise a peak exposure concentration to induce inactivation of spores, as reported by Fu et al. (2012). This methodology may not be successful in targeting all major problematic strains. There have been no reports thus far conducted which investigate whether the treatment times and their corresponding times of exposure are sufficient to eradicate multiple clinical *C. difficile* strains. It is therefore possible that exposure to a peak concentration of a limited time may result in the persistence of a few resistant spores. The effects of decontamination could therefore be rendered useless, permitting re-colonisation.

Labas et al. (2008) reviewed the effects of hydrogen peroxide in the disinfection of *Escherichia coli*. This group utilised a modified Series-Event model, which assumes a unit of microbial damage equates to an individual event. An accumulation of these 'damage-causing' events results in the inactivation, with a kinetic constant throughout. The surviving organisms are then calculated. This group found a 3-$\log_{10}$ CFU increase in the inactivation of *Escherichia coli* from 15 ppm to 300 ppm after 2.5 hr exposure of hydrogen peroxide. The experimental data matched their Series-Event model well. The authors concluded the inactivation was dependent on the concentration of hydrogen peroxide utilised, as well as increasing the concentration results in the reduction of an initial lag phase.

The literature is sparse with regards to investigations of *C. difficile* inactivation kinetics. Studies rarely compare more than one concentration of hydrogen peroxide against time, and infrequently research a range of isolated strains. This makes comparisons between findings increasingly difficult, as the concentrations of vaporised hydrogen peroxide used are often peak, rather than applied at a steady state. Therefore, there is a call for the collection and presentation of these data to allow for the identification of the parameters required to induce *C. difficile* spore inactivation.

1.6 Research aims and objectives

This introduction has thus far discussed some of the problems encountered by the healthcare industry. Surprisingly, despite the reports of *C. difficile* contamination in the hospital environment, few studies have been designed to optimise its isolation, recovery, and resuscitation. There are a number of techniques that could be employed and these are infrequently compared. It is important to monitor and identify *C. difficile* strains that are dominant and prevalent in healthcare facilities, and PCR ribotyping is a method used in the UK to characterise *C. difficile* strains. Epidemiological studies identifying strain predominance within a geographical region are also infrequently conducted. This information could be crucial in identifying the migration of strains around a country. With respect to cleaning regimes and eradication of *C. difficile* contamination, hydrogen peroxide vapour is currently being explored as a method for decontamination of healthcare facilities. However, there is a lack of kinetic inactivation data pertaining to *C. difficile* strains. The work conducted into the application of these liquid and vaporised hydrogen peroxide exposure approaches in a controlled environment is scarce.

Therefore, the main aims and objectives of the work conducted in this thesis are as follows:

i. To evaluate five different agar media prepared in RODAC plates in a novel method, based on their recovery of chemically damaged *C. difficile* spores from stainless steel tiles.
ii. To identify the differences in recovery of *C. difficile* with combinations of these five media with selective and germination agents based on their application in laboratory experiments and within hospital environments.
iii. To establish and optimise *C. difficile* environmental sampling techniques.
iv. To explore techniques in the resuscitation of *C. difficile* spores following recovery from environmental sampling.

v. To optimise and utilise PCR ribotyping as a method of characterising *C. difficile* strains present in the environment of healthcare facilities and compare these with those isolated directly from patient faecal specimens.

vi. To establish the prevalence of strains isolated from an NHS Trust with respect to spatial and temporal distribution in an epidemiological context.

vii. To identify the effects in *C. difficile* ribotype distribution within an NHS Trust following a change in the method of detection from faecal specimens.

viii. To investigate the use of controlled concentrations of hydrogen peroxide vapour as a means of *C. difficile* spore inactivation.

ix. To assess the times and concentrations of hydrogen peroxide exposure to elicit *C. difficile* spore inactivation.

x. To compare the inactivation kinetics of different ribotypes of *C. difficile* with both vaporised and liquid-form hydrogen peroxide.

1.7 Structure of the thesis

The thesis comprises of six chapters, which have been summarised:

- Chapter 1 introduces the thesis with a brief overview, highlighting potential areas of research development. It also reviews the relevant literature, with a summary of the major contributions of work to the field.
- Chapter 2 describes the model system developed to establish which media would be the most successful in the recovery and resuscitation of *C. difficile* spores following damage after chemical stress with a germicide, ChlorClean.
- Chapter 3 explains the importance of environmental sampling, with three separate studies to recover *C. difficile* from hospital sites. This chapter also explores different sampling tools, including the culturing of samples onto various agar media.
- Chapter 4 reviews *C. difficile* ribotype distributions in an NHS Trust from two distinct cohorts, exploring spatial and temporal prevalence of strains. This chapter also explores changes in ribotype diversity following the replacement of the traditional diagnostic technique with an accurate novel method.
- Chapter 5 demonstrates how controlled application of hydrogen peroxide can elicit a significant reduction in the viability of *C. difficile* spores. This chapter investigates the inactivation kinetics of spores through varying the concentration used. Hydrogen peroxide is exposed in liquid and vapour-form to ribotype 027 and three additional *C. difficile* strains.
- Chapter 6 concludes the work presented throughout the thesis and recommends future work.

Chapter 2. Media for the Isolation of *C. difficile* from the Hospital Environment

2.1 Introduction

There is significant information in literature pertaining to the best solid agar medium for the initial isolation and growth of *C. difficile* from faecal specimens or for the subsequent confirmation of the bacterium's presence (Wilcox 2006). There are also a number of studies which have utilised different methods for the recovery of *C. difficile* from various environments, for example to report on hospital contamination rates (Clabots et al. 1991; Wilcox et al. 2000; Guerrero et al. 2011). However, there are no reported studies of the most successful or ideal medium, and/or its selective agents, when conducting direct contact plate environmental sampling. It is well documented that *C. difficile* spores are able to persist on surfaces in the environment for long periods of time (Fekety et al. 1981; Kim et al. 1981; Gerding et al. 2008). These surfaces include those found in hospitals and other healthcare facilities. Resistant spores are able to survive for many months (Wilcox 1996). Therefore, it is necessary to identify a suitable medium that supports the growth of stressed *C. difficile*, facilitating the recovery, resuscitation and allowing the germination of its spores which may be damaged after experiencing these stresses.

The benefits of the different supplements used in *C. difficile* selective media have been outlined in Chapter 1, and therefore when deciding upon the five media to be used in this study, it was important to consider a range of selectivity agents. The purpose of germinants in media preparation is to aid the bacterial spores in progressing through germination and form colonies on the surface of an agar plate, which can then be enumerated. The media used here are listed in Section 2.2.3.1. In order to ensure that not all five media selected contained antibiotics for *C. difficile* selectivity, two were chosen that were considered non-selective but prepared with germination enhancers (sodium taurocholate and lysozyme).

In the UK, chlorine-releasing agents are used for routine disinfection of hospital wards and the neighbouring toilet and bathroom areas. The most common is a product called ChlorClean, its active ingredient is sodium dichloroisocyanurate (NaDCC); it is used at a concentration of 1000 ppm. Other studies have shown this cleaning product to be successful at decreasing the

abundance of *C. difficile* spores on surfaces at concentrations of either 1000 ppm or above (Ungurs et al. 2011). The series of experiments in this chapter will utilise sub-lethal concentrations of ChlorClean and observe changes in spore viability.

2.1.1 Aim of this study

This study aimed to identify the most effective media for the recovery and growth of *C. difficile* from healthcare sites. This chapter outlines how five media were used to recover *C. difficile* spores in the laboratory using a model system to mimic the hospital environment with the use of a germicide product, ChlorClean, and pick-up efficiency from the surface of a stainless steel tile. It was important to identify whether a particular combination of selective or germination agents was preferential in the recovery of spores. The work devised within this chapter describes how stressing the spores to different concentrations of ChlorClean in liquid suspension affect spore viability.

2.2 Materials and Methods

2.2.1 Anaerobic chamber

The anaerobic chamber used to culture *C. difficile* throughout this study was the Anaerobic Workstation – mini MACS [Don Whitley Scientific Ltd., UK]; anaerobic gas and oxygen-free nitrogen [BOC Ltd., UK].

2.2.2 Preparation of C. difficile spores on solid media

Stocks of *C. difficile* spores were prepared from a modified protocol based on that described by Shetty et al. (1999) and Wheeldon et al. (2011). Ribotype 027 was isolated from a faecal specimen of an infected *C. difficile* patient. Cryogenically-preserved stocks were sub-cultured onto BHI blood agar and anaerobically incubated for six days at 37°C encouraged the sporulation of *C. difficile*. Plates were removed and left in aerobic conditions over-night. The spore-vegetative cell mixture was collected and washed in 50% (v/v) IMS/PBS solution. Samples were then centrifuged at room temperature for 20 min at 4200 rpm [Beckman-Coulter, Allegra-X-22R] to separate the spores from cell debris. The pellet was re-suspended in PBS and heat-shocked for 20 min at 60°C to ensure only *C. difficile* spores were viable. Samples were serially diluted in PBS and plated onto BHI agar supplemented with 0.1% sodium taurocholate prior to incubation for 24 hr before being enumerated using the method of Miles et al. (1938). Spores were stored in PBS at 4°C until use; storage was never longer than two months. The spores were visualised under a scanning electron microscope (SEM), as described in Chapter 5.

2.2.3 RODAC plates for C. difficile recovery

One method is common for environmental sampling and recovery of *C. difficile*: the use of RODAC plates [Becton, Dickinson and Company Diagnostics, USA]. This requires the use of different media for direct surface contact. The media were prepared with an 'agar base' and the addition of supplements, such as an antibiotic mixture which prohibits growth of all bacteria except that of *C. difficile*. The use of supplements can be to enhance *C. difficile* spore germination or to inhibit the growth of the other competing bacterial species.

2.2.3.1 Composition of solid media

CCAB: Cycloserine-cefoxitin-amphotericin B with 0.1% sodium taurocholate. Prepared from Columbia agar [Oxoid Ltd., UK] supplemented with 0.1% (w/v) sodium taurocholate. Wilson et al. (1982) demonstrated the importance of high-grade sodium taurocholate to aid in the germination of *C. difficile* spores and subsequent colony formation. Following sterilisation by autoclaving, an antibiotic mixture of cycloserine (250 mg/l) and cefoxitin (16 mg/l) was added [BioConnections, UK]. Amphotericin B (0.5 mg/l) and 5% (v/v) sheep blood [both Oxoid Ltd., UK] were also added as described by Martirosian et al. (2005).

CCEY: Cycloserine-cefoxitin agar with egg yolk, also known as Brazier's Medium. Solid agar was prepared as directed and autoclaved at 121°C for 15 min. Once cooled, 5% (v/v) egg yolk emulsion and an antibiotic mixture of cycloserine (250 mg/l) and cefoxitin (8 mg/l) were added [all BioConnections, UK]. This is the most commonly used agar medium in laboratories for the isolation of *C. difficile* from faecal samples. The selectivity is provided by the antibiotics in the same concentrations as above.

CDMN: *Clostridium difficile* moxalactam-norfloxacin agar. Prepared from the *C. difficile* Oxoid base and autoclaved at 121°C for 15 min before supplementing with 7% (v/v) defibrinated horse blood, and an antibiotic mixture comprising moxalactam (64 mg/l) and norfloxacin (24 mg/l) [all Oxoid Ltd., UK].

FAA: Fastidious anaerobe agar [BioConnections, UK]. Medium was prepared according to the manufacturer's directions, with the addition of 1% Agar Bacteriological (Agar No. 1) [Oxoid Ltd., UK] and 0.1% sodium taurocholate.

GS-BHI: Glucose-supplemented Brain-Heart Infusion agar with 10 mg/l lysozyme and 0.1% sodium taurocholate. The composition of the medium was modified from Kamiya et al. (1989). BHI agar was prepared as directed with the addition of 1.5% Agar Bacteriological (Agar No. 1). Supplements of 0.8% (w/v) glucose, 0.05% (w/v) L-cysteine-HCl, 1% (w/v) soluble starch, 10 mg/l lysozyme and 0.1% sodium taurocholate were also added prior to autoclaving.

2.2.3.2 Preparation and application of RODAC plates

Agar was poured aseptically into RODAC direct contact plates. Each plate was filled with 12 ml of media. Each RODAC plate was gently pressed to a horizontal surface, applying constant uniform pressure using a 200 g weight to ensure contact of the whole agar surface with the sample area. The plates were pressed to a surface for a period of 30 sec, and then transferred to a 37°C anaerobic incubator for 48 hr.

2.2.4 RODAC plates on stainless steel tiles

To mimic the recovery of *C. difficile* from a surface, following sub-lethal damage induced by exposure to a disinfectant, a known concentration of spores was applied onto a stainless steel tile. This was then coated with ChlorClean [Guest Medical Ltd., UK] before recovery by tamping using different solid media in RODAC plates. This action imitated the sampling of bacteria from healthcare facilities' surfaces.

2.2.4.1 Preparation of the stainless steel tiles

Stainless steel tiles (30 cm x 20 cm, Grade 2B, 2 mm thick) were sterilised by autoclaving and washed with pure Teepol [Teepol, UK] and water before drying in a Class II Laminar Flow Cabinet. Tiles were soaked for 15 min in 1% (v/v) Teepol solution, a further 15 min in general purpose grade acetone [Fisher Scientific Ltd., UK] and finally thoroughly washed with 1500 ml distilled water. Tiles were left to dry thoroughly for 15 min in the laminar flow cabinet.

2.2.4.2 Application of C. difficile spores and ChlorClean

The chlorine-releasing agent used here was ChlorClean. ChlorClean tablets were dissolved in 1000 ml ultra-pure water for 1000 ppm; this was diluted accordingly for the desired concentration of germicide. A 0.1% (v/v) Tween 80-spore suspension (6-$\log_{10}$ CFU) was prepared and thoroughly mixed by vortex. The purpose of the surfactant Tween 80 was to aid the spreading of spores over a surface. Of this mixture, 200 µl was applied to a 14 cm x 14 cm area on the stainless steel tile and spread in a uniform pattern until dry. An equal volume of ChlorClean (either 100 or 1000 ppm) was added, and evenly dispersed in the same manner.

2.2.4.3 C. difficile spore recovery onto RODAC plates

The media listed in Section 2.2.3.1 were prepared in RODAC plates. Four plates were placed in quadrants within the 14 cm x 14 cm area on the stainless steel tiles for 30 sec, with 200 g weight to ensure even surface contact. The same area was sampled again a further three times using fresh plates of the same media. This was to investigate how many spores were still recoverable and viable after the initial pick-up. Plates were incubated anaerobically for 48 hr at 37°C before enumerating.

2.2.5 Effects of ChlorClean concentration and exposure time on C. difficile spores

Having studied the recovery of *C. difficile* spores with high concentrations of the germicide, the next series of experiments conducted were to investigate how a range of ChlorClean concentrations (10, 50, and 100 ppm) can affect the number of viable spores over time, stressing them to different extents. In order to examine the impact of ChlorClean interacting with *C. difficile* spores over a defined time, this was carried out over 60 min.

2.2.5.1 Preparation of liquid suspension of C. difficile spores and ChlorClean

A 0.1% (v/v) Tween 80-*C. difficile* spore mixture was prepared. This mixture was individually added to the ChlorClean concentrations and aliquots taken at times (min) t = [0, 1, 5, 10, 15, 30, and 60]. The samples were then centrifuged at 21,000 x g for 5 min before removing the supernatant and re-suspending the pellet in an equal volume of PBS.

2.2.5.2 C. difficile spore recovery

Spores were recovered from the suspension by applying and spreading 50 µl of sample onto 55 mm Petri dishes prepared with BHI agar and 0.1% sodium taurocholate plates until dry. These were placed in the anaerobic workstation overnight at 37°C, before enumeration and calculating CFU.

2.2.6 Statistical analyses

Minitab 16 [Minitab Inc., USA] was utilised to perform statistical analyses on the data obtained from these experiments. Both Minitab 16 and Microsoft Excel

were used to create graphical representations of these data. Significance was set to $P<0.05$. The *F*-test was used with a one-way analysis of variance. A two-sample t-test compared differences of mean CFU.

2.2.7 Ethical issues

Ethical approval was obtained for the use of the strain in this chapter. All information regarding patient data was made anonymous prior to use.

2.3 Results

2.3.1 Comparison of media for the recovery of C. difficile spores

To date, only a few reports have compared different *C. difficile* media and their selectivity and ability in recovering spores from environmental sites (Buggy et al. 1983; Wilcox et al. 2000; Sorg & Sonenshein 2008; Rousseau et al. 2010). These groups used a limited number of media, with research groups often using the same medium, with little to no guidance as to why they were chosen, nor were they compared to other media in the experiments conducted. Therefore, the aim of this work was to determine how the different supplements in selective media alter the recovery of *C. difficile* from physical environmental surfaces. This is in contrast to the extensively studied *C. difficile* isolation from faecal samples (Borriello & Honour 1981; Levett 1985; Health Protection Agency 2008).

C. difficile spores require sodium taurocholate to complete germination and develop into colonies (Wilson et al. 1982). It was therefore decided to incorporate this as a germination agent in three of the five media tested. Antibiotics are standardly used in media as *C. difficile* is naturally resistant and therefore their incorporation would increase selectivity. Over the past twenty years, only a few groups have studied *C. difficile* spore survival from stainless steel surfaces in an endeavour to mimic the processes observed within a healthcare facility (Pinto et al. 2009; Ungurs et al. 2011). Based on the lack of literature at the time of these studies being conducted, it was therefore decided to attempt to apply *C. difficile* spores onto stainless steel tiles and subsequently recover them.

In order to investigate both the spore survival and media selectivity with regards to recovering the damaged spores, an experiment was designed where spores were aseptically evenly dispersed onto stainless steel tiles and subsequently recovered using direct contact plates which were prepared with different media and selective agents. To optimise the media in the least complicated manner, the tiles were exposed to only the spore stock of *C. difficile*, and not a soil culture or mixed preparation consisting of other organisms. This could have been conducted by emulating biological soiling with 0.3% bovine serum albumin, as demonstrated (Otter & French 2009). This would eliminate the

possibility of competition from other bacteria on the solid agar surface. This would serve to give a clear indication of how well the media could recover *C. difficile* from a surface with no other selection pressures. It was also important to use a spore stock prepared of PCR ribotype 027 due to the severity, relapse rate, and mortality associated with this particular strain (Kuijper et al. 2008).

The application of 200 µl of a 6-$\log_{10}$ CFU spore stock onto the stainless steel tiles can theoretically produce a maximum possible recovery on a given RODAC plate of 4.41-$\log_{10}$ CFU. This recovery demonstrates the limitations of this methodology, as it is impossible for 100% of the spores applied to the surface to be recovered with a simple tamping of a RODAC plate. This was calculated using the concentration of the spore stock with the areas of the exposed tiles and the contact plates themselves.

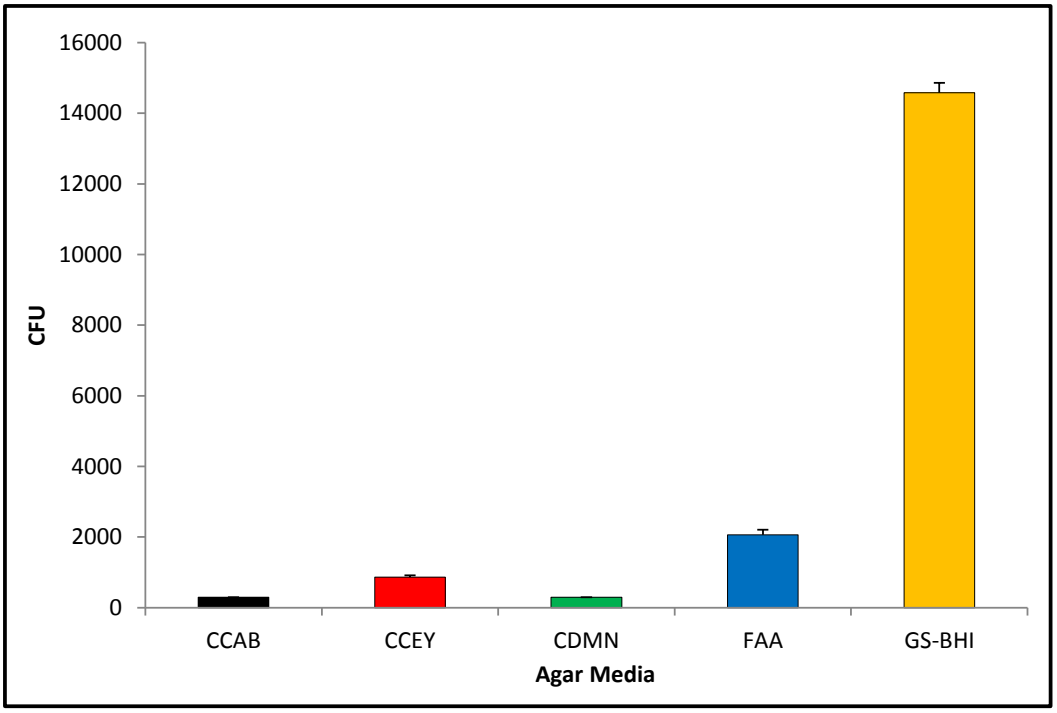

Figure 2.1: Recovery of *C. difficile* PCR ribotype 027 spores on various agar media following tamping from stainless steel tiles with RODAC plates.

The bars indicate averages from eight replicates in one independent experiment and the error bars indicate the standard errors of the means. CFU: colony-forming units.

GS-BHI agar was the most successful medium in this study (Figure 2.1), picking up 57.2% of the expected recoverable spores. The lack of antibiotics in this medium does not appear to inhibit its selectivity in the pick-up of spores from the stainless steel tiles, providing nutrients and necessary supplements for the

germination into countable colonies. FAA recovered 8.1% of the surface spore concentration, and despite additionally comprising of only sodium taurocholate, it recovered more spores than the selective media. The selectivity of the antibiotic-containing media was expected to result in a higher pick-up rate than found due to their use by many research groups; the recovery ranged from 1.17% to 3.38% of the expected. The combination of cycloserine and cefoxitin, accompanied with egg yolk in the CCEY agar was more successful than that of CCAB, containing the relatively uncommon antibiotic, amphotericin B. Surprisingly, despite its success rates in previous studies conducted in healthcare facilities, recovering spores from the surfaces in the environment, CDMN recovered substantially fewer spores than the other media (Alfa et al. 2008). In addition to this, statistical analysis has shown only CDMN and CCAB were similar (P=0.106) in their ability to recover *C. difficile* spores; all other media are significantly different from one another (P<0.05).

Having established the maximum numbers of recovery for each solid agar with application of spores onto the tiles, the next step was the exposure of the *C. difficile* ribotype 027 spores to ChlorClean by spreading both solutions onto stainless steel tiles. This was to investigate the effects of a common chlorine-based cleaning agent used in hospitals to eradicate problematic bacteria, such as MRSA and *C. difficile*. A sub-lethal concentration was used to ensure the spores were in a moribund state, rather than inactivating them completely. It also served to identify differences in media selectivity following imposing stress onto the spores, not only in pick up efficiency, but also for the subsequent germination of spores.

This experiment was carried out using the methodology described previously (Section 2.2.4), and the spreading of ChlorClean, prepared at 100 ppm, onto autoclaved stainless steel tiles. Figure 2.2 shows the counts of *C. difficile* spores recovered from an initial tamping of the agar onto the stainless steel tiles and the five media following exposure to this concentration of the germicide.

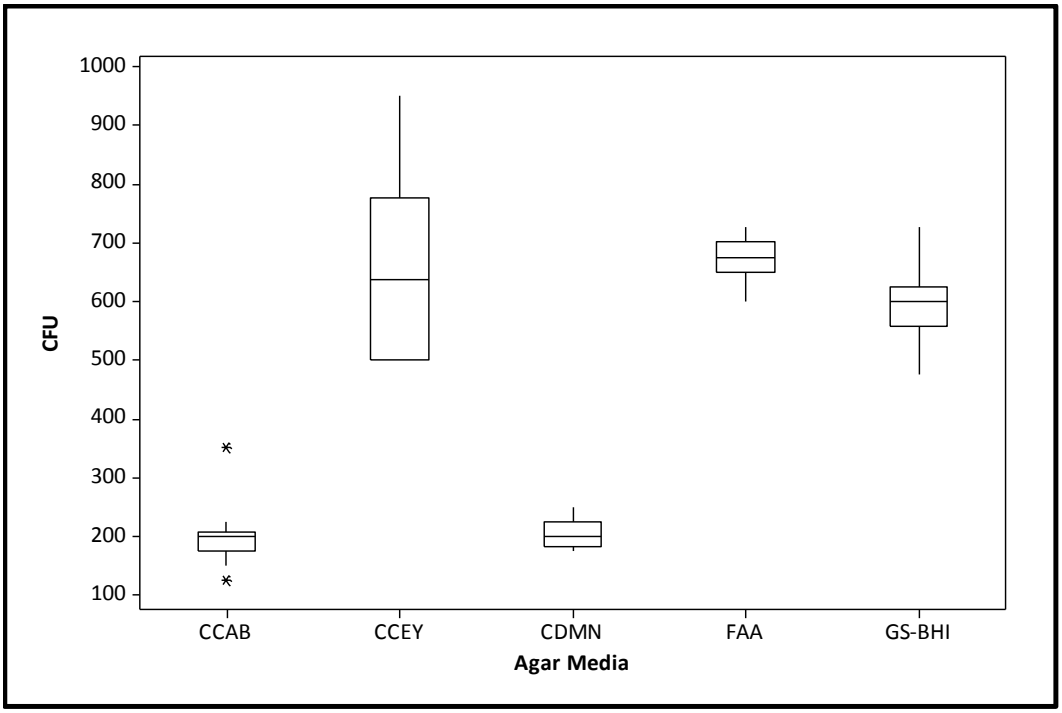

Figure 2.2: Distribution of the five agar media and the ability to recover *C. difficile* spores from stainless steel tiles following exposure to 100 ppm ChlorClean.

The bars indicate averages from a minimum of sixteen replicates in one independent experiment the averages from one independent experiment. '*' - denotes outliers. CFU: colony-forming units.

There is a difference between the counts, however, overall CCEY appears to be the most successful medium at recovering damaged *C. difficile* from one tamping using RODAC plates from stainless steel tiles. Despite the mean CFU recovered from FAA being slightly higher, the data show no significant difference between CCEY and the non-selective FAA (P=0.615).

Of the *C. difficile* selective media, CCEY agar clearly yields the highest spore recoveries; CDMN and CCAB recovered only 0.82% and 0.77% respectively of the number of *C. difficile* spores present on the surface. In the previous experiment with no exposure to a germicide, these two media picked up 1.17% of the spores applied onto the tiles. Although a decrease was expected with the addition of ChlorClean, the efficiency of spore recovery using the maximum possible CFU and the initial control data show CCAB led to the recovery of 65.8% and CDMN 69.6% (Table 2.1).

With the non-selective media, following exposure to ChlorClean, FAA appears to be better at resuscitating damaged *C. difficile* spores, however the pick-up efficiency of these media was much lower than that of the selective media:

32.7% (FAA) and 4.1% (GS-BHI). Despite its initial 57.17% recovery of spores from a single tamping with no germicide exposure, GS-BHI agar was only able to pick up 2.32% upon inducing spore stress. Nevertheless, the non-selective media were once again found to be more capable of recovering spores than CDMN and CCAB.

Statistical analysis has identified significant similarities between different media from this experiment. CCAB and CDMN counts (P=0.360) differed from those obtained from any of the other media tested. CCEY agar was found to be similar to FAA (P=0.615) and GS-BHI (P=0.099), although the counts obtained from FAA and GS-BHI showed these two media to be statistically different.

Agar Media	**No ChlorClean**	**100 ppm ChlorClean**	**Success Rate (%)**
CCAB	298 ± 1	196 ± 11	65.8
CCEY	863 ± 54	655 ± 35	75.9
CDMN	299 ± 1	208 ± 6	69.6
FAA	2061 ± 147	673 ± 9	32.7
GS-BHI	14583 ± 281	591 ± 13	4.1

Table 2.1: Colony counts of *C. difficile* recovered from stainless steel tiles with RODAC plates prepared with different agar media.

Spores were recovered with and without exposure to 100 ppm ChlorClean. Data represent mean ± standard errors of the mean. Recoveries was calculated using means of 100 ppm ChlorClean and means of controls as total possible counts recoverable for given medium.

Following the initial tamping of the agar media onto the stainless steel tiles to recover the *C. difficile* spores, freshly prepared RODAC plates corresponding with the same media, were subsequently tamped onto the same area three more times (Figure 2.3). The method for using and incubating the RODAC plates was the same as previously described.

For all media, repeated tamping of a given area resulted in the number of spores recovered decreasing suggesting that fewer spores were remaining on the face of the tile, but also the ability of the media to actually make contact and have the spores adhere to its surface was declining (Figure 2.3). CCAB and CDMN agar recovered the least spores, and with repeated tamping, demonstrated somewhat of a steady decline in pick-up.

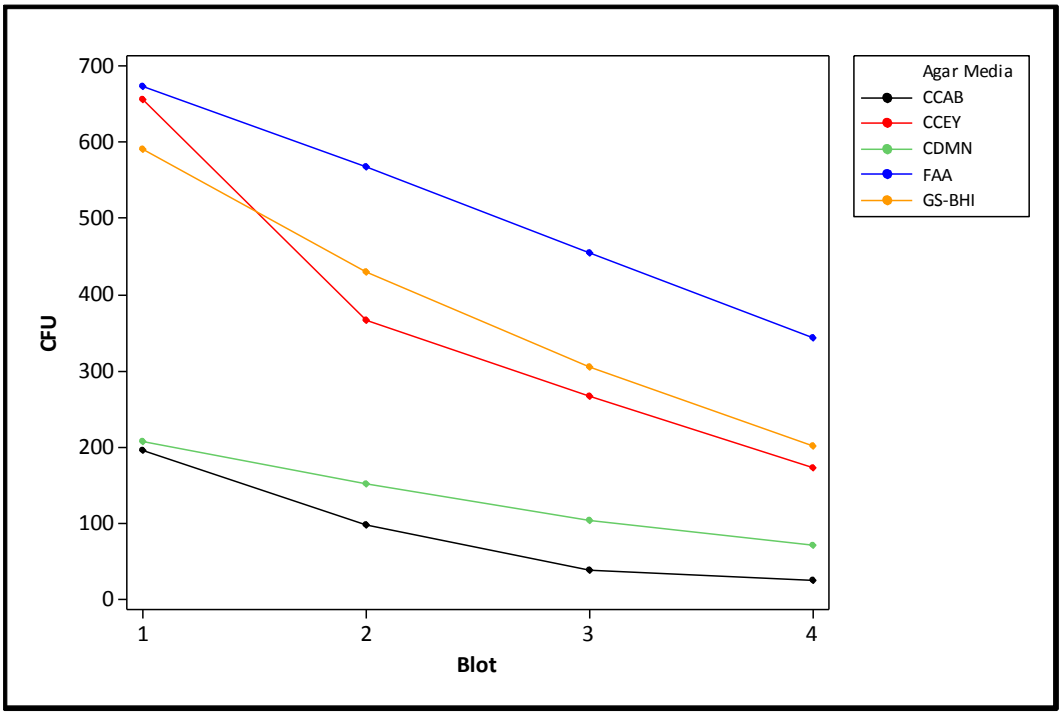

Figure 2.3: *C. difficile* spores recovered as colonies by five different agar media from stainless steel tiles following exposure to 100 ppm ChlorClean.

Areas of recovery were blotted three further times. Data points indicate averages from a minimum of sixteen replicates in one independent experiment. CCAB (black), CCEY (red) and CDMN (green) are selective media; FAA (blue) and GS-BHI (orange) are non-selective media. CFU: colony-forming units.

Regression analysis for all of the media was calculated based on the average counts produced for each agar medium. The data produced suggested that following a further tamping of the tiles with either CCAB or CDMN, the recovery of *C. difficile* spores would no longer be expected. Interestingly, the regression analysis also showed CDMN and CCAB possess similar trend lines and appear to recover at least three times fewer spores with each tamping than the other media. These two media also produced similar rates of recovery with no germicide applied onto the tiles. It has also been estimated that a sixth blot would be required in order to recover the maximum capable of CCEY and GS-BHI, and seven for FAA. These figures are the theoretical number of tamping required to recover the entire population. This does not mean the media in question would have been able to recover all of the spores applied onto the surface of the tile by the corresponding tamping. However, merely judging by the decline in the number of spores recovered with each subsequent RODAC plate, it could be inferred that that particular medium would be unlikely to be able to resuscitate and germinate any more *C. difficile* spores.

When comparing all of the data generated in Section 2.3.1, statistical analyses show that for each of the five media, there is a statistical difference between the counts obtained from exposing the *C. difficile* spores to the germicide and those from the initial recovery test ($P<0.05$). Therefore, these data show the application of a NaDCC has either had an impact on the ability for the media to pick up and recover the spores or reduced the number of spores viable and possible to adhere to the surface of the agar plate to germinate; most importantly, it has an overall impact in that it is killing spores.

2.3.2 Recovery of C. difficile spores with an increased germicide concentration

The experiment described above allowed the testing of the different media to be compared based on their recovery of *C. difficile* spores after exposure to 100 ppm ChlorClean disinfectant. The aim of this study was to identify a medium which would possibly be able to recover the maximum number of viable *C. difficile* spores from a hospital environment. Therefore, to attempt to mimic cleaning routines in hospitals, which utilise ChlorClean at 1000 ppm, two of these five media (CCEY and GS-BHI) were tested for recovery efficiency at this concentration. The choice of media was based on the high levels of recovery exhibited by CCEY agar in the previous experiment with the exposure of ChlorClean at a sub-lethal level, and the less studied non-selective medium (GS-BHI), containing two different *C. difficile* spore germinating agents.

The methodology described in Section 2.2.4 was utilised as before, altering only the concentration of the germicide. The CFU counts observed for CCEY agar at the two different concentrations of ChlorClean are compared in Figure 2.4. There was no significant difference in spore recovery ($P=0.090$); the variances were similar (24052.6 for 100 ppm and 22572.9 for 1000 ppm). The data obtained in the previous experiment comparing CCEY agar with the other media in this study showed there was no statistical difference between CCEY and the two non-selective *C. difficile* media. Taken together, these data demonstrate that this medium is likely to be able to recover damaged *C. difficile* spores from a hospital environment and allow them to fully germinate, regardless of disinfectant strength.

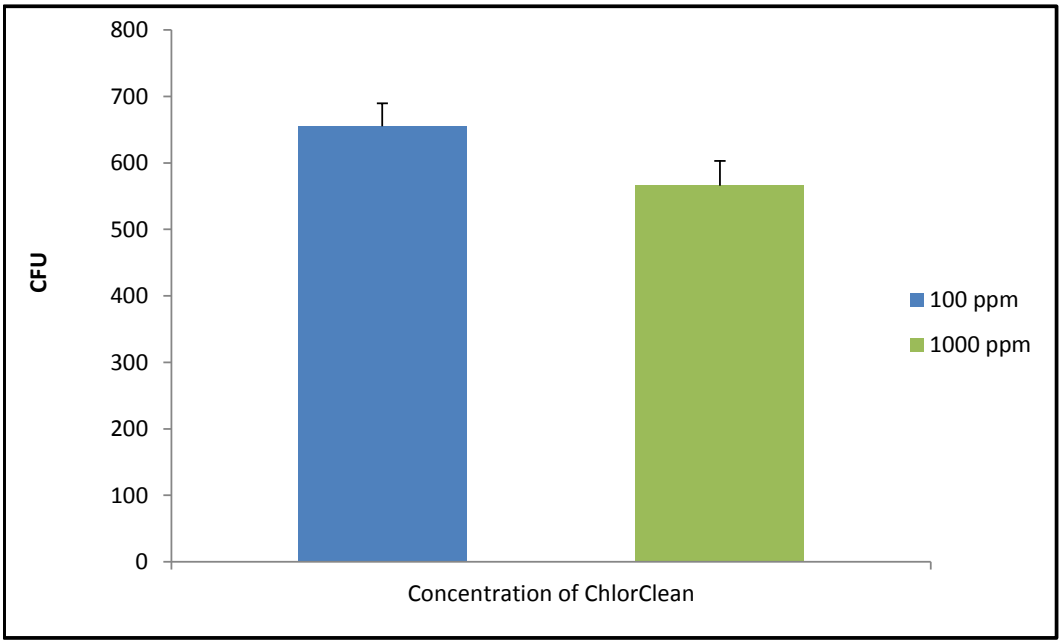

Figure 2.4: *C. difficile* spores recovery from stainless steel tiles following exposure to different ChlorClean concentrations (100 ppm and 1000 ppm) using CCEY agar in RODAC plates.

Concentrations: 100 ppm (blue) and 1000 ppm (green). Bars indicate averages from a minimum of sixteen replicates in three independent experiments and the error bars indicate the standard errors of the means. CFU: colony-forming units.

C. difficile spore recovery with GS-BHI agar was recorded in the same manner (Figure 2.5). Despite a relatively small variation in the mean number of colonies recovered, there was a significant difference between the two different germicide concentrations investigated (P=0.023). Therefore, despite its pick-up efficiency following 100 ppm ChlorClean in the initial stainless steel tiling experiment, it appears application of ChlorClean at an increased concentration reduces spore recovery; the variances between these two experiments (3570.7 for 100 ppm and 26022.8 for 1000 ppm) differed almost ten-fold. Application of an increased concentration could therefore act to increase the inconsistency of spore recovery. Analysis of *C. difficile* spore recovery subsequent to stress exerted from the increased concentration of NaDCC for CCEY and GS-BHI agar suggest the relative pick-up abilities to be similar (P=0.305).

These studies show that *C. difficile* spores are able to survive the application of 1000 ppm ChlorClean when applied onto a solid surface. These data also demonstrate that CCEY and GS-BHI media are able to yield high recovery of spores, allow efficient germination and proliferation for calculations using CFU.

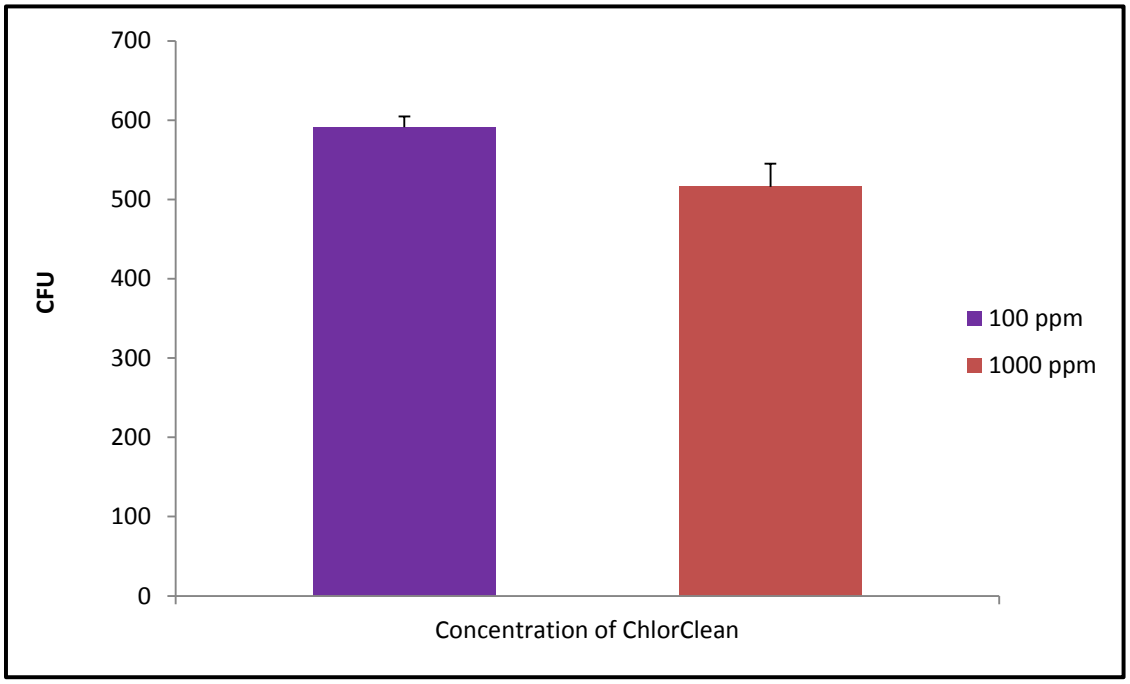

Figure 2.5: *C. difficile* spores recovery from stainless steel tiles following exposure to different ChlorClean concentrations (100 ppm and 1000 ppm) using GS-BHI agar in RODAC plates.

Concentrations: 100 ppm (purple) and 1000 ppm (red). Bars indicate averages from a minimum of sixteen replicates in three independent experiments and the error bars indicate the standard errors of the means. CFU: colony-forming units.

2.3.3 Investigating exposure of ChlorClean to C. difficile spores over time

Thus far, the experiments outlined in this chapter have been based on studies investigating the effect of exposing spores to ChlorClean and then subsequently recovering by means of applying a RODAC plate containing agar media. This study has demonstrated the effects ChlorClean has on *C. difficile* spores over time, even at sub-lethal concentrations. The advised working concentration for this product is 1000 ppm, as previously stated. For the purpose of this experiment, doses of 10, 50, and 100 ppm were employed again for investigating the effects of sub-lethal concentrations; these experiments were conducted in liquid suspension. This would also provide an insight into how changes in lower concentrations of ChlorClean can affect the proportion of viable spores.

C. difficile spores were prepared as previously described (6-$\log_{10}$ CFU) and exposure to an equal volume of 10, 50, or 100 ppm ChlorClean. At specific time intervals, these samples were centrifuged, the pellet washed before re-suspending in an equal volume of PBS and then plated and spread onto BHI agar with 0.1%

sodium taurocholate and incubated anaerobically overnight; colonies were enumerated and expressed as CFU (Figure 2.6).

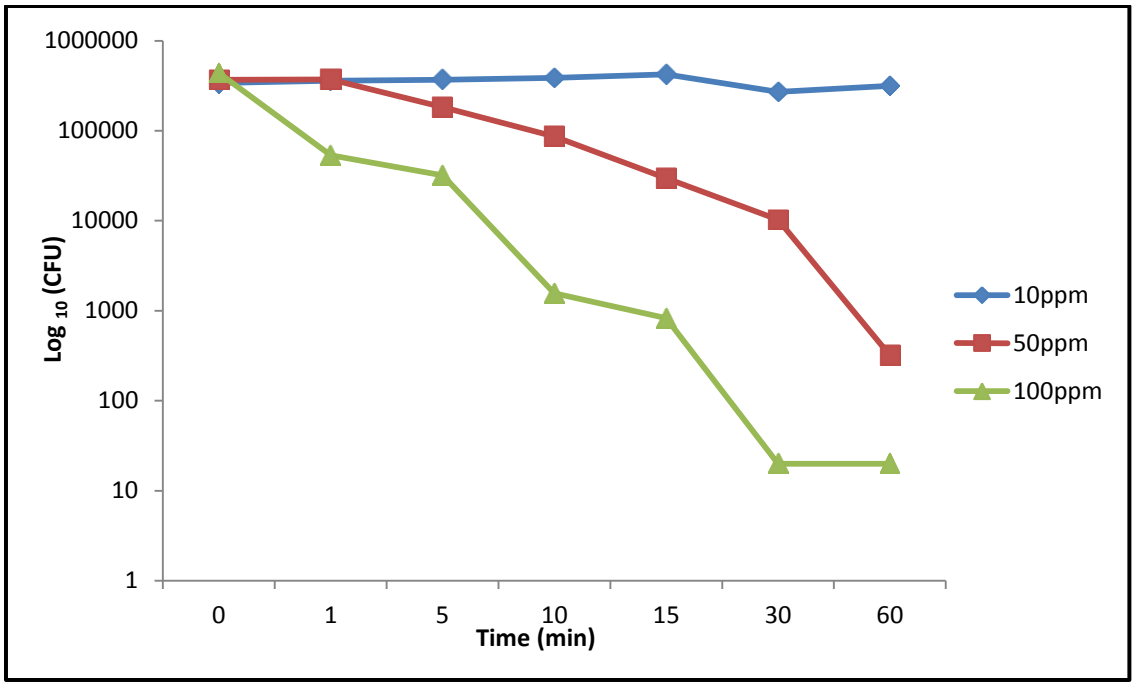

Figure 2.6: Semi-logarithmic scale representation of ribotype 027 spore recovery following exposure to different ChlorClean concentrations over 60 min.

Concentrations: 10 ppm (blue), 50 ppm (red) and 100 ppm (green). The data points indicate the averages of a single experiment carried out with a minimum of three repeats. CFU: colony-forming units.

The application of 10 ppm ChlorClean does not appear to have a great effect in reducing the number of *C. difficile* spores. However, the CFU was lower than the initial inoculum and greater than with 1 ppm ChlorClean (data not shown). Over time, the addition of 10 ppm had little effect; the counts remained similar. Increasing the germicide concentration to 50 ppm elicited a reduction of the spore population, despite being twenty times more diluted than the recommended working concentration. Almost a 2-$\log_{10}$ CFU reduction was seen over the initial 30 min, with a further 2-$\log_{10}$ CFU reduction after 60 min ChlorClean exposure from the recovery at t=0. Despite the preparation being ten times diluted than with cleaning, 100 ppm ChlorClean resulted in a steep decline in the number of spores recovered over time. A 3-$\log_{10}$ CFU reduction was seen after 10 min exposure and after 30 min, the recovery is negligible, with one colony recorded over three repeats. There was a marked difference of 4-$\log_{10}$ CFU between the counts following exposure to 10 and 100 ppm ChlorClean after 60 min.

2.4 Discussion

Despite the diversity of solid media used for culturing and propagation of *C. difficile* from faecal samples, to date, there is no definitive selective media recommended for the recovery of the bacterium from environmental sites. Different research groups tend to use either one or two media in their work, each with its own merits with respect to supplementary agents. Thus far, there is little guidance provided in the literature on media for environmental sampling, and no reports which compare different media for *C. difficile* isolation from potentially contaminated surfaces.

Within this chapter, the importance of investigating selective media for the recovery of *C. difficile* spores has been highlighted. Five different media were tamped onto stainless steel tiles contaminated with *C. difficile* ribotype 027 spores, both with and without the use of a sub-lethal concentration of a chlorine-containing germicide, ChlorClean. All the media chosen had been identified in literature as being successful in *C. difficile* spore recovery and germination, either from surfaces in healthcare facilities or in the laboratory. The effect of directly adding sub-lethal concentrations of ChlorClean to the *C. difficile* spores has also been investigated in this chapter. This was to identify how contact time with sub-lethal levels of the germicide can affect the number of *C. difficile* spores which can be recovered.

The results from this study show that whether in the presence of the germicide or not, the success rates of agar media differ in their abilities to recover *C. difficile* spores. However, a difference is clearly observed in the recovery following the killing of spores with ChlorClean. Stressing the spores as such would then allow for the identification of the medium most successful at resuscitation. When investigating recovery success, Pinto et al. (2009) suggested that a maximum of 56% of the spores applied onto a surface can subsequently recovered from stainless steel discs. This would imply that of the 6-$\log_{10}$ CFU of *C. difficile* spores initially applied to the stainless steel tile, 4.41-$\log_{10}$ CFU would be expected to be the maximum recoverable with the use of direct agar media contact. Based on this estimation, when investigating the different media, GS-BHI was the most

successful agar at recovering *C. difficile* spores from the stainless steel tiles with 57.17% of the applied spores recovered by the medium. Upon applying 100 ppm ChlorClean, the medium recovered fewer spores; only 2.32% of the original inoculum could be recovered with single tamping of media. Using GS-BHI agar to recover *C. difficile* spores following the exposure to 1000 ppm ChlorClean resulted in recovery of 2.02% of the initial inoculum. Therefore, although GS-BHI agar appeared to be the most successful medium tested for *C. difficile* spores recovery from contaminated stainless steel tiles, there was a significant effect on the CFU recovered following ChlorClean application. The spore germinants that were present in GS-BHI agar could therefore enable the spores recovered to complete germination into colonies. It is also possible that the lack of antibiotics could result in a lack of *C. difficile* selectivity when used in a setting with other competing spores.

Despite the recovery of spores being notably lower with FAA than GS-BHI agar in the control study, FAA still recovered significantly more counts than the selective *C. difficile* media investigated (2061 ± 147). The number of spores recovered when tamping the contaminated surface with FAA medium showed that not only was it similar to that of CCEY agar with the exposure of 100 ppm ChlorClean, but it was the medium recovering the highest mean number of colonies (673 ± 9) under conditions of germicide exposure.

When testing CCEY agar for spore recovery from the contaminated surface, the exposure of spores to 100 ppm ChlorClean again resulted in a 25% reduction in CFU, and when increasing the chlorine agent to 1000 ppm, there is a further reduction of 14%. These results suggest that despite the significantly low recovery of spores from the initial inoculum (3.38%), upon stressing the spores CCEY agar is still able to recover a similar proportion (2.57%); with the application of 1000 ppm ChlorClean, recovery remained at 2.22%. The maintenance of spore recovery at increasing ChlorClean concentrations was unique among the media compared here.

Martirosian et al. (2005) reported 12.2% and 36% recovery of *C. difficile* samples with CCAB after environmental sampling. CCEY agar was used by Shapey

et al. (2008), who reported following hospital sampling, 24% of isolates were positively identified as *C. difficile*. The combination of moxalactam and norfloxacin in CDMN was shown to improve selectivity and the recoverability of *C. difficile* over cycloserine-cefoxitin fructose agar with a 20% higher isolation rate (Aspinall & Hutchinson 1992). Alfa et al. (2008) also reported a rate of 33% positive cultures (*n*=102) recovered from toilets of CDAD patients, despite the introduction of an optimal cleaning technique devised by this group, with UV markers. Wheeldon et al. (2008) tested five different bile salts in a series of experiments to investigate the recovery of *C. difficile* spores, identifying the most successful combination to be FAA with 0.1% sodium taurocholate. Kamiya et al. (1989) used two compositions of GS-BHI agar to recover *C. difficile* spores following a series of heat and alkali treatments: either 10 mg/l lysozyme (success rate: 10-47% and >90%) or 0.1% sodium taurocholate (<1% and <6%). Despite the low success rate with the bile salt, for the purpose of this work it was thought a combination of the supplements might aid in the germination of spores.

A decline in bacterial spore recovery with the addition of a chlorine-based cleaning agent is expected, as this is the purpose of the product. However, the level of which the media were unable to recover *C. difficile* was surprising. CCAB and CDMN were able to recover 65.8% and 69.6% of the original recovered CFU with the addition of ChlorClean, however for the non-selective *C. difficile* media, FAA and GS-BHI, the efficiency was much lower: 32.7% and 4.1%, respectively. This is despite the success rates reported in literature.

The use of antibiotics in the media preparation has not necessarily increased the proportion of *C. difficile* spore recovery and despite being integral to their composition, CCAB and CDMN agar were unable to recover as many spores as the other tested media. Amphotericin B, a little known antibiotic with respect to media, but often administered and linked to *C. difficile* infection has not been as capable in the selection of *C. difficile* spores as expected (Gerding et al. 1995). This is interesting as the other two antibiotics within CCAB, cycloserine and cefoxitin, were able to elicit higher levels of recovery in CCEY agar. A low recovery of spores was also observed with the use of the moxalactam and norfloxacin in CDMN agar;

the antibiotics were presumed to aid in selectivity of *C. difficile* spore recovery, however this did not appear to hold true when comparing these media to CCEY, or even the non-selective agars.

These data suggest that the incorporation of antibiotics could have elicited somewhat of an inhibitory effect on the ability of *C. difficile* recovery. Perhaps, it could also be inferred that the use of antibiotics may select for completely viable spores, permitting these to germinate, as opposed to spores which have endured physical damage for example and are less lusty but are not inactivated. The effect of this is that it would result in the number of CFU being lower as spores that are not entirely viable would not be selected. This could therefore be problematic if attempting to recover *C. difficile* spores from surfaces in a healthcare environment.

The ability of GS-BHI agar to recover *C. difficile* as successfully as it had could be attributed to the addition of lysozyme and sodium taurocholate. Lysozyme use in a media has been well-documented within research groups and found to subsequently increase the recovery of *C. difficile* through spore germination stimulation (Wilcox et al. 2000; Verity et al. 2001). The addition of sodium taurocholate into agar bases also increases spore recovery. Buggy et al. (1983) identified up to six times more viable *C. difficile* spores were recoverable with the incorporation of sodium taurocholate into media. The use of this bile salt could also be the reason why the second non-selective media in this study, FAA, was able to recover *C. difficile* spores and complete germination as successfully.

The addition of a chlorine-based cleaning product is vital in the overall reduction of *C. difficile* spore contamination on a hard surface; the data obtained in this chapter emulated previous results. Ungurs et al. (2011) investigated concentrations of a NaDCC-based germicide of a minimum of 1000 ppm, with an upper value of 6000 ppm. For the purpose of this study, ChlorClean was selected as it is commonly used in the routine clean of a healthcare facility, and its incorporation of NaDCC has been shown to be more effective against the survival of *C. difficile* spores than a standard hypochlorite solution (Fawley et al. 2007). The work here also used lower concentrations to subject the spores to stresses, which would not result in a complete ablation of the number of viable spores. In

order to compare the different media investigated, it was therefore important to demonstrate a low level of stress, but not too great an amount.

A study conducted by Wheeldon et al. (2008) reported that with 1000 ppm of available chlorine, there was a significant 2.76 to 2.96-$\log_{10}$ CFU reduction of spores recovered on FAA with 0.1% (w/v) sodium taurocholate and 5% (v/v) defibrinated horse blood, with a contact time on the surface for 15 to 30 min. Another group, Perez et al. (2005), identified more than a 6-$\log_{10}$ CFU reduction in spore viability with a sodium hypochlorite solution of 1000 ppm chlorine with a contact time of only 15 to 20 min. Comparing the data from these two studies, suggests variations may well exist between studies and experiments. Much like other decontamination studies of this nature, this work has demonstrated the use of chlorine-based cleaning products, such as ChlorClean, is vital in the cleaning process for the reduction of viable spores. The results here have shown there to be statistically significant differences with CFU recovered when comparing even a sub-lethal concentration to that of a control.

Another aspect of *C. difficile* spore recovery investigated in this study was that of repeated tamping of agar media prepared in RODAC plates onto the contaminated surface of the stainless steel tile. The results demonstrate that the total number of spores, which are exposed to the surface of the agar following the exposure to ChlorClean, to form colonies, declines with each additional further tamping. This could be due to the number of viable spores capable of adhering to the agar decreasing with each subsequent contact of the agar. Data from Ungurs et al. (2011) suggests that with the spreading of *C. difficile* spores onto a stainless steel tile and attempting to remove them with a chlorine-releasing agent akin to ChlorClean, there was a decrease in the viability of spores. This group reported as little spore reduction with 1000 ppm NaDCC. When the concentration was increased to 3000 ppm, they reported a reduction of 1.51 to 2.59-$\log_{10}$ CFU reduction between 20 and 120 min exposure. This was higher still with 6000 ppm: 2.39-$\log_{10}$ CFU reduction at 2 min with total kill of all spores by 20 min. These data suggest a much higher concentration is required with direct contact onto a surface in order to elicit a response or substantial reduction in spores. The results

from this group confirm the result from this body of work: longer exposure time of chlorine results in more inactivation of *C. difficile* spores.

The effect of ChlorClean over time has also been studied in this chapter, and these data support that a longer exposure time to the germicide proves to be more lethal. This study also aimed to identify the effects of employing sub-lethal concentrations of ChlorClean to *C. difficile* spores over a time course of 1 hr in liquid culture, as opposed to dried onto a surface. These data demonstrate there is a clear difference in the inactivation of *C. difficile* spores and the subsequent proportion which can be recovered using the chosen sub-lethal concentrations of ChlorClean. The higher the concentration of ChlorClean applied to the spores, the more significant the reduction in viability observed. There was no $\log_{10}$ CFU reduction observed over the 1 hr time period for 10 ppm, but with 50 ppm, a 1-$\log_{10}$ CFU reduction was recorded after only 10 min exposure. By the end of the time course, 50 ppm ChlorClean had resulted in a 3-$\log_{10}$ CFU reduction of *C. difficile* spores. This was also observed with 100 ppm, however, the initial recovery of spores at t=0 for 50 ppm was 5.57-$\log_{10}$ CFU, with 100 ppm as 4.62-$\log_{10}$ CFU. This suggests that the effect of adding ChlorClean impacts the viability of spores instantly; there is no delay in impact.

It can therefore be assumed that the spores are placed under stress with the addition of ChlorClean, either when deposited onto stainless steel tiles as above, or upon contacting with the disinfectant liquid form. These results also suggest that with more contact time with the germicide, there is an overall reduction in the number of viable spores present in solution and therefore able to successfully complete germination.

Numerous studies have shown that using NaDCC concentrations of 1000 ppm, as well as above this, are able to successfully elicit reductions in the spore titre recovered subsequent to exposure (Jeanes et al. 2005; Perez et al. 2005; Dawson et al. 2011; Ungurs et al. 2011). The results from this study differ in that they have shown that even with lower levels and doses of the cleaning agent, statistically significant reductions in spore viability can be observed. Despite this being seemingly obvious, the 3-$\log_{10}$ CFU reduction of spore recovery with 100

ppm from *t*=0-60 min was faster than expected. This would suggest the level of chlorine activity in the product is still sufficiently high enough to reduce the viability of the *C. difficile* spores and in turn inactivate them. This is not to suggest such low concentrations in healthcare facilities should be employed, but it confirms that, even at lower levels, there is some reduction of spore viability. Nevertheless, it is important to note that too high a concentration of ChlorClean could compromise safety. As it is used in routine cleaning, the patients, healthcare workers, and cleaning staff could be put into damage with exposure to high concentrations of ChlorClean.

The experimental work presented in this chapter is based on spore recovery data obtained from the use of one strain of *C. difficile*, ribotype 027. This particular strain was selected for use in this project due to its and persistence within healthcare facilities and in the community (Bauer et al. 2011; Health Protection Agency 2011). It is important to bear in mind that the case may well be that differences exist between strains in susceptibility to a range of disinfectants and cleaning treatments, or perhaps more specifically to the NaDCC-based product used, ChlorClean (Dawson et al. 2011). Recent studies investigating *C. difficile* spore recovery following various cleaning treatments have used different strains: Ungurs et al. (2011) used *C. difficile* strain NCTC 11209, while Wheeldon et al. (2008) used ribotype 027 as did Horejsh & Kampf (2011) and Siani et al. (2011).

Identification of the most successful medium in recovering damaged and stressed spores would therefore provide information into the components required to recover the spores from the hospital or healthcare environment, and its surfaces. The results found in this chapter illustrate the importance of identifying a means of recovering *C. difficile* from the environment using agar media. As discussed above, CCEY agar appears to be the most successful; in terms of statistical analysis, this agar was capable of recoveries the same as the non-selective media GS-BHI and FAA. CCEY agar also possessed the additional factor of antibiotics incorporated within the medium, which appeared to aid in selectivity when stress was applied onto the *C. difficile* spores. Despite the findings of FAA and GS-BHI agar successfully recovering *C. difficile* spores in this study, it must be

born in mind that when utilising these in the healthcare environment for detection of *C. difficile* contamination, there would be an absence of antibiotics. This could result in a lack of selectivity for *C. difficile* and may allow the thriving and proliferation of other organisms. Therefore, although it has been documented as the most successful medium when attempting to isolate and culture *C. difficile* from faecal samples, this work suggests that CCEY agar would also be the most suitable when conducting environmental sampling.

2.5 Conclusions

- A model system was developed to compare the spore recoveries between five different agar media, with and without a germicide. GS-BHI agar recovered over 57% of the *C. difficile* spores applied to the tile surface, but only 2% with ChlorClean usage. FAA was also successful in spore recovery, despite the lack of antibiotics for means of selectivity (8%).
- CCEY agar significantly recovered *C. difficile* spores (655 ± 35 CFU) more efficiently and consistently than the other two selective media, CCAB (196 ± 11 CFU) and CDMN (208 ± 6 CFU) with 100 ppm ChlorClean. This was the most successful selective media.
- Despite the addition of 1000 ppm ChlorClean, CCEY and GS-BHI agars were able to recover *C. difficile* spores, 565.6 and 516.1 CFU, respectively.
- Exposing ribotype 027 spores directly to liquid ChlorClean (10, 50, and 100 ppm) resulted in inactivation of spores as the concentration increased. Fewer spores germinated and form colonies over time. With a twenty-fold dilution of the recommended concentration, a 3-$\log_{10}$ CFU reduction of spores can occur within 60 min, demonstrating a significant reduction can be elicited with a smaller dose of ChlorClean.

Chapter 3. Environmental Sampling of *C. difficile*

3.1 Introduction

The role of environmental contamination has been implicated in the spread of HCAI, however there is still considerable controversy surrounding this, in addition to a number of complicating factors. Reports in literature suggest rooms of *C. difficile* infected patients are frequently contaminated with the infecting organism and that the proportion of environmental samples from such rooms that test positive for *C. difficile* ranges between 9% to 59% (Barbut et al. 2009). A report has also suggested that in rooms inhabited by patients who are neither infected nor colonised with *C. difficile*, up to 8% of environmental samples could be positive for the bacterium (Kim et al. 1981). Roberts et al. (2008) sampled the air in a bay inhabited with elderly patients, identifying *C. difficile* spores counts of 53–426 CFU/m^3 contaminating the air. These spores would then fall through the air and settle on surfaces in wards and isolation rooms, capable of persisting on surfaces for up to five months; it is important to identify methods that result in their eradication (Kim et al. 1981).

Different techniques are utilised for the recovery and isolation of bacterial contamination. Three techniques were employed in the work described here for the recovery of both *C. difficile* spores and vegetative cells. Direct contact or RODAC plates have been used in other studies as a means for recovering bacteria from environmental sources (Buggy et al. 1983; Lemmen et al. 2001; Alfa et al. 2008). Preparing these plates with different media and also the incorporation of antibiotics and other selective agents allows for the recovery of specific bacteria. These have been explored in the previous chapters of this thesis. IMS washing or alcohol shocking with ethanol to isolate *C. difficile* is a practice commonly used with both clinically and environmentally obtained samples, selecting for spores only. *C. difficile* faecal samples are routinely exposed to a mix (1:1) of either IMS or ethanol for this purpose before isolation of the bacterium. The mixed culture is then plated out onto selective medium comprising antibiotics and incubated to allow for the bacterial growth. This is usually CCEY agar, if not a variation (Wilcox 2006). Samples have also been transferred into selective or enrichment broths to aid in the isolation of *C. difficile* following environmental pick-up (Arroyo et al.

2005). Sponges or swabs are inoculated into these solutions, usually consisting of antibiotics to aid bacterial selectivity.

Layering two different media in a single Petri dish is a practice utilised within the food industry, but less so within the hospital environmental sampling field (Wu et al. 2001). The top, non-selective agar layer, acts to supply microorganisms with an environment for any injured or damaged cells to thrive following application to the surface and subsequent incubation (Wu et al. 2001). The antibiotic and selective agents present within the bottom layer would then eliminate all microbial growth except the selected bacterium. This would allow their growth as well as the simultaneous inhibition of other microorganisms (Kang & Fung 2000; Wu et al. 2001). It was also suggested in literature that the non-selective medium poured on top would not hinder the characteristic pigmentation and morphology of the colonies which would be produced by microorganisms, and therefore this was also inferred its use in this thesis (Wu 2008). Creating an agar consisting of layering two different media, both with distinguishing characteristics, would allow the slow diffusion of antibiotics up through the non-selective media, without selection pressure on those spores not affected by cleaning routines.

3.1.1 Aim of this study

The main purpose of the work described in this chapter was to identify which *C. difficile* strains are present in the environment in healthcare facilities, particularly 'high' contact sites. Patients, staff, and visitors frequently physically contact these areas. Based on the different media investigated in the previous chapter, two sampling campaigns were conducted to investigate the differences exhibited between the laboratory-based recovery from surfaces and that of the hospital. This intended to distinguish which of these media were best for the recovery of *C. difficile* spores in a controlled study, as well as with a higher level of germicide present. In the final section of this chapter, these media were used to resuscitate *C. difficile* spores recovered from the environment with pre-moistened sponges. This used the above described layering media technique.

This study aimed to determine which medium is the most effective for the recovery of *C. difficile* spores from healthcare facilities. RODAC plates were used

for this purpose along with the use of environmental sponges. Identifying the strain distribution in wards of two healthcare facilities within the UHL Trust was a further aspect of study within this chapter. This was carried out through determining the ribotypes of the isolates obtained from these sampling studies.

3.2 Materials and Methods

Three distinct environmental sampling campaigns were carried out for this study for *C. difficile* contamination. They have been differentiated here by the methods used to recover bacteria from wards. The first two campaigns utilised RODAC plates. In the first campaign, CDMN agar alone was used for the recovery of *C. difficile*; the second was conducted with the five different media used in the previous chapter of this thesis (Chapter 2). The third environmental sampling campaign made use of sponge-sticks pre-moistened with neutralising buffer [3M Healthcare, UK] to recover bacteria from sites prior to enrichment and resuscitation on different preparations of the five aforementioned agar media.

3.2.1 Sampling with RODAC plates: Campaign 1

Sampling was carried out at two hospitals in the UHL Trust: the LRI and LGH, with Pawel Wolyniec [Loughborough University]. In both hospitals, the sampling was conducted in a single bay around beds (Figure 3.11). These were selected as they were closed prior to being cleaned by using steam followed by hydrogen peroxide release. Treatment was carried out with a device that emitted a vapour from an aqueous solution of 5% hydrogen peroxide, (<50 ppm) silver ions and (<50 ppm) orthophosphoric acid (Department of Health 2008).

Following the re-opening of these wards, sampling was conducted. The bay selected in the LRI was a part of an Ophthalmic Surgery Ward (Ward 37, Bay 3), and the bay in the LGH was a part of an Acute Medicine Ward (Ward 6, Bay 3). Weekly sampling was conducted in both hospitals before the morning routine clean for a 15-week period. RODAC plates containing the medium CDMN were pressed down onto a range of surfaces with a 200 g weight for 30 sec.

3.2.1.1 Sampling locations

Sampling was carried out in 71 locations in both wards (Figure 3.12 and Figure 3.13, pages 116 and 117). These locations were: floors, floor spaces under beds, tops of lockers, tops of bedside tables, bottoms of bedside tables, arms of chairs, wall rails, toilet floors, underneath the toilet lids, a toilet support bar, and windowsills.

3.2.1.2 Incubation of plates and C. difficile recovery

Following sampling, the plates were transferred into a 37°C anaerobic incubator for 48 hr. Colonies were counted and recorded; any possessing the characteristic grey pigmentation and irregular-shaped *C. difficile* morphology were sub-cultured onto BHI blood agar and incubated for a further 48 hr. Diagnostic tests were then performed to confirm the identity of the bacterium. These tests are based on colony morphology, odour produced and yellow-green fluorescence under UV light. RODAC plates were placed back into the anaerobic workstation for a further 72 hr to allow for additional growth, with checks performed every 24 hr. Distinctive colonies recovered from further incubation were also propagated.

3.2.1.3 Bacterial identification kit

The API 20A (rapid ID32 A) strips kit [bioMérieux, France] is a standard diagnostic method used widely to identify and determine the identity of common anaerobic bacteria. Groups have reported use of this kit to determine the anaerobic species detected following environmental sampling (Kaatz et al. 1988; Lindstrom et al. 1999). Through a series of twenty-nine different biochemical tests using a range of enzymes present in individual cupules, colour changes indicate a positive or negative result for each reaction. These outcomes are then scored using an identification table provided with the kit. Eighty different anaerobic organisms have been investigated by the manufacturers, and their results for each biochemical test recorded. Samples were prepared as described in the guidelines provided with this kit.

3.2.1.4 Bacterial 16S sequencing

Another method used to distinguish the species of the bacteria present on the RODAC plates was using universal 16S primers. These target the conserved region of the 16S rRNA gene, providing an accurate substitute to bacterial identification by phenotypic methods as well as re-classification of bacteria (Clarridge 2004).

Universal 16S primers, Bact-8F {5'-AGA GTT TGA TCC TGG CTC AG-3'} and Bact-1492R {5'-CGG CTA CCT TGT TAC GAC TT-3'}, were used as described by Baker et al. (2003). DNA was extracted using the phenol/chloroform technique

described later in this thesis (Section 4.2.4). The samples were quantified as before, and then treated as described in the guidelines provided with the QIAquick Gel Extraction Kit Protocol: using a micro-centrifuge [Qiagen, UK]. Below are the conditions for the PCR employed:

Process	**Temperature (°C)**	**Time (sec)**	**Cycles**
Initialisation	95	300	1
Denaturing	95	30	30
Annealing	55	60	30
Extension	72	60	30
Further extension	72	300	1

Table 3.1: PCR protocol for bacterial 16S sequencing.

3.2.1.5 Analysis of PCR products

Samples were diluted to 30 pMol prior to sequencing by The Sequencing Centre [University of Dundee].

3.2.1.6 Statistical analyses

Data gathered from the environmental sampling were analysed using Microsoft Excel's statistical functions and Data Analysis Tool Pack. The results obtained from the bacterial 16S sequencing were converted with Chromas v2.33 [Technelysium Pty Ltd., Australia] into FASTA sequences and the species identified by comparing with published sequences with the online database GenBank [National Center for Biotechnology Information, USA].

3.2.2 Sampling with RODAC plates: Campaign 2

Sampling was carried out once in the LRI, within the *C. difficile* isolation ward. A map and legend of the beds are shown in Figure 3.11. The following solid media were used: CDMN, CCEY, CCAB, FAA, and GS-BHI.

3.2.2.1 Sampling locations

Patients testing positive with *C. difficile* infection based on diagnostic tests from faecal specimens were transferred into the *C. difficile* isolation ward in individual rooms. For this study, two of these occupied rooms were sampled.

Five RODAC plates, one of each solid medium, were placed and pressed down with a 200 g weight for 30 sec in a given location. The sites sampled were:

floor entrance, floor under bed, bed rail, back of bed frame, sink, table top, table base, top of contaminated materials bin, floor behind door and bed controls. Following a daily routine clean of the rooms and an additional 30 min wait, to allow the detergent and germicides used to dry efficiently, sampling of these areas was repeated. The ward toilet (inside and outside of the rim, floor near toilet, toilet rails and sink) used by other patients, as well as the sluice (inside and outside rim, floor near sink, sink and draining board) for disposing of bedpan waste, were sampled once with the five media.

3.2.2.2 Incubation of RODAC plates

The same method as outlined in Section 3.2.1.2 was utilised.

3.2.2.3 C. difficile recovery

After sampling, the RODAC plates were subjected to an alcohol wash. The surface of each plate was washed individually with 1 ml IMS repeatedly, collecting all colonies into an Eppendorf tube. This suspension was mixed via vortex and left at room temperature for 45 min. Of this mixture, 0.5 ml was centrifuged at 15,000 *x g* for 5 min and the pellet washed and re-suspended in FA broth containing cycloserine and cefoxitin (250 mg/l and 8 mg/l), with 0.1% sodium taurocholate (CCFA broth), as an enrichment step. Following anaerobic incubation for 10 days at 37°C, these were cultured onto CCEY agar plates. Any colonies resembling the *C. difficile* morphology as previously described were sub-cultured further onto BHI blood agar to confirm presence of the bacterium.

Of the 1 ml IMS-culture suspension, 50-75 µl was plated onto four different agar media in an attempt to resuscitate the bacterium (CCEY, BHI blood agar, FAA with 0.1% sodium taurocholate, and GS-BHI agar with 10 mg/l lysozyme and 0.1% sodium taurocholate). These were then anaerobically incubated at 37°C for 48 hr. These media were chosen based on differences in antibiotic mixtures, additional supplements as well as their agar bases. Any *C. difficile*-like colonies were sub-cultured as above.

Positive *C. difficile* samples obtained from both methods of recovery were cultured in liquid media prior to chromosomal DNA extraction and strain identification by PCR ribotyping as described in Section 4.2.4.

3.2.3 Sampling with pre-moistened sponge-sticks: Campaign 3

Investigations into other sampling tools were carried out, and pre-moistened sponges, containing neutralising buffer to aid bacterial recovery, were decided upon due to the larger surface area that could be covered, and the ability to make contact with non-flat surfaces. Sampling was carried out on one occasion in the same *C. difficile* isolation ward in the LRI as with the second sampling campaign.

The resuscitation of viable *C. difficile* spores was identified as a problem from the previous sampling campaigns (Sections 3.3.1 and 3.3.2), and therefore different media for recovering the bacterium were utilised. Based on the five media which had been previously used (CCEY, CDMN, CCAB, FAA, and GS-BHI), combinations were created whereby a non-selective medium was poured on top of a selective, or antibiotic-containing medium (Table 3.2). Different volume ratios (ml) of the agar mixes were prepared, as well as each medium poured individually.

	FAA			**GS-BHI**		
CCAB	5:15	10:10	15:5	5:15	10:10	15:5
CCEY	5:15	10:10	15:5	5:15	10:10	15:5
CDMN	5:15	10:10	15:5	5:15	10:10	15:5

Table 3.2: Volume ratios of layered media utilised in the resuscitation of *C. difficile* isolates.

Layering was carried out by the pouring and setting of CCAB, CCEY, or CDMN, and over-laying FAA or GS-BHI to a total of 20 ml. Each medium was also prepared alone (20 ml).

3.2.3.1 Sampling locations

In this sampling study, pre-moistened sponges were used to wipe three surfaces within five occupied patient rooms: sink area and taps, floor near patient bed and the bed rails used by patients.

3.2.3.2 Treatment of pre-moistened sponge-sticks

Following contact with surfaces in the patients' rooms, the sponges were immediately immersed and sealed in individual bags to retain moisture. To each

individual sponge, 10 ml PBS with 0.01% (v/v) Tween 80 solution was added before homogenising at 260 rpm for 5 min using the Stomacher 400 Laboratory blender [Seward, UK], as previously conducted (Dubberke et al. 2007). The solution was transferred into a fresh vial before use.

3.2.3.3 C. difficile recovery

Samples were thoroughly mixed via vortex for 1 min before adding 1.5 ml pure ethanol (1:1) and mixing for a further 1 min. The remaining solution was then stored at 4°C. The samples were left for 45 min for sufficient alcohol shock, 100 µl of this solution was aseptically applied and spread onto each of the 23 different agar combinations. Plates were incubated at 37°C anaerobically for 48 hr to allow sufficient growth. Checks were made daily for 72 hr and potential *C. difficile*-positive colonies were sub-cultured onto BHI blood agar and confirmed as described above.

3.2.4 Ethical issues

Ethical approval was obtained for all sampling campaigns conducted in this chapter. All information regarding patient data was made anonymous prior to use.

3.3 Results

3.3.1 Recovery from sampling with RODAC plates: Campaign 1

Bacterial counts were recorded from the CDMN plates following environmental sampling in both the LRI and LGH wards. The average counts calculated for each location have been shown (Figures 3.1 and 3.2).

3.3.1.1 Leicester General Hospital

Results from the LGH ward (Figure 3.1) show increases in the counts of bacterial contamination obtained from 8 of 11 locations sampled during week 6 of 15, with counts from the toilet support bars reaching 220 colonies. This location returned the highest number of colonies for 5 of the 15 weeks that sampling was carried out. The toilet floor also yielded high numbers of colonies, with a global average of 42.69 colonies per round of sampling, and median of 40.88. This median value is over four times greater than the colonies counted from the toilet seats, 9.35.

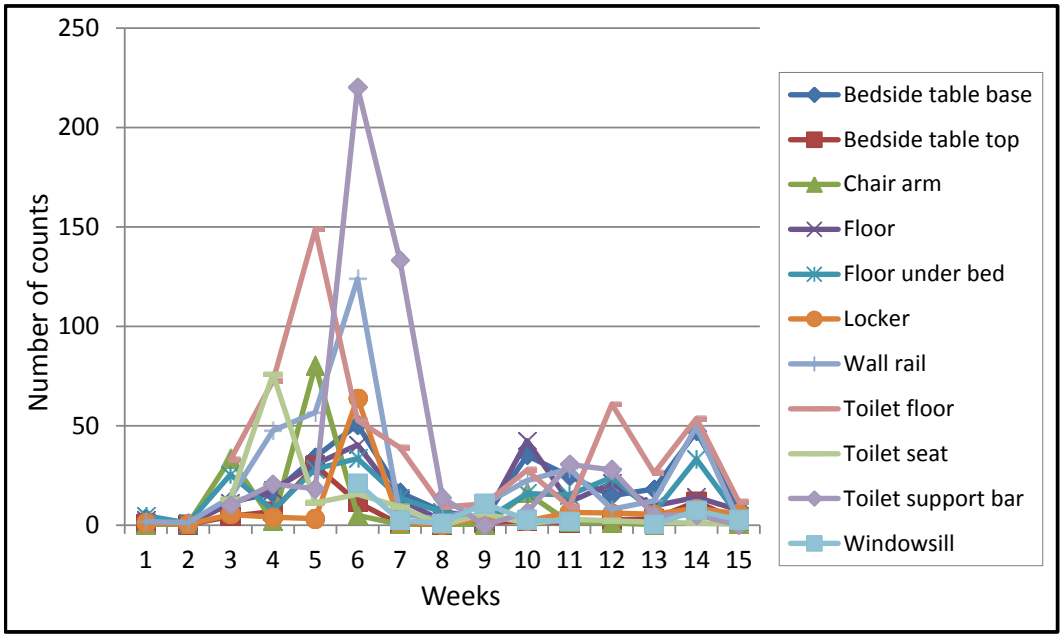

Figure 3.1: Average colony counts observed from a selection of sites using CDMN RODAC plates following sampling from an LGH ward over a 15-week period.

Very low recoveries of anaerobic organisms were recorded for certain areas. These were the windowsills, with an average of 5.56, and the top of the bedside table, with an average of 5.22 colonies per week. These locations consistently produced very low counts, except for weeks 5 and 6 of this sampling

period. The top of the bedside table had its highest count in week 5, with 30 colonies recovered. In week 6, the counts for these locations increased to 21 and 11.5 colonies, respectively. Following this, both returned to averages of 0-3 colonies recovered the following week.

The bedside table base averages do not appear to reveal a trend or pattern of any kind; colony counts from this location were recorded as low as 0.6 during week 2 and as high as 50.5 colonies during week 6. There is a fluctuation in recovery for this site, but no correlation can be determined.

However, a general pattern can be seen overall when analysing the data collected from the LGH in the second week of sampling. There was a decrease in recovered colonies for all locations so much so that the highest average was 1.33 colonies on the sampled wall rails, although no explanation can be given for this. The following week, all locations reported increases in recovered colonies on the CDMN plates ranging from a 10-fold rise for the wall rail to a 55-fold elevation on the arms of the chairs. The latter decreased in the following week, as the remainder of sites had increased colonies recovered through week 6.

Overall, weeks 7-8 showed a decrease in bacterial recovery. Despite the highest value of this fortnight period being 133.3 colonies recovered from the toilet support bars, the general trend was a fall in the numbers recovered. The following five-week span (weeks 9-13) showed no consistent pattern in terms of increases and decreases of bacterial recovery. During this period, no significantly high counts were observed other than 60.83 colonies being recovered in week 12 for the toilet floor. All other counts through these weeks ranged from 0.0 to 42.45. The final two weeks of the time period (weeks 14-15) show a respective increase in total recovered colonies for week 14 and decrease for the final week, with the highest number recovered from the latter at 11.83 colonies.

3.3.1.2 Leicester Royal Infirmary

The results of sampling over a 15-week period in the LRI ward are presented in Figure 3.2. Key areas of high recovery of bacteria using the CDMN plates are shown to be the bedside table base, which had an average of 9.2 colonies

per week recorded, the toilet support bar (13.22) and the floor of the toilet (7.51). In contrast to this, it was found that areas of low recovery were the top of the bedside table (0.76) and the top of the lockers (1.34) colonies per week.

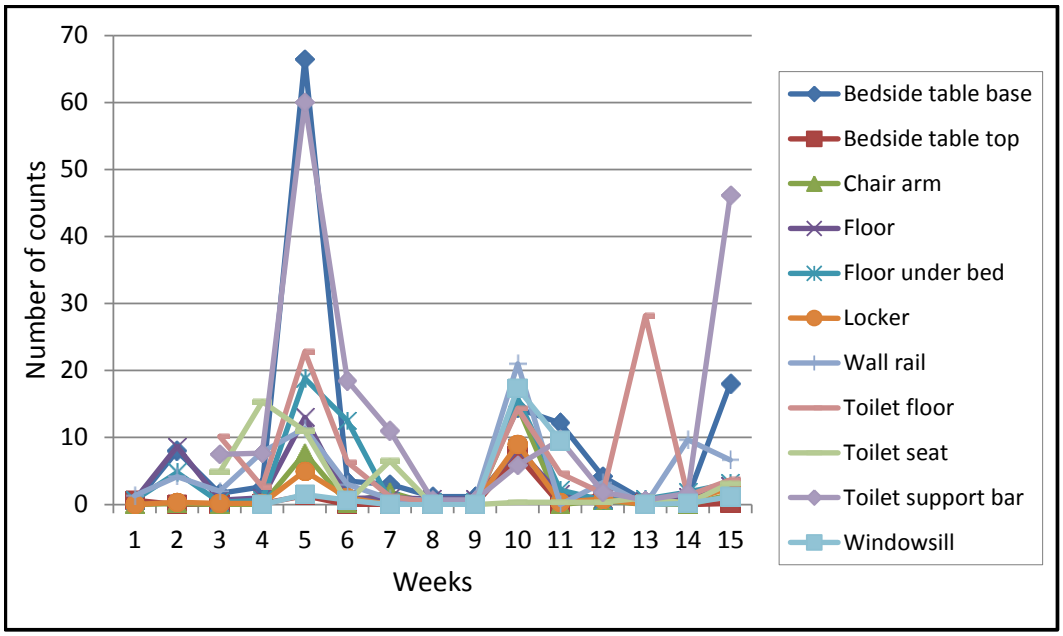

Figure 3.2: Average colony counts observed from a selection of sites using CDMN RODAC plates following sampling from an LRI ward over a 15-week period.

The two highest numbers of colonies recovered from all sites were both found in week 5. Counts of 66.5 and 60 were observed for the bedside table base and toilet support bar, respectively. The third highest was 46.2 colonies, also from the toilet support bar. There were many instances in which no colonies were recovered from specific sites; the arm of the chair adjacent to the patients' bed failed to recover bacteria for 8 of the 15 weeks.

There was a general increase in the overall recovery of anaerobic growth in week 2, which subsequently fell in week 3. This steady increase of an average of 0.3 additionally recovered colonies continued through the following week. Week 5 of the period, however, recovered the highest counts for 4 of the 11 chosen sites. This resulted in 13 colonies collected from the floor, 22.83 from the toilet floor, 66.5 from the base of the bedside table and the 60 from the toilet support bar, as referred to previously. The total counts fell from week 5 to week 6; the weekly average total of 219 recovered from all sites in week 5 decreased to 46.9 colonies, a significant decrease of 78.5%. Weeks 8 and 9 were recorded as identical in

terms of recovering colonies, both recovering total highs of 1.17 for the base of bedside table, and no growth recorded for the respective tops, arms of chairs, toilet seats, and windowsills.

A rise in counts similar to that observed for weeks 2-3, also occurred almost two months later in weeks 10-11. Following this, no general patterns were recorded until week 15 where rises in numbers of colony growth were recorded at all sites. There were no known reasons as to why this would have occurred. In the final week of the study, 3 of the 11 chosen locations recovered levels of bacterial growth that were within the highest three numbers for their given site.

3.3.1.3 Identifying bacterial growth on RODAC plates

After the RODAC plates were used for environmental sampling, they were incubated for 48 hr at 37°C in the anaerobic chamber, and then were removed and the colonies counted.

Initially, those colonies suspected to be *C. difficile* based on their morphology (about 1 mm in diameter) were selected from the CDMN plates and re-plated onto fresh blood plates. As single colonies had been selected from these plates and sub-cultured further, it was assumed that pure cultures of *C. difficile* were isolated. These samples were allowed a further 48 hr in anaerobic conditions and these cultures were subject to the characteristic *C. difficile* tests described earlier in this report (Section 3.2.1.2). Through the diagnostic tests, it was found that not all of the colonies selected from the CDMN agar plates were *C. difficile*, failing the determining tests. This was despite the media being a specific agar-based product combined with particular antibiotic supplements, moxalactam and norfloxacin, which are recommended for *C. difficile* isolation. Based on the differentiation observed with colony morphologies, identification of the microorganisms was conducted.

3.3.1.4 API 20A strips kit

The API 20A (rapid ID32 A) strips kit is a standard diagnostic method commonly used to identify anaerobic bacteria, as described in Section 3.2.1.3. Colonies were chosen from four isolated locations, sampled with CDMN plates

from the LRI. The colonies chosen were selected because they closely resembled the previously described colony morphology expected for samples of *C. difficile*, but failed the characteristic identification tests. In addition to this, to ensure reproducibility, a known strain of *C. difficile*, ribotype 027, was used in this procedure as a positive control (Figure 3.3). The colour changes observed were identical to those recorded by the manufacturers for *C. difficile*.

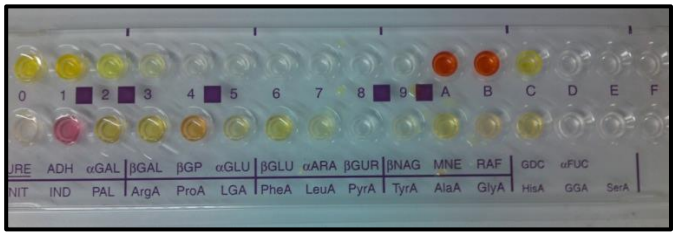

Figure 3.3: API 20A (rapid ID 32 A) strip for ribotype 027.

The colours represent a positive or negative result for a biochemical test. This was used as a positive control and was a known strain of *C. difficile*.

Sample G9 was collected from the top of a locker. However, the kit was not able to produce a conclusive result in terms of identifying the bacterium based on the list of eighty tested anaerobes, due to conflicting potential species requiring specific colour changes. Samples 10 and 11 obtained from the top and bottom of one bedside table, respectively, were also tested. These both produced inconclusive results too, as the colour changes observed did not map any specific species listed. Based on the inconclusive results of the biochemical reactions for these three samples, the kit was unable to aid in the classification of the anaerobes isolated from the RODAC plates.

Sample 22 was collected from the base of a different bedside table within Ward 37. The colour changes due to biochemical reactions for this organism indicate the bacterium was *Clostridium clostridioforme.* However, despite this one positive result, as well as the positive control simultaneously carried out, there was an overall lack of irrefutable results with this method of identifying and confirming anaerobic organisms.

This test was largely ambiguous and variations of colours often made it hard to distinguish whether a given result could be construed as positive or

negative for a given organism. No further samples were prepared for this method of classification.

3.3.1.5 Bacterial 16S sequencing

Based on the lack of conclusive results from the API 20A strip kits, another technique was investigated. The second method chosen to classify the colonies obtained from the CDMN plates using in the first environmental sampling campaign was bacterial 16S sequencing. There were twelve colonies selected from the RODAC plates prepared with CDMN used in environmental sampling; chosen for their differences in colony morphology, and obtained from both hospital sites, as described in Table 3.3. This was to identify the bacteria that were capable of growing on CDMN agar.

The sequencing procedure was able to identify facultative anaerobes that would not have been detected with the use of the API strips, as these are limited to known anaerobes alone. This study has aided in the identification of facultative anaerobes present in the hospital environment, as well as the potential inability of the CDMN plates to recover these whilst lacking the desired selectivity.

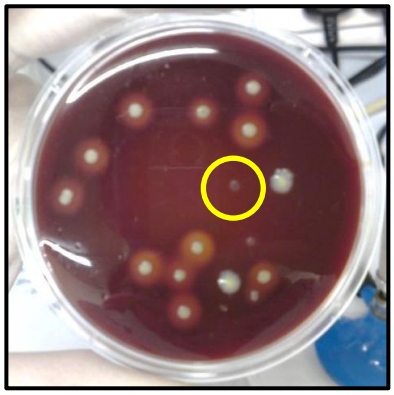

Figure 3.4: *C. difficile* colony formation on a CDMN RODAC agar plate.

C. difficile colony is highlighted within the yellow circle. Other colonies were deemed as contaminants.

Subsequent to discovering the different morphology of *C. difficile* colonies when growing on the CDMN plates following recovery from the environment, the identities of a further three positive *C. difficile* isolates were confirmed. These possessed the characteristic dull, grey morphology seen with sample 56* (Figure 3.4). These were samples 22 and 23 from the LRI ward from the top and bottom of

the same bedside table, in addition to sample G52 from the LGH, isolated from a toilet support bar (Section 3.6). Conducting PCR ribotyping for these four samples identified all isolates as ribotype 027.

Sample	**Location**	**Appearance of colony**	**Species**
6	Arm of chair	Medium, with haemolysis (4 mm)	*Staphylococcus epidermidis*
7	Floor	Medium, with haemolysis (4 mm)	*Staphylococcus epidermidis*
28a	Base of bedside table	Small, pale grey (2 mm)	*Staphylococcus haemolyticus*
31	Floor	Large, yellow (5 mm)	*Staphylococcus saprophyticus*
*56**	Underside of toilet seat	Dull grey † (3 mm)	*Clostridium difficile*
G2	Floor under bed	Small, pale grey (3 mm)	*Staphylococcus saprophyticus*
G8	Floor under bed	Large, yellow (6 mm)	*Enterococcus faecium*
G9	Top of locker	Large, with haemolysis (6 mm)	*Staphylococcus epidermidis*
G22	Base of bedside table	Medium, yellow (4 mm)	*Staphylococcus epidermidis*
G23	Top of bedside table	Medium, grey (4 mm)	*Lactobacillus fermentum*
G31	Floor	Large, with haemolysis (6 mm)	*Staphylococcus epidermidis*
G32	Floor under bed	Small, yellow (2 mm)	*Staphylococcus epidermidis*

Table 3.3: Descriptions of colonies selected for bacterial 16S sequencing.

† - indicates the colony selected had serrated edges. All others were smooth-edged. All samples possessed a maximum identity of >95%.

3.3.2 Recovery from sampling with RODAC plates: Campaign 2

The previous environmental sampling study identified difficulties with the use of just one medium in the RODAC plates. It was also thought the locations chosen for sampling were not ideal for recovering *C. difficile*. Therefore, the five media investigated for *C. difficile* recovery in the laboratory subsequent to exposure to ChlorClean were prepared and used in a second hospital sampling campaign. The ward chosen for this study was the *C. difficile* isolation ward in the LRI as it was expected that *C. difficile* contamination would be abundant. Sampling was carried out in two private isolation rooms as well as a shared toilet area and the sluice for waste disposal. The isolation rooms were sampled before, and thirty minutes after, a daily routine cleaning procedure. This was to investigate the effects of the standard routine cleaning technique carried out.

After conducting the sampling, any positive *C. difficile*-like colonies were sub-cultured onto BHI blood agar plates for the diagnostic tests to confirm presence of the organism. The positive isolates are represented by blue bars in Figure 3.5.

The low recovery of putative *C. difficile* colonies, and overall high bacterial counts on the plates made it difficult to visually assess the number of *C. difficile* colonies that had formed following the tamping. Therefore, all of the plates were subjected to an IMS wash and treated as previously described plating onto four freshly prepared agar plates (BHI blood, CCEY, FAA, and GS-BHI) as shown (Figure 3.6). BHI blood agar was chosen for its use in diagnostic tests for confirming presence of *C. difficile*; CCEY agar for its ability to isolate *C. difficile* within a sample when found amongst a range of other faecal bacteria due to its antibiotic selectivity. Conversely, FAA and GS-BHI agar were chosen specifically because they did not contain antibiotics but had aids to spore germination. These media were also successful in *C. difficile* recovery in Chapter 2. The numbers of positive *C. difficile* isolates identified after this enrichment step increased the totals, as shown by purple bars in Figure 3.5.

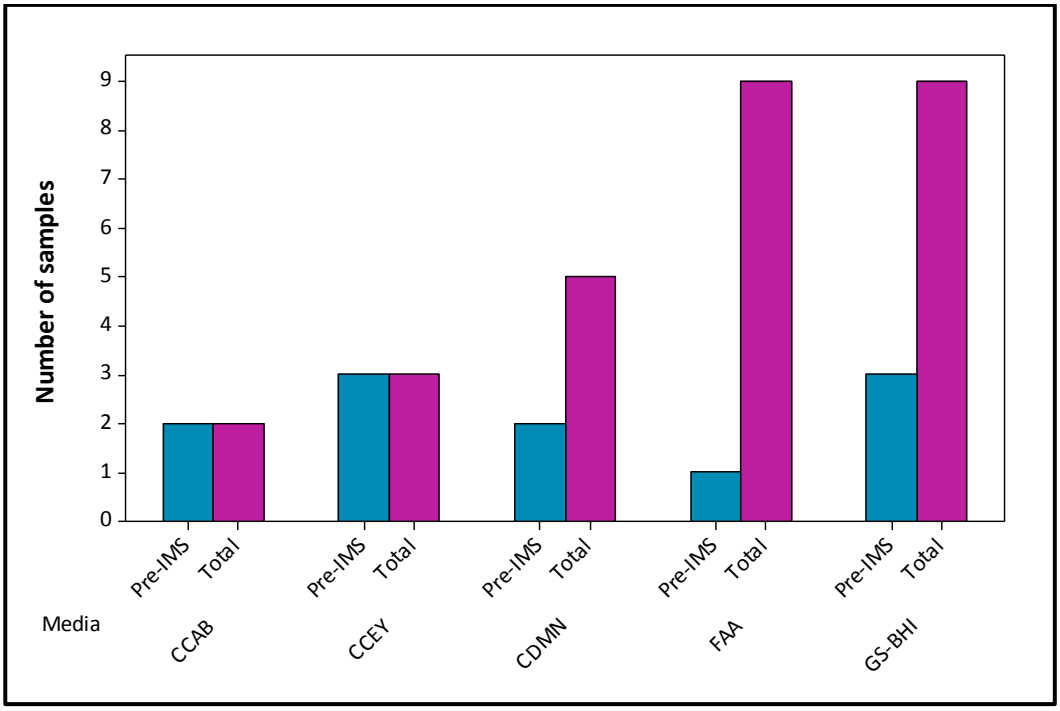

Figure 3.5: Recovery of *C. difficile* isolates with and without IMS washing of RODAC plates.

'Pre-IMS' refers to the number of plates with positive *C. difficile* colonies observed upon visual inspection. 'Total' is the final number of *C. difficile* positive samples obtained following IMS washing.

				Resuscitation media after IMS washing			
Location	**RODAC plate**	**PCR Ribotype**	**Positive before IMS**	**FAA**	**BHI blood**	**GS-BHI**	**CCEY**
Side room 1 (pre-clean)							
Table base	GS-BHI	103		+	+	+	
Top of yellow bin	FAA	103		+			
Top of yellow bin	CDMN	010			+		
Floor behind door	FAA	a		+	+	+	
Side room 1 (post-clean)							
Floor entrance	GS-BHI	103		+	+		
Table top	GS-BHI	103	*				+
Side room 2 (pre-clean)							
Floor entrance	GS-BHI	103		+	+	+	+
Floor under bed	CCEY	103	*				+
Floor under bed	CDMN	a			+		
Bed rail	CCEY	103	*				+
Back of bed frame	CDMN	103	*				+
Back of bed frame	GS-BHI	103	*				+
Back of bed frame	FAA	010		+	+	+	+
Sink	CDMN	103	*				+
Sink	FAA	103	*				+
Table top	GS-BHI	a		+	+		+
Floor behind door	FAA	103				+	
Bed controls	CCAB	103	*				+

				Resuscitation media after IMS washing			
Location	**RODAC plate**	**PCR Ribotype**	**Positive before IMS**	**FAA**	**BHI Blood**	**GS-BHI**	**CCEY**
Side room 2 (post-clean)							
Floor entrance	CCEY	103	*				+
Sluice							
Draining board	CDMN	103		+			
Sink	FAA	027		+			
Sink	GS-BHI	027		+			
Floor near sink	CCAB	027	*				+
Floor near sink	FAA	081		+	+	+	
Toilet							
Toilet rim (outside)	FAA	b		+	+	+	
Toilet rails	GS-BHI	103				+	
Sink	FAA	103			+		
Sink	GS-BHI	103	*				+

Table 3.4: Distribution of samples and corresponding ribotypes with RODAC plates.

'*' – Individual colony was identified as *C. difficile* from visual selection before IMS washing. '+' – Isolation via IMS washing step and resuscitation on the specified agar. 'a'/'b' – unknown ribotypes.

The data from this resuscitation procedure show the additional IMS washing step has a profound effect on the number of isolates classified as positive for *C. difficile*, with the RODAC plates prepared with the media CDMN, GS-BHI and FAA. Only three isolates were confirmed as *C. difficile*-positive with the use of the liquid enrichment technique, CCFA broth with 0.1% sodium taurocholate. In total, of the 202 RODAC plates used, 25 recovered *C. difficile* (12.4%). Furthermore, 20 of the 42 sites were *C. difficile*-positive (47.6%).

The most striking improvements in recovery are with the two non-selective media: GS-BHI agar and FAA. An additional six isolates were obtained from the original GS-BHI agar plates and eight more from the initial FAA plates used in the hospital. The medium CDMN recovered *C. difficile* on two RODAC plates prior to the IMS washing stage, and subsequently a further three when the enrichment technique had been used. CCEY and CCAB did not exhibit any differences in the numbers of RODAC plates successfully germinating *C. difficile* spores following the IMS wash and recovery.

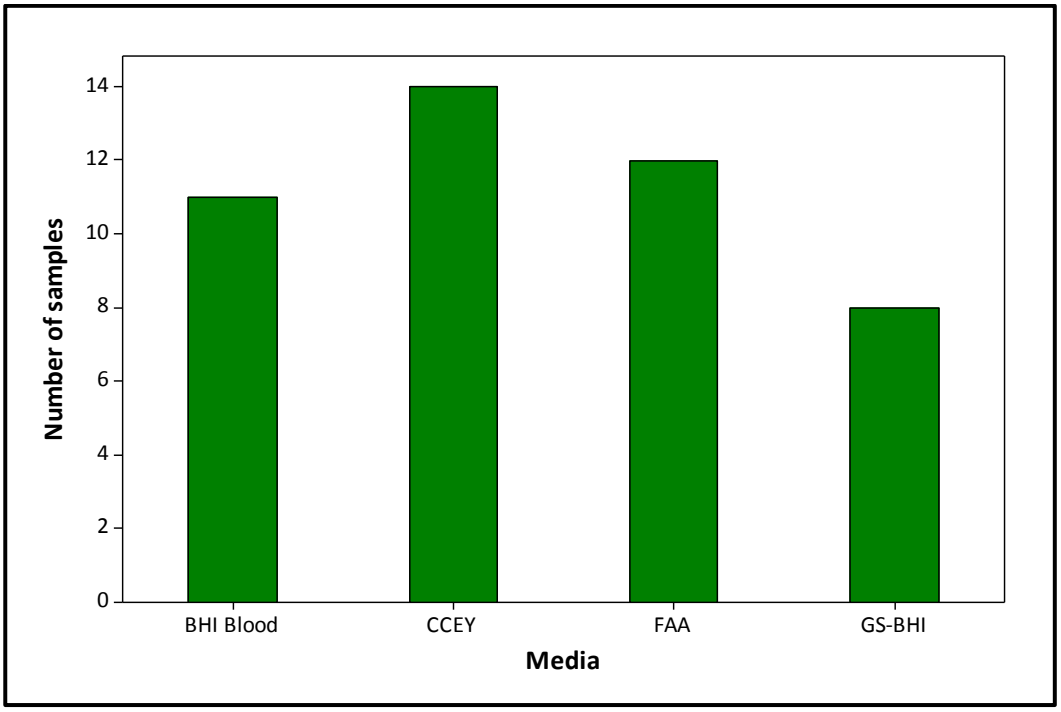

Figure 3.6: Recovery of *C. difficile* isolates after IMS washing and resuscitation onto agar media.

The use of CCEY agar led to the recovery of only two isolates of *C. difficile* directly from the RODAC plates tamped onto surfaces within the ward, and the

additional IMS wash step failed to germinate any additional spores. Despite this, the data from this study show that CCEY agar appears to be highly successful when used as a resuscitation medium, recovering fourteen additional isolates of *C. difficile*. BHI blood agar and FAA led to the recovery of eleven and twelve positive *C. difficile* isolates respectively after samples were IMS washed and plated out, and on GS-BHI, there were eight. There were isolates that were positive on more than one resuscitation medium (Figure 3.6).

Following the confirmation of *C. difficile*, from either the initial visual checks of the RODAC plates used in the sampling or the additional resuscitation technique, the putative *C. difficile* isolates were cultured and the chromosomal DNA extracted. The isolates were contextualised by strain using PCR ribotyping identified from this round of sampling (Figure 3.7).

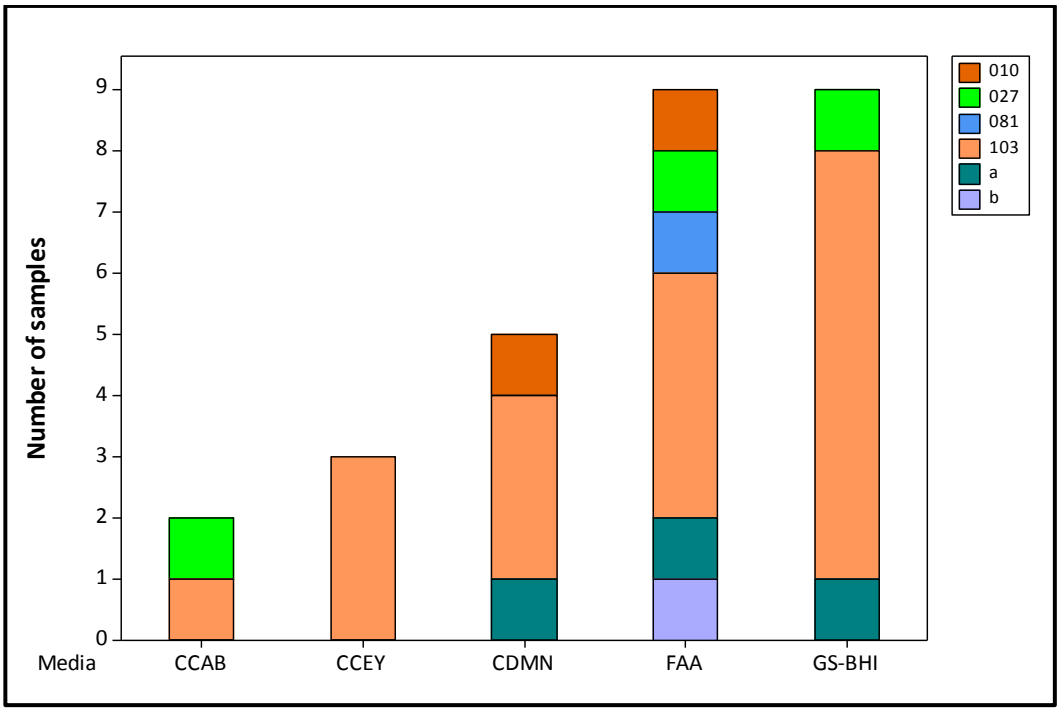

Figure 3.7: Ribotypes of isolates and the initial recovery agar media from RODAC plates.

These data suggest the clear prominence of ribotype 103 within the side rooms and additional sites sampled, as it was recovered on all five prepared media. Before the routine cleaning, eight out of the ten sites sampled recovered *C. difficile*. Only three of the five media recovered the expected dominant ribotype 027, however these were all localised in the sluice area sampled (Figure 3.8). It

was expected that ribotype 027 would be present throughout all tested sites due to the reports of its ubiquity (Morgan et al. 2008). The sluice was also found to possess ribotype 081, as only identified solely with FAA, and 103, with one sample recovering each of these strains.

With respect to the tested media, the overall recoveries were somewhat lower than anticipated based on the results reported in literature. However, FAA and GS-BHI agar each recovered nine positive samples. Between these two media, at least one of each ribotype was found. Both media detected the presence of the unknown ribotype 'a'. This strain was identified in both side rooms sampled, but only before the routine clean (Figure 3.8). Following the cleaning process, repeat sampling was unable to recover this particular type again. Unknown strain 'b' was only found once with FAA prepared in the RODAC plates and in the toilet. The toilet area was a site with only this strain and ribotype 103 present. Of the five sites sampled within the sluice, three were *C. difficile*-positive; the same proportion was identified within the toilet.

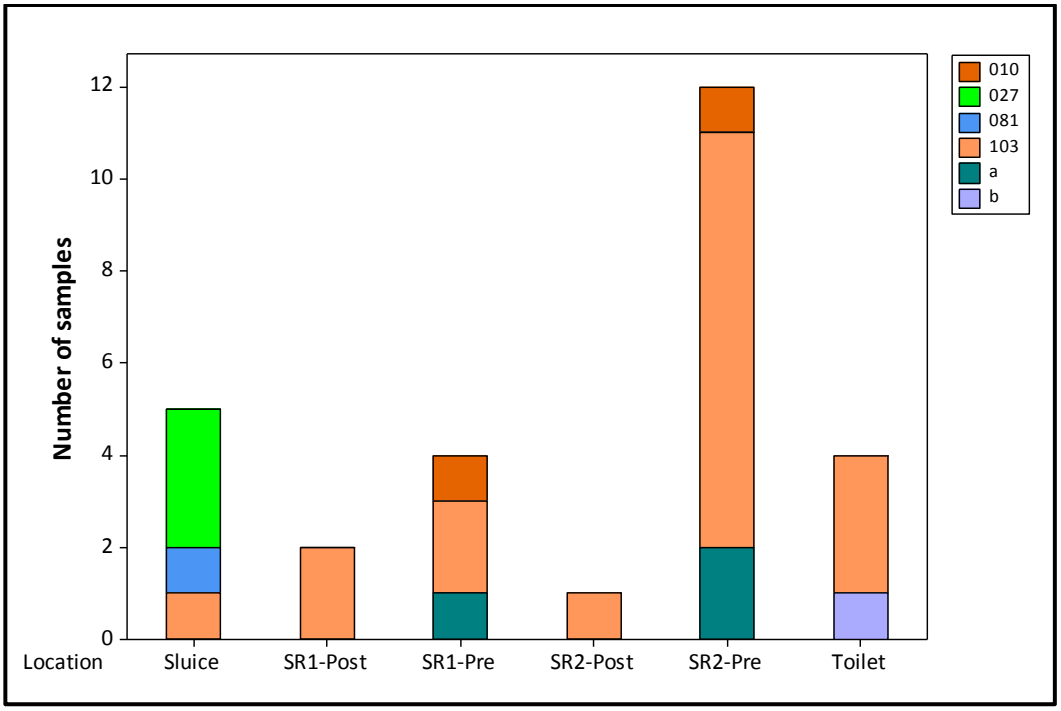

Figure 3.8: Distribution of ribotypes recovered in the *C. difficile* isolation ward, according to site.

'SR': identification assigned to *C. difficile* isolation side room number. 'Pre' and 'post' refer to whether the sampling was conducted before or after routine cleaning.

The clearest result from this body of data is the difference in recovery from the second side room selected for sampling (Figure 3.8). The data strongly imply that the number of *C. difficile* spores present on surfaces in that room have been reduced by the cleaning method employed. In addition to this, the findings suggest that certain ribotypes react differently to cleaning, ribotype 103 may be resist to cleaning, whereas ribotype 010 may be sensitive to the cleaning agents. Ribotype 010 was isolated from both sided rooms before cleaning; it was recovered from neither afterwards.

The data also suggest that the strain types do not differ between rooms (ribotypes 010, 103 and 'a'). Patients in these isolation rooms are often bed-bound and immobile; therefore, they cannot be responsible for the transfer of these strains. It could be inferred that the movement between rooms by either the cleaning or the hospital staff could result in the transfer of the *C. difficile* ribotypes.

3.3.3 Recovery from sampling with pre-moistened sponge-sticks: Campaign 3

The paucity of *C. difficile* isolated from the previous two environmental sampling studies gave rise to the idea of adopting another technique for bacterial

recovery. The use of RODAC plates in environmental sampling limits the sampling area. This restriction therefore could be a reason as to why such low levels of recovery were found. Using a pre-moistened sponge would give the benefit of allowing a larger surface area to be covered as well as the flexibility of sites that were not flat. 'High' contact sites chosen were the sink area and taps, the floor near the patients' beds and the bed rails used by patients.

For the purpose of this study, pre-moistened sponges were used to sample one of these three surfaces in five occupied *C. difficile*-positive patient rooms in the isolation ward, at the LRI. The sponges were used before adding 10 ml PBS with 0.01% (v/v) Tween 80 solution and homogenising as described by Dubberke et al. (2007). The samples were thoroughly mixed both before and after the addition of 1.5 ml pure ethanol (1:1). After 45 minutes at room temperature, 100 µl was spread aseptically onto each of the 23 selected agar combinations. Putative *C. difficile* colonies were subjected to the standard characteristic confirmation tests.

The preparation of the double-layered agar media was to maximise the recovery of spores from the environment that may have been subjected to stresses, such as desiccation or exposure to cleaning products. The top agar contained no selective agents or antibiotics, as the presence would stimulate further stress onto the spores; this non-selective media also encourages the spore resuscitation. The antibiotics in the lower layer then diffuse up to the surface of the top layer to induce selectivity. This interval of time between the resuscitation and antibiotic reaching inhibitory concentration potentially permits the maximum *C. difficile* recovery. To compare the effects of different proportions of each combination, the volume ratios of the agar mixes were varied as shown (Table 3.2).

The data show no single media combination utilised in this work was the most effective for recovery of *C. difficile*. Additionally, not all media were able to recover *C. difficile* when the samples were applied directly onto them; only CCAB and the two non-selective media (FAA and GS-BHI agar) were successful (Figure 3.9). Analysis of the total bacterial counts for the RODAC plates used show FAA and GS-BHI agar to be the least selective with *C. difficile* growth, culturing a variety of other microbial species. It was therefore expected that these two would be able

to produce high numbers of success; however only five of the total fifteen samples prepared grew on GS-BHI and only one on FAA.

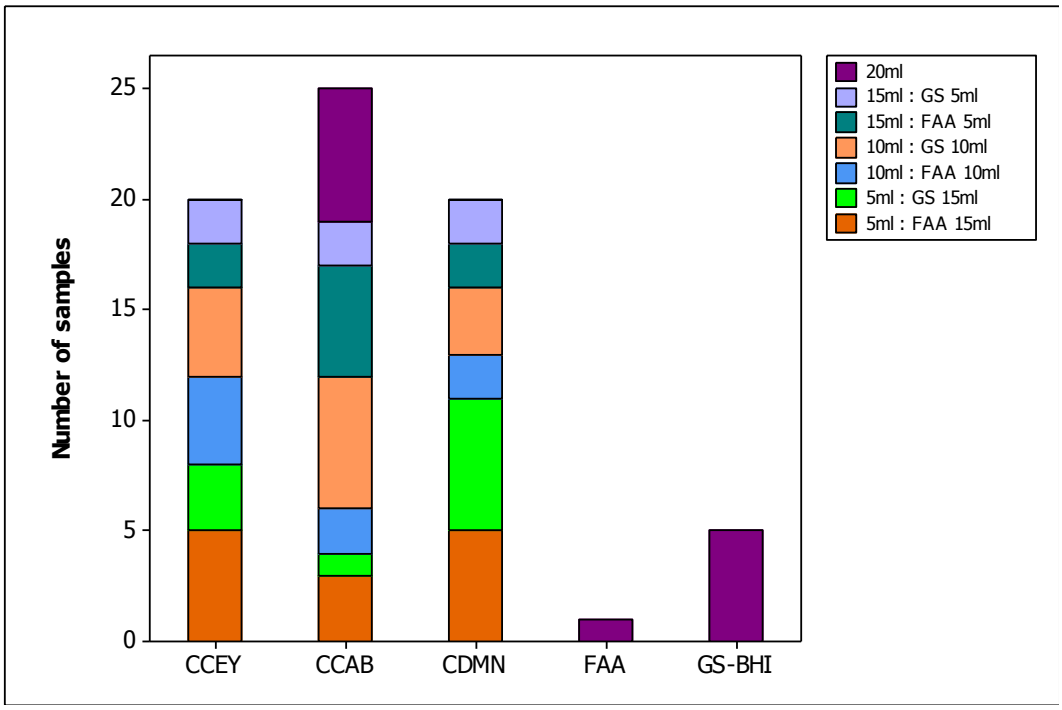

Figure 3.9: *C. difficile* isolates obtained after recovery from double-layered *C. difficile* agar media.

Generally, there appears to be little difference between the abilities of the layered media to recover *C. difficile*. All three of base media in conjunction with the various layered combinations successfully grew *C. difficile* from the samples obtained. Minor distinctions were noted, for example, CCAB only recovered one sample when used in the ratio of 5 ml to 15 ml with GS-BHI agar; five fewer than CDMN. Comparing this one positive with the five obtained with GS-BHI agar alone (20 ml) could suggest that the combination of the two media is not optimal for bacterial growth. Furthermore, with the ratio reversed and triple the quantity of antibiotic-containing media used, only two samples were recovered. However, when used in equal ratio, the combination of CCAB and GS-BHI resulted in six positive samples. Therefore, the results from this novel methodology of *C. difficile* recovery demonstrate there may well be a link between the volumes of agar media used and the recovery success.

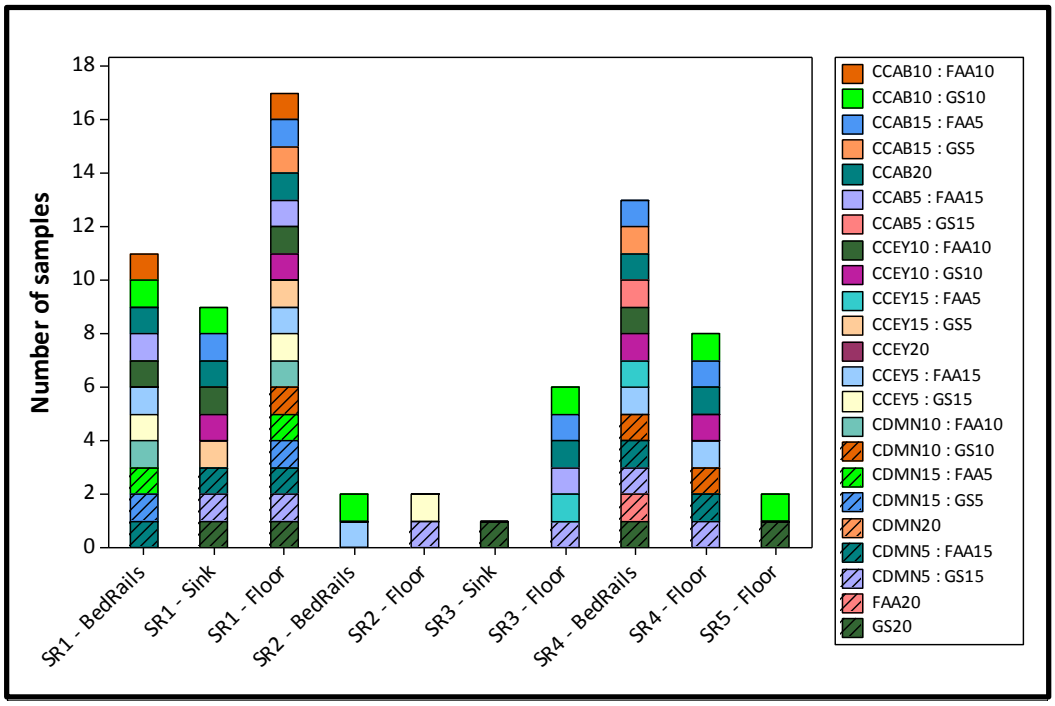

Figure 3.10: *C. difficile* isolates obtained after recovery with layered agar media by site.
'SR': identification assigned to *C. difficile* isolation side room number.

Figure 3.10 depicts an overview of all the sites sampled in the third sampling campaign for which *C. difficile* colonies were confirmed on the media after spreading. Of the 15 sites selected for sampling, 10 were *C. difficile*-positive (66.7%). Side room 1 was the only room from which *C. difficile* was recovered from all three sites. This could be attributed to the fact that this patient was the most mobile, standing to use the sink area with little assistance from the ward staff, for example. Side rooms 2, 3 and 4 were *C. difficile*-positive at two of the three sampling sites, and only one of the three sites in side room 5. The bed rails and floor were the two *C. difficile*-positive locations in side room 4, and similarly were the sites with the higher recovery of *C. difficile* within side room 1.

The floor was the one site to be *C. difficile*-positive from all five sampled rooms. This again could be due to the movement of cleaning and hospital staff and transmission through the air. Sampling of the bed rails led to the recovery of the second and third highest counts overall (from side rooms 4 and 1, respectively). *C. difficile* growth was also recovered in side room 2, which corroborates the expectation that 'high' contact areas are reservoirs for *C. difficile*. Patients in side rooms 3 and 5 were immobile, and therefore the lack of movement could have

resulted in reduced contact with the bed rail for help in moving compared with the other patients. This could explain why there was less *C. difficile* recovered from the sink areas as the risk of spread is subsequently reduced if these areas are not touched by the contaminated patients.

This study also involved identifying the ribotypes of the faecal samples from the patients occupying the five isolation rooms, and comparing these with the strains obtained from the three surrounding sites. Table 3.5 demonstrates a clear presence of ribotype 027 in the isolation side rooms sampled as well as directly from the patients themselves. This comparative study has shown the strain identified from a patient is the same as those strains isolated from sites around them.

Patient	**Faeces**	**Bed rail**	**Sink**	**Floor**
1	027	027	027	027
2	Unavailable	027	---	027
3	027	---	027	027
4	027	027	---	027
5	Unavailable	---	---	027

Table 3.5: Comparison of ribotypes from patients' faecal samples with those identified from the surrounding sites. Patient numbers also refer to side room number. '---' refers to no positive *C. difficile* isolate was obtained from site.

3.4 Discussion

C. difficile presence has been reportedly widespread, nosocomially as well as outside the hospital environment (al Saif & Brazier 1996; Beggs et al. 2008; Weaver et al. 2008; Sethi et al. 2010). Detection of this contamination in combination with characterisation typing methods, enforces knowledge in outbreaks as well as underlying prevalence of strains. With respect to hospital settings, sampling of *C. difficile* permits investigation into how the landscape of infection changes, in addition to detecting where potential 'hot spots' may lie. Identification of which sites in a hospital ward are more likely to be infected with *C. difficile* also helps ascertain where cleaning may need to be targeted and therefore assists in shaping cleaning regimes.

There are many methods discussed in literature for isolating *C. difficile* from the environment. Sampling with direct contact plates prepared with selective media has been shown to recover *C. difficile* (Fekety et al. 1981; Buggy et al. 1983; Danforth et al. 1987; Clabots et al. 1991; Lemmen et al. 2001; Martirosian et al. 2005; Alfa et al. 2008). Previous work within this thesis has demonstrated the importance of germination agents present within an agar medium in its ability to recover *C. difficile* (Chapter 2). Within this chapter, emphasis has been placed on *C. difficile* selective media and its role in resuscitation, building on the findings initially found in this thesis, demonstrating the optimisation required in order to germinate damaged spores before strain characterisation. Furthermore, the work presented here investigates whether a novel technique in *C. difficile* resuscitation could be used in its isolation from environmental sampling.

Prior to the environmental sampling studies carried out in this chapter, preliminary work was conducted. All five agar media were prepared in RODAC plates and used to recover microorganisms in a hospital ward. The results indicated higher counts were obtained with the use of CDMN agar (data not shown). Therefore, as this agar was able to recover the most bacterial counts, it was assumed the probability of picking up *C. difficile* would also be increased and it was subsequently utilised as the lone medium in RODAC plates for the first sampling campaign conducted at the LRI and LGH.

In-depth studies similar to the first sampling campaign described in this chapter are infrequently conducted. Conducting sampling in such a manner in addition to the processing, culturing and analysis of isolates can be time-consuming and deemed as not cost-effective. However, the work carried out here has shown that routine cleaning of wards may be ineffective in eradicating bacterial contamination. Sampling was conducted prior to routine manual cleaning, and recovery of microorganisms was nevertheless detected.

With respect to the sites sampled in the LGH, locations with high numbers of bacterial growth were the floors and support bars in the toilets, and the wall rail in the bay area. Areas with the lowest recovery at this ward were the bedside table tops and the windowsills. The high levels of recovery may be attributed to increased use of the toilet facilities in the ward, as these communal sites would be utilised by all patients throughout the day. This would result in an increased probability of transmission within this area and a high transfer efficiency of microorganisms. The wall rails are classified as a 'hard to reach' area, in the sense that the cleaning staff may not necessarily be able to clean these areas easily due to the height at which they are placed, in addition to the fact that patients may stay in this ward for long periods of time (Barbut & Petit 2001). Therefore, in an attempt to minimise the disturbance to patients, sites may not be cleaned as thoroughly, increasing the rates of bacterial accumulation. The top of the bedside table is an area that would be cleaned more often than any other site as this is used by patients throughout the day and is subject to spillages of food and drink. These would be cleaned up more efficiently, resulting in a reduction in the numbers of bacteria, as observed. As an area which would not have many people making contact with it, and thus when cleaned would theoretically be free from bacterial transmission through touching, the windowsills were another site with little recovery of colonies.

For the samples collected at the LRI, the toilet support bars, base of the bedside table and toilet floors led to the recovery of *C. difficile*. This could be due to the high usage of the support bars, and toilet areas in general in comparison to the rate of cleaning. Therefore, with more patients using these areas, there is an

increased chance for bacterial colonisation and subsequent pick up during sampling (Davies et al. 2011). The base of the bedside table would be wiped down and cleaned less frequently, as spillages of food and drink on the surfaces would be removed, but there would be fewer circumstances where the base would be cleaned. The results from these data suggest that in 'hard to reach' areas such as the wall rails and floor spaces under beds, there were fewer bacterial colonies recovered. These locations would be expected to have lower counts of bacteria as cleaning staff, patients and healthcare workers are unlikely to frequently come into contact with these sites (Davies et al. 2011).

A potential explanation for the numbers of bacterial contamination on the sites identified within this sampling campaign is that in the UK, cleaners of wards are provided with a specific list of areas to clean. An emphasis is placed on the cleaning of visual contamination on floors and within toilets (Dancer 2008). It has also been reported that the responsibility of cleaning certain frequently touched sites such as bed rails, door handles and switches, including the nurse call button, falls onto the ward nurses (Dancer 2004). However, whether this was true for the hospital wards used in the sampling studies within this thesis is unknown. Kramer et al. (2006) also discussed the importance of bacterial contamination on sites within hospitals and the effect of its persistence. This report demonstrated how the transmission of most HCAI is through contact with the patient and/or healthcare workers and inanimate sites. Therefore, poor cleaning of these sites may be a latent source for harbouring and transmission of pathogens.

Based on the counts from the samples recovered in the LGH, concerning changes and differences in recovery numbers over the course of the sampling period, there appeared to be some periodic changes occurring. General increases in counts were observed roughly every four weeks, for example, weeks 5 and 6 saw high counts, which then fell. The counts rose again through the later weeks in the study, particularly in weeks 10 and 14 (excluding a brief increase in week 12 for the toilet floor). This four-week cycle indicates that general rises in bacterial contamination are seen on a monthly basis. There was an anomaly observed during weeks 7 and 8 of the sampling, recovering fewer colonies consistently. It

could be speculated that fewer patients were present in the bay during this time, and thus the chance of bacterial transmission was reduced, but these data were not available at the time of conducting this study and so conclusive findings cannot be reported.

Considering the data collected from sampling within the LRI, there were three notable instances in the fifteen weeks investigated in this current study whereby there was an increase in counts observed. These all occurred periodically, every five weeks, with rises in weeks 5, 10, and 15. Similarly, to the LGH, this is indicative of a cyclical pattern. There are distinct patterns observed for changes; there appears to be more fluctuation in recovery from the earlier periods of sampling, with counts settling during the latter season. However, the lack of information regarding the ward occupancy over time hinders the derivation of conclusions from these findings.

Overall, there appear to be fewer colonies recovered in total from the LRI than LGH, with averages ranging from 0.76 to 13.22 and 5.22 to 42.69 colonies, respectively. This has been consistently viewed when analysing the data. However, the ophthalmic ward in the LRI did have a greater patient turnover. This ward housed patients prior to surgery that waited no more than one day, and so cleaning staff accessed this site more often; bed areas were cleaned thoroughly following the discharge of a patient. In comparison, the LGH ward where sampling took place is one whereby a patients' stay can exceed weeks, and therefore, bed areas may not be cleaned as thoroughly trying to minimise disruption to the patient. The increase in periodic cleaning and times spent within the LRI ward could potentially be attributed to the low values of recovery, as reported by Wilcox & Fawley (2000). This group identified how frequent cleaning of a site may play a role in reducing the bacterial colonisation. In conjunction with the data collected throughout this chapter, it could therefore be concluded that regular cleaning of sites in and around patients could reduce the transmission and persistence of bacterial contamination.

This study also showed that the presence of antibiotics within a medium when used for environmental sampling does not necessarily restrict all other

bacterial growth. A number of different organisms were able to grow on the CDMN agar in RODAC plates, not just *C. difficile*. Diagnostic *C. difficile* tests identified that despite the media being a specific agar-based product combined with particular antibiotic supplements, moxalactam and norfloxacin, specifically developed for *C. difficile* growth, this was not observed. In addition, there were a variety of other colony morphologies recorded following sampling and incubation of the plates suggesting a lack of agar selectivity.

The API 20A strip kit was utilised on four unknown samples that had been recovered from the CDMN plates and purified. Despite it being a widely used diagnostic method, this technique failed to identify three of the four chosen colonies (Kaatz et al. 1988). Of the one positive isolate identified, *Clostridium clostridioforme*, its presence and infection is not frequently reported. Finegold et al. (2005) suggest that this bacterium consists of a mixture of three different clostridial species: *Clostridium bolteae*, *Clostridium clostridioforme*, and *Clostridium hathewayi* due to its variability when subjected to both tests, of phenotypic and antimicrobial susceptibility nature. Few studies have been conducted into *Clostridium clostridioforme*, its symptoms and effects, but a link has been shown to bacteraemia and *Clostridium hathewayi* (Woo et al. 2004). The API strip kit was able to identify this particular anaerobic bacterium, but little is known about its potential severity in the hospital environment.

The tests were highly time-consuming, requiring incubations of four to four and a half hours following addition of the sample chosen for classification. The kit also proved to be less than efficient with respect to cost. Of the large proportion of the colonies growing on the CDMN agar plates, about 90% were determined as not *C. difficile* based on colony morphology with the majority of these isolates failing characteristic tests. It was important to conclude what organisms were capable of growing on this media, as well as in the anaerobic conditions.

The classification of the anaerobes on the CDMN plates with this kit was inconclusive, as three out of four chosen colonies failed to yield significant colour changes, indicative of biochemical changes. This inhibited profiles to be created using the provided identification table. This could be due to the kit being limited

to eighty different anaerobic microorganisms, and as discovered using the bacterial 16S sequencing, the bacteria recovered from the CDMN plates were facultative anaerobes. In addition, of the list of species the kit is able to detect, none of the bacteria classified with the bacterial 16S sequencing would have been identified. Consequently, in order to determine any unidentifiable colony morphologies, this study has found bacterial 16S sequencing would be the most reliable technique. Developments in technology over the past twenty years have allowed for this method to emerge as being of high importance in terms of identifying bacterial species (Baker et al. 2003).

From the results obtained in this current study, it can be concluded that a high proportion of the recovered bacteria were *Staphylococcus epidermidis*, 50% of the tested samples (n=12). Furthermore, these samples do not possess the same morphology, varying in size as well as the ability to lyse the blood surrounding the colony. It can be concluded that its presence is due to it being shed continuously from the skin of patients, visitors, and healthcare staff members. The same conclusion can be drawn with respect to *Staphylococcus saprophyticus*, positive in 16.7% of the tested samples. This too possessed a range of size and colours in terms of morphology. All species identified were facultative anaerobes. Furthermore, the presence of antibiotics had no effect in inhibiting the growth of these *Staphylococcus* species, suggesting their resistance to the media used. Moxalactam has previously been shown to exhibit sensitivity to both *Staphylococcus aureus* and *Staphylococcus epidermidis* (Chang et al. 1988). Additionally, Dancer (2008) stated the resistance of staphylococci to desiccation was recognised, with MRSA contamination capable of surviving within a hospital site for over a year after initial inoculation. Furthermore, there have been reports to suggest the use of 1000 ppm chlorine-based products in manual cleaning does not result in effective reduction of MRSA (French et al. 2004; Jeanes et al. 2005).

Sample 56*, the first positive *C. difficile* colony isolated, was an important find as its colony morphology does not appear characteristic of *C. difficile*, nor did the colony fluoresce yellow-green when the original sample plate was exposed to UV light. This isolate did fluoresce, however, when re-plated onto a BHI blood

plate, prior to DNA extraction and sequencing. This result of the colony not fluorescing when growing on a RODAC plate has not been documented prior to this study; no other groups have reported the colony morphology of *C. difficile* differing depending on how the media on the plate was exposed to the bacterium, in particular when collected and recovered from the environment. Based on these results, positive samples of *C. difficile* were identified from the environment, as classification via colony morphology became more accurate. Furthermore, upon sub-culturing the chosen colony from sample 56* onto a BHI blood plate, the characteristic *C. difficile* odour was detected, though this was not found on the RODAC plate (Fordtran 2006). There have been no prior publications suggesting a difference in the odour of *C. difficile* when comparing samples recovered via a RODAC plate and with direct culture application onto a BHI blood plate.

Bacterial 16S sequencing therefore proved to be a robust, efficient means of identifying the unknown organisms growing on the plates collected from hospital sampling as conclusive results were obtained from all samples. Furthermore, the level of confirmation for all organisms was above the accepted thresholds in terms of percentage identity. It also showed the medium CDMN is capable of recovering facultative anaerobes, which are able to tolerate the conditions optimal for *C. difficile* growth. The identification of the colony morphology *C. difficile* presents with when recovered from direct contact on CDMN agar prepared in RODAC plates had been recorded (Figure 3.4). This was then used as the definitive description following subsequent sampling episodes to identify *C. difficile* recovery. Strain analysis of the four *C. difficile*-positive isolates from this sampling campaign confirmed the samples as ribotype 027.

Due to the lack of *C. difficile* isolates recovered from the first sampling campaign in combination with the findings from the previous chapter in this thesis (Chapter 2), it was decided that all five media explored would be prepared in RODAC plates and used for a second round of sampling. In addition to this, the location of *C. difficile* sampling was changed to a *C. difficile* isolation ward. The motivation for this was to increase the chances of recovering the bacterium.

Furthermore, literature suggested that *C. difficile*-infected patients inhabit rooms that can recover from between 9-59% *C. difficile* isolates (Barbut et al. 2009). This is in comparison up to 8% *C. difficile* contamination recorded from rooms where no *C. difficile* had been reported, either through infection or colonisation (Kim et al. 1981). However, this could be due to aerosolisation of spores and subsequent settling over time, as the authors report *C. difficile* spores are capable of persisting on surfaces for up to five months.

Patients testing positive for *C. difficile* following a toxin test were isolated in side rooms in the LRI ward. An increased probability of picking up these spores from sites within the isolation ward and subsequent germination was expected. Two side rooms as well as a shared toilet area were selected for sampling. The disposal area for patient bedpans, the sluice, was also sampled with these media prepared in RODAC plates. In order to ascertain how successful ChlorClean and routine cleaning of these side rooms were, the isolation rooms were sampled both before and after a daily standard clean.

Data previously obtained in this project with respect to the pick-up and germination of *C. difficile* spores following damage by ChlorClean identified the non-selective media FAA and GS-BHI as the most successful. It is possible the incorporation of antibiotics in the media CCEY, CDMN, and CCAB resulted in an additional stress against spores damaged by exposure to the cleaning agent.

Following incubation, it quickly became apparent that the non-selective media, FAA and GS-BHI agar, did not discriminate bacterial pickup, recovering overall bacterial counts ranging from zero to 498, many of which were not identified as *C. difficile* or *C. difficile*-like. The selective *C. difficile* media exhibited perhaps more selectivity than predicted with RODAC plates prepared with CCEY recovering up to five colonies. This could also be attributed to the antibiotic-containing media affecting the selection pressure against less viable spores, affecting the germination into vegetative cells and colony formation. Differences in susceptibility to antibiotics with species of *Listeria* have been observed. Changing the temperature at which the antibiotic-containing agars are incubated has been shown to affect selectivity (Curtis et al. 1989). Although it is not

suggested that the incubation temperature would have affected the growth of *C. difficile* in this study, it could be inferred that the addition of antibiotics has had some effect on the number of colonies present on a RODAC plate following sampling.

The overall recovery numbers from the RODAC plates were somewhat lower than expected, however. This was based on the success rates reported in literature by the research groups who had previously utilised these agar media, which range from 12% to up to 90% *C. difficile* recovery (Table 1.1) (Kamiya et al. 1989; Martirosian et al. 2005; Alfa et al. 2008; Shapey et al. 2008; Wheeldon et al. 2008). Electing an isolation ward was based on the prospect of a site heavily contaminated with *C. difficile*, which would be seemingly easier to recover from and therefore culture for strain identification.

Prior to IMS washing, GS-BHI agar led to the recovery of the highest number of *C. difficile*-positive samples with the RODAC plates directly contacting the surfaces in this sampling campaign (n=3). This was equal to CCEY, the agar concluded overall as the most selective following the results from Chapter 2 of this thesis. The results from this chapter also demonstrated CDMN and CCAB recovered the same number of positive samples of *C. difficile*, which was also seen with this campaign (n=2). Despite the ability of FAA to recover 32.7% of *C. difficile* spores in the laboratory tests following chemical damage with sub-lethal concentrations of ChlorClean, this success did not translate well with use in the hospital environment (n=1). Nevertheless, FAA was the only agar to pick up all of the different ribotypes found when conducting this sampling campaign.

Only 3/25 *C. difficile* isolates were identified with the use of CCFA broth. All 25 were found with RODAC plates and the other means of resuscitation. This lack of selectivity with the enrichment broth is not concurrent with previous findings in literature (Riley et al. 1987; Arroyo et al. 2005; Thitaram et al. 2011). Riley et al. (1987) reported that the use of an alcohol shock technique (9.6%) in the isolation of *C. difficile* was twice as efficient as attempting to directly culture following sampling (5.5%), whilst a selective enrichment broth was three times more successful (14.2%) in the resuscitation of *C. difficile* (n=218). Reports of 15.9%

(*n*=345) *C. difficile* with an enrichment broth (cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate) were also stated by Thitaram et al. (2011). The same broth was used by Arroyo et al. (2005), with a 94% isolation rate. It is noteworthy, however, that all of these studies were based on the recovery of *C. difficile* from faecal specimens whereas the use of this technique within this chapter was from damaged and stressed spores isolated from the environment. The use of an enrichment broth was suggested only when fewer *C. difficile* spores were expected to be present, reporting a frequent over-growth of other microorganisms (O'Farrell et al. 1984).

Based on the difficulties encountered with the identification of individual *C. difficile* colonies on RODAC plates containing other bacterial growth, sometimes over-run with contamination, the method of *C. difficile* selection and recovery was modified. Personal experience and literature demonstrated that with faecal samples it is essential to use an alcohol shock to ensure only *C. difficile* spores survive and are isolated from within the plethora of organisms (Borriello & Honour 1981; Riley et al. 1987; Clabots et al. 1989). Therefore, this notion was applied to the environmental isolates. Each RODAC plate was washed with IMS to induce an alcohol shock and, theoretically, kill any other bacterial cells that had grown on the surfaces of the plates. This led to an increase in the numbers of samples testing positive with *C. difficile* and would therefore be advisable as a technique to be used after sampling with RODAC plates. This practice has been previously reported with environmental sampling and also demonstrated as a necessary step in isolation in this thesis (Kaatz et al. 1988; al Saif & Brazier 1996; Alfa et al. 2008).

The agar media used in the resuscitation of *C. difficile* after IMS washing were selected because of its use in laboratory culturing of *C. difficile* (CCEY and BHI blood), or its success in recovery identified in this thesis (Chapter 2 and this chapter) (FAA and GS-BHI). The number of positive *C. difficile* cultures obtained from RODAC plates prepared with GS-BHI agar increased from three to nine, and for FAA this was one to nine. This supports the previous findings that the non-selective agars are more successful at the pick-up and germination of *C. difficile*

spores. Lysozyme has been shown to significantly increase the recovery of *C. difficile* when integrated into a selective medium which contains bile salts (Wilcox et al. 2000). Although not selective media, FAA and GS-BHI agar did contain sodium taurocholate. It has been well documented that the incorporation of this bile salt into *C. difficile* media has the effect of increasing the proportion of *C. difficile* spores germinating into CFU up to 1.7-$\log_{10}$ higher (Wilson et al. 1982). Therefore, it could be possible to attribute the increase in recovery to the presence of germination agents within the agars, enabling the spores to thrive and complete their growth cycle.

The additional step of IMS washing also helped increase the number of positive samples obtained from CDMN prepared RODAC plates from two to five. This result shows the effects of incorporating this alcohol shock can extend to selective media as well as non-selective. It has been reported that the incorporation of such a shock results in the reduction of competing flora, allowing *C. difficile* to be isolated somewhat easier (Brazier 1998). However, with the media CCEY and CCAB, the washing of the colonies with IMS did not induce any more *C. difficile* positive cultures, and so the numbers of samples remained unchanged. These two media recovered far fewer overall bacterial counts; for example, a RODAC plate used to sample the floor entrance in the second side room with CCEY recovered only one colony, which was confirmed as *C. difficile*. It could be inferred that the selectivity of the antibiotics was successful in that no other bacteria were able to proliferate on the surface of this particular RODAC plate.

The data from this study also identified that there were differences in the ability of an agar to resuscitate *C. difficile* following the IMS wash step. CCEY agar was the most successful with respect to colony formation and enabling *C. difficile* growth. Fourteen samples grew on CCEY agar from the original 42 RODAC plates used for sampling. This medium is used for the isolation of *C. difficile* from faecal specimens, specifically selective of *C. difficile* over other clostridial species. This study has demonstrated that this selectivity extends to samples obtained environmentally.

The media FAA and BHI blood agar recovered twelve and eleven *C. difficile* samples, respectively. The lack of selective agents in these two agars meant that these results were relatively unexpected. BHI blood agar possesses no selective or germination agents to propagate *C. difficile* from the IMS shocked cultures. The main use of this agar is in the identification of *C. difficile* in diagnostic testing and in the maintaining of samples through sub-culturing.

GS-BHI agar recovered the highest number of positive samples from the RODAC plates; eight samples spread post-sampling onto this medium isolated *C. difficile.* It is therefore possible that this medium is less able to resuscitate *C. difficile* spores, but more successful in initially picking-up the spores from a surface and germinating them, as seen in the previous chapter of this work.

All five media used for this sampling campaign recovered ribotype 103. Little knowledge exists in literature on this particular strain; it does not appear to be clinically relevant or dominant in UK hospitals. This makes its presence particularly noteworthy. The spread of this ribotype in all sites sampled could be attributed to the dissemination or transfer by healthcare and/or cleaning staff between rooms, particularly since patients present in these side rooms are usually rendered immobile. In addition to this, there were few occurrences of ribotype 027 recovered from this sampling campaign, despite it featuring heavily in literature as a problematic strain (Roberts et al. 2008; Dawson et al. 2011; Health Protection Agency 2012). Localised within the sluice area, this shows that either the media were unable to pick up and germinate the *C. difficile* spores of this type or it was not present within the side rooms or toilet at the time of sampling. This could also explain the presence of ribotype 081 in the sluice alone.

Conducting the routine cleaning procedure evidently has an effect on the presence of ribotype 010; found in both side rooms before cleaning, but in neither after. This non-toxigenic strain has been reported in pigs, cats and dogs (Terhes et al. 2006; Keel et al. 2007; Koene et al. 2011). It accounted for 0.04% of the *C. difficile*-positive samples submitted for ribotyping to the HPA during the period of 2007/2008 (Health Protection Agency 2008). A recent report proposes the spread of *C. difficile* cannot be accredited entirely to contact with infected patients

(Walker et al. 2012). This group interestingly discovered that even with consenting to the persistence of ward contamination after the discharge of a *C. difficile*-positive patient, there was no increase in the infection rates.

From this sampling campaign, there was a distinct lack of positive samples obtained, both before and after the enrichment stage. Although, the incorporation of the enrichment aided in increasing the total number of positive samples, the overall number of RODAC plates that successfully recovered at least one viable *C. difficile* colony was too low for meaningful statistical analyses to be performed.

The RODAC plates were not IMS washed immediately following the sampling. Having attempted to identify the maximum potential *C. difficile* colonies, the plates were individually sealed and refrigerated to restrict further bacterial growth. A common method for preservation of faecal samples before isolation of the bacterium is storage at 4°C (Freeman & Wilcox 2003). It is possible, however, that the spores present in a faecal sample differ from those recovered environmentally in situations such as these, in which the spores have been exposed to more stresses, and storage at this temperature could be another potential stress.

Little has been found as to the mechanisms of how *C. difficile* spores react when exposed to stresses, but studies have been carried out into such effects on *Bacillus subtilis*. The effect of hypochlorite or other such agents are capable of rendering spores defective and incapable of successful germination because of the damage these agents cause to the inner membrane of the spores (Young & Setlow 2003; Fawley et al. 2007). Evidence has also shown that germinant receptors can be distressed and therefore damaged. Comparisons are often drawn between *C. difficile* and *Bacillus subtilis* spores due to the homology of their genes; *Bacillus subtilis* germination has also been well studied (Paredes-Sabja et al. 2011).

This particular method of sampling in an attempt to gauge the presence of *C. difficile* and the distribution of ribotypes was not as successful as anticipated. This was based upon previous studies reporting success in the recovery of *C. difficile* spores (Kamiya et al. 1989; Martirosian et al. 2005; Alfa et al. 2008; Shapey et al.

2008; Wheeldon et al. 2008). Further development into the sampling technique employed would be required to maximise the number of samples recovered. Additionally, resuscitation methods following sampling also required optimisation. This consequently effected the resuscitation of the damaged spores themselves. The findings from this section have demonstrated there may be further work in manipulating and modifying the media used to resuscitate *C. difficile*.

From the results obtained from the previous two rounds of environmental sampling, and the lack of recovered *C. difficile*, a further third campaign was conducted, with a different technique. The main restriction identified with the use of a RODAC plate is the maximum area that can be sampled. The probability of recovering *C. difficile* from a site is therefore reduced with a smaller sampling area. Therefore, expanding this site would potentially increase the proportion of *C. difficile*-positive samples recovered, if the bacterium is indeed present. Subsequently, alternate methods for sampling were explored and the use of pre-moistened sponges in the same *C. difficile* isolation ward was chosen. This would allow for coverage of a greater surface area as well as more 'hard to sample' and rounded-edged areas with a RODAC plate (Otter et al. 2009). It was still assumed that sampling in the isolation ward would recover the highest number of positive samples.

Following this sampling campaign, all fifteen sponges were individually homogenised before being alcohol shocked and then plated onto the surfaces of the non-selective top layers. Samples were spread on solid agar rather than immersing cultures in the non-selective agar in its molten forms and subsequently poured onto the antibiotic-containing media to reduce the possibility of any further sub-lethal damage to the spores and cells. The alcohol shock was thought to illicit enough injury to the other bacterial contamination.

Of the sponges used in the sampling campaign, *C. difficile* was successfully isolated from 66.7% (n=15). Use of pre-moistened sponges such as those applied in this work, previously produced *C. difficile* from 27% samples collected over six healthcare facilities (Dubberke et al. 2007). The recovery recorded was 28% for

Otter et al. (2009) following hydrogen peroxide vapour treatment of the sampling sites.

The layering of two different media, an antibiotic-containing base with a non-selective top media, has been employed in the food industry (Wu 2008). Wu (2008) suggested the presence of a thin layer of non-selective agar (5 ml) over antibiotic-containing media (14 ml) aids in the bacterial growth, demonstrating success with *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Yersinia enterocolitica* (Wu et al. 2001). Duan et al. (2006) also reported overlaying an equal volume of 10 ml non-selective medium over a selective medium was an effective alternative method for recovery of bacteria.

The five different media (CCAB, CCEY, CDMN, FAA, and GS-BHI) which have been used throughout these studies, were combined to produce a range of layered agars (Table 3.2). The use of a layered media tempers the stress that antibiotics may impose directly. The damaged cells are able to survive and grow, germinating due to the presence of sodium taurocholate in the lower layer, when incubated (Wilson et al. 1982; Buggy et al. 1985). The supply of nutrients and antibiotics from the base layer would interact with the cultured cells to resuscitate microorganisms, in this case, *C. difficile*. The antibiotics are thought to also inhibit the formation of colonies not *C. difficile*, as suggested with findings from other bacteria (Kang & Fung 2000; Wu et al. 2001; Wu & Fung 2003). This aids in the resuscitation of weaker spores from hostile environments, as exhibited in the hospitals, such as from cleaning products and an aerobic setting. The use of this layering technique is expected to be significant as the combination of the two media acts to allow those damaged spores present on the surface of a sampling site to be detected. Ordinarily, these may not have necessarily been successful in full germination to cells if selection pressures from antibiotic use in the media alone resulted in inhibition. The presence of the non-selective media and allowing the samples to be exposed to this first would have theoretically permitted the cell growth before selection can be applied.

In this study, not only was a thin layer of non-selective media used over an antibiotic-containing preparation, but the inverse with a thicker layer, as well as equal volumes of both. No conclusive evidence was produced with the samples of *C. difficile* collected in this study to indicate a particular ratio was important in ensuring recovery of the bacterium. The numbers of positive samples recovered were also too low to warrant conducting statistical testing. This work in isolation is not sufficient to conclude the success of layering media in *C. difficile* recovery, however, the scarcity of studies pertaining to the optimisation of *C. difficile* resuscitation from environmental isolates suggests this is a potential area for further investigation. Nevertheless, there may be a link in the proportions of antibiotic concentrations required. The positive isolates recovered indicate the spores can be resuscitated with this layering media technique. However, experimental work in the laboratory exposing known concentrations of cultures to the surfaces of these agar media may provide an insight into the specifications required for conclusive results.

The selection of the *C. difficile* isolation ward for sampling was for the same reason as with the second campaign: to maximise the chances of recovering the bacterium. Attempting to pick up *C. difficile* in a ward where the patients have been previously tested positive for the bacterium and are under-going treatment for its disease would suggest that the sites themselves would be at higher risk of contamination (Kim et al. 1981; Barbut et al. 2009). Exposures of the sites following diarrhoeal incidents results in aerosolised spores and therefore the presence of *C. difficile* (Roberts et al. 2008).

The work in this study identified the presence of ribotype 027 within hospital wards, amongst other strains, albeit non-epidemic strains. Environmental sampling by Shapey et al. (2008) identified the prevalence of ribotypes 001, 027 and 106, as well as 16 other strains. Rotimi et al. (2003) reported the isolation of *C. difficile* from Kuwaiti hospitals: ribotype 078 from one hospital site alone, while ribotype 097, an infrequently reported strain, was restricted to another. Many authors, however, fail to report the identity of ribotypes isolated from such studies,

providing only the total number of successful samples collected (Barbut et al. 2009; Otter et al. 2009). This hinders any comparisons that can be drawn with this work.

The presence of ribotype 027 throughout all the sampled side rooms either would suggest the transfer of strains or perhaps suggests the general strain abundance. Comparing the ribotypes from the patients to their individual side rooms could suggest that regardless of potential strain transfer by hospital, clinical or cleaning staff, the patients themselves were carrying this ribotype prior to admission. As described above with the second sampling campaign, many of the patients present in the side rooms sampled were immobile and were not permitted to move from room to room if able enough. The faecal samples analysed from the patients present in the rooms were all obtained before the admission into the *C. difficile* isolation ward. The infection had already been contracted. The contamination of ribotype 027 in the rooms could well have been from the current patients residing. This is supported by a report by Hota (2004), which suggested a link exists between strains infecting a patient and those within the surrounding environment.

Comparing the ribotype distribution obtained from the two sampling campaigns conducted in the *C. difficile* isolation ward in the LRI, only a small proportion of ribotype 027 was identified from the second sampling campaign. This contrasts with all of the samples from the third campaign, confirmed as 027. A method that can be used for further contextualisation of strains to a more discriminatory level than a ribotype is MLVA typing (van den Berg et al. 2007). This technique would provide information as to whether the same MLVA type of the ribotype 027 strain was being carried by the patient and present in their surroundings, as identified in the third campaign, or perhaps that the repeated reports of ribotype 027 and its underlying presence within healthcare facilities is responsible for its detection from each room investigated.

Despite this, it is possible for more than one ribotype to reside within a given site; the second sampling campaign demonstrated the presence of multiple strains in one enclosed room, however, the sponges recovered only one type per room. Although, the total number of samples collected differs between the two

studies, 15 samples were collected with the pre-moistened sponges and 42 from the RODAC plates, the proportion of positive samples identified from the sponges was greater. This suggests further advantage of the sponge technique for *C. difficile* pick-up over the use of RODAC plates in addition to the aforementioned surface area sampled.

The initial sampling campaign conducted within this chapter was designed to identify whether a difference in the recovery of *C. difficile* is affected with the use of a deep clean with hydrogen peroxide. Issues with the identification of *C. difficile*, in addition to the disadvantage of utilising only one RODAC plate per site sampled proved problematic. The results from this chapter have identified the use of agar media within RODAC plates may not be ideal, however, optimisation of the layering of media in combination with the use of pre-moistened sponges has suggested a potential avenue in the selection and isolation of environmental *C. difficile*.

In the previous chapter of this thesis, RODAC plates were utilised for the pick-up of one strain of *C. difficile* from an area where the spores had been applied and therefore its presence was known. In addition to this, the spores were stressed with only one factor, a germicide. With environmental recovery of *C. difficile*, there are a number of stresses exerted onto the spores, such as desiccation, disinfectants, and more importantly, aerobic conditions. The findings from this chapter have demonstrated that regardless of these additional pressures, a range of ribotypes were able to tolerate these stresses. This therefore suggests that further work is required to investigate the different strains present within the healthcare facilities and methodologies into their eradication, as investigated in Chapters 4 and 5.

3.5 Conclusions

- RODAC plates prepared with different media were placed in 'high' and 'low' contact sites. Temporal patterns were observed with respect to bacterial contamination over time.
- The *C. difficile* medium, CDMN, was not as selective as expected and so techniques to germinate and isolate the bacterium were necessary. Bacterial 16S sequencing was identified as a key tool in differentiating between species recovered from environmental sampling.
- Five *C. difficile* media were used in recovery from two isolation rooms before and after routine cleaning, in addition to a shared toilet area and sluice (47.6% *C. difficile*-positive sites).
- Fewer positive cultures were identified post-clean, indicating a reduction in spore viability with the use of ChlorClean, as supported in literature.
- Different ribotypes were found to reside within a single patient isolation room.
- The importance of IMS washing and/or alcohol shocking to inhibit other microbial flora from environmental isolates was highlighted. This increased the number of positive samples isolated from the sampling campaigns.
- Sampling with sponges was the most successful in terms of *C. difficile* recovery (66.7% *C. difficile*-positive samples). Investigations into layering of non-selective media over antibiotic-containing agar were inconclusive, although a link in proportions is suspected.
- Ribotype 027 was identified in both patient faecal samples and the surrounding isolation rooms, indicating potential patient-environment contamination.

3.6 Appendix

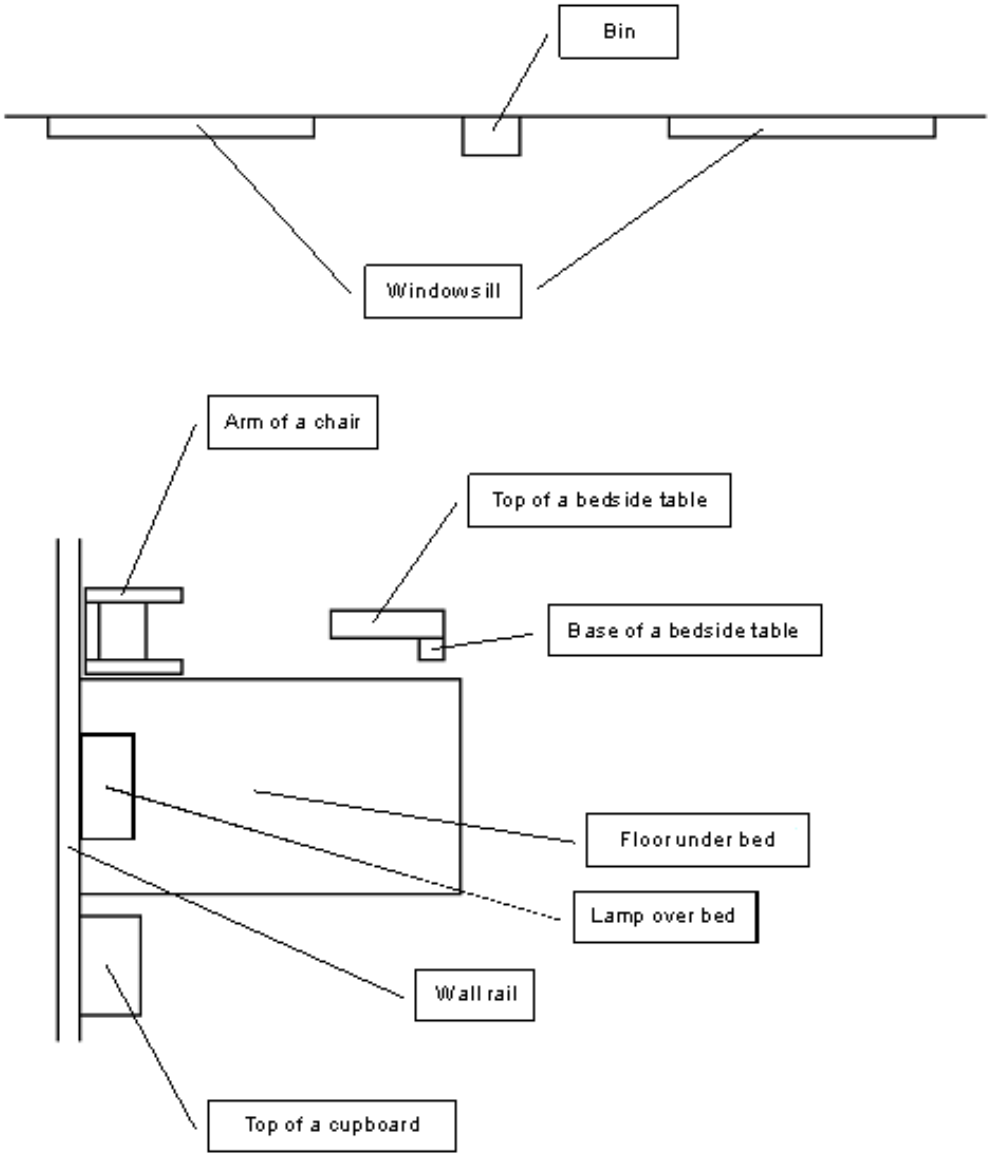

Figure 3.11: Map of a bed in the UHL Trust.

Areas identified show where sampling occurred with Campaign 1. Sampling Campaigns 2 and 3 were conducted in isolation rooms, as such, with the addition of a personal sink. Image by Pawel Wolyniec.

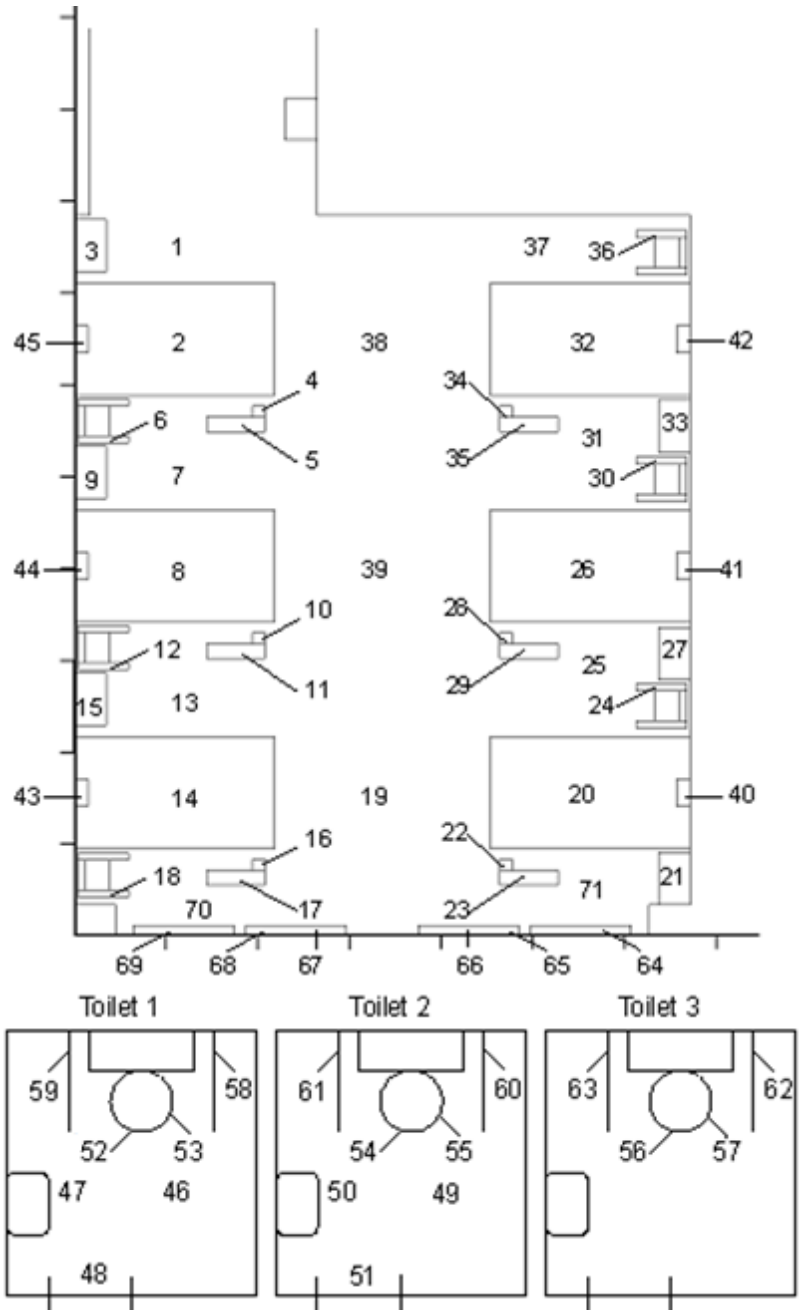

Figure 3.12: Map of the LGH ward sampled in Campaign 1.

Numbers indicate notation for plate identification. Image by Pawel Wolyniec.

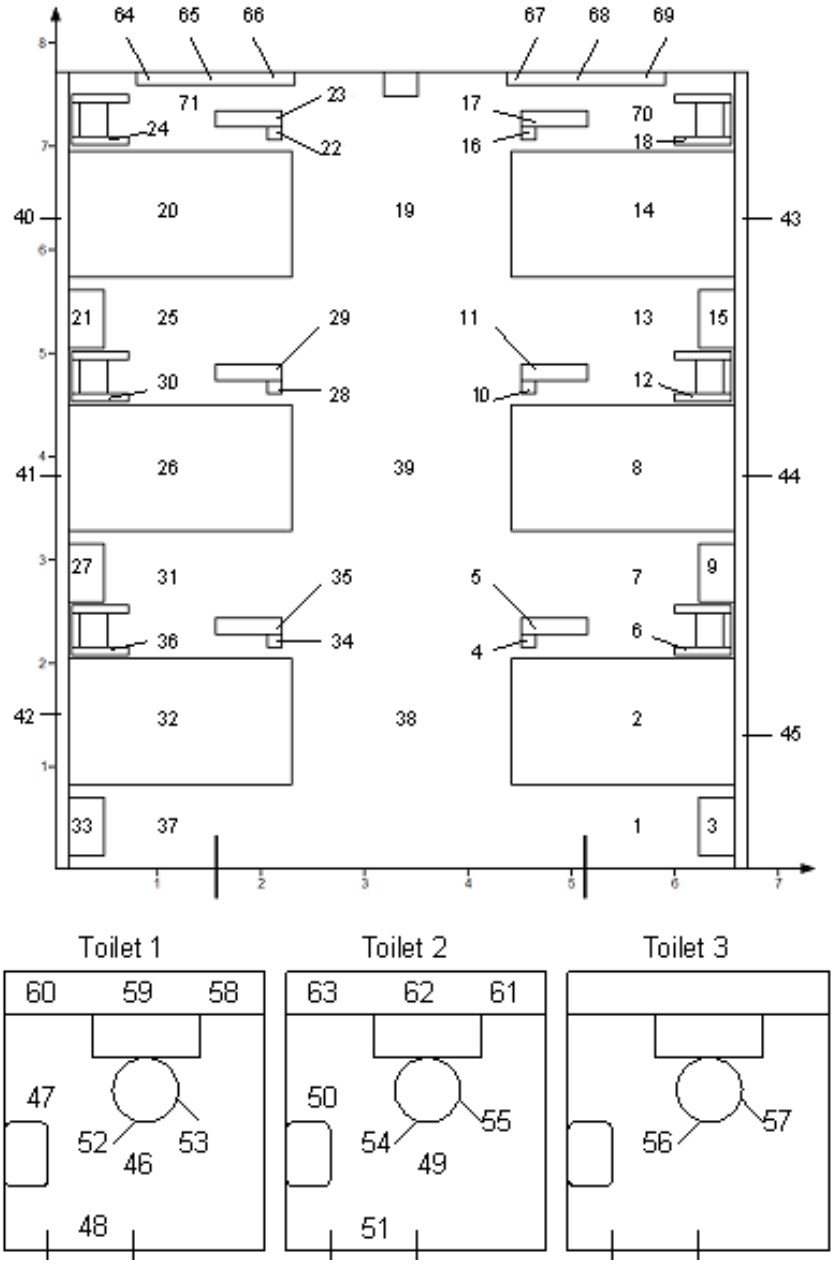

Figure 3.13: Map of the LRI ward sampled in Campaign 1.

Numbers indicate notation for plate identification. Image by Pawel Wolyniec.

Sample	**Genbank ID**	**Full Species Identity**
6	FJ357583.1	*Staphylococcus epidermidis* strain BBEN-01d 16S ribosomal RNA gene, partial sequence 1432 bp
7	EU419922.1	*Staphylococcus epidermidis* strain RW35 16S ribosomal RNA gene, partial sequence
28a	AP006716.1	*Staphylococcus haemolyticus* JCSC1435 DNA, complete genome 2685015 bp
31	EU162006.1	*Staphylococcus saprophyticus* isolate PGO16 16S ribosomal RNA gene, partial sequence 1196 bp
*56**	AM180355.1	*Clostridium difficile* 630 complete genome
G2	EU162006.1	*Staphylococcus saprophyticus* isolate PGO16 16S ribosomal RNA gene, partial sequence 1150 bp
G8	FJ749756.1	*Enterococcus faecium* strain IMAU60025 16S ribosomal RNA gene, partial sequence 1434 bp
G9	FJ613561.1	*Staphylococcus epidermidis* strain EIV-3 16S ribosomal RNA gene, partial sequence
G22	AE015929.1	*Staphylococcus epidermidis* ATCC 12228, complete genome
G23	FJ844987.1	*Lactobacillus fermentum* strain IMAU20081 16S ribosomal RNA gene, partial sequence
G31	FJ357583.1	*Staphylococcus epidermidis* strain BBEN-01d 16S ribosomal RNA gene, partial sequence 1440 bp
G32	FJ613572.1	*Staphylococcus epidermidis* strain EIV-14 16S ribosomal RNA gene, partial sequence 1190 bp

Table 3.6: Full species information following bacterial 16S sequencing of isolates collected in Campaign 1.

Chapter 4. Distribution of *C. difficile* PCR Ribotypes in an NHS Trust

4.1 Introduction

Several methods have been developed in order to contextualise and categorise the different sub-types of *C. difficile*. These include multi-locus sequence typing, multi-locus variable-number tandem-repeat analysis, pulsed-field gel electrophoresis, restriction endonuclease analysis, amplified fragment length polymorphism and surface layer protein A gene sequence typing (Killgore et al. 2008). Of the possible approaches, PCR ribotyping is the most commonly used in the UK and Europe (Kuijper et al. 2007; Bauer et al. 2011). This technique targets the 16S and 23S rRNA interspacer regions of the bacterial gene with specific primers (Brazier 2001). A ribotype is assigned according to the number of copies of this gene and their sizes which range from 250 to 600 bp (Stubbs et al. 1999). It has been reported that there are over 430 recognised official ribotypes (W Fawley, 2012, pers. comm., 20 Mar).

Different ribotypes of *C. difficile* exhibit different phenotypes, with respect to virulence factors, toxin production, and susceptibility to antibiotics, amongst others. The differentiation of bacterial strains is important as it can also identify the prominent in a sample cohort. Routine monitoring of strains could aid in identifying which particular ribotypes are currently in the environment and therefore can be considered responsible for causing a disease outbreak. It has been reported that ribotype 027, the most commonly isolated ribotype of *C. difficile* worldwide, causes a higher level of disease severity than any other ribotype (McDonald et al. 2005; O'Connor et al. 2009). Different research groups have debated this, but what is certain is its dominance.

Traditionally, PCR products from ribotyping are separated on a high-resolution agarose gel, and the sizes of the bands are estimated with gel analysis software, with size standards used as markers. The separation of DNA can differ between gels which leads to the analysis of sizes of bands being subjective (Zaiss et al. 2009). Recently, capillary gel-based PCR ribotyping electrophoresis has been used by research groups as well as the HPA in the UK (Indra et al. 2008; Wei et al. 2011; Xiao et al. 2012). This incorporates a fluorescent primer in the PCR mixture and a fluorescent standard marker for each sample. Computer outputs in the form

of chromatograms have peaks corresponding to fragment sizes allowing greater precision and enabling standardisation of strains between laboratories (Indra et al. 2008).

Data on the PCR ribotypes identified within a Trust or a healthcare facility are not routinely collected in the UK. This is due to time and costs which arise from the isolation and culturing of the bacterium. However, collating such information would make for a discriminatory dataset, which would in turn provide the framework to enable further understanding of the disease epidemiology. Currently in the UK, the HPA has a programme in place, which requires NHS Trusts to disclose the number of *C. difficile*-positive patients. The HPA also conduct mandatory surveillance of *C. difficile* infection rates and routinely release these data (Health Protection Agency 2008). Specific subsets of samples are sent to the HPA following periods of increased incidences or outbreaks, for example, and are all ribotyped to produce an overview of strains present in the regions of the UK. The data have shown that since the start of the scheme, ribotypes 002, 015, and 078 have become more abundant over the periods 2007/8. They then gained even greater prominence in subsequent years (Health Protection Agency 2012).

4.1.1 Aim of this study

In this section of the thesis, a robust method for PCR ribotyping was established. The specific aim was to investigate the ribotypes present within an NHS Trust over a period, to determine their abundance, and their geographical locations, with respect to hospital or within community sites. The work presented in this chapter is from two different sampling cohorts. In the first study, faecal samples tested as *C. difficile*-positive in the UHL Trust over a four-month period with a reported increased incidence were characterised by ribotype (August to November 2009). The method of *C. difficile* detection from faecal samples changed in January 2011 within the UHL Trust. Specimens were therefore obtained both two months prior and following this enforced change to evaluate any patterns in the distribution of strains.

The optimisation of PCR ribotyping was also imperative for addressing aims in other chapters of this thesis, in the investigation into the diversity of

strains collected from environmental sampling in hospitals (Chapter 3) and subsequently exposure of hydrogen peroxide to a range of strains (Chapter 5).

The findings from this chapter aim to help better understand the *C. difficile* epidemiology within our local NHS Trust. It will also show how more comprehensive studies can identify trends that may not be reported with the current practice of submitting only a proportion of patient faecal samples for strain analysis.

4.2 Materials and Methods

4.2.1 C. difficile culturing on solid medium

These are the solid media used for growth of *C. difficile* in a laboratory.

4.2.1.1 Selective C. difficile medium

CCEY agar: Prepared as directed and autoclaved at 121°C for 15 min. Once cooled, 5% (v/v) egg yolk emulsion and an antibiotic mix of cycloserine (250 mg/l) and cefoxitin (8 mg/l) were added [all BioConnections, UK].

This agar also known as Brazier's Medium, was used to select for *C. difficile* from faecal specimens following an alcohol shock (Brazier 1993). The use of egg yolk emulsion in media preparation is to aid in the detection of lecithinase and lipase enzymic activity. Indicators of positive results of the enzymes are a shiny surface and a halo around colonies. It has been documented however that *C. difficile* produces neither of these characteristics; the addition of the supplement proves useful with respect to confirmation and identification (Wilson et al. 1982).

4.2.1.2 Non-selective C. difficile medium

BHI blood agar: Prepared as directed from Brain-Heart Infusion and 1% Agar Bacteriological (Agar No. 1), prior to autoclaving as described above. This was supplemented with 7% (v/v) defibrinated horse blood [all Oxoid Ltd., UK].

This medium was used to confirm *C. difficile* presence and for diagnostic tests, as described by George et al. (1979).

4.2.1.3 Preparation of agar plates

Following preparation of the media, as described above, the agar was poured aseptically into 55 or 90 mm Petri dishes [Fisher Scientific Ltd., UK].

4.2.2 C. difficile culturing in liquid medium

There were two different broths used to grow *C. difficile* cultures: BHI and FA broth [BioConnections, UK]. 37 g of BHI broth or 22.8 g FA broth was dissolved in 1000 ml of ultra-pure water and autoclaved at 120°C for 15 min. All liquid media were pre-reduced with overnight incubation in the anaerobic chamber.

4.2.3 PCR ribotyping

PCR ribotyping is a method used to differentiate between *C. difficile* strains through the individual patterns produced from the 16S to 23S bacterial interspacer regions, and has been utilised throughout this project. There has been evidence to show the dominance of particular ribotypes in a given geographical location, in addition to there being consistency between the strain possessed by a patient and their surrounding areas (Rotimi et al. 2003; Goorhuis et al. 2008; Cheknis et al. 2009). Therefore, PCR ribotyping has been used in this work to contextualise faecal specimens as well as samples collected from hospital environments in other parts of this thesis to help understand what strains are being found in the UHL Trust.

4.2.3.1 Faecal specimen collection

The samples utilised in this study were collected by hospital or healthcare staff directly from patients possessing symptoms associated with *C. difficile* infection. Following confirmation of *C. difficile* within the UHL Microbiology Laboratory [Leicester Royal Infirmary] using the diagnostic methods described below, the specimens were kindly donated for the use in this work.

4.2.3.2 Methods for C. difficile detection in faecal samples

From 2008 and 2010 in the UHL Trust, the method for confirming *C. difficile* was an ELISA-based assay [Techlab Inc., USA]. This detected the production of *C. difficile* specific toxins, enterotoxin (A) and/or cytotoxin (B), from diarrhoeal faecal samples.

The method of detection changed in January 2011 to a GDH antigen detection assay [Techlab Inc., USA], which acts to screen out *C. difficile*-negative samples. The GDH-positive specimens are then further analysed using a toxin EIA to detect the presence of toxins within the faecal sample. Molecular methods are also used in the form of a PCR-based test (GeneXpert *C. difficile* assay) which tests for PCR ribotype 027 specifically through a series of signals [Cepheid, USA].

All samples were selected based on whether the patient was over the age of 2 years and had a loose stool. All loose faecal samples obtained from patients aged

65 or over were subject to testing. For faecal specimens positive for either toxin, aliquots were stored in sealed vials at 4°C aerobically. This method of storage has been shown to have little effect on the viability of *C. difficile* spores and toxins (Freeman & Wilcox 2003). Strains were isolated and stored in cryopreservation glycerol stocks at -80°C. All samples were obtained from the three major hospitals in Leicester: LRI, LGH and GGH, as well as community sites, consisting predominantly of GP surgeries or other smaller healthcare facilities.

4.2.3.3 C. difficile isolation and identification

For the purpose of *C. difficile* isolation from patient samples, the protocol was the same regardless of which of the two above methods were used to confirm diagnosis.

A faecal sample (1 g) was suspended in 1 ml IMS in the ratio 1:1, mixed via vortex using Vortex Genie 2 Shaker [Scientific Industries, USA] and left at room temperature for 30 min. This selected viable *C. difficile* spores and eliminated potential growth of non-spore forming faecal organisms. Using an inoculation loop, 50-75 µl was then added to CCEY agar plates before incubating overnight at 37°C in the anaerobic chamber. Single colonies were selected and plated out onto BHI blood agar plates. These were then incubated overnight in the anaerobic chamber at 37°C.

4.2.3.4 C. difficile propagation

To confirm *C. difficile* presence on the BHI blood agar plates, characteristic tests were carried out; positive colonies should appear about 4-6 mm irregular in diameter with edges slightly fanning out, raised, opaque and whitish grey in colour. Under long-wave UV light (365 nm), a greenish-yellow colour is fluoresced (Figure 4.1) (Brazier 1998). When cultured, *C. difficile* samples also possess a characteristic horse manure odour.

C. difficile-positive cultures were grown anaerobically overnight at 37°C in 3 ml of pre-reduced FA broth. Cultures were then sub-cultured in 8 ml BHI broth and incubated under previous conditions, or until the optical density (OD550) reached 0.8-1.0. This was measured using a UV-Visible Spectrophotometer Model

6715 [Jenway, UK] set at a wavelength of 550 nm with cuvettes [Scientific Laboratory Supplies Ltd., UK].

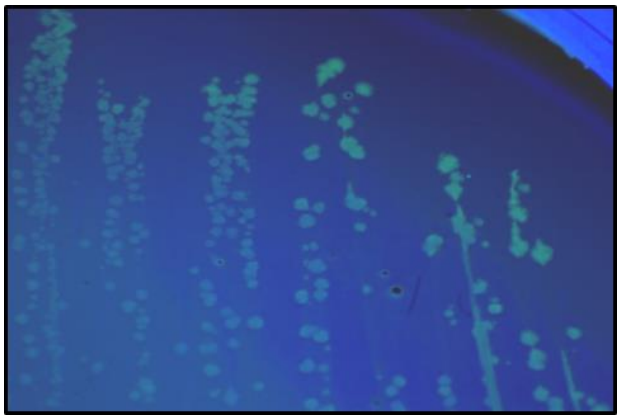

Figure 4.1: *C. difficile* colonies on BHI blood agar.
Colonies fluoresce a greenish-yellow colour under UV light. Photograph by Dr Danish Malik.

4.2.3.5 Chromosomal DNA extraction

DNA extraction was modified from a standard protocol (Sambrook & Russell 2001). Briefly, cells were centrifuged at 21,000 x *g* for 5 min at room temperature. The pellet was washed with 0.1 x saline-sodium citrate buffer and centrifuged as before, then re-suspended in 0.8 ml of lysis buffer (10 mM Tris.Cl pH 8.0, 1 mM EDTA, 1% SDS and 0.2 mg proteinase K). Cell pellets were incubated for 90 min at 37°C after which an equal volume of 4°C phenol:chloroform:isoamyl alcohol (25:24:1) was added for 2 min prior to centrifugation at 21,000 x *g* for 10 min at 4°C. The aqueous layer was recovered and the step repeated with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous layer was again recovered and the DNA precipitated by adding 2 volumes of 4°C propan-2-ol and 0.4 volume of 7.5 M ammonium acetate, then centrifuged at 21,000 x *g* for 30 min at 4°C. The pellet was washed with 1 ml of 4°C 75% ethanol, centrifuged as before for 15 min then air-dried and re-suspended in 50 µl of Tris.HCl pH 8.5. DNA quantity and quality was analysed using a NanoDrop ND-8000 spectrophotometer [ThermoScientific Ltd., UK].

4.2.3.6 PCR amplification

PCR reactions of each sample were prepared as follows (50 µl): 200 µM dNTPs, 10 x reaction buffer with NH_3, 1.5 mM $MgCl_2$, and 0.5 U *Taq* polymerase [all

Bioline, UK], 0.5 µM of the 16S rRNA gene primer, fluorescently labelled with carboxyfluorescein (FAM) {5'-CTG GGG TGA AGT CGT AAC AAG G-3'} and 0.5 µM of the 23S rRNA gene primer {5'-GCG CCC TTT GTA GCT TGA CC-3'} [Applied Biosystems, UK and vhbio, UK, respectively] (Stubbs et al. 1999). Template DNA (100 ng) was added. Table 4.1 shows the conditions for *C. difficile* PCR ribotyping, amplified with a SensoQuest Gradient Thermal Cycler [Geneflow Ltd., UK].

Process	Temperature (°C)	Time (sec)	Cycles
Initialisation	94	360	1
Denaturing	94	60	35
Annealing	57	60	35
Extension	72	60	35
Further extension	72	420	1

Table 4.1: PCR protocol for PCR ribotyping.

4.2.3.7 Agarose gel-based PCR ribotyping

PCR products were visualised under UV light after 3% agarose gel electrophoresis using Hi-Res super agarose [AGTC Bioproducts Ltd., UK] in TAE buffer containing 0.5 µg/ml ethidium bromide [Sigma-Aldrich, UK], and with reference to HyperLadder IV 100-1000 bp molecular weight markers [Bioline, UK].

4.2.3.8 Capillary gel-based PCR ribotyping

PCR fragments were run using an Applied Biosystems ABI 3730 genetic analyser by Protein Nucleic Acid Chemistry Laboratory [University of Leicester].

Briefly, a 20-1200 bp fluorescent GeneScan ladder was added to each sample prior to denaturation at 95°C for 2 min, storing on ice and analysed with the sequencer prepared with a 50 cm capillary, loaded with a POP7 gel. The samples were injected with 1.6 kV over 15 sec with a total running time of 103 min at 8 kV run voltage [all Applied Biosystems, UK].

4.2.3.9 Statistical analyses

The sizes of the peaks from chromatograms following capillary PCR ribotyping were resolved using Peak Scanner v1.0 [Applied Biosystems, UK]. Peaks that were at least 10% of the highest peak of the sample in question and between 250 and 650 bp were accepted as genuine fragments. Minitab 16

[Minitab Inc., USA] was used to create graphical representations of these data and to perform subsequent statistical analyses. Chi-squared defective tests were used to compare strain presence between cohorts. Significance was set to $P<0.05$.

4.2.3.10 C. difficile reference ribotypes

Reference strains of *C. difficile* ribotypes 027 and 106 were kindly donated by Professor Anthony Hart, Royal Liverpool University Hospital, and PCR ribotype 012 (CD 630) from Professor Neil Fairweather, Imperial College London. Confirmation of officially accepted ribotypes was carried out by Dr Warren Fawley and Professor Mark Wilcox, University of Leeds.

4.2.4 Ethical issues

Ethical approval was obtained for the ribotyping distribution studies conducted in this chapter. All information regarding patient data was made anonymous prior to use.

4.3 Results

4.3.1 Capillary gel-based PCR ribotyping compared with agarose gel-based

Three reference strains (PCR ribotypes 012, 027, and 106) and a representative of each of the ribotypes identified in this study were analysed using both agarose gel-based and capillary-based electrophoreses techniques. The ribotype profiles obtained matched to known profiles with both methods; there were similar numbers of DNA fragments and corresponding sizes (Figure 4.2 and Figure 4.3). The capillary-based system was found to detect fragments that were difficult to differentiate on an agarose gel. The capillary-based technique was investigated for its reproducibility with five isolates and three biological replicates each. The data obtained were consistent in all cases with respect to fragment size, in bp and number. The variations in fragment size recorded for an individual fragment ranged from 0.0-0.16 bp. Therefore, the majority of samples from this study were PCR ribotyped using the capillary-based system only. Representatives of each ribotype were sent to the CDRN reference laboratory [HPA, Leeds, UK] to assign them to their official designated nomenclature.

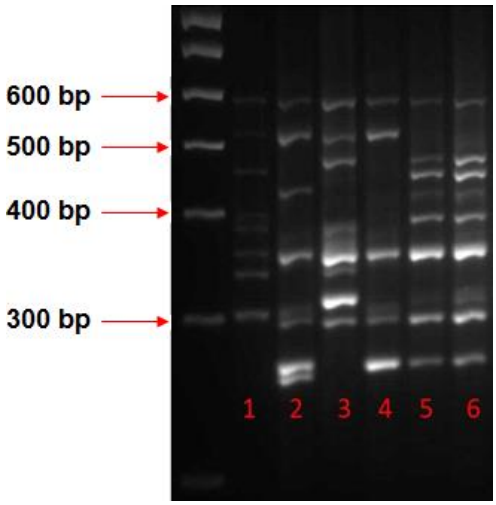

Figure 4.2: Agarose gel-based electrophoresis image of *C. difficile* PCR ribotype profiles.

Lane 1: PCR ribotype 012. Lane 2: Unknown ribotype. Lanes 3-5: PCR ribotype 106, NC11204, PCR ribotype 027. Lane 6: Faecal sample from study identified using the capillary-based system as ribotype 027.

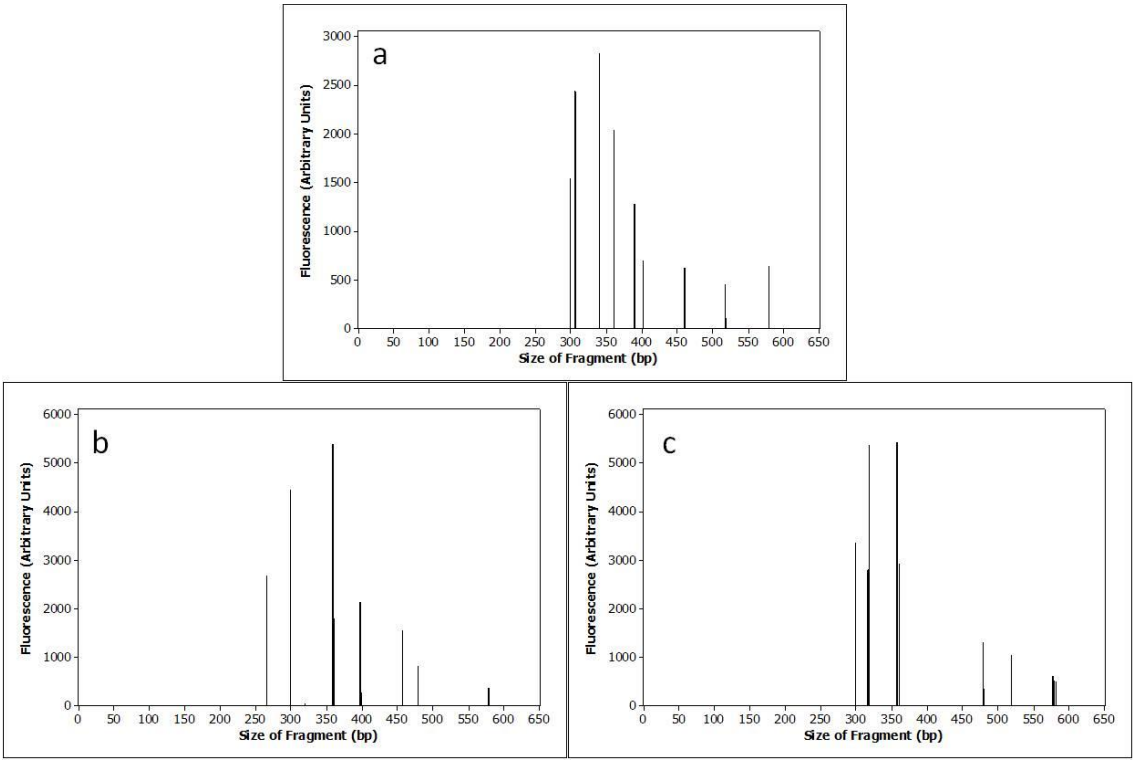

Figure 4.3: Capillary-based outputs of reference PCR ribotypes.

Peaks represent DNA fragments (base pairs), corresponding to bands which are visible on an agarose gel. Peak intensity is shown as fluorescence. Chromatograms correspond to the PCR ribotypes identified in Figure 4.2. (a): PCR ribotype 012 (lane 1), (b): PCR ribotype 027 (lane 5), (c): PCR ribotype 106 (lane 3).

4.3.2 Ribotype distribution between August to November 2009

The aim of this study was to describe the genetic diversity observed within one NHS Trust with a 'snapshot' of ribotype distribution over a short period of time. No previous studies have concentrated on a cohort of samples continuously collected over a fixed episode. This unique study was carried out in an attempt to recognise any emerging trends in distribution or abundance.

Faecal samples that appeared to be *C. difficile*-positive following an ELISA-based test, detecting the presence of toxins A and/or B, were collected from all patients who presented with symptoms and/or a positive *C. difficile* test in the UHL Trust over a four-month period (August to November 2009). This study aimed to establish the number of different ribotypes present within the UHL Trust and their relative abundance. Samples were acquired from the three major hospitals in Leicester, and from community samples that were collected from care homes, smaller hospitals, or predominantly GP surgeries.

4.3.2.1 Strain collection and characterisation

After significant effort, *C. difficile* was isolated from 203 of 215 toxin A/B-positive faecal samples. It is possible that the remaining twelve samples from which *C. difficile* was unable to be isolated from could have been false positives from the initial ELISA-based assay. Of the 203 isolated that successfully grew *C. difficile* onto the selective CCEY agar plates, 187 could be sub-cultured onto the non-selective BHI blood agar plates. From these, 181 samples grew when inoculated in BHI broth in preparation for the extraction of chromosomal DNA, as described in Section 4.2.3.5. Good quality DNA was obtained from 179 samples, all of which were assigned a PCR ribotype; 83% of the original 215 faecal specimens. This has been reported as an acceptable success rate (Health Protection Agency 2009).

4.3.2.2 Ribotype prevalence

Figure 4.4 shows the 11 different PCR ribotypes identified from the samples in this study. The most prevalent was ribotype 027, with 63 samples. There were four other frequently identified ribotypes: 014/020 (*n*=24), 002 (*n*=23), 015 (*n*=16) and 078 (*n*=15). Other ribotypes were confirmed: 107 (*n*=12), 013 and 087 (*n*=7), 005 (*n*=6), 026 (*n*=4), and 081 (*n*=2).

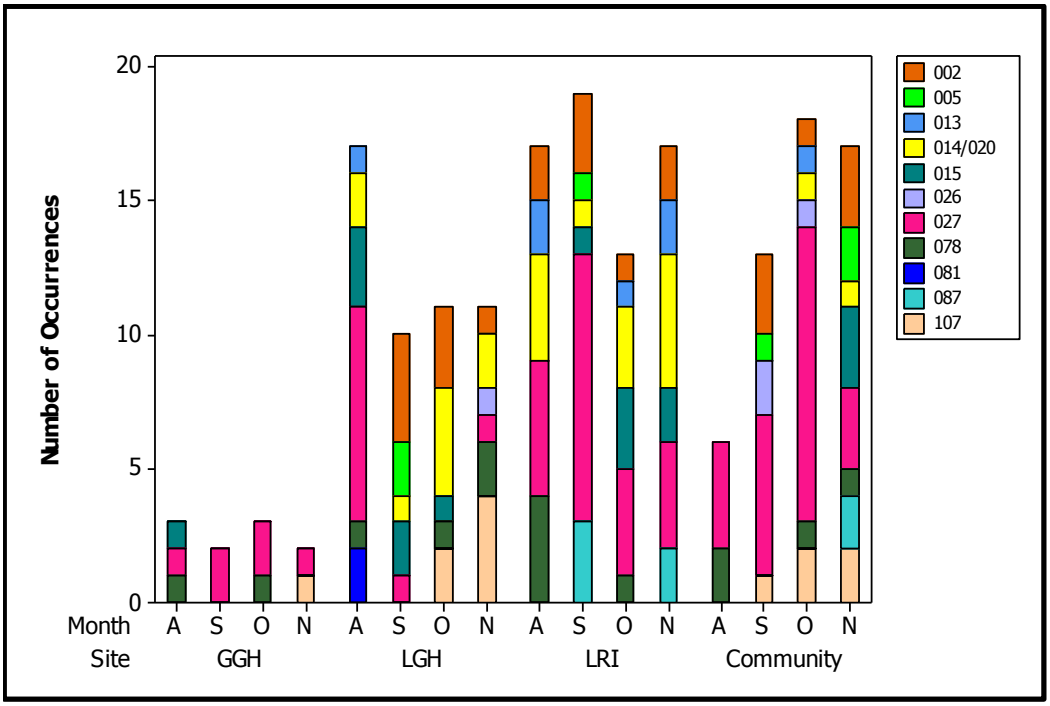

Figure 4.4: PCR ribotypes by month and site from the first cohort.

Distribution of *C. difficile* ribotypes from UHL Trust between August and November 2009.

4.3.2.3 Ribotype temporal diversity

The relative proportions of ribotypes varied per month over the four-month period of study (Figure 4.5). For example, ribotype 107 was predominantly identified in October and November (P=0.020), and made up 15% of all the November isolates but none in August. Ribotype 078, in contrast over this four-month period, was found eight times in August but only three times in November (P=0.017).

Ribotype 027 was identified as the most prevalent strain, occurring in all of the sampled months. The apparent decrease in strain abundance by month was not statistically significant (P=0.057). The other ribotypes in the study were consistently isolated over the four-month period. The ribotype cluster 014/020 was the second most dominant ribotype, isolated 6, 2, 8 and 8 times respectively; there were no significant changes in proportion with time (P=0.235).

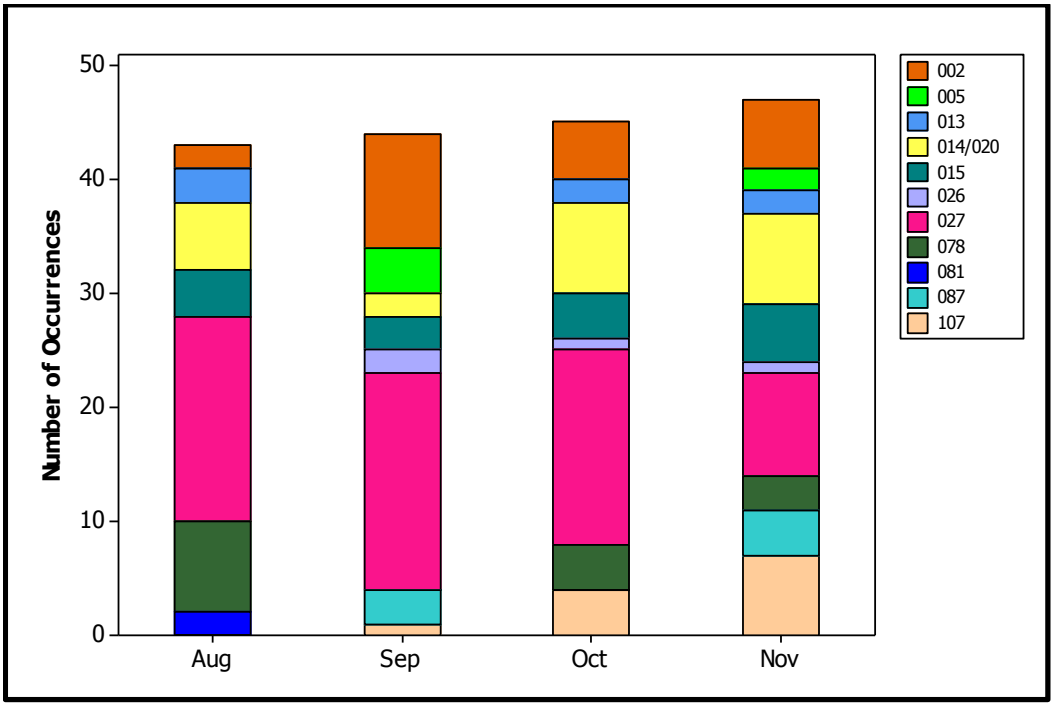

Figure 4.5: PCR ribotypes by month from the first cohort.

Distribution of *C. difficile* ribotypes from UHL Trust between August and November 2009.

4.3.2.4 Ribotype spatial diversity

Multiple ribotypes were found in all three major hospitals and in the community sites, with most ribotypes being distributed throughout the sites. In the LRI, the 66 isolates represented 8 ribotypes, the 10 isolates from the GGH belonged to 4 ribotypes and the 49 from the LGH 10 ribotypes. The 54 isolates from the community samples also grouped as 10 different ribotypes. Despite the LRI accounting for the highest number of isolates recovered, there were fewer ribotypes present at this site than at the LGH. There were consistently higher rates of ribotype 027 in the LRI than in other hospitals (P=0.031).

Figure 4.6 illustrates the dominance of ribotype 027 in all three main hospitals despite its varying proportion for each site; 60%, 20% and 35% of the isolates were from the GGH, LGH and LRI, respectively (P=0.024). The proportion of this strain within the samples obtained from the patients in the community was also high (44%). The ribotype cluster 014/020 was predominantly isolated in the LRI and LGH (P=0.027), but was overall the next most abundant strain. Ribotypes 015 (P=0.698) and 078 (P=0.597) were distributed evenly throughout the sites, having been identified in all four sample sets.

Localisation of ribotypes was observed with particular sites. PCR ribotype 087 was found only in the LRI and from community samples (P=0.048), and ribotype 081 only in the LGH (P=0.147). Despite not being dominant in any specific site, ribotype 107 was absent from the LRI (P=0.048). This study did not identify any ribotypes that were exclusively identified in the community.

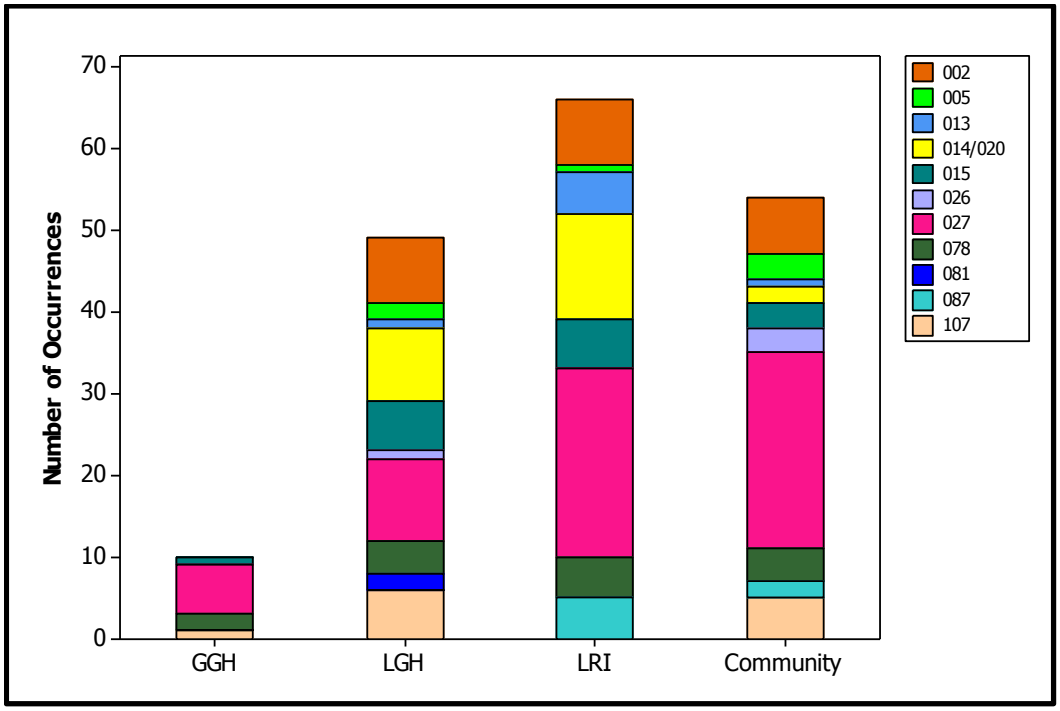

Figure 4.6: PCR ribotypes by site from the first cohort.

Distribution of *C. difficile* ribotypes from UHL Trust between August to November 2009.

4.3.3 Ribotype distribution with changes in method of C. difficile diagnosis

The motivation for this study was to determine if a change in the faecal detection method from the ELISA-based assay to the GDH and EIA-based test affects the proportion of *C. difficile*-positive samples and whether this in turn changes the ribotype distribution.

For the second *C. difficile* PCR ribotyping study in this chapter, samples were collected from the UHL Trust and another study of four months investigating the diversity of *C. difficile* ribotypes was conducted.

4.3.3.1 Strain collection and characterisation

Faecal samples were treated in the same manner as in the first cohort, isolating the bacterium prior to extracting the chromosomal DNA and assigning a PCR ribotype.

As before, following significant effort, *C. difficile* was isolated from 162 of 174 GDH- and PCR-positive faecal samples. Once again, this could be attributed to false positives. From 162 isolates which grew onto CCEY agar plates, 157 were then sub-cultured onto BHI blood agar plates. From these, 142 samples were propagated when inoculated in BHI broth prior to the extraction of chromosomal DNA (Section 4.2.3.5). Good quality DNA was obtained from 136 samples, before the assigning of a PCR ribotype; 78% of the original 174 faecal specimens.

4.3.3.2 Ribotype prevalence

The twenty-five individual PCR ribotypes found in the samples investigated in this study and the relative number of occurrences is shown below (Figure 4.7). There were frequently identified ribotypes: 014/020 (*n*=24), 015 and 027 (*n*=20), 106 (*n*=15), 078 (*n*=13) and 005 (*n*=6). Other ribotypes were found to a lesser extent. One instance of an unknown ribotype was also found; this strain did not match any of the known PCR ribotypes within the CDRN database at the time of this study.

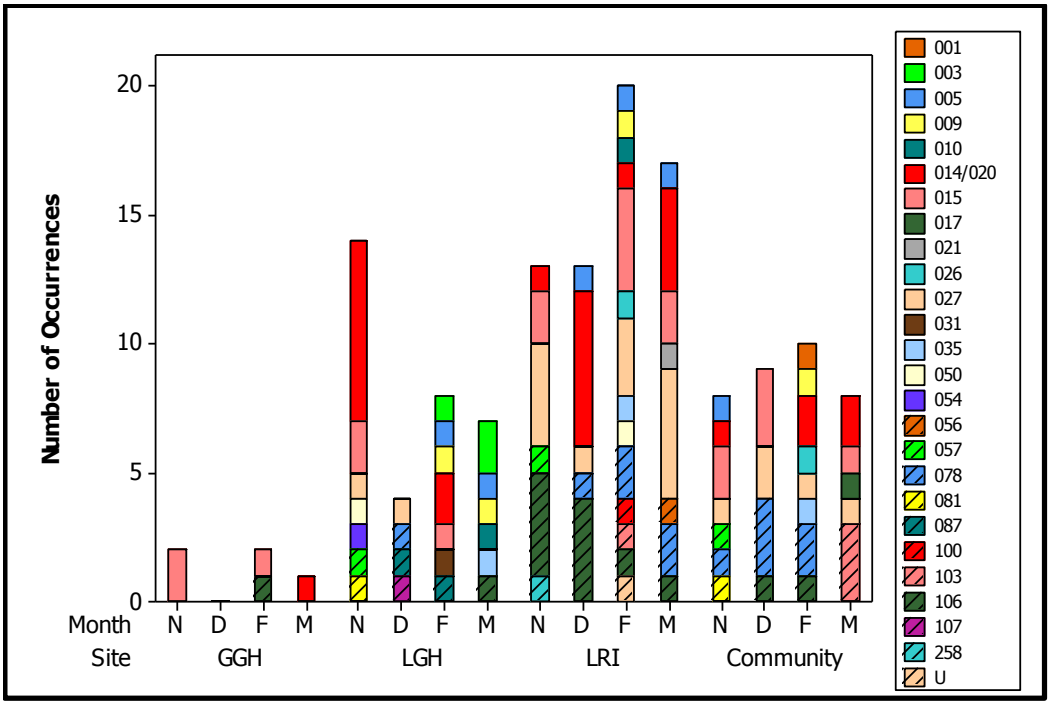

Figure 4.7: PCR ribotypes by month and site from the second cohort.

Distribution of *C. difficile* ribotypes from UHL Trust from November to December 2010 (ELISA-based assay) and from February to March 2011 (GDH-based testing).

4.3.3.3 Ribotype temporal diversity

Before the change in diagnosis was enforced, the samples obtained in November and December amassed 11 and 9 different ribotypes, respectively. Following the change in methodology, which incorporated the GDH-based test, 17 different ribotype profiles were identified in February and 15 in March. The month following this change possessed the greatest variation in strains identified; seven different strains were found in the community-isolated samples, 2 in the GGH, 7 in the LGH and 14 from the LRI. The proportions of PCR ribotypes successfully assigned for the samples obtained from each of the months were as follows: November (37/41), December (26/27), February (40/59) and March (33/47). Therefore, in addition to the relative number of samples that were *C. difficile*-positive increasing in February and March, the relative proportion of samples that were assigned a PCR ribotype decreased.

With respect to strain temporal distribution, five strains were recovered in both February and March, but these strains were not identified in either November or December (ribotypes 003, 009, 026, 035, and 103).

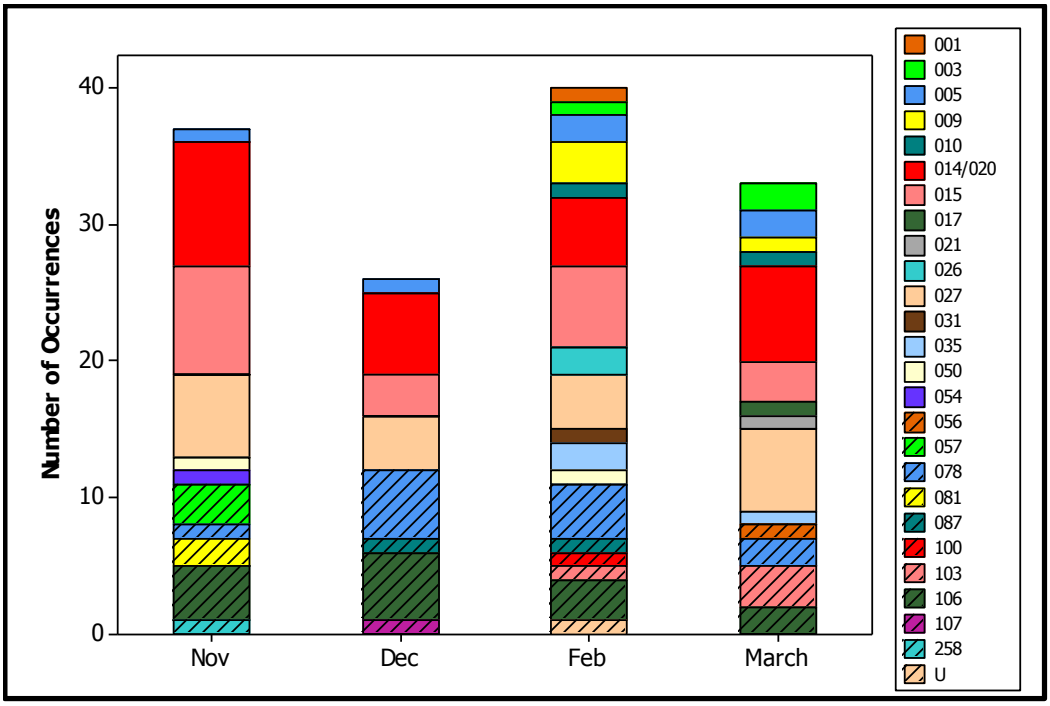

Figure 4.8: PCR ribotypes by month from the second cohort.

Distribution of *C. difficile* ribotypes from UHL Trust from November to December 2010 (ELISA-based assay) and from February to March 2011 (GDH-based testing).

Some ribotypes were found in one month only, with ribotypes 054, 057, 081 and 258 found exclusively in November and 107 in December. February was the only month in which ribotypes 031, 100 and an unknown strain of *C. difficile* were identified while ribotypes 017, 021, and 056 were isolated during March. Therefore, 11 of the 25 different ribotypes found in this cohort were exclusive to their respective months.

However, certain strains were identified in all months of the study: ribotypes 005, 014/020, 015, 027, 078, and 106. With the exception of 078, which has a significant difference between the months November and December (P=0.046), the proportions of each of these ribotypes are statistically significantly similar between months (P>0.05). This suggests that certain strains of *C. difficile* are recovered consistently, irrespective of time. For example, the proportions of ribotype 027 found in each month are significantly similar (P=0.777) and thus remaining constant between months. The temporal patterns are shown in Figure 4.8.

4.3.3.4 Ribotype spatial diversity

The composition of ribotypes found in this study varied by site. The numbers of ribotypes identified from the samples were as follows: community (35/44), GGH (5/9), LGH (33/46), and LRI (63/75). Of the three major hospital sites, the LRI was the most diverse with 18 different ribotypes identified from 63 isolates. The other hospitals, the LGH and GGH, found 17 and 3 different ribotypes from 33 and 5 isolates, respectively. The 35 isolates from community sites were classified into 13 ribotypes. The spatial breakdown of ribotypes is shown in Figure 4.9.

Of the four most dominant strains identified in this study, 014/020 (n=24), 015 and 027 (n=20) and 106 (n=15), three of these were found at all sites. The only exception was that ribotype 027 was not recovered at the GGH, but this is not a statistically significant result (P=0.204), and is likely due to the small sample size of the GGH. These dominant strains were therefore recovered evenly across all sites with no noted patterns of diversity, and of the less frequent ribotypes, 005, 014/020, 015, 078 and 106 were all found to have statistically significantly similar counts for all sites (P>0.05).

Of the 25 different ribotypes found in this cohort, nine were localised in one site only. The LRI was the only host to ribotypes 021, 056, 100 and Unknown, while the LGH was the only site from which ribotypes 003, 031, 054, 087 and 107 were recovered. Ribotype 017 was recovered from just community sites and not in the three major hospitals. All ribotypes found within the GGH were observed at other sites, but again, this can be attributed to its sample size. Furthermore, of these 10 ribotypes that were exclusive to their respective site, 7 were found exclusively in either February and/or March.

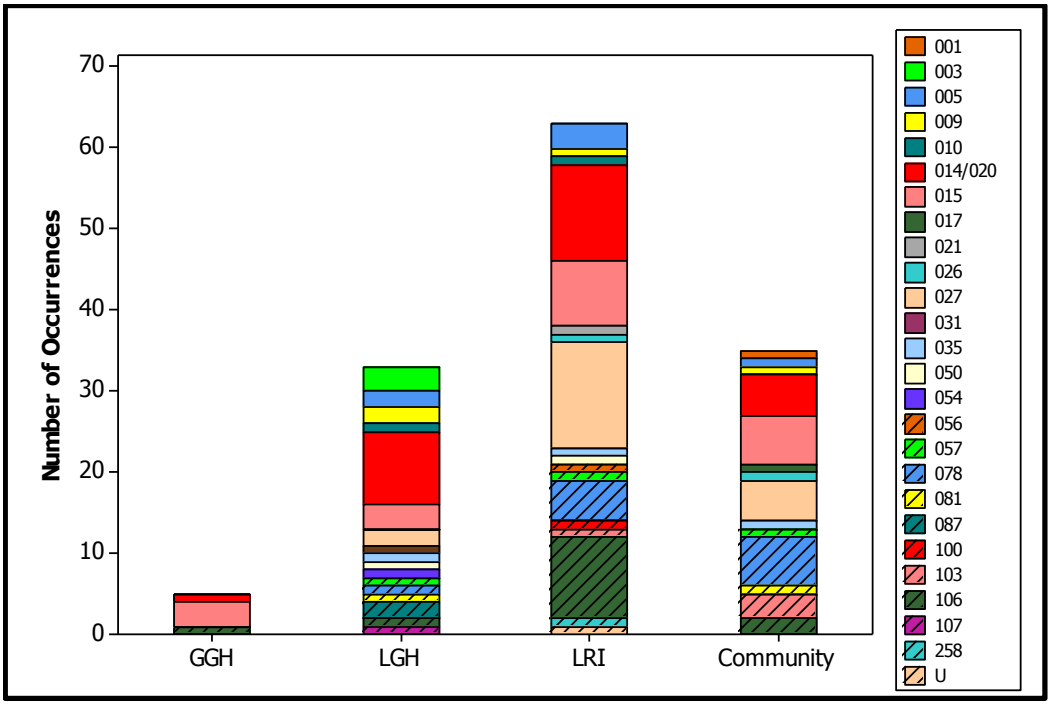

Figure 4.9: PCR ribotypes by site from the second cohort.

Distribution of *C. difficile* ribotypes from the UHL Trust from November to December 2010 (ELISA-based assay) and from February to March 2011 (GDH-based testing). 'U' – unknown ribotype.

The largest hospital, the LRI, was dominated by ribotypes 027 (*n*=13), 106 (*n*=10) and 014/020 and 015 (*n*=8). This constitutes over half of the ribotype 027 isolates and two-thirds of the 106 isolates from the entire cohort. Statistically, however, the proportions of 027 (*P*=0.204) and 106 (*P*=0.288) show that these strains are not significantly distributed across all sites.

4.3.3.5 Cases involving multiple samples per patient

The samples obtained from this second cohort were analysed further by means of investigating the patients. Of all the samples tested and assigned a PCR ribotype, thirteen patients presented with a positive *C. difficile* specimen more than once within the four-month period. Seven of these had the samples collected from the same site and were found to be the same ribotype.

Three of these seven patients were all positive with isolates from the LRI, with ribotype 106. All of the patients had moved within the site between collections as the wards from which the specimens were collected differed. The time between the collections of samples was either one, three or four weeks.

One patient was identified as *C. difficile*-positive at two different sites within the community (once in November and again in February). On both occasions, the isolated strain was ribotype 078.

A separate incident occurred with a community sample collected in December identified as 027 and then a second from the same patient in the LRI also identified as carrying the same strain months later in February.

Finally, in December, one patient possessed ribotype 107 when in the LGH. Upon release and months later in February, the patient tested *C. difficile*-positive again within the community setting, for ribotype 035. These strains have not been noted as serious, widespread strains.

4.4 Discussion

The most common method for characterising *C. difficile* strains in the UK and Europe is PCR ribotyping. It has become increasingly common when ribotyping to utilise a capillary-based gel electrophoresis method as it has been shown to reliably assign strains unambiguously and more accurately (Indra et al. 2008; Janezic et al. 2011). For the purpose of this work, this technique has been used throughout the thesis to characterise isolates collected directly from patients in addition to those obtained from sampling the environment.

Surveys on PCR ribotype distribution and prevalence have been carried out across Europe. These continent-wide studies allow the identification of major disease-causing strains, comprising of data from a number of different countries and mapping which ribotypes are problematic. Continuous monitoring like this would eventually permit the movements of strains to be tracked. The ECDIS (European *Clostridium difficile* infection study) has investigated *C. difficile* infection on such a scale (Barbut et al. 2007; Bauer et al. 2011). Barbut et al. (2007) collected *C. difficile*-positive samples from 38 hospitals in 14 European countries over a two-month period. Sixty-six different ribotypes were identified; the most prevalent overall were 001 (13%), 014 (9%) and 027 (6.2%). Investigations like this highlight differences in abundance between countries too: ribotype 027 was far more abundant in countries such as the Netherlands (40%) and Belgium (31.4%) (Barbut et al. 2007). Bauer et al. (2011) collected samples from 34 countries, identifying 65 individual profiles; ribotypes 014/020 (16%), 001 (10%) and 078 (8%) were the most prevalent in this study. These two reports have shown the importance of ribotyping to identify patterns in strain diversity. In addition to this, they have ascertained although specific ribotypes may be dominant overall, relative ribotype composition varied with both country and time (Barbut et al. 2007; Bauer et al. 2011; Kachrimanidou & Malisiovas 2011).

Despite PCR ribotyping being used in this study as well as others in determining *C. difficile* strain diversity, it is not a practice routinely carried out within all NHS Trusts. This is due to the extensive labour, and limitations associated with finances and time that would be encountered for each *C. difficile*-

positive sample. The introduction of the CDRN has meant that a proportion of samples that are *C. difficile*-positive, in addition to those collected during an outbreak, must be submitted for strain identification. These results are published online quarterly, identifying important trends in ribotype distribution and prevalence within England and Northern Ireland. It permits an overall monitoring of ribotypes from the start of the study in 2007, allowing in depth analyses into strains such as ribotypes 002, 015, and 078. These three strains have increased in abundance over the period of 2007/8, and even more so in 2008/9 and 2009/10 (Health Protection Agency 2011). Despite the increasing emergence of these new *C. difficile* ribotypes the most prevalent ribotype in the UK, across Europe and in North America remains ribotype 027.

From the first study, 83% of the samples in the cohort were assigned a PCR ribotype, and from the second, 78%. It has been previously reported that with the failure rates of *C. difficile* detection tests and assays used on faecal samples to aid in the diagnosis of a patient, these recovery and ribotype rates are consistent and are acceptable values (Health Protection Agency 2009).

Through the analysis of the data, there was a clear dominance of ribotype 027 from August to November 2009 in the UHL Trust, accounting for 35% (n=179). These data show that although it is still abundant, its prominence in healthcare facilities is somewhat declining, making way for other ribotypes to emerge as dominant strains (Health Protection Agency 2009; Hensgens et al. 2009). It has been suggested that the success of this strain in the hospital environment and environment is due to its ability to persist in these sites, despite changes in cleaning techniques (Best et al. 2010).

Other ribotypes found in this cohort were the ribotype cluster 014/020, grouped together due to their similarity both genetically and when run with agarose gel-based electrophoresis (Janezic et al. 2012). This was also abundant in two of the tested hospital sites (LGH and LRI), and its increasing prominence is also consistent with the national data available from the HPA (Health Protection Agency 2009).

Another nationally emerging strain is 078. This strain was identified in all of the three hospitals, as well as the community, in this study. Originally isolated from porcine and bovine sources, ribotype 078 has been since reported in healthcare facilities, both in the UK and internationally (Goorhuis et al. 2008; Health Protection Agency 2009). From this cohort, it was predominantly found in August. In contrast to this, the little known ribotype 107 was identified as more abundant in the later months of the study in October and November.

Several trends were recorded within the less abundant ribotypes. Three ribotypes (002, 005, and 013) were not found in the GGH, although this could be due to the number of isolates from this site being low. Ribotype 087 was identified exclusively in the LRI and within the community, which could suggest the potential of strain transfer between these environments. It could be possible that these patients along with those with the ribotypes 014/020, 027, 078, and 107 contracted *C. difficile* whilst hospital inpatients, before being discharged into the community, where they were later confirmed as *C. difficile*-positive. Although there were only two ribotype 081 samples identified, these were from the LGH and both in August, which suggests strain transfer between patients. The movement of strains may clarify why similar ribotypes are found in the hospital sites as well as the isolates from GP surgeries. All strains identified from the community samples were also found from patients within at least one of the three main hospitals. This finding reinforces the suggestion of strain transfer to the community following admission within the hospitals in the UHL Trust.

The second study described in this chapter was carried out in November and December 2010 and February and March 2011. This study set out to identify any patterns of PCR ribotype distribution with changes in the method of *C. difficile* detection from faecal samples.

There was a clear difference noted in the number of profiles produced between the two methods, the initially utilised ELISA-based technique, and the subsequently established GDH-based and EIA-based assays. The change was implemented in the UHL Trust in January 2011, and it has since been announced that the Department of Health require all NHS Trusts to change their method of *C.*

difficile detection (H Patel 2011, pers. comm., 14 Jan). No previous studies have investigated the diversity in PCR ribotypes identified before and after implementing this change, which strengthens the novel nature of this work.

A key reason for the implementation of a new technique was to improve diagnosis and subsequently deliver treatment that is more efficient. There is currently no gold standard for *C. difficile* detection from faecal samples, and it has been reported that the most accurate procedure for identifying its presence is through culturing onto selective medium (Bartlett & Gerding 2008). This can take a number of days and can be costly, and since these time and financial limitations exist, for the purposes of rapid diagnosis for healthcare workers and clinicians, a quick laboratory-based test is preferable. The combination of the high sensitivity-low specificity GDH-based assay, which targets for *C. difficile* itself, and the low sensitivity-high specificity EIA, detecting the presence of toxins within the faecal samples, aims to eliminate the possibility of false positive and negative results (Tenover et al. 2011).

The data presented here show the numbers of samples recorded as *C. difficile*-positive increased following the change in methodology. However, an increase in the number of samples failing to grow on both the selective and non-selective plates when culturing was also observed. Another finding from this study is the increase in diversity of ribotypes identified. More strains were isolated from all of the sites investigated, including those less reported, such as ribotypes 035 and 054. Despite the combination of sensitivity and specificity tests used, it is possible that the GDH-assay methodology of detecting *C. difficile* is identifying non-toxigenic strains. This would explain the increase in less clinically relevant ribotypes.

Some strains were present in all months of this study (005, 014*/020, 015*, 027*, 078* and 106). An asterisk (*) identifies one of the most prevalent strains in England (Health Protection Agency 2012). Ribotypes 014/020, 015 and 106 were also identified in all three main hospitals in the UHL Trust as well as from the community samples. Data from the HPA show that these dominant strains are

present nationwide, as well as within the UHL Trust, demonstrating consistency with results from this study.

These national reports suggest the overall decrease of strains (014/020, 023, and 027) and increase of others (002, 005, 015, and 078) (Health Protection Agency 2012). Bar 014/020, which demonstrates an increase in this ribotype cluster from the time between these two cohorts, the result from these studies agrees with these national averages. There appears to be a shift in the proportions of the most prevalent strains in the UK.

Ribotype 026 was identified in this second study, but not the first. Not much is known in regard to this strain compared to the other previously described strains. The strain was identified in the community as well as the LRI in this study. This ribotype accounted for 4% of the cohort of this study, and 1% of the total samples tested in the East Midlands, found exclusively between January and March 2011 (Health Protection Agency 2012). Accounting for 0.8% of all the samples tested by the CDRN in both 2009/10 and 2010/11, the proportion of the ribotype has not changed over the past two years with respect to its national prevalence. However, this was the first quarter in which it was identified in the East Midlands (Health Protection Agency 2012). It could be inferred that this strain was therefore spreading throughout the country prior to the study, and migrated into the East Midlands region over this period.

Statistical significant differences were observed with the prevalence of ribotype 078 between months (November and December) in this second study, as well as ribotype 015 between sites (GGH and LGH). This could also be attributed to strain migration both temporally and spatially.

Thirteen patients tested positive for *C. difficile* more than once and had PCR ribotypes assigned to their samples. Of these, three patients were in separate wards in the LRI and had carriage of PCR ribotype 106, a strain often isolated in the UK; the times of specimen collections also differed (John & Brazier 2005). A case was also recorded of ribotype 078 identified from a patient twice, once in November and again in February. These four patients demonstrate strains can

repeatedly cause *C. difficile*-associated diarrhoea, and may persist despite treatment.

Ribotype 027 was found at a statistically similar proportion overall across all eight months studied in this chapter. The same was also seen with the ribotype cluster 014/020. These data therefore demonstrate these strains to be consistently prevalent within the UHL Trust, prevailing despite the twelve-month gap between sample collections. The prevalence of ribotype 078 across all of the sites tested was also statistically similar in both cohorts. There were clear differences in the relative proportions of ribotypes 014/020 and 027 in the first cohort; both were more evenly distributed in the second. The persistence of these strains is therefore an indication that they are established within all areas of the UHL Trust.

The work completed in this chapter demonstrates how monitoring a subset of samples can aid in the detection of newly emerging strains. Studies of this nature are not often conducted, so clinicians and healthcare practitioners are not aware of which strains are carried by patients in their hospital environment. A further benefit of monitoring is that it could help establish the spread of particular *C. difficile* strains between multiple hospitals, and their surrounding community. It would also allow the identification of persistent strains within these sites. This in turn provides an insight into the distribution and diversity as a useful aid in strategic planning of patient management and treatment.

4.5 Conclusions

- Official ribotypes were assigned to *C. difficile*-positive faecal samples using a recently developed capillary-based gel electrophoresis method, before analysis was conducted into where and when the samples had been collected. This was deemed more discriminatory than the traditional agarose-based approach.
- The first study found that although ribotype 027 was still dominant within the UHL Trust, other ribotypes including 002, 014/020, 015, and 078 were prevalent at multiple sites.
- With the second study, there was a clear difference in the profiles produced between the two methods, the ELISA-based and GDH-based tests. There was an increase in the diversity of ribotypes, including less well-reported strains, found after the change in *C. difficile* detection. However, the number of samples from which *C. difficile* could not be isolated following a positive result with the GDH-based assay also increased. Furthermore, 11/25 ribotypes identified were month-exclusive. The six most dominant strains were found in all months, four of which are recorded as the most prevalent in the England.
- National data from the CDRN suggests the prevalence of ribotypes 027 and 014/020 are decreasing and 002, 005, 015, and 078 are increasing. Apart from the increase observed with 014/020 between these two cohorts, the results from these studies agrees with these averages.

Chapter 5. Inactivation of *C. difficile* Spores Using Hydrogen Peroxide

5.1 Introduction

C. difficile forms spores which are resistant to standard cleaning protocols within healthcare facilities (Gerding et al. 2008). These reside on surfaces within the hospitals and are able to persist for many months (Kim et al. 1981). As *C. difficile* can be transmitted orally, a patient touching these contaminated surfaces and subsequently putting their hands in contact with their mouth will put them at risk of contracting the infection. Results in Chapter 3 showed that the strain carried by a *C. difficile*-infected patient was also found in their surrounding environment. Therefore, the monitoring of bacterial contamination in a healthcare facility is important so that appropriate interventions can be made if such environments prove to be heavily contaminated by pathogenic organisms.

Such investigations permit the identification of problematic strains. Correct strain characterisation also helps in the recognition of new, emerging ribotypes. In addition to this, the findings of clinically relevant strains, including ribotype 027, with an increased ability to sporulate with the application of cleaning products is worrying (Fawley et al. 2007; Akerlund et al. 2008). The findings from these studies suggest there may be lapses in efficiency with manual cleaning, especially in areas that are hard to reach as well as with hospital machinery. Cleaning may also be rendered futile due to the slow settling rates of aerial *C. difficile* spores. This suggests that the method of cleaning with disinfectants and detergents in hospitals may need addressing.

Recently, hydrogen peroxide has been investigated as an adjunct decontamination method to manual cleaning. Studies have demonstrated the effectiveness against bacteria and bacterial spores both in clinical settings and with inactivation curves, as well as its benefits as an antiseptic and disinfectant, whilst remaining non-toxic and non-corrosive (Rogers et al. 2005; Barbut et al. 2009). Hydrogen peroxide vapour can be generated readily and it can be dispersed relatively easily thereby lending itself to decontamination of large enclosed spaces. There has been increasing interest shown into its usage to decontaminate healthcare facilities. It has been demonstrated to successfully reduce surfaces from bacteria causing HCAI, such as MRSA and *C. difficile* (Dryden

et al. 2008; Cooper et al. 2011). There are a number of hydrogen peroxide decontamination systems available, which can be purchased and subsequently used within healthcare facilities. These use either hydrogen peroxide in its vapour form or as a dry-mist with silver cations to decontaminate (Boyce et al. 2008; Barbut et al. 2009).

However, an inherent problem with the use of vapour decontamination in hospitals is the time taken before patients can be admitted into wards again following hydrogen peroxide exposure. The concentrations at which these machines are operated have been shown to successfully reduce the bacterial contamination (Hall et al. 2007; Hardy et al. 2007; Otter et al. 2007). However, these concentrations also increase the time before the wards or operating rooms can be used again, as the concentration has to fall below 1 ppm before it is deemed safe for patient re-admission (Boyce et al. 2008).

The range of concentration of vaporised hydrogen peroxide predominantly used is within the region of 1000 ppm, with these machines ranging from 500-2000 ppm. Interestingly, there is a paucity of reports in the literature using hydrogen peroxide vapour at concentrations below 100 ppm. In addition, there are reports to suggest the use of hydrogen peroxide vapour can result in degradation or corrosion of particular materials due to its characteristic as an oxidising agent (Rogers et al. 2005; Maillard 2011). Therefore, investigating the efficiency of hydrogen peroxide vapour at lower concentrations would demonstrate the effectiveness at reducing *C. difficile* contamination.

As previously stated, there are about 430 *C. difficile* strains, of which only 20% have been suggested as currently problematic in clinical facilities (W Fawley 2012, pers. comm., 20 Mar). It could be argued, however, that all strains have the potential to become 'problematic'. Other ribotypes are found underlying and present to a lesser extent in other sources, for example food, water and soil samples (al Saif & Brazier 1996; Rodriguez-Palacios et al. 2007). Despite this large number of problematic and disease-causing strains present within healthcare facilities, the potential resistance of certain ribotypes to hydrogen peroxide vapour at the concentrations employed for decontamination has not been investigated. In

addition to this, the inactivation kinetics of *C. difficile* with hydrogen peroxide is not well studied. Only a few research groups have studied how concentrations of hydrogen peroxide affect the spore recovery with respect to time (Lawley et al. 2010; Fu et al. 2012). There have been no reports to date on the effects of hydrogen peroxide on multiple *C. difficile* strains, including the exposure times and concentrations required for inactivation of clinical strain.

5.1.1 Aim of this study

This study was designed to obtain inactivation data of *C. difficile* spores at lower levels of hydrogen peroxide decontamination. For the basis of this work, a purpose-built environmental chamber was designed and commissioned to generate a known concentration of hydrogen peroxide and expose its vapour in a controlled manner. It also monitored temperature and humidity.

Experimental work within this chapter was devised to demonstrate how changing the hydrogen peroxide concentration can affect the numbers of *C. difficile* ribotype 027 spores recovered. Three additional *C. difficile* strains were used in this chapter: ribotypes 014, 103, and 220. Ribotype 014 was obtained from a faecal specimen (Chapter 4), ribotype 103 following a round of environmental sampling (Chapter 3), and ribotype 220 was isolated from an estuary sample. The work presented here will explore how these different ribotypes respond to the direct liquid application of hydrogen peroxide to a lawn of bacterial growth. A key aim of this chapter was to compare the fractional survivals of these ribotypes and identify whether different times of vaporised exposure were required. The work presented here would also identify whether these strains respond differently to the exposure of hydrogen peroxide as a disinfectant.

5.2 Materials and Methods

5.2.1 Strain selection for hydrogen peroxide exposure experiments

Throughout this thesis, samples have been collected and characterised by strain using PCR ribotyping (Chapter 4). In order to conduct the work carried out in this chapter and investigate how *C. difficile* spores respond to the exposure of hydrogen peroxide, it was necessary to select a range of strains representative of the diversity of strains, which would exist within a healthcare facility. This was to ensure that data collected more accurately represents what would be found in the environment.

Four strains were collected from different origins. Ribotypes 014 and 027 are clinically relevant strains, often reported in literature as linked to sources of disease (Bauer et al. 2011). These two strains were isolated from faecal specimens from individuals within the UHL Trust who were infected with *C. difficile* (samples collected from work conducted in Chapter 4).

An aim for the whole project was to identify which strains of *C. difficile* are prevalent in hospitals, demonstrating resistance to cleaning and disinfection treatments. The selection of ribotype 103 was due to its isolation during environmental sampling (conducted in Chapter 3). This strain was not present within faecal specimens collected from the patient residing in the particular side room. This may suggest that this particular strain may have withstood previous cleaning regimes and may have arisen from a previous infected patient occupying the room. Ribotype 103 is not well studied, no reports have been made with respect to its link with disease; there is little information regarding its isolation, with respect to geography, as well as its virulence.

The final strain selected was ribotype 220. This strain was not identified from either faecal samples or environmental sampling throughout this thesis; the sample was isolated from an estuary sediment sample by Katherine Hargreaves [University of Leicester]. It was selected because it is not clinically relevant and its site of isolation differs to the other strains, as it too is non-clinical. Again, there is little known about this strain.

5.2.2 C. difficile spore inhibition with liquid-phase hydrogen peroxide

This section outlines the protocol for investigating the growth of a bacterial lawn following exposure to filter paper discs immersed in different concentrations of hydrogen peroxide. As the final part of this thesis will focus entirely on hydrogen peroxide vapour decontamination, this experiment was devised as a preliminary screening test to identify strains showing differences in peroxide resistance.

BHI agar with 0.1% sodium taurocholate plates were prepared and poured as described in Chapter 2. Grade 3 filter paper discs [Whatman, UK] were cut out with a standard desk hole-punch and autoclaved. A 0.1% (v/v) Tween 80-*C. difficile* spore mixture (6-$\log_{10}$ CFU) was prepared (Chapter 2); 50 µl was spread onto the agar plates. Discs were immersed into liquid hydrogen peroxide, concentrations of 1.5, 3, 4.5, 6, or 10% (w/v) for 5 sec, and placed in the centre of the agar plates. Hydrogen peroxide in its liquid form (3-6%) has been reported to be an effective disinfectant (Andersen et al. 2006). The plates were incubated in the anaerobic workstation overnight at 37°C. By measuring the diameter of the circular area that was clear from any visible colony growth, the area (cm^2) or zones of inhibition were calculated. These were assumed as regions where lawns were unable to grow and therefore, the spores were taken to have been inactivated.

5.2.3 C. difficile spore inactivation with vaporised hydrogen peroxide

This section briefly outlines the methodology developed by Claire Shaw [Loughborough University] for the inactivation of spores using hydrogen peroxide vapour, described in the PhD thesis by the same (Shaw 2013). The techniques were optimised with *Bacillus subtilis*, a non-pathogenic bacterium frequently used in disinfection studies (Gardner & Shama 1998; Meszaros et al. 2005). The strain utilised was ATCC 6633, purchased from the American Type Culture Collection. *Bacillus subtilis* is easier to cultivate and therefore was ideal for preliminary experiments rather than using the more problematic *C. difficile* for method development.

A hydrogen peroxide decontamination chamber was designed and commissioned to permit generation of controlled concentration of hydrogen peroxide (in the range 0-100 ppm) to spores in a controlled manner. These data could then be used to establish the inactivation kinetics of hydrogen peroxide exposure to *C. difficile* spores.

5.2.3.1 Deposition of C. difficile spores onto membranes

In order to prepare *C. difficile* spore viability bio-indicators, a method was devised whereby a known volume of a given concentration of spore solution was filtered through 0.22 µm isopore membrane filters [Millipore, UK], adapted from Kai et al. (1999) and Kaláb et al. (2008). A single membrane filter was placed on the base of a 13 mm syringe filter holder [Sartorius, UK], moistened with 30 µl deionised water to ensure there was no movement or drying out when autoclaving. The top of the filter holder was secured tightly. A small amount of non-absorbent cotton wool was used to bung the syringe hole at the top of the holder. Once assembled, the holder was entirely wrapped in aluminium foil and autoclaved at 121°C for 15 min.

A 6-$\log_{10}$ CFU *C. difficile* spore solution was prepared, with 1 ml filtered through each membrane. This was conducted by unwrapping the foil from the filter holder after sterilisation, and removing the cotton wool. A disassembled 2 ml syringe [DB Plastipak, USA] was placed in the top of the filter holder, filled with 1 ml of the stock solution. The spore solution was filtered through the membrane, and the filtrate discarded. To remove any residual water, the filter holder was placed into a bung on top of a Büchner flask and a vacuum of 0.5 bar was achieved in the flask using a vacuum pump. The membrane was aseptically removed from the filter holder by dismantling it to its two constituent parts, placing it directly into a vial containing recovery liquid (as described in Section 5.2.3.6) or onto double-sided adhesive tape, affixed to a sheet of acetate film. The latter could then be exposed to hydrogen peroxide in the environmental chamber. The used filter holder was autoclaved as before and washed using deionised water prior to reassembly.

5.2.3.2 Viewing C. difficile spores under scanning electron microscope

To capture images of the *C. difficile* spores, they were deposited on the membrane, as described above. The membrane was then immersed in 2% (v/v) glutaraldehyde overnight, before removal and washing for 30 min. Washing was repeated twice. The membranes were then dried in a laminar flow cabinet and attached onto 13 mm aluminium stubs using double-sided carbon sticky pads [Agar Scientific, UK]. Membranes were then gold-coated (approximately 30 nm thick) in a sputter coater for 90 sec at 20 mA [Polaron SC7640]. Samples were then visualised [Hitachi scanning electron microscope (S3000H)].

5.2.3.3 Exposing C. difficile spores in a desiccator

In order to establish whether the reduction in the number of *C. difficile* spores recovered was due entirely to the exposure of hydrogen peroxide in the decontamination chamber, the spores were initially subjected to a desiccator. These were conducted as control experiments into how the spores react to exposure to the membranes and the subsequent recovery. It was important to ensure the humidity conditions the spores would experience within the chamber were mimicked. A saturated sodium bromide solution [Fisher Scientific, UK] was prepared with deionised water to generate a relative humidity of 60%. The solution was poured into the base of a desiccator and left at room temperature overnight; a humidity sensor (OM-62) [Omega, UK] was placed in the top of the desiccator to monitor the surrounding environment. Membranes were prepared as described in Section 5.2.3.1 and exposed for 5 min, 45 min, and 7 hr. The spores were recovered and enumerated as explained later in this chapter (Section 5.2.3.6).

5.2.3.4 Hydrogen peroxide decontamination chamber

C. difficile spores were exposed to hydrogen peroxide vapour in the environmental chamber (Figure 5.1 and Figure 5.2). The chamber comprised a hydrogen peroxide generation unit, an exhaust unit and three exposure boxes connected in series. Hydrogen peroxide solution of the required concentration was fed at a pre-determined flow-rate using a syringe infusion pump (WU-74900-05) [Cole-Parmer Instrument Co., UK] fitted with a 60 ml syringe onto a hotplate maintained at a temperature of 130°C.

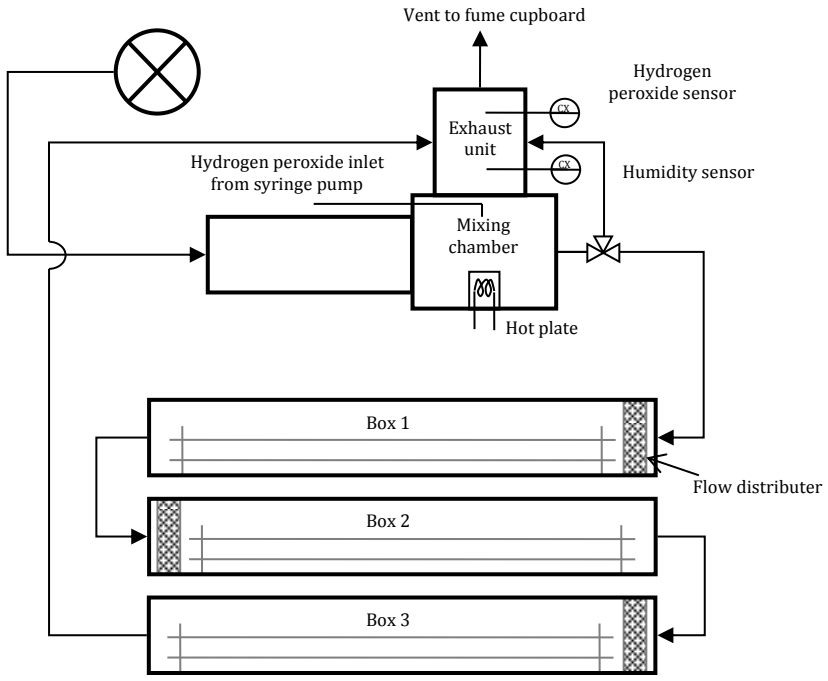

Figure 5.1: Flow diagram of the hydrogen peroxide vapour decontamination chamber.

Hydrogen peroxide left the syringe pump at a controlled flow rate, entering the mixing chamber. A heated plate flash evaporated the liquid into its vapour form, which was then combined in the airflow and fed into the boxes in series. Membranes with *C. difficile* spores were attached to acetate films, placed within the boxes, and subsequently exposed. Image by Claire Shaw.

The required concentration of hydrogen peroxide was prepared and fed into the chamber. Upon evaporation, the hydrogen peroxide was mixed into the airflow generated by a fan (ACM150) [Vent-Axia, UK]. The velocity of the gas phase was maintained at 7 m/sec as measured at the outlet of the exhaust unit using an anemometer (Model 300) [Kestrel Ltd., Canada]. From the vapour generation unit, the air-hydrogen peroxide mixture flowed into the first of the three Tecavinyl PVC exposure boxes. These were identical in construction: height 100 mm, width 133 mm, and length 665 mm. At the entrance to each of the boxes was a 30 mm thick reticulated foam gas flow distributer to ensure mixing of the hydrogen peroxide vapour throughout the box with the air.

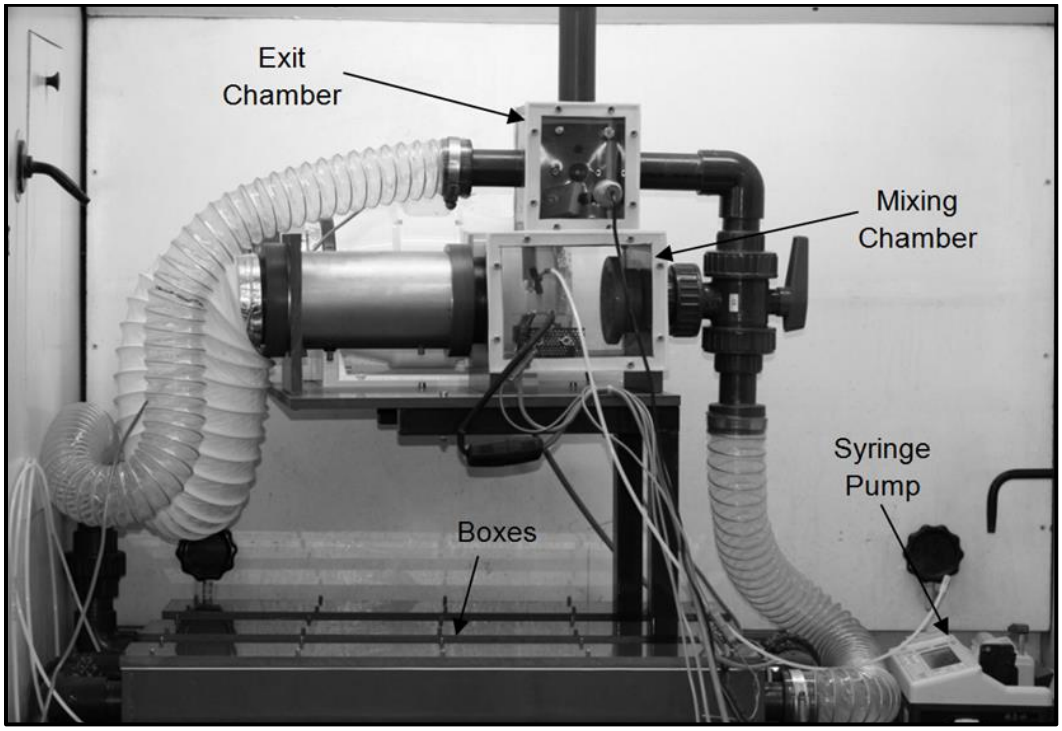

Figure 5.2: Hydrogen peroxide vapour decontamination chamber.

Hydrogen peroxide leaves the syringe pump at a controlled flow rate, entering the mixing chamber. A heat plate converts the liquid to its vapour form, which is then fed into the boxes in series. Membranes with *C. difficile* spores were attached to acetate films, placed within the boxes, and subsequently exposed. Photograph by Claire Shaw.

The lids of the boxes were secured with wing nuts; the third box in series could be opened to remove samples without affecting the exposure and airflow in the two other boxes. On exiting from the last of the three chambers, the air-hydrogen peroxide mixture flowed into the exhaust unit. This contained a hydrogen peroxide sensor (Model A11-34) [ATI Ltd., UK] and a combined humidity and temperature logger (Model OM-62) [Omega Ltd., UK]. The hydrogen peroxide data were recorded using an EasyLog USB data logger [Lascar Electronics, UK]. The hydrogen peroxide sensors were routinely checked, and if required, the sensors were re-calibrated using an in-house calibration bath (this work was carried out by Claire Shaw).

The establishment of steady state conditions within the environmental chamber took approximately 2 hr. The entire environmental chamber was located inside a fume cupboard and the gases exiting the chamber were safely vented.

5.2.3.5 Exposure of hydrogen peroxide vapour to C. difficile spores

To generate a hydrogen peroxide vapour concentration of 10 ppm, 6% (w/v) hydrogen peroxide was fed into the evaporation unit at a rate of 4 ml/hr. For a vapour exposure of 90 ppm, 6% hydrogen peroxide was applied at 15 ml/hr. For 50 ppm, 3% was pumped at 15 ml/hr. These concentrations were monitored constantly as stated, the data collected and analysed following each experimental run.

For each time point investigated, five replicate spore-covered membranes were prepared as described. For the purpose of control tests, five further replicate membranes were prepared, and transferred directly into the recovery solution without being exposed to the environmental chamber. The membranes for exposure were placed into the boxes for a specified time, before removal and subsequent recovery of spores.

5.2.3.6 C. difficile spore recovery

In order to remove the spores from the surfaces of the membranes, a spore recovery liquid was used. If conducting a control sample to enumerate the spore recovery before exposure, membranes were transferred into 30 ml universal vials [Sterilin, UK] containing 10 ml PBS, 0.05% (v/v) Tween 80, and 0.2 mg bovine liver catalase [Sigma-Aldrich, UK], with five 2 mm glass pre-sterilised Ballotini impact beads. The addition of catalase was to counteract any hydrogen peroxide which may have adsorbed onto the membranes, as previously outlined by Johnson et al. (2005). Ballotini balls were added to aid in the recovery of *C. difficile* spores, detaching them from the membrane through impact. Membranes were vortex-mixed for 5 min at 3,000 rpm and serially diluted as required in PBS before assessment of viability. This was conducted by pour-plating solutions into BHI agar with 0.1% (w/v) sodium taurocholate, mixing the molten agar and sample thoroughly before setting. Agar plates were anaerobically incubated at 37°C for 48 hr prior to enumeration. This was used to calculate the $\log_{10}$ CFU reduction of spores based on the concentration of the initial spore solution.

5.2.4 Statistical analyses

Data gathered from the hydrogen peroxide exposure experiments were analysed using Microsoft Excel's statistical functions and Data Analysis Tool Pack. Microsoft Excel was also used to create the graphical representations of the data. The Series-Event models and the *D*-values (decimal reduction times) were obtained using the Goal Seek feature in Microsoft Excel. Additional statistical analysis was conducted with Minitab 15. Significance was set to $P<0.05$. The *F*-test was used with a one-way analysis of variance. A two-sample t-test compared differences of mean CFU.

5.2.4.1 Series-Event models

The Series-Event models used in this chapter were calculated based on the equations and methodology described by Labas et al. (2008) with the use of liquid hydrogen peroxide, and more recently by Malik et al. (2012) in the inactivation of *Bacillus subtilis* with vaporised hydrogen peroxide.

5.2.5 Ethical issues

Ethical approval was obtained for the use of the strains in this chapter. All information regarding patient data was made anonymous prior to use.

5.3 Results

5.3.1 C. difficile spore morphology

Following the production of spore collections, preparations of ribotype 027 spores were submitted for visualisation under SEM. This was to investigate the morphology of the spores which were to be used for the work carried out in this chapter, as well as those used in Chapter 2. It was also conducted to check the purity of the spores and confirm the stocks consisted predominantly of spores, rather than cells or cell debris.

Membranes were prepared as described in Section 5.2.3.1. SEM images were taken in order to ensure *C. difficile* spores were adherent and evenly distributed on the membrane surface. It was necessary for the spores to be uniformly spread on the membrane surface, as this would ensure equal exposure of hydrogen peroxide vapour to spores.

The SEM images (Figure 5.3) show a membrane laden with *C. difficile* at different magnifications. The spores were approximately 2-3 μm in length and the holes in the membrane were 0.22 μm in diameter, and therefore, the spores were successfully captured and adhered to the surface of the membrane.

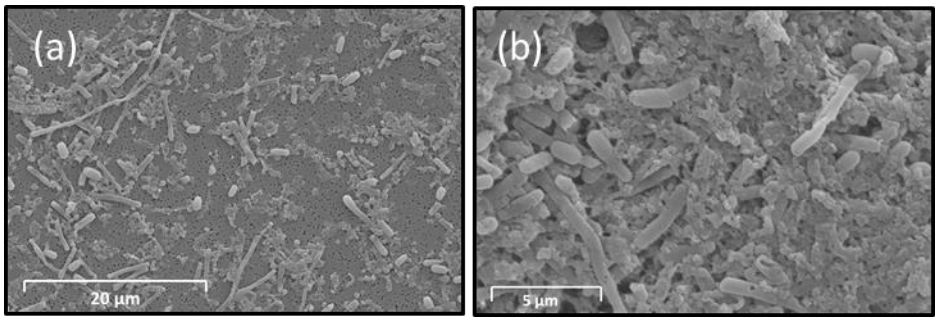

Figure 5.3: Scanning electron microscope images of *C. difficile* ribotype 027 spores following deposition onto membrane surfaces via filtration.

Spore solutions were prepared at 6-$\log_{10}$ CFU. Magnification: (a) 3000x; (b) 9000x. Photographs by Claire Shaw.

Increasing the concentration of spores resulted in a more even spread on the membrane surfaces (images not shown). This was important as it suggests that with the application of ChlorClean (Chapter 2) or hydrogen peroxide (this chapter), the spores would be equally exposed to the decontamination agent.

5.3.2 Exposure of different C. difficile strains to hydrogen peroxide solution

A study was devised to obtain information into how different strains of *C. difficile* would react to the exposure of hydrogen peroxide vapour for the decontamination work later in this chapter. The experiment was based on the inhibition of *C. difficile* spores with the application of liquid hydrogen peroxide. The basis behind the exposure of the hydrogen peroxide to the ribotypes was to gain an insight into whether there was a difference in susceptibility to hydrogen peroxide between the strains before exposing them to the vapour form.

There was little difference observed with the zones of inhibition throughout 1-6% hydrogen peroxide between ribotypes 014, 027, and 220, irrespective of the peroxide concentration in which the discs were immersed (Figure 5.4). Analyses also showed the means for these were not statically significantly different. PCR ribotype 103, however, differed; the zone of inhibition for this strain was consistently smaller than the other strains.

With a hydrogen peroxide concentration of 1.5%, the range of clearing was between 1.4-1.7 cm^2 for all strains except ribotype 103 (0.2 cm^2). The means were also statistically different, for example at 1.5%, for ribotypes 027 and 103 (P=0.022); the difference between ribotypes 103 and either 014 or 220 were both (P=0.040). This would therefore suggest that there is a difference in the susceptibility of ribotype 103 to hydrogen peroxide at these lower concentrations.

Irrespective of the hydrogen peroxide concentration used, the area of inhibition was significantly similar for ribotypes 014 and 220; P-values ranged from 0.397 to 0.896. This would imply these strains are alike in their susceptibility to the liquid form of hydrogen peroxide.

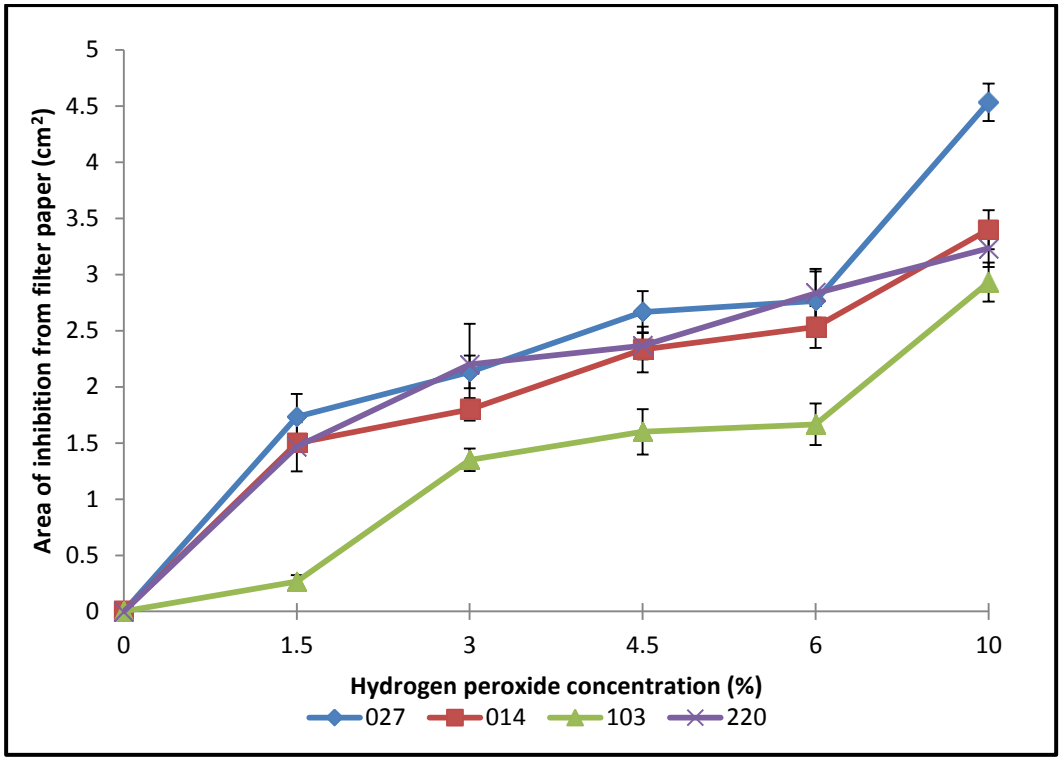

Figure 5.4: Area of inhibition exerted on the growth of various *C. difficile* strains from filter paper discs immersed in liquid hydrogen peroxide.

Strains used: Ribotype 027 (blue diamond), ribotype 014 (red square), ribotype 103 (green triangle), and ribotype 220 (purple cross). Data points indicate the averages of a single experiment carried out in triplicate, and the error bars indicate the standard errors of the mean.

Applying the maximum concentration of hydrogen peroxide (10%), showed a significant difference between the area of clearing for ribotype 027 and the other strains tested: ribotype 027 with ribotype 014 (P=0.018), ribotype 103 (P=0.021), and ribotype 220 (P=0.005). This suggests that ribotype 027 may be less resistant to the application of hydrogen peroxide, exhibiting greater susceptibility than the other strains at the 10% peroxide concentration level.

The results from this experiment show that when exposed to the liquid form of hydrogen peroxide, there is a statistical difference between how *C. difficile* spores of different ribotypes respond. At lower concentrations, ribotype 103 appears more resilient to the hydrogen peroxide, however for the peroxide concentration of 10%, the areas of inhibition are similar to that of ribotypes 014 and 220. The opposite was observed with ribotype 027, despite similar inactivation response as ribotypes 014 and 220 at lower concentrations, this strain was more susceptible to the presence of 10% hydrogen peroxide solution.

Therefore, there may be a difference expected between the strains, upon exposure to hydrogen peroxide vapour in the decontamination chamber.

5.3.3 Recovery of ribotype 027 spores following exposure in a desiccator

The basis behind exposing PCR ribotype 027 spores to a desiccator was to gain insight into the number of spores that survive and are recovered from the membranes. The work conducted earlier in this thesis (Chapter 2) demonstrated that the *C. difficile* spore recovery from a stainless steel tile was not 100% when attempting to use agar media and tamping onto the tile surface. The objective of this work was to identify the inactivation of spores due to hydrogen peroxide. Therefore, it was important to calculate the maximum possible recovery without hydrogen peroxide exposure.

Spores were recovered from the membranes after 5 min in the desiccator, 45 min and 7 hr. These time points were selected to demonstrate the effects of time on the recovery of spores, with 7 hr the longest time of exposure used throughout these experiments. There was a 1-$\log_{10}$ CFU reduction of spores applied onto the membranes to the spores which were recovered after all three time points. There was no statistical difference in the recovery of spores from the membrane surfaces irrespective of the time in the desiccator, with 5 min and 45 min (P=0.293) and 5 min and 7 hr (P=0.796). Therefore, this suggests that with the addition of 6-$\log_{10}$ spores onto the membranes, irrespective of the time the spores are left on the surface of membranes in the desiccator, the proportion of spores that cannot be recovered remains the same; the variances between the times were also similar.

The results from this experiment show that when exposed to air in a desiccator, with no additional stress for example hydrogen peroxide, there is a quantifiable reduction in the number of *C. difficile* spores recovered. This difference was then used to correct all other spore recoveries following hydrogen peroxide exposure in later experiments described in this chapter.

5.3.4 Inactivation of ribotype 027 spores with hydrogen peroxide vapour

This section outlines the results obtained with experimental runs where *C. difficile* spores were exposed to different concentrations of hydrogen peroxide vapour. These experiments were carried out to investigate the changes in $\log_{10}$ CFU observed with a range of concentrations and over time. PCR ribotype 027 spores were used in this work to identify the optimal time for exposure to elicit an efficient $\log_{10}$ CFU reduction with a given concentration of hydrogen peroxide. Once determined, these parameters were then used in the final experiments in this chapter. As 50 ppm was used in the preliminary experiments, to explore an evenly spread range of concentrations, 10 ppm and 90 ppm were also investigated. Ribotype 027 spores were used throughout this section as it is frequently used in disinfection and decontamination studies.

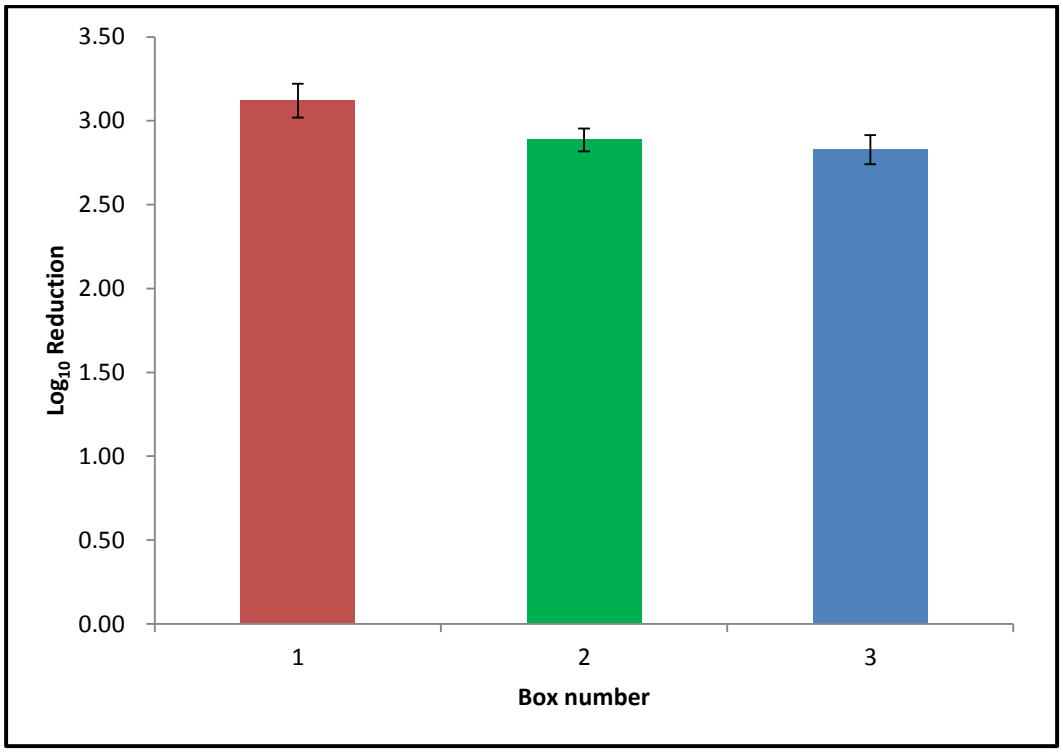

Figure 5.5: *C. difficile* PCR ribotype 027 spores reduction from exposure to 50 ppm hydrogen peroxide for thirty minutes in the exposure chamber.

Boxes are numbered in series. Bars indicate the averages of five repeats and the error bars indicate the standard errors of the means.

Preliminary experiments conducted at 50 ppm investigated the flow rate of the hydrogen peroxide through the three boxes in series. This was to ensure that the exposure was equal throughout all three boxes (Figure 5.5). Each of the three

boxes exposed five membranes with ribotype 027 spores to 50 ppm hydrogen peroxide vapour for 30 min. Statistical analyses show there was no statistical significant difference in the $\log_{10}$ CFU recovery obtained from the boxes; boxes 1 and 2 (P=0.107); boxes 1 and 3 (P=0.089); boxes 2 and 3 (P=0.641). This finding ensures there would be no significant difference in the recovery of the spores between the boxes selected for exposure.

Exposing the ribotype 027 spores to 10 ppm, 50 ppm, and 90 ppm hydrogen peroxide vapour was important to identify the rates of *C. difficile* spore inactivation for a concentration less than 100 ppm. For the purpose of the Series-Event models, an 'event' is determined by a degree of damage imposed onto a given cell. An accumulation of these in series results in death of the cell. The experimental data generated in this chapter has identified, for all concentrations of hydrogen peroxide, the total number of hits for the inactivation of *C. difficile* spores determined was four.

The data generated for 10 ppm hydrogen peroxide exposure shows there are three distinct regions of inactivation over a 7 hr period where efficient $\log_{10}$ CFU reduction was observed (Figure 5.6). A Series-Event model has also been determined to help predict the time frame in which spores may inactivate. There is a period of initial decline in spore survival before inactivation begins as seen in the first hr of the experiment. This is followed by an exponential decrease in the $\log_{10}$ CFU recovered (t=1 to t=4), where a 2-$\log_{10}$ CFU reduction is observed. Finally, the few remaining hardy spores resist the effects of hydrogen peroxide towards the end of the exposure period. This is represented by the slight decline of less than 0.5-$\log_{10}$ CFU between the latter 3 hr of the experiment (t=4 to t=7). Therefore, this suggests that after 4 hr of exposure to 10 ppm hydrogen peroxide, there is no difference in the spores killed. To inactivate all of the spores, according to the Series-Event model, it may be required for the exposure time to be lengthened much past 8 hr. Nevertheless, the application of 10 ppm hydrogen peroxide does appear to have an overall effect on the presence of ribotype 027 spores. Comparing the counts between t=0 and t=7 identifies a highly significant difference (P=0.000).

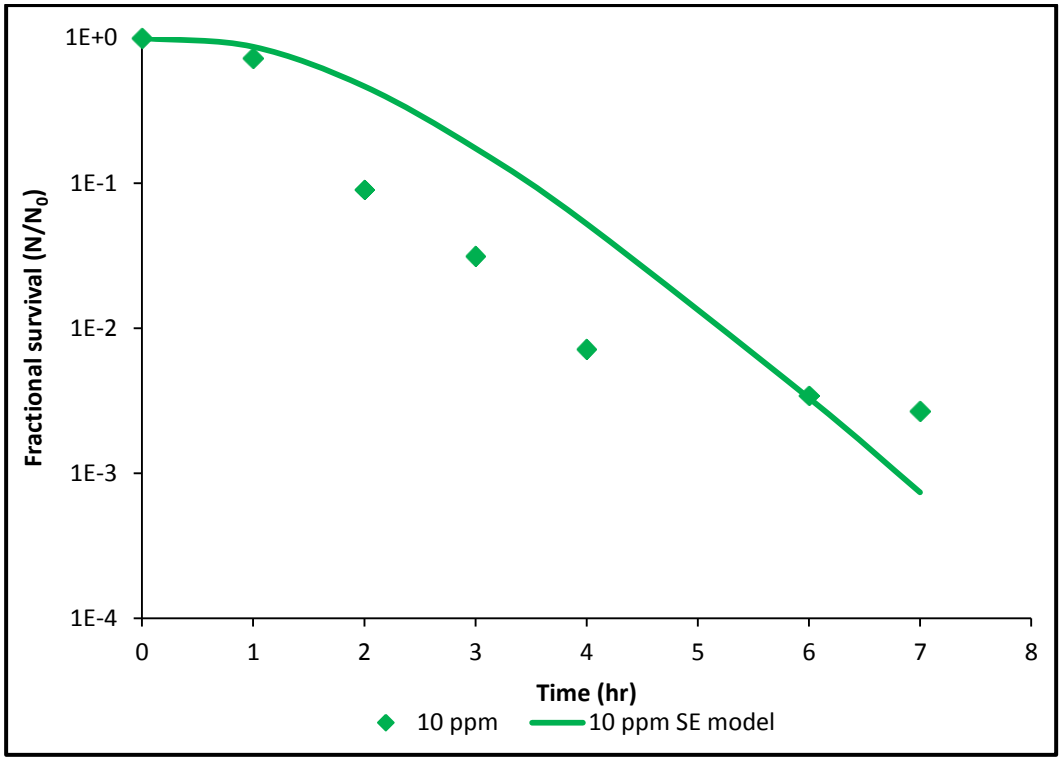

Figure 5.6: Semi-logarithmic scale representation of *C. difficile* ribotype 027 spore inactivation as a function of time. Comparison of a modified Series-Event model prediction (solid line) with experimental data at 10 ppm hydrogen peroxide.

Four experiments were conducted with different times of exposure. Data points indicate the averages of ten to twenty-five replicates.

Figure 5.7 shows the fractional survival curves and respective Series-Event models obtained with 50 ppm and 90 ppm hydrogen peroxide exposure. As with the 10 ppm data, these results also show the viability of spores decreases with increased times of exposure. There is the initial decline in $\log_{10}$ CFU counts where the weaker spores are affected by the concentration of peroxide used.

With respect to 50 ppm, the increased concentration shows there is a statistically significant difference when comparing 10 ppm and 50 ppm after 60 min (P=0.008). After exposure for 2 hr, there is a further 1.3-$\log_{10}$ CFU reduction when compared to the end of the 10 ppm experiment (at t=7). The counts between t=0 and t=120 identifies significant difference (P=0.000), with a total reduction of 3.9-$\log_{10}$ CFU.

The final experiment carried out in this series was the exposure of spores to 90 ppm over a period of 80 min, which elicited a 5.9-$\log_{10}$ CFU reduction. The rate at which the spores were inactivated with this concentration was much faster than

observed with the previous two concentrations; the exponential decline occurred in less time than with 10 ppm or 50 ppm and resulted in fewer spores remaining by 60 min. Most of the remaining resistant spores appear to be inactivated by 80 min. Extending the Series-Event model past the data points shown with 90 ppm in Figure 5.7 suggests complete inactivation of spores may be observed by 90 min. Comparing the recovery at 60 min, there was a significant difference between the CFU at 10 ppm and 90 ppm (P=0.007).

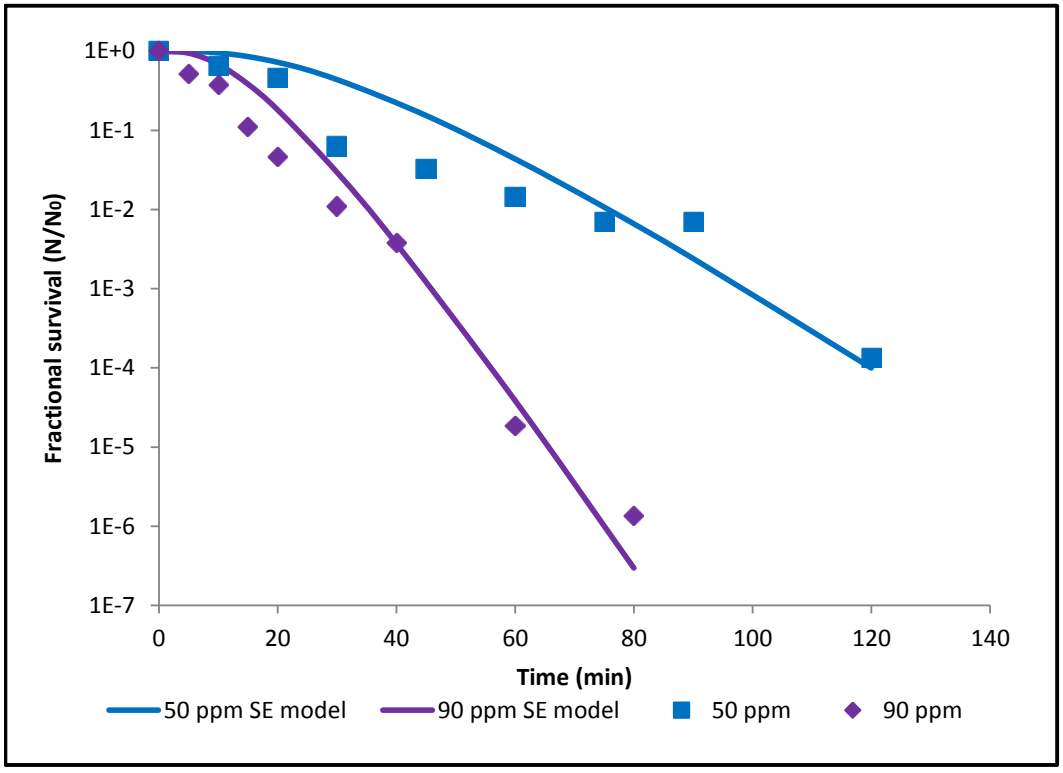

Figure 5.7: Semi-logarithmic scale representation of *C. difficile* ribotype 027 spore inactivation as a function of time. Comparison of modified Series-Event model predictions (solid lines) with experimental data at 50 ppm and 90 ppm hydrogen peroxide.

Seven experiments were conducted with different times of exposure. Data points indicate the averages of ten to twenty-five replicates.

Calculating the D-values, identifies the average time required for a 1-$\log_{10}$ CFU reduction of *C. difficile* spores. This value is frequently determined in food microbiology, as shown by Meszaros et al. (2005). Using the Series-Event models devised in this work and the derivation of an equation in Malik et al. (2012), the application of 10 ppm was calculated to require 3.5 hr of exposure, 50 ppm needed 50 min, and with 90 ppm, 21.8 min was necessary.

The results from these experiments show that time of hydrogen peroxide exposure is linked to a reduction in spore recovery. There is an early stage where the weaker spores are inactivated followed by an exponential decline in a large proportion of the spores used. Finally, the persistence of the more resistant spores can be observed towards to end of the experiments, and these are the spores which require the most attention with respect to disinfection. In order to prevent re-colonisation, these spores need to be eradicated. Therefore, the longer the spores were exposed to the hydrogen peroxide, the larger the $\log_{10}$ CFU reduction was observed, as the hardier spores were beginning to be inactivated.

In addition to this, increasing the concentration of hydrogen peroxide vapour also has an effect on the $\log_{10}$ CFU counts. There was a greater reduction of spores recovered at the same time points as the concentration increased, for example, there were a greater number of inactivated spores at 60 min with 90 ppm than with 10 ppm. These data suggest the greatest and fastest $\log_{10}$ CFU reduction of spores was with 90 ppm, where a significant 5.9-$\log_{10}$ CFU reduction was observed over the course of 80 min.

5.3.5 Inactivation of C. difficile strains with hydrogen peroxide vapour

The purpose of this final experiment was to inactivate *C. difficile* spores obtained from different PCR ribotypes with hydrogen peroxide vapour in the decontamination chamber. This experiment also aimed to identify whether there were differences in the responses to counts obtained between the *C. difficile* strains, with respect to the rates at which the spores were killed.

As conducted in Section 5.3.4, this study used a concentration of 90 ppm hydrogen peroxide to elicit a reduction in the number of spores recovered. The protocol remained unchanged as 90 ppm invoked the greatest $\log_{10}$ CFU reduction in ribotype 027 spores, resulting in the inactivation of the hardier spores in less time. In order to establish whether a difference could be observed between liquid hydrogen peroxide and its vapour form, the same strains from Section 5.3.2 were used.

C. difficile spores for ribotypes 014, 027, 103, and 220 were exposed for 40 min in the decontamination chamber (Figure 5.8). There was more killing of the non-clinically relevant ribotypes 103 and 220, than the problematic strains (ribotypes 014 and 027). Modified Series-Event models were created for all strains used in the experiment; ribotypes 014 and 027 mapped better than the non-clinical strains. In Section 5.3.4, the ribotype 027 inactivation data appears to follow a somewhat sigmoidal curve which deviates from the model during the experiment, mapping better initially and towards the end of the inactivation. However, over this shorter time period, the model appears to map better with the data.

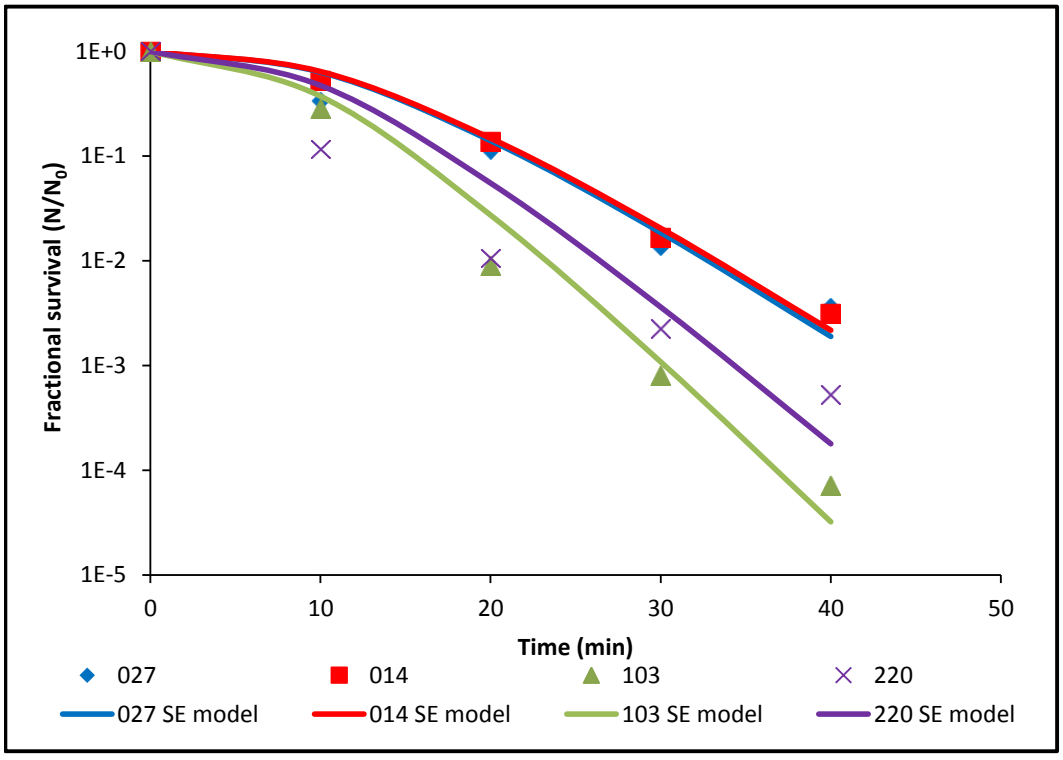

Figure 5.8: Semi-logarithmic scale representation of *C. difficile* strains and spore inactivation as a function of time. Comparison of modified Series-Event model predictions (solid lines) with experimental data at 90 ppm hydrogen peroxide.

Strains used: Ribotype 027 (blue diamond), ribotype 014 (red square), ribotype 103 (green triangle), and ribotype 220 (purple cross). Data points indicate the averages of five to ten replicates.

The three distinct inactivation stages observed with the previous experiments were also noted with these data. All ribotypes appeared to have an initial inactivation of the weaker spores by 10 min, with ribotype 220 resulting in 0.9-$\log_{10}$ CFU reduction. At 20 min, there was a significant difference in the

recovery of ribotypes 014 and 027 compared to the non-clinically relevant strains, as observed when comparing ribotypes 104 and 103 (P=0.001).

Further to this, at the end of the experiment, with 90 ppm hydrogen peroxide for 40 min, ribotypes 014 and 027 had 2.5- and 2.4-$\log_{10}$ CFU reductions, respectively. This significantly contrasts with the recovery observed with the other strains tested, ribotype 220 (3.3-$\log_{10}$) and ribotype 103 (4.2-$\log_{10}$ CFU reduction). These data show that there was a large difference in the spores recovered following inactivation compared to the recovery of ribotypes 014 and 027, with this strain exhibiting more resistance to the vapour decontamination. This decrease in spore recovery demonstrates that the susceptibility of these strains to hydrogen peroxide is greater than with the clinical strains.

In addition, it could be predicted using the Series-Event models that between 50-60 min, ribotype 103 would be completely inactivated and ribotype 220 could have a 4-$\log_{10}$ CFU reduction at this concentration, should the experiment be extended. The D-values were determined for these strains: 22.1 min (ribotype 014), 21.8 min (ribotype 027), 15.4 min (ribotype 103) and 17.6 min (ribotype 220). These showed that the more susceptible the strain, the quicker the spore recovery was reduced by 1-$\log_{10}$ CFU.

The results from this experiment show that when exposed to 90 ppm hydrogen peroxide, there is a significant reduction in CFU counts for all as the time of exposure is increased. These data also demonstrate there is a statistical difference in susceptibility observed between the four strains investigated, with the clinically relevant strains more resistant to the exposure of hydrogen peroxide vapour.

5.4 Discussion

Various procedures have been put into place to control the spread of infection in healthcare facilities such as isolating infected patients, ensuring hands are washed thoroughly after contact with infected patients, minimising the administration of high-risk antibiotics, as well as daily cleaning (Boyce et al. 2008). Routine cleaning with a sodium hypochlorite solution has been successful in the control of CDAD (Kaatz et al. 1988; Mayfield et al. 2000; McMullen et al. 2007). Despite this, there is evidence to suggest routine cleaning is not entirely effective, with surfaces in hospitals still contaminated with bacteria irrespective of cleaning (Oie et al. 2007; Otter et al. 2010). These surfaces and medical equipment can act as bacterial reservoirs, increasing the probability of cross-contamination by healthcare workers, and subsequently contributing to the spread rather than eliminating it. There are also areas such as keyboards, monitors, electrical beds and handsets where it may be difficult to clean (Andersen et al. 2006). It must be noted that this is a daunting and extremely challenging task, with certain disinfectants and detergents being rendered useless due to difficulties in accessing smaller components within machinery, or being incompatible with materials (Davies et al. 2011).

The use of coupons infected with bacterial spores for use in decontamination studies is common (Meszaros et al. 2005; Pottage et al. 2010; Davies et al. 2011). However, a recent report into *Listeria* cells and the application of a bacterial load identified a more uniform technique for infecting materials in such a manner with the filtration onto membranes; this was confirmed with SEM (Bayliss et al. 2012). This group concluded that spores were layered more homogenously; use of this method in this chapter ascertained this, as shown in Figure 5.3 of this thesis.

Before conducting experimental runs exposing *C. difficile* spores to the decontamination chamber, it was important to run control tests to test the natural loss in spore recovery and the decontamination chamber to ensure the flow of hydrogen peroxide interspersed within the air was constant, and at a steady state from the initial box to the third. Using a desiccator, *C. difficile* ribotype 027 spores

were exposed to the relative humidity at which the environmental chamber would be during an experimental run. A decrease was observed when comparing the number of spores applied to the surface of the membrane and the number which could then subsequently be recovered. Although this was found with *Bacillus subtilis* spores by Shaw (2013), the proportion of loss has not been widely reported. The 1-$\log_{10}$ CFU reduction identified in this study was then applied to all subsequent recoveries from the decontamination chamber to correct accordingly.

Preliminary work was conducted whereby the decontamination chamber exposed membranes to 50 ppm hydrogen peroxide vapour for 30 min. These findings showed the counts obtained from the boxes were statistically similar (P>0.05). This confirmed the distribution of air and hydrogen peroxide vapour mixture was equal throughout all three boxes within the chamber. Shaw (2013) demonstrated the same with *Bacillus subtilis* spores, and established there was no more than a 5% drift in the hydrogen peroxide concentration when exposing for longer periods of operation.

There are two different hydrogen peroxide systems commonly used in cleaning: vapour decontamination and in the form of a dry-mist. Boyce et al. (2008) reported 25.6% (n=43) of samples collected from surfaces in a hospital in Connecticut were positive for *C. difficile*; recovery after exposure of vapour from 30% liquid hydrogen peroxide [Bioquell, UK] fell to 0%. This group also reported a fall in the reports of *C. difficile* associated disease (1.89 to 0.88 cases per 1,000 patient-days, corresponding to the pre-intervention and intervention periods, respectively). The entire process of decontamination took 3-4 hr for an individual room and about 12 hr for an entire ward.

Utilising a 5% hydrogen peroxide dry-mist disinfection system (Sterinis) [Gloster Sante Europe, France], and a single cycle of decontamination, Barbut et al. (2009) reported a 4.2-$\log_{10}$ CFU reduction on *C. difficile* spore-infected coupons. Furthermore, Andersen et al. (2006) reported a 6-$\log_{10}$ CFU reduction of *Bacillus* spores with a dry-mist hydrogen peroxide system (Sterinis). The group conceded that this complete inactivation only occurred with three 3 hr decontamination cycles.

Fu et al. (2012) were the first group to conduct a comparative study into the effects of using these two techniques. This group used a variety of microbes, including an undisclosed *C. difficile* strain, to demonstrate the inactivation of spores. The authors conceded the dry-mist technique resulted in an uneven distribution of hydrogen peroxide, concluding cycle times required further investigation.

The experiments in this thesis have not used such cycle times in disinfection. The use of a constant concentration of hydrogen peroxide over a distinct time period identified how long was required in order to elicit an efficient $\log_{10}$ CFU reduction in the applied spores. The need for these long cycle times reported in literature ensures the inactivation of bacterial spores, in particular, those that are more resistant. The persistence of these spores was observed in the third stage of inactivation and towards the latter time points of the vapour decontamination experiments in this chapter.

Rodriguez-Palacios & Lejeune (2011) investigated the *D*-values of three strains of *C. difficile* spores following heat treatment, however, to date, no studies have identified the *D*-values for the *C. difficile* spores from different ribotypes for a range of hydrogen peroxide concentrations. The benefit of obtaining this value is that it aids in the design of hydrogen peroxide decontamination processes. The results in this chapter have identified increasing the concentration of hydrogen peroxide decreases the time required to elicit a 1-$\log_{10}$ CFU reduction.

The findings from this work have also demonstrated that increasing the time of exposure leads to a greater decline in *C. difficile* spore inactivation. This work used 10, 50, and 90 ppm, and the results were supported with the faster *D*-values with increasing the hydrogen peroxide concentration. With the application of 10 ppm hydrogen peroxide vapour, a 2.6-$\log_{10}$ CFU reduction was observed with spores of ribotype 027 over the course of 7 hr. Increasing this concentration to 50 ppm with the same strain resulted in a 3.9-$\log_{10}$ CFU reduction within a shorter time period of 2 hr. A slightly greater reduction was obtained with 90 ppm (5.9-$\log_{10}$ CFU) after exposure for 80 min. This demonstrates that although, as expected, increasing the concentration of hydrogen peroxide increases the rate of

killing and decreases the time required for exposure, lower concentrations are able to elicit significant kill efficiencies.

For all of these fractional survival curves, there were three sections of inactivation observed, as described with UV inactivation of *Bacillus* spores by Mamane-Gravetz & Linden (2005). Initially, there was a stage of spore reduction, an exponential stage where most of the spores were inactivated, and a final region, which consisted of the eradication of the most resistant spores. It was found that the longer the time of hydrogen peroxide exposure, the increased possibility of inactivating these resistant spores.

A study by Otter & French (2009) investigating the effects of inactivation of a number of nosocomial bacteria with hydrogen peroxide vapour identified results similar to those in this chapter: longer exposure times result in an eventual greater number of inactivated spores. This work demonstrated inactivation of *C. difficile* ribotype 106 spores with 30 min of 30% (w/w) liquid hydrogen peroxide (6.4-$\log_{10}$ CFU). These data also correlate with the findings of Pottage et al. (2010) and the inactivation of MS2 bacteriophages with hydrogen peroxide.

Lawley et al. (2010) identified that with 400 ppm hydrogen peroxide vapour, 5-20 min of exposure was required to reduce the viability of *C. difficile* spores, stating prolonged exposure resulted in complete inactivation of the 6-$\log_{10}$ spores applied. The work in this thesis showed that more than 80 min of 90 ppm hydrogen peroxide would be able to exhibit the same.

However, the method used by Lawley et al. (2010) made use of a peak concentration of exposure, whereas the concentration of hydrogen peroxide vapour administered in this work was continuous. Many studies in which spore inactivation is reported, utilised an exposure concentration that has fluctuated over the time, reaching a peak concentration for a short time (Johnston et al. 2005; Hall et al. 2008; Fu et al. 2012).

Furthermore, irrespective of the range of bacterial inactivation studies conducted, not exclusive to those investigating *C. difficile* alone, there are many studies where the authors have neglected to disclose all of the parameters for the

hydrogen peroxide vapour exposure. The lack of details with respect to concentrations used and the corresponding times of exposure therefore prevent in-depth comparisons being drawn between the findings presented in this chapter with those in the literature.

Despite utilising different *C. difficile* strains within their reports, neither Shapey et al. (2008) nor Barbut et al. (2009) concluded how the application of hydrogen peroxide had had an effect on these subtypes. Shapey et al. (2008) referred to how, with the environmental sampling prior to the dry-mist application, ribotypes 001, 027 and 106 were collected; these are all clinically relevant strains. However, the group did not report which strains of *C. difficile* were responsible for the contamination they identified when sampling post-exposure. This was also observed when analysing a study by Barbut et al. (2009), who did not conclude the effect hydrogen peroxide bore on the different ribotypes. Of the strains identified for the recovery experiments, two were variants of ribotype 027 (one historical and the other epidemic), and the third a reference strain (VPI 10463), sub-typed as toxinotype 0. This strain has not been assigned a ribotype and therefore it is not known if this is a clinically relevant strain; toxinotype 0 could be ribotype 001, 003, 012, or 014/020, amongst many others (Rupnik et al. 2001; Mukherjee et al. 2002; Janezic et al. 2012). It is not clear whether the application of hydrogen peroxide induced inactivation at the same rate between strains. Furthermore, the individual $\log_{10}$ CFU reductions obtained for these strains by Barbut et al. (2009) following the use of hydrogen peroxide was not provided. Therefore, it is not known whether a statistical difference was observed in spore inactivation between strains. Conversely, the work presented in this thesis outlines the concentration and times of exposure for the strains used, as well as identifying differences in responses to hydrogen peroxide.

Exposing *C. difficile* strains to different concentrations of liquid hydrogen peroxide through a simple zone of inhibition assay identified differences exist between ribotypes and their responses to the agent. At lower concentrations of hydrogen peroxide (1-6%), ribotypes 014, 027, and 220 were statistically similar with respect to susceptibility; ribotype 103 was significantly more resistant. This

was observed with the smaller zone of clearing around the filter paper disc in the centre of the bacterial lawn. Increasing the concentration to 10% revealed ribotype 027 as highly susceptible to the application, despite its reputation as a problematic and difficult to eradicate strain.

Interestingly, however, these preliminary data were not indicative of the responses of the strains when exposed to vaporised hydrogen peroxide. The striking variation observed was the difference in susceptibility of ribotype 103; with liquid contact, this strain was the most resistant. This strain was isolated following environmental sampling (Chapter 3), with its spores having resisted desiccation and decontamination in the hospital. However, following exposure in the hydrogen peroxide chamber, ribotype 103 was recognised as the most susceptible of those tested. The results from this strain suggested a reduction of 4.2-$\log_{10}$ CFU after 40 min at 90 ppm. It could be predicted using the Series-Event model that within a further 20 min exposure, complete inactivation of the 6-$\log_{10}$ spore solution would be observed.

Lawley et al. (2010) used 6-$\log_{10}$ spores obtained from a ribotype 017 sample, an epidemic strain in Poland (Pituch et al. 2006). This group demonstrated complete inactivation of these spores with 10% hydrogen peroxide solution, with >99% inactivation within 1 min exposure. This research group also reported a 75% reduction in spore viability with the use of 1% hydrogen peroxide over 20 min. These findings concur somewhat with the results identified from the work in this thesis, which shows that increasing the concentration of hydrogen peroxide solution exposed to *C. difficile* spores increases the rate of inactivation.

Furthermore, there has been an investigation into the effects of liquid hydrogen peroxide and sporulation rates of different strains of *C. difficile* (Fawley et al. 2007). The results from this study suggest the application of a hydrogen peroxide product, G-Force [JohnsonDiversey, UK], increased sporulation of ribotypes 001, 010, and 027. This product bore no evident effect on the *C. difficile* spores, which is inconsistent with the findings in this study as well as an *in vitro* study by Perez et al. (2005). This group obtained a complete inactivation of 6-

log_{10} CFU reduction with 70,000 mg/l hydrogen peroxide with the Virox system within 13 min [Virox Technologies Inc., USA].

Johnston et al. (2005) used *C. botulinum* spores to identify linear inactivation kinetics, comparing the log_{10} CFU surviving after exposure for a specified time, as conducted in this thesis. This group were able to demonstrate exposure with less than 10 min was sufficient to induce inactivation, using a maximum hydrogen peroxide concentration of 355 ppm, which is significantly greater than the vapour concentrations used in this work.

In addition, the findings from the liquid hydrogen peroxide experiments identified ribotypes 014 and 027 as susceptible to its exposure. The vapour decontamination did not concur with this. Ribotypes 014 (2.5-log_{10} CFU reduction) and 027 (2.4-log_{10}) were the most resistant strains after 40 min at 90 ppm. The calculated *D*-values for these strains emphasise the rate at which the recovery decreases by 1-log_{10} CFU. Unfortunately, the lack of derivation of these values by other research groups hinders the comparison of these data.

As these were the two clinically relevant strains investigated, it is vital to note that with respect to the vapour decontamination studies, they were resistant. This would be important to bear in mind when deciding on which concentration of hydrogen peroxide and for what time period, as these are two of the most problematic strains, clinically (Chapter 4). No other studies have compared clinical strains in such a manner to identify the times and concentrations required to elicit spore inactivation.

Based on the findings from this work, it could be suggested that the current treatment times used with hydrogen peroxide systems may not be adequate for the reduction of a range of clinical *C. difficile* strains. The paucity of reports investigating the effects of different ribotypes suggests this is an area requiring further research. The results from this chapter have identified significant differences exist in the responses of ribotypes to hydrogen peroxide decontamination, whether in liquid form or vaporised.

Furthermore, despite a vast number of reports demonstrating complete inactivation of *C. difficile* spores, the lack of control of hydrogen peroxide vapour at a fixed level could result in only the peak concentration affecting the spores for a limited time. This means higher concentrations of exposure are required to elicit this reduction. The identification of three regions of spore inactivation in this work suggests that constant application of hydrogen peroxide could be vital in the reduction of viable spores, targeting both the susceptible and resistant spores, whilst maintaining a lower exposure concentration.

An important aspect in the work carried out in this chapter was to provide an insight into whether there is a link between different *C. difficile* strains and the responses elicited following hydrogen peroxide exposure. The experiments were also designed to identify whether inactivation of *C. difficile* spores can be carried out with lower hydrogen peroxide concentrations. This work suggests that a difference does exist between strains, with the clinically relevant strains more challenging to eradicate with hydrogen peroxide vapour. However, susceptibility was demonstrated even with lower concentrations. Further work into responses of clinical strains to vapour decontamination, with an extension into the time of exposure would be beneficial in accurately determining the parameters for complete inactivation.

There has been a call in the literature for adequate cleaning prior to hydrogen peroxide decontamination. Otter et al. (2007) and Boyce et al. (2008) have shown the application of pre-cleaning of contaminated surfaces with cleaning agents and disinfectants before the application of hydrogen peroxide vapour disinfection is necessary. Although in this work there was no biological soiling added to the spores to simulate this contamination, previous findings have created such a simulation, stating hydrogen peroxide decontamination should not be applied in isolation and must be used in conjunction with an established cleaning procedure with regards to disinfection. This is due to the spores being protected by various types of organic matter, affecting the inactivation.

Hospital decontamination is an important albeit difficult problem to tackle. The resistant nature of *C. difficile* spores makes it even more challenging to reduce

the probability of cross-contamination and infection. The reported success of hydrogen peroxide vapour decontamination with the range of HCAI researched to date indicates its incorporation into cleaning practice may aid in the reduction of problematic pathogens as well as inducing inactivation of *C. difficile* spores. The work in this chapter also demonstrated that with lower concentrations of hydrogen peroxide, significant $\log_{10}$ CFU reduction could still be obtained, and affect the most clinically relevant strains. This is the first study to identify how a low controlled concentration of hydrogen peroxide vapour can inactivate spores of a range of *C. difficile* strains to date. There is a need for data of this kind to be collected to provide an insight into the application of hydrogen peroxide in hospital cleaning. Awareness into the responses of different ribotypes increases the probability of eradicating all problematic clinically relevant strains.

5.5 Conclusions

- A protocol was optimised to assess the inactivation of *C. difficile* strains after liquid hydrogen peroxide exposure. Measurements were taken at multiple time-points to analyse the rate of kill on BHI agar with sodium taurocholate. Ribotypes (014, 027, 103, and 220) were found to respond differently; ribotype 103 was the least susceptible. Clinically relevant and problematic strains 014 and 027 were highly susceptible.
- Following the application of spores onto membranes prior to vapour exposure, a 1-$\log_{10}$ CFU loss of ribotype 027 spores was observed.
- An environmental decontamination chamber was used to expose a specific concentration of hydrogen peroxide vapour in a controlled environment. With ribotype 027 spores, it was demonstrated that even at 10 ppm, significant inactivation of spores occurs. Increasing the concentration of exposure also increases the number of spores inactivated.
- Use of the environmental chamber at 90 ppm with the above named strains identified a marked difference in susceptibility. Ribotypes 014 and 027 were the most resistant, followed by 220. Inversely to the liquid peroxide results, ribotype 103 was the most susceptible.
- There have not been studies of this kind conducted previously; this is the only study to demonstrate spore inactivation differs between *C. difficile* strains.

Chapter 6. Conclusions and Future Work

6.1 Key findings of this work

The studies presented in this thesis were designed to identify the strains of *C. difficile* prevalent within UK healthcare facilities and to try to determine why they are so prevalent. Using the UHL Trust as a case study, this work required several optimisation steps. In order to investigate the ribotypes present in both patients and their surroundings, methodologies were optimised for the environmental recovery of *C. difficile* spores as well as PCR ribotyping for characterisation of strains. A further aspect of the work was to use hydrogen peroxide in both its liquid and vapour form to investigate the kinetics in *C. difficile* spore inactivation. It is unknown as to whether there is a reason for the prevalence of particular strains that persistently were recovered in the environment. Could it be that their survival was due to resistance to disinfection agents typically deployed in these sites?

In this section, the main conclusions from the work presented in this thesis are summarised:

An aim of this work was to optimise a range of different *C. difficile* agar media and analyse the recovery efficiencies in subsequent environmental sampling. Most of the medium comparative studies have been conducted with reference to the recovery of *C. difficile* from faeces, and therefore the media and conditions identified as ideal for these particular circumstances should not be regarded as necessarily optimal in environmental recovery. Work in this thesis described the development of such a comparison, which was used in the resuscitation of environmental *C. difficile*. This comparative study involved five different media and spore recovery following exposure to a germicide; investigations of this kind are infrequently conducted. A methodology for the media optimisation in the environmental isolation of *C. difficile* spores is presented, in which three antibiotic-containing agar media (CCAB, CCEY, and CDMN) and two non-selective (FAA and GS-BHI) were compared. The work within Chapter 2 explored the stressing of spores with a germicide in liquid suspension and on stainless steel tiles. It was also explored whether any of the media could recover *C. difficile* despite these stresses.

This study was designed to identify which media would be the most capable in the recovery of spores from the hospital environment, and whether a particular combination of selective and germination agents was more successful. The recovery of ribotype 027 spores was analysed with different *C. difficile* agar media, which have been reported as successful in the environment as well as in the laboratory culturing of *C. difficile*. The media displayed differences in both recovery and selectivity to ChlorClean. It was shown that with direct contact of stainless steel tiles contaminated with *C. difficile*, GS-BHI and FAA recovered the highest proportion of *C. difficile* spores. These two media possessed only the base agar media and supplements to aid in spore germination.

This work has also strengthened reports in literature that allude to the importance of sodium taurocholate with a base agar media. With sub-lethal concentrations (100 ppm) of the germicide, high recoveries of *C. difficile* spores were obtained using all five media. Of the selective media, CCEY agar recovered *C. difficile* spores significantly more proficiently than CDMN and CCAB. These results have also shown that CCEY and GS-BHI agars were able to recover *C. difficile* spores from environments where the spores had been exposed to 1000 ppm ChlorClean.

With direct liquid exposure of ChlorClean at 10, 50 and 100 ppm to *C. difficile* ribotype 027 spores, it was found that fewer CFU were recovered with an increase in the concentration. In addition, the longer the exposure time, the fewer spores recovered. The effect of 10 ppm appeared to have very little effect overall on the spore reduction, however, with 50 and 100 ppm a marked inactivation was identified within 1 hr. In addition, the link between the contact times of germicide and spores in order to effectively induce inactivation was demonstrated, as supported throughout literature.

Despite the results of the laboratory-based experiments, it was hypothesised the use of an agar media with no selectivity properties in the form of antibiotics would recover a plethora of microorganisms when applied to surfaces in a hospital ward. A further aim of this work was to identify techniques that could be used in the resuscitation of *C. difficile* from hospital environments. The use of

CDMN agar in direct contact sampling did not yield high recovery of *C. difficile*-positive isolates, despite preliminary environmental sampling (not presented) demonstrating this media recovered the highest number of colonies. On the contrary, analysis of a sub-set of the colonies present on the surface of the agar plates were identified as constituting a variety of microbial species, including *Staphylococcus haemolyticus* and *Staphylococcus epidermidis*. This finding suggests that agents widely held to be selective for *C. difficile* have been found to lack selectivity when applied in the environment. Four positive isolates were confirmed from this first sampling campaign as ribotype 027, suggesting the presence of this strain environmentally (Chapter 3).

Routine cleaning with germicides is effective in reducing the number of *C. difficile*-positive samples isolated, as demonstrated in this thesis. A sampling campaign using the five agar media in two *C. difficile* isolation rooms (before and after a standard cleaning episode), toilet area, and sluice was conducted; 47.6% sites were *C. difficile*-positive. The results demonstrated that within an isolation room, multiple ribotypes could be identified; ribotypes 010 and 103 were present in both isolation rooms. Despite an overall decrease in numbers of putative *C. difficile* isolated after cleaning, there was clear evidence to suggest that routine cleaning fails to completely eradicate *C. difficile* spores from the environment, with ribotype 103 remaining after cleaning. Little is known about this strain, however its predominance at the time of the study indicates its resistance to the cleaning regimes implemented in the ward. These outcomes emphasise the need to ensure cleaning techniques and methodologies are tested against a range of strains and are capable of inactivating and eradicating multiple ribotypes of *C. difficile*.

More commonly used in the isolation of *C. difficile* from faecal specimens to inhibit other microbial flora, the practice of alcohol shocking was also used to aid in *C. difficile* isolation following environmental sampling from RODAC plates. Samples were then evenly dispersed onto four agar media (CCEY, BHI blood, FAA, and GS-BHI); CCEY agar recovered the most positive isolates. This agar medium is predominantly used in *C. difficile* isolation from faecal samples. However, this

work has also demonstrated its success in the enrichment and subsequent resuscitation of environmentally isolated spores.

Through this work, it was found that selective agents within media that are successful in certain circumstances, for example in the isolation of *C. difficile* from faeces, appear to act in an inhibitory manner in the recovery of stressed spores, as demonstrated from the experiments conducted with the stainless steel tiles. Nevertheless, without the presence of these antibiotics, one would isolate a number of microorganisms from the environment. Therefore, the incorporation of selective and non-selective agars was a potential method for isolation and recovery of *C. difficile*, as explored within Chapter 3.

An area of interest is whether *C. difficile* infection contracted by patients is due to its presence in the vicinity, or whether environments are contaminated with *C. difficile* because of the patients residing within them. In the final sampling campaign (Chapter 3), faecal specimens from patients were collected and ribotyped before their admission to the ward; all patients were infected with ribotype 027. Upon sampling in their individual side rooms with pre-moistened sponges, the same strain was detected for all sites. This strongly suggests a potential for strain correlation and contamination between patient and environment.

Double-layered agar media has been used in the food industry to recover bacteria, but has not been reported prior to this work in the resuscitation of *C. difficile*. This work demonstrated environmental sampling with pre-moistened sponges and subsequent resuscitation of *C. difficile* on a double-layered agar media results in a higher rate of recovery than direct sampling with the agar media in RODAC plates. The findings in this work have also identified that the combination of two distinctive media may act in conjunction, with the non-selective medium resuscitating damaged spores before the presence of the selective antibiotics in the lower layer diffuses onto the surface of the medium. This would then theoretically inhibit the microbial growth of other bacteria, isolating *C. difficile* alone.

Through conducting two of the three sampling campaigns with RODAC plates prepared with *C. difficile* agar, the results from this thesis suggest that perhaps this method is not ideal in the recovery of environmental *C. difficile* (12.4% of the RODAC plates used recovered *C. difficile*). An increased recovery was observed with the use of pre-moistened sponges (10/15 sponges were positive) and subsequent resuscitation onto these media prepared in a layered manner. Although there were no conclusive results into the optimum concentrations of antibiotics or volumes of agar required, a link may have been established.

The third aspect of this thesis was to determine the strains of all *C. difficile* isolates collected. This was conducted using capillary gel-based PCR ribotyping, a technique explained in Chapter 4. This modern system outperformed the traditional agarose gel-based method by producing more accurate and precise ribotyping profiles. The generation of a data file and chromatogram with the banding patterns that would be observed with an agarose gel image permits comparisons of isolates between laboratories. Differences of ±4 bp could be detected, which enhanced and aided in the assignment of strains. The findings from this thesis correlate with current literature suggesting this method of ribotyping should be employed by all typing laboratories.

Two sampling cohorts were investigated, identifying ribotype distributions, both spatially and temporally, within the UHL Trust. As expected due to its national presence at the time, there was a prevalence of ribotype 027 throughout all the sites sampled within the first cohort. Many other dominant ribotypes were also identified as common throughout the three major hospitals and community-based practices: ribotypes 002, 014/020, 015 and 078. These outcomes correlate with the national statistics by the HPA, suggesting the presence of particular strains occurs on a wider geographical basis than within one region alone.

The second epidemiological study conducted was designed to investigate any changes in ribotype distribution due to the implementation in *C. difficile* detection from faecal specimens. Screening of isolates was modified from ELISA-based tests to GDH-based assays, a method which has since been enforced in all NHS Trusts. These changes were implemented to improve the accuracy and

efficiency in diagnostics. Based on the findings, the number of samples from which *C. difficile* could not be isolated increased with the use of the GDH-based assay. Nevertheless, the presence of ribotypes 005, 014/020, 015, 027, 078, and 106 in all months of the study was noted, correlating with the predominant national strains. Interestingly, despite reports of ribotype 027 declining, the data from this thesis suggests a similar proportion of the strain was identified throughout the time sampled. This persistence would suggest ribotype 027 is well established in the UHL Trust. This was also observed with ribotypes 014/020 and 078, correlating with literature. The outcomes from this study emphasise the importance of designing studies of this nature to investigate the effect in ribotype proportion and diversity.

There was also an increase in the number of ribotypes identified after implementing the change in diagnosis in January 2011, perhaps most curiously, in strains that are less well reported. These less clinically dominant strains were not detected in the first sampling study, and few reports refer to them in literature. For example, the aforementioned strain, ribotype 103, was infrequently isolated from faecal specimens collected through the epidemiological studies described, obtained only following the modification in the method of detection. The presence of many of these strains has not been reported on a national scale. This may be attributed to the detection of non-toxigenic strains with the GDH-based assay, which would have not been identified using the ELISA-based assay.

Determining which strains were present within areas of the UHL Trust aided in the decision of the ribotypes to target in the fourth and final aspect of this thesis: to investigate whether exposure to hydrogen peroxide has an effect on the recovery with respect ribotype. As demonstrated in Chapter 5, an environmental decontamination chamber was designed for the application of hydrogen peroxide vapour in an enclosed space. The concentration of hydrogen peroxide vapour administered was monitored throughout and remained constant. Current studies often only specify the peak concentration of hydrogen peroxide reached during environmental release, with incomplete information regarding the time of peak concentration exposure, or of details on cycle times and total length of exposure.

The hydrogen peroxide vapour concentrations utilised (10, 50, and 90 ppm) showed the rate of inactivation of ribotype 027 spores was dependent on the hydrogen peroxide concentration employed. The findings from this thesis demonstrated that even at 10 ppm, there is significant reduction in spores recovered. Longer exposure times were also found to result in greater inactivation, increasing the time in which to eliminate the population of spores that were particularly hardy. Series-Event modelling for these three hydrogen peroxide concentrations represented the experimental data reasonably well.

An assay to identify differences in ribotype susceptibilities to liquid hydrogen peroxide was devised, using ribotypes 014, 027, 103, and 220. Results showed statistical differences exist between strain susceptibilities, with the clinically relevant strains 014 and 027 the least resistant to liquid hydrogen peroxide, with ribotype 103 the most resistant.

Inversely, exposure of vaporised hydrogen peroxide at 90 ppm identified a marked difference in susceptibility. Ribotype 014 was the most resistant, followed by 027, with 103 demonstrating the fastest inactivation. This susceptibility of ribotype 103 was interesting to observe due to the resistance demonstrated to the liquid-phase hydrogen peroxide and its predominance in the *C. difficile* isolation ward in the second sampling campaign irrespective of routine cleaning. This is the only study to demonstrate spore inactivation and kinetics differ between *C. difficile* strains, in particular prevalent and lesser-known isolates. Initiatives like those presented in this thesis would aid in the development of a system to reduce contamination of HCAI from healthcare facilities, whilst using lower, safer concentrations of hydrogen peroxide.

6.2 Proposed future work

The following section includes several recommendations for future work based on the results presented in this thesis:

Arguably, the most important aspect of *C. difficile* environmental sampling is the method by which the spores can be resuscitated and germinate. In order to develop the work conducted in this thesis, it may be useful to cultivate spore solutions of various strains and repeating the described recovery experiments. This would help identify if there are any differences, with respect to which ribotypes can be resuscitated onto the double-layered agars, or whether ribotype 027 grows preferentially on these media combinations.

This thesis has provided evidence to suggest the application of antibiotics in an agar medium could inhibit the growth of damaged *C. difficile* spores. Studies have identified the optimal concentrations of cycloserine and cefoxitin for *C. difficile* from faecal specimens, although whether these are the ideal concentrations for resuscitation of environmental isolates have yet to be studied. Additional work into modifying the concentrations of antibiotics in the layered media may provide information into the requirements of damaged *C. difficile* spores following recovery. Comparative studies in resuscitation could be conducted with different proportions of the recommended concentrations of antibiotics.

General protocols used to track the presence and spread of *C. difficile* infection within a ward could be adapted to gain more epidemiological data. This would be vital in extending the studies conducted in Chapter 4. Investigating the strains present in a healthcare facility following an outbreak or case of *C. difficile*, and specimen analysis from surrounding patients could help control the spread of *C. difficile* infection. Sampling around the infected patients could answer the question: is there an association with the strains found from a patient and their surrounding areas?

Studies into the diversity of strains through the monitoring of patients following the initial diagnosis of *C. difficile* could also be conducted. The

characterisation of strains following the confirmation of *C. difficile* infection and tracking of patient movement between wards, as well as in and out of hospitals, could provide a unique viewpoint into whether the strains residing within the gut remain the same throughout infection, and potentially re-infection. The data collected could provide interesting information into the prevalence of strains within infected patients.

Based on the findings in this thesis, and as supported with current literature, the dominance of ribotype 027 in UK is declining, with previously infrequent strains increasingly identified. Conducting epidemiological studies to investigate shifts in ribotype distribution provides information on a localised geographical basis. In conjunction with the regional and national data provided by the HPA, this would provide the framework for further investigative epidemiological findings into strain proportions.

The second epidemiology study was designed to investigate the effects of a change in the *C. difficile* detection from faecal specimens. Utilising a small sample of the faecal isolate, retrospective analysis of strains could be carried out. It may be that non-toxigenic *C. difficile* strains identified with the GDH-based assay would not have been identified with an ELISA-based assay. A PCR test specifically detecting the toxin genes would clarify this. Conducting the alternate detection test would permit a comparison between these diagnostic techniques. This could answer the important question: was the increase in detection and diversity of lesser-known ribotypes entirely due to changes method of detection increasing the number of non-toxigenic strains?

A conclusion from Chapter 4 suggested there was an increase in non-cultured isolates was observed with the GDH-based assay; the samples were positive with the diagnostic tests, however, *C. difficile* could not be cultured onto selective agar in the laboratory. Therefore, building on this knowledge would enable more understanding into whether the tests might be creating a bias and increasing the number of false positives. Corroborating the findings of the ELISA-based assays may demonstrate the differences in proportions of false positive isolates. No comparative study of this nature has been reported, and therefore the

outcome would be of interest. An insight into the effect these changes can have on the diversity of strains identified from such epidemiological studies would be gained.

Further investigation into the strains used in the hydrogen peroxide studies at a genetic level may identify genes affecting resistance, or susceptibility, to exposure of decontamination agents. Do particular strains possess genetic mutations enabling the resistance to standard cleaning methods? An interesting strain with which to commence investigations would be ribotype 103. This strain was collected from the environment, persisting in isolation rooms despite routine cleaning. Upon exposure to liquid hydrogen peroxide in the laboratory, this strain exhibited the greatest resistance to inactivation, even more so than ribotypes 014 and 027. Do these lesser-known, but very much present, strains carry virulence factors that allow them to reside on environmental surfaces irrespective of routine cleaning with disinfectants?

Exposure of hydrogen peroxide vapour to different strains could also identify whether the resistance with the clinical ribotypes is observed with other dominant isolates, for example, ribotypes 015 and 078 that were found throughout the epidemiology studies. Investigations into the inactivation kinetics of a range of strains could help shape the optimal conditions needed for a reduction in *C. difficile* prevalence.

Another area for potential development of this project is through the collection of field data with large-scale environmental decontamination. The use of biosensor plates exposed to strains of *C. difficile* and hydrogen peroxide vapour, allows the comparison between the spatial and temporal distribution data with those obtained from the laboratory-based experiments.

Finally, hydrogen peroxide vapour decontamination systems in current literature do not account for room sizes. The work conducted in this thesis provides informative kinetic data. Used in conjunction with computational modelling, these data could accurately calculate the required concentration and time of exposure to result in decontamination.

6.3 Concluding remarks

Healthcare facilities possess many sites where pathogenic bacteria are capable of persisting irrespective of the use of disinfectants in routine cleaning, with findings from this work have shown the persistence of *C. difficile* spores. This thesis has outlined the importance of identifying the strains present on these surfaces. It is evident that the literature and knowledge of selective media in sampling and resuscitation of *C. difficile* spores is fundamental. Comparative studies are rare and therefore the ideal selective media has yet to be identified. This work has identified the use of pre-moistened sponges in recovery and subsequent application onto a double-layered agar media for isolation is a potential method to be explored in environmental recovery of *C. difficile*. In addition, it has also been shown how monitoring of strains within a single NHS Trust over a number of months and with a change in *C. difficile* diagnostics can identify differences in ribotype prevalence.

With the application of hydrogen peroxide, this thesis has demonstrated the possibility in reducing *C. difficile* from both healthcare equipment and surrounding environments in a controlled, safer manner with lower exposure concentrations. Identification of the disease-causing and persistent strains uncovers the importance of local surveillance into which types require attention to control spreading of epidemic *C. difficile* infections. This work has uncovered evidence to suggest there are differences in the responses of clinical and non-clinical isolates to hydrogen peroxide exposure.

Reports conducted so far have yet to thoroughly investigate the *C. difficile* spore inactivation kinetics with controlled hydrogen peroxide vapour decontamination. However, with the development and implementation of such a system, it is possible to gain more understanding into this important aspect of *C. difficile* contamination, spread and infection, and thereby aid in the development of more effective strategic techniques.

Publications relating to these studies

Shan, J., Patel, K.V., Hickenbotham, P.T., Nale, J.Y., Hargreaves, K.R., Clokie, M.R.J., 2012. Prophage Carriage and Diversity within Clinically Relevant Strains of *Clostridium difficile*. *Applied and Environmental Microbiology*, 78(17), pp.6027–6034.

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