THE UNIVERSITY *of* EDINBURGH

Clostridium difficile in south-east Scotland

An analysis of severe, recurrent and community-associated disease with a report on the emergence of PCR ribotype 078

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Contents

Declaration

The author performed all the investigations and procedures presented in this thesis, unless otherwise stated.

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Abstract

Clostridium difficile infection (CDI) has proven to be a constantly evolving disease periodically posing new diagnostic and clinical dilemmas. Different regions of the world have reported specific local genomic characteristics of the infecting strains, which may be related to variation in disease presentation and outcome. This study was performed to determine the clinical and molecular features of severe, recurrent and community-associated disease in the Lothian region of Scotland, UK among patients diagnosed from August 2010-July 2011. Three hundred and thirty-five patients with laboratory confirmed CDI were studied for epidemiological features, clinical presentation, and laboratory markers. They were followed up for one year to determine recurrence and mortality. Four hundred and thirty-two episodes were recorded. Ribotypes, presence of toxin genes and MLVA subtypes of isolates from these episodes were determined. During the course of the study, PCR ribotype 078 was identified as an important emerging type and concerns of "hypervirulence" were raised when an outbreak was recorded in 2012. This ribotype was studied to compare its clinical and molecular characteristics with other endemic ribotypes and between its own outbreak-related and endemic subtypes. Asymptomatic children were also sampled to determine their role as pools of potential pathogens.

Severe episodes accounted for 40.4% of total and 29.3% patients had multiple episodes on record. One-year mortality was 32.8% of which CDI was listed on 25.5% death certificates. Ribotype 078 was confirmed in 6.8% episodes. Community-associated disease was identified in 25.3% patients, which differed significantly from hospital-associated disease in the number of antibiotics and gastrointestinal manipulation prior to CDI. Endemic PCR ribotype 078 caused significantly less recurrent disease and more community- associated disease when compared to the most prevalent ribotype 001. Patients who died from ribotype 078 within 30d had a lower Charlson comorbidity index than ribotype 001 counterparts suggesting that the former may infect healthier patients. MLVA subtyping of ribotype 078 proved useful in identifying epidemiological relationships during the outbreak. CDI had contributed to the death of 50% of all patients infected with the outbreak related ribotype 078 strain compared to 14.3% of those infected with the

endemic strains. This study documents the changing epidemiology of CDI in the region and demonstrates differences in epidemic and endemic disease.

Abbreviations

CA-CDI	Community-associated CDI
CCEY	Cyclosine cefoxitiv egg-yolk agar
CCI	Charlson co-morbidity index
CDI	Clostridium difficile infection
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
d	Days
DNA	Deoxyribonucleic acid
FAM	Proprietary name
GI	Gastro-intestinal
HA-CDI	Hospital-associated CDI
HCA-CDI	Healthcare-associated CDI
HCF	Healthcare facility
IBD	Inflammatory bowel disease
MIC	Minimium inhibitory concentration
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable number tandem repeat analysis
MRSA	Methicillin resistant *Staphylococcus aureus*
NED	Proprietary name

PCR	Polymerase chain reaction
PET	Proprietary name
PFGE	Pulse field gel electrophoresis
PMC	Pseudomembranous colilits
PPI	Proton pump inhibitor
RAPD	Random amplified
RNA	Ribonucleic acid
STRD	Summed tandem repeat difference
VIC	Proprietary name
VNTR	Variable number tandem repeats
VRE	Vancomycin resistant enterococci

Chapter one
Introduction

1.1 General microbiology

1.1.1 *C.difficile*: A brief history

The organism was first identified as a Gram-positive rod from the stool samples of babies in the year 1935, and was difficult to grow under standard conditions. It was initially christened *Bacillus difficilis* (Hall and O'Toole, 1935) but was later re-named *Clostridium difficile.* However the clinical disease potential of this bacterium was confirmed even later in 1978 when experiments showed that the toxin produced in the faeces of patients with pseudomembranous colitis (PMC) and post operative diarrhoea was identical to the toxin of *Clostridium difficile* (George et al., 1978, Larson et al., 1978)

Over the years, with better understanding of growth requirements, it has been fairly easy to cultivate under the right anaerobic conditions, but the exact pathogenesis still eludes us. Of its two anatomical forms, the vegetative form is known to be quite susceptible to antimicrobials and disinfectants, but once converted to the spore bearing form it can be quite resistant to common hospital disinfectants and as a result it persists in hospital environment as well as laboratory surfaces as demonstrated by numerous studies (Mayfield et al., 2000, Vohra and Poxton, 2011b, Kaatz et al., 1988, Dumford et al., 2009). Its potential to cause outbreaks has been well documented (Hall et al., 1985, Barbut et al., 1994, Graf et al., 2009) though in the preceding few years the incidence has reportedly declined (HPA, 2012). However it remains endemic in many hospitals and its reported increase in incidence among the non- hospitalized community has been a subject of a substantial number of studies (Huang et al., 2009b, Fellmeth et al., 2010, Riley et al., 1986). It produces three toxins namely toxin A, toxin B which are strongly associated with symptomatic *C.difficile* infection (CDI) (Steele et al., 2012), and in addition a third toxin called the binary toxin is also produced in some strains with some studies suggesting its presence in severe cases of CDI (Barbut et al., 2005, Bacci et al., 2011). It is however, a multifactorial disease and factors such as use of antimicrobials,

administration of agents which reduce the production of stomach acid, multiple comorbidities, prolonged hospital admission and advanced age have been considered predisposing factors in various studies (Bloomfield et al., 2012).

1.1.2 Characteristics of *C.difficile*

C. difficile is a Gram-positive sporulating bacillus with an anaerobic lifestyle. It may, sometimes, appear Gram-variable. The cells are thin (0.5μm wide) and 3-5μm long. Spores, when present, are sub-terminally located. It grows well on enriched media like blood agar but clinical samples often necessitate the use of selective media due to the mixed flora of the sites where *C. difficile* generally inhabits. Cycloserine, cefoxitin, egg yolk (CCEY) agar is a commonly used medium. Colonies on CCEY are yellowish, flat, with an irregular edge and possess a ground glass appearance. On blood agar the colonies are non haemolytic 2-4mm in diameter, with a dull surface and a mottled appearance. *C.difficile* produces a distinctive unpleasant odour, which is likened to "horse manure".

1.1.3 Pathogenicity

Pathogenicity of *C. difficile* is dependent on the presence of two high molecular weight toxins. Toxin A is a 308 kDa enterotoxin which increases fluid accumulation in ligated rabbit ileal loops and toxin B is a 270 kDa cytotoxin which produces cytopathic effects in tissue cultures. They both belong to the family of large clostridial cytotoxins (LCT) and their actions have been proposed to be synergistic (Rupnik et al., 2001). They both perform the function of glucosyltransferases and can inactivate the Rho, Rac and Cdc42 present in the intestinal cells. Some pathogenic strains are seen which elaborate toxin B but not toxin A (Toyokawa et al., 2003, Stabler et al., 2006). The third toxin, called the binary toxin has also been found in 6-15% strains (Voth and Ballard, 2005). Other virulence factors like adhesins and enzymes have been proposed but their role remains doubtful (Poxton et al., 2001).

1.2 Clinical presentations

1.2.1 Spectrum of infections

CDI can present with mild self-limiting diarrhoea. However, it can also cause severe, life-threatening infection, which can be rapidly fatal. Between these two extremes there is a spectrum of more or less severe syndromes characterised by variable degrees of diarrhoea, abdominal pain, fever and leucocytosis. Fulminant (or severe complicated) CDI is characterized by the presence of inflammatory lesions and pseudomembranes in the colon (giving it the name pseudomembranous colitis - PMC). Toxic megacolon may also occur leading to bowel perforation, sepsis, shock and death.

However asymptomatic carriage can occur and studies have demonstrated colonisation rates varying from 4.2% to 15.3% in healthy non-hospitalised adults. Rates vary from the population studied (Kato et al., 2001).

1.2.2 Severe infections

Early identification of patients who are at high risk for severe CDI may help clinicians to alter the modifiable factors and hence improve outcomes. For example surgical prophylaxis in high-risk patients may involve use of lower risk antibiotics.

Various markers for severe disease have been proposed using variable end points. The commonest end point used is mortality up to 30d from diagnosis or development of toxic megacolon or pseudomembranous colitis. Markers like total leucocyte count, serum albumin, serum creatinine and CRP have been studied. Other risk factors like age, presence of co-morbidities, length of stay and immunosuppression have also been subjects of analysis and guidelines have been published on the identification and management of patients with severe CDI (HPA, 2008) However two recent systematic reviews have concluded that the studies published so far have several potential limitations. The majority are retrospective employed single stage stool testing procedures, and some did not use multivariable analyses to adjust for confounders. Poor sample size and reliance on medical records can be additional limitations (Abou Chakra et al., 2012, Bloomfield et al., 2012).

1.2.3 Recurrent infections

One of the characteristics features of *C.difficile* is its propensity to cause recurrent infections. Recurrences occur in 15 to 35% of cases (Fekety et al., 1997, Barbut et al., 2000). Recurrences of *C. difficile*-associated diarrhoea can be a serious clinical problem, increasing the length of stay and overall cost of hospitalisation (Spencer, 1998). Patients may experience three or more episodes adding to their overall morbidity.

The pathogenesis of recurrences may be explained either by the endogenous persistence of *C. difficile* spores or by the acquisition of a new strain from an exogenous source. Reports have previously shown that 38 to 56% of recurrences of *C. difficile*-associated diseases were due to re-infections (Barbut et al., 2000). These numbers will no doubt vary with the discriminatory capacity of the technique used to determine strain type, since techniques like MLVA are known to be more discriminatory than ribotyping (van den Berg et al., 2007).

The predisposition of patients to develop recurrent CDI has not yet been accurately determined, although a number of studies have investigated the subject. Some of these suffered from paucity of numbers and hence lacked the power to detect significant differences between recurrent and non-recurrent cases. A recent large-scale population-based study has performed an analysis of the medical records of 363 patients with clinical recurrence of CDI and proposed a score to predict CDI recurrence following first-ever CDI diagnosis. This score includes factors like severity of initial disease, past health care exposure, stool frequency, CRP level, age, previous MRSA colonisation, admission to a gastroenterology unit, etc. to determine the risk of development of a repeat episode of CDI. The score ranges from -2 to 15 and the authors propose it can be used to make clinical decisions which would reduce the risk of recurrence (Eyre et al., 2012).

1.2.4 Place of acquisition

Although the major burden of CDI lies in the hospitals, patients in the community are also at risk for CDI, albeit at a considerably lower rate than those who are hospitalised.

Considering that healthy adults who had not been exposed to antimicrobial agents for the preceding four weeks, when examined for intestinal carriage of *C. difficile*, revealed an overall carriage rate of 7.6% by faecal culture and typing of their isolates by PCR ribotyping and pulsed-field gel electrophoresis (PFGE) demonstrated clusters of carriers colonised by a single type, it is very likely that community-associated CDI can exist and even cross-transmit in close-knit groups (Kato et al., 2001).

The community-associated CDI rates in the United States have been reported as 7.7 cases per 100,000 person years, and studies from the UK have reported community-associated CDI prevalence of 1.29 per 10,000 population (Fellmeth et al., 2010). More recent studies by the Centre for Disease Control and Prevention found similar community rates, but an increased severity of the disease (Centers for Disease and Prevention, 2005, Centers for Disease and Prevention, 2008).

Proposed sources of CDI in the community include soil, water, pets, animals used for food, meats, vegetables and salads (Bouttier et al., 2010, Gould and Limbago, 2010, Harvey et al., 2011, Keessen et al., 2011, Metcalf et al., 2010, Bakri et al., 2009a). However, even though strains related to human infection have been found from food-related sources, causation of CDI from food and food products has never been proven. Nevertheless there is an observed increase in rates of CDI, which could be due a variety of reasons including heightened awareness. Continuous vigilance and investigation may reveal hitherto unknown factors to connect the unknown pieces of the story.

Although a number of good quality studies have recently been published characterising the factors associated with CA-CDI (Khanna et al., 2012c, Kuntz et

al., 2011), very few have evaluated the molecular epidemiology of the strains associated with CA-CDI. Hence this is an area where more information is needed.

1.2.5 Predisposing factors

1.2.5.1 Antibiotics

Although 6-45% patients have been reported to develop CDI without known exposure to antibiotics (Fellmeth et al., 2010, Khanna et al., 2012c), the role of antibiotics in the aetiology of CDI has been established since many years.

The aetiology of CDI following antibiotic exposure has been studied with the help of 16S ribosomal RNA sequencing. The microflora of the gut is made up of a large number of microorganisms, which can be disrupted by the use of antibiotics. Since *C. difficile* can be resistant to a multitude of antibiotics, it can multiply unhindered in the intra-colonic environment made diversity-deficient by antibiotics. Recent studies have revealed that the effect of antibiotics on the microflora can last for several days even after the last dose of the offending antibiotic. Susceptibility to CDI can persist for a variable amount of time depending on the half-life of the drug administered. In a hamster model, clindamycin treatment led to a much longer period of susceptibility to infection as compared to cephalosporins (Merrigan et al., 2003b, Merrigan et al., 2003a).

In a recent multicentre case-control study from the Netherlands conducted between March 2006 and May 2009, 337 hospitalised patients with diarrhoea and a positive toxin test were compared with 337 patients without diarrhoea. A control group of patients with diarrhoea due to a cause other than CDI was also included. Their results suggested that in the month prior to CDI, patients with symptoms more frequently used an antibiotic compared with non-diarrhoeal patients (77% versus 49%). During antibiotic administration and in the first month after cessation of the therapy, patients had a 7-10-fold increased risk for CDI (OR 6.7-10.4). The highest risk for CDI was found during the first month after antibiotic use and this gradually

decreased from one month to three months after cessation of antibiotic therapy (Hensgens et al., 2012a).

Restoration of colonisation resistance of the normal flora is therefore a key factor in the prevention of CDI in patients (Rupnik et al., 2009). Figure 1.1 explains the effect of antibiotics on the human gut and the resultant susceptibility to *C. difficile.*

A recent study compared the efficacy of faecal transplantation with Vancomycin in the treatment for recurrent CDI and found that the Simpson's Reciprocal Index of diversity of fecal microbiota obtained from nine patients with recurrent CDI before faecal transplantation was consistently low and increased after infusion to become comparable with that of the donors.

In addition phylogenetic microarray profiles of each sample showed that after donor-faeces infusion there were quantitative changes in relevant groups of intestinal bacteria ($P<0.05$) and included xincluded increased numbers of Bacteroidetes species and of clostridium clusters IV and XIVa and decreased numbers of Proteobacteria (van Nood et al., 2013).

Figure 1.1 Effects of antibiotics on gut flora

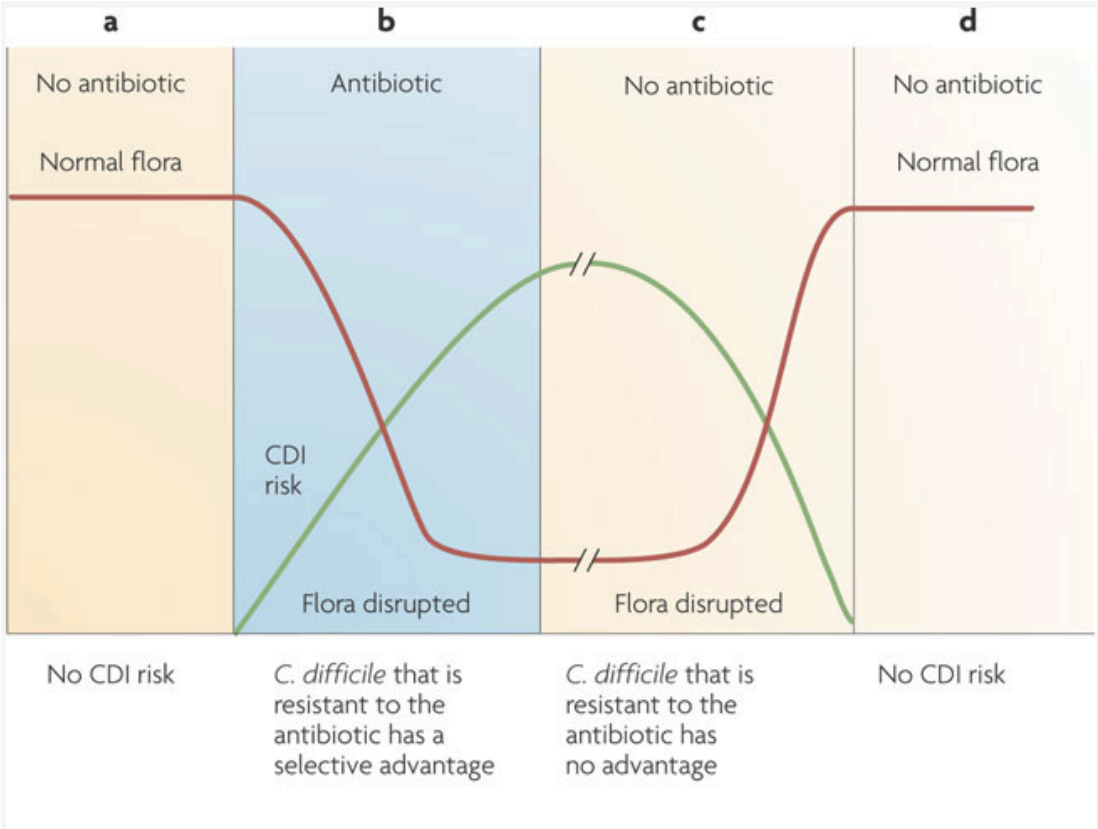


Figure adapted from Maja Rupnik, Mark H. Wilcox & Dale N. Gerding *Clostridium difficile* infection: new developments in epidemiology and pathogenesis Nature Reviews Microbiology 7, 526-536 (July 2009)

(a) Resistant to CDI in the absence of antibiotics.

(b) During antibiotic treatment *C. difficile* can overgrow in the absence of competing normal flora, since it is likely to be resistant to the antibiotic being administered

(c) Once the antibiotic stops, the microflora remains disturbed for a variable period of time (indicated by the break in the graph), depending on the antibiotic given, hence susceptibility to antibiotics remains even after cessation of the offending antibiotic. During this time, patients can be infected with either resistant or susceptible *C. difficile.*

(d) Colonisation resistance to *C. difficile* is restored once the normal microbiota regenerates

1.2.5.2 Age and gender

C. difficile infection mostly affects the elderly population, although cases are being seen in younger patients including children and pregnant women (Hojsak et al., 2012). Children reported to develop CDI usually have predisposing factors like immunosuppression and malignant disease, and exposure to multiple antibiotics classes (Kim et al., 2012).

However the burden of infection lies in the ageing population. Studies evaluating risk factors have suggested that increasing age >75 are associated with the development of recurrent or severe disease (Eyre et al., 2012). Data is accumulating that community-associated disease may affect a younger population

An evaluation in an urban, tertiary care hospital of 89 CDI inpatients divided by age < 60 yr of age (younger) and those > or = 60 yr (elderly) identified no difference in mortality or morbidity in elderly individuals with CDI when compared with younger persons with CDI. The response to standard treatment was similar in both groups. However, the elderly patients were more likely to have an elevated total leucocyte count (60% vs. 26%, $p < 0.05$), and were more likely to have hospital-associated disease (89% vs. 50%, $p < 0.0001$)(Brandt et al., 1999).

However another study found age over 70 years, co-morbid illness and CDI recurrence as significant risk factors for severe disease and a poor outcome (Andrews et al., 2003).

Hence there are mixed reports on the effects of age on CDI recurrence severity and mortality. Gender differences have only rarely if ever been reported.

1.2.5.3 Acid suppressing agents

In the normal human stomach, the low pH due to normal gastric acid secretion acts an as defence against microorganisms which may enter the body via the mouth. Inhibition of this gastric acid removes this innate immune mechanism against ingested bacteria and spores, increasing the risk of some forms of gastroenteritis. Hence it is suggested that gastric acid suppression may also increase the risk of CDI.

A number of studies have investigated a possible link between acid suppression therapy and CDI in various patient populations but have reported conflicting results.

In a report of two communities population based case control studies in the United Kingdom the first included 1672 cases of CDI between 1994 and 2004. Each case was matched to 10 controls. In the second study community-associated cases were matched to non-hospitalised controls. The adjusted rate ratio of CDI with current use of proton pump inhibitors was 2.9 (95% CI 2.4-3.4) and with H2-receptor antagonists the rate ratio was 2.0 (95% CI, 1.6-2.7, hence concluding that the use of acid-suppressive therapy was associated with an increased risk of CA-CDI (Dial et al., 2005).

Another population-based, nested case-control study of linked health care databases in Canada, from 1 April 2002 through 31 March 2005 identified patients ≥ 66 years of age who were hospitalized for CDI within 60 days of antibiotic administration in the community. One thousand three hundred and eighty-nine case patients were matched with 12,303 control subjects. PPI use by case patients and control subjects was categorized as current (within 90 days), recent (91-180 days), or remote (181-365 days). Case patients were found to be no more likely than control subjects to have received a PPI in the preceding 90 days (AOR 0.9; 95% CI 0.8-1.1). No association between hospitalization for CDI and more remote use of PPIs was found (Lowe et al., 2006).

A more recent report compared the outcomes of patients with CDI to establish an association with PPI use. Demographic data and outcomes, including severe, severe-complicated, and recurrent CDI and treatment failure were compared in a cohort of patients with CDI who were treated with acid suppression medications with these outcomes in a cohort with CDI that was not exposed to acid-suppressing agents. On univariate analysis, patients taking acid suppression medications were significantly older (69 vs 56 years; P<.001) and more likely to have severe (34.2% vs 23.6%; P=0.03) or severe-complicated (4.4% vs 2.6% CDI; P=.006) infection than patients not undergoing acid suppression. On multivariable analyses, after adjustment for age and co-morbid conditions, acid suppression medication use was not associated with

severe or severe-complicated CDI. In addition, no association between acid suppression and treatment failure or CDI recurrence was found. Hence patients on acid suppression treatment were not more likely to experience severe or severe-complicated CDI, treatment failure, or recurrent infection (Khanna et al., 2012a).

From the above studies it seems that there is yet no conclusive evidence on the effect of acid suppressive medication and the incidence and severity of CDI.

1.2.5.4 Colonisation with other hospital acquired pathogens

There have been reports of association of CDI with other primarily hospital associated pathogens like MRSA and VRE carriage. A study evaluating the risk factors for CDI and VRE found that antimicrobial exposure, number of antimicrobials, days of antimicrobial use, third-generation cephalosporins, clindamycin, patient age, length of admission, severity of underlying illness, enteral feedings, environmental contamination, and contamination of the hands of health care workers were associated with their carriage (Gerding, 1997).

A recent report found that patients with CDI were more likely to be colonised with MRSA in medical wards as compared to surgical wards (Lavan et al., 2012).

Since these pathogens share risk factors with CDI acquisition, it is possible that they could contribute to a risk evaluation assessment of patients who are likely to develop CDI or its complications.

1.2.5.5 Underlying GI pathology

Underlying gastro-intestinal pathology including inflammatory bowel disease, cancer of the GI tract, and other structural and physiological abnormalities have been known to disrupt the normal functioning of the gut and hence could pose an increased risk for the development of CDI. A number of studies have tried to evaluate this risk but no conclusive evidence has yet been found.

In some previous studies, no statistically significant association was found between complications and history of bowel surgery (Morrison et al., 2011) or enteral feeding. However, at least one study found small bowel obstruction as a significant risk factor

on multivariable analysis (OR 3.33, p value 0.014)(Henrich et al., 2009). Another case control study involving 35 patients with fulminant CDI, found operative therapy within the last 30 days (P = .03) and a history of inflammatory bowel disease (P = 0.04) independent risk factors for the development of fulminant CDI on multivariate analysis (Greenstein et al., 2008).

1.2.5.6 Immunosuppression

As the factors already mentioned, immunosuppression has been studied in various aetiological studies. However, the definition of immunosuppression varies from study to study, hence a general comparison cannot be made between studies. However, immunosuppression was one of the factors that predisposed to the development of severe *C. difficile* colitis ($p< 0.05$) in a study compare risk factors for severe colitis (Rubin et al., 1995).

An evaluation of the prevalence of risk factors treatment and outcome of CDI in patients with human immunodeficiency virus (HIV) infection including 124 patients grouped as HIV-infected with CDI, HIV-seronegative patients with CDI and HIV-infected patients without CDI revealed that the prevalence of CDI in HIV-infected patients was more than in HIV-seronegative patients (P = 0.02). The clinical symptoms of CDI were more severe in HIV-infected patients than in controls, though the CD4+ cells did not influence the outcome of CDI. No difference in the survival curves of AIDS patients with or without CDI, stratified according to age, sex and CD4+ cell count was observed (Tumbarello et al., 1995).

Another study defined immunosuppression as presence of human immunodeficiency virus (HIV), leukaemia, lymphoma, organ transplant, neutropenia, immunosuppressive drug use or systemic corticosteroids for >1 month and found a statistically significant correlation with severity of CDI (OR 2.7; 95% CI 1.5, 4.9) (Pepin et al., 2007). However a number of other studies did not find a statistical correlation (Gujja and Friedenberg, 2009, Labbe et al., 2008, Morris et al., 2002).

Of note, the markers of severity appear to be different between immunosuppressed and immunocompetent patients as suggested by a recent study, which evaluated 29

immunosuppressed patients with CDI. The white blood cell count, platelet, and albumin levels were found to be the same in the severe and non-severe immunosuppressed CDI patients although those with severe CDI were older and had evidence of renal dysfunction (Pant et al., 2011)

1.2.5.7 Analysis of co-morbidities

A number of co-morbid conditions like renal disease, gastrointestinal conditions, COPD, diabetes and cancer have been individually studied as predisposing conditions for severe and/or recurrent disease. However the commonest marker used for analysing the co-morbid condition has been the Charlson co-morbidity index (CCI)

Although other co-morbidity indices are available (Index of co-existent disease, geriatric index of co-morbidity, functional co-morbidity index, total illness burden index) none appears to be distinctly superior to others. Since Charlson Index was consistently associated with outcome it has been used in a number of studies reporting CDI related research.

The Charlson Index was proposed in 1987 as a means for quantifying the prognosis of patients enrolled in clinical trials (Charlson et al., 1987). It was based on one-year mortality data from internal medicine patients admitted to a single New York Hospital and was initially validated within a cohort of breast cancer patients. The index encompasses 19 medical conditions weighted 1–6 with total scores ranging from 0–37. When it was being developed, a stepwise backward proportional hazards Cox Regression model was used to calculate the relative hazard of patients with the co-morbidity to a relative risk of death within 12 months. A weight was then assigned to each condition based on the relative risk (RR); for example, RR <1.2 = weight 0, RR 1.2<1.5 = weight 1, RR 1.5<2.5 = weight 2, RR 2.5<3.5 = weight 3, and for 2 conditions (metastatic solid tumour and AIDS) = weight 6

Criticism for the use of the CCI is that it was developed as a research tool using a variety of different conditions and hence its applicability in routine practice is likely to be cumbersome. Also it includes only 19 conditions, so there may be others

associated with the risk of dying which are not included. Nevertheless the CCI has been consistently associated with severity due to CDI (Bloomfield et al., 2012).

1.3 Disease in children

Although paediatric disease has been reported since the 1980s, it has been a relatively recent area of interest.

1.3.1 Theories for low pathogenicity

The low incidence of CDI in children has been observed since many years though recent studies are reporting an increase in incidence. Proposed theories for the low paediatric incidence include absence of toxin receptors in the neonatal gut and neutralisation by colostrum (Wolfhagen et al., 1994). However none of these have been substantiated and the structure of the receptors to *C. difficile* toxins A and B is still not defined.

1.3.2 Incidence

Studies have reported that CDI in children is a real and increasing problem. The annual rate of paediatric hospitalisations with CDI in the United States was reported to increase from 7.24 to 12.80 cases/10,000 hospitalisations from 1997 through 2006. Although incidence was lowest for newborns (0.5 cases/10,000 hospitalizations), incidence for children <1 year of age who were not newborns (32.01 cases/10,000 hospitalisations) was similar to that for children 5-9 years of age (35.27 cases/10,000 hospitalisations) (Zilberberg et al., 2010)

In addition, the CDI rate has been reported to be up to 15 times more among children with cancer compared with those without cancer. Children with cancer accounted for 21% of all paediatric CDI cases (Tai et al., 2011).

1.3.3 Pool of potential pathogens

In addition to increasing incidence of disease, theories have been proposed on the possible link between asymptomatic carriage in children and the maintenance of a pool of potential pathogens in the community (Rousseau et al., 2012) since at least some studies have reported that the presence of *C. difficile* in stools of children even after antibiotic therapy may not significantly associated with the onset of antibiotic-

associated diarrhoea (Elstner et al., 1983). Although the transmission of *C. difficile* from children to adults has however never been proven, molecular typing of 28 *C. difficile* isolates from children in Oxfordshire, United Kingdom, identified eight toxigenic genotypes, of which six were also found in 83 (27%) concurrent adult CDI cases, suggesting that children could transmit of some adult disease-causing genotypes (Stoesser et al., 2011).

1.4 Laboratory diagnosis

The laboratory diagnosis of CDI is generally made by non-culture techniques, they being less labour intensive than culture. Direct toxin detection from stool is considered the most convenient method, as the presence of toxin is a prerequisite to confirm the pathogenicity of the strain. Culture based methods, if used, must then also go through a toxin detection ritual if pathogenicity is to be established.

Toxin detection by the tissue culture cytotoxin assay is often regarded as the gold standard but currently the most common technique is enzyme immunoassay (EIA). Earlier EIA kits performed only toxin A detection but after the pathogenic potential of A^-B^+ *C.difficile* strains was established, kits using a combination of toxin A and toxin B detection are routinely used. More recently direct PCR from the stool sample to detect the toxin gene has been found to have a greater sensitivity than toxin EIA. A study recent reported that 44% of all cases were detected by PCR only as opposed to cytotoxin assay, and the proportion of cases of non ribotype 027 detected by PCR only differed significantly from patients infected with ribotype 027 strains ($P < 0.05$). However no significant difference was found in the severity of illness and outcome among patients that tested positive for CDI by both tests compared to those that tested positive by PCR only (Kaltsas et al., 2012).

Glutamate dehydrogenase (GDH), encoded by the *gluD* gene, is a metabolic enzyme produced by *C. difficile.* It is known to be present in the stool sample of patients infected with *C. difficile* and has been used as a marker for the presence of *C.difficile*. However since it is present in both toxigenic and non-toxigenic strains, the detection of GDH must be followed up with a more specific test like toxin EIA to

detect direct toxin or PCR to detect the toxin gene. GDH has been found to be highly conserved among *C. difficile* ribotypes (Carman et al., 2012).

The two-stage algorithm toxin EIA/PCR was more specific compared to the GDH/PCR combination when compared with either of the two reference tests cell cytotoxin and cytotoxigenic culture reference methods. However the latter was more sensitive. The PPV for the toxin EIA-based algorithm was higher (89.0-90.8%) than the GDH based algorithm (59.6-80.7%) (Wilcox and Planche, 2011).

At the present time, there is considerable diversity in the practice of laboratory diagnosis of CDI. A multi-step approach to diagnosis using either GDH and EIA combination or GDH EIA/ toxin gene PCR culture combination algorithm is recommended (Wilcox et al., 2010).

1.4.1 Antimicrobial susceptibility

Traditionally anaerobes have been considered susceptible to metronidazole. Practice guidelines recommend metronidazole or vancomycin as first line therapy (HPA, 2008). However, in a study comprising 632 infected cases treated with metronidazole, failure of therapy was reported in 14 (2%) cases (Sanchez et al, 1999). Recent reports of metronidazole resistance state that 2-6% isolates may have an MIC greater than the susceptible breakpoint of 16μg ml^{-1} (Bishara et al., 2006, Brazier et al., 2001, Johnson et al., 2000). A recent report examined the genome of C.difficile strain stably resistant to metronidazole by whole genome sequencing and comparative genomic analysis and found single nucleotide polymorphism (SNP) level variation within genes affecting core metabolic pathways such as electron transport, iron utilization and energy production (Lynch et al., 2013)

1.5 Molecular biology

After the genome of the *C. difficile* strain 630 was sequenced, the results facilitated a good understanding of the molecular structure of the organism (Sebaihia et al., 2006). Although belonging to the genus *Clostridium*, the genome of *C. difficile* shares only 15% of its coding sequences (CDSs) with other clostridia like *C. perfringens, C. tetani* and *C. botulinum.* About 50% of its CDSs are unique to itself.

Its G+C content is extremely variable throughout its length probably because of a number of mobile genetic elements, which comprise about 11% of its genome. The *C. difficile* genome possesses an element closely related to the conjugative transposon Tn916 that mediates tetracycline resistance. Other transposons, which occupy places, are CTn2, CTn*4* and CTn*5,* which are related to the vancomycin resistance coding transposon Tn*1549*. However resistance studies revealed that although present these do not encode vancomycin resistance in the organism.

More recently the genomes of other pathogenic ribotypes have been sequenced and data regarding the differences between strains is emerging. Whole genome sequencing was used in one study to genetic variation and virulence among 30 different *C. difficile* isolates, to determine macro and microevolution of the species. According to their results it appears that horizontal gene transfer and large-scale recombination of core genes has been involved in the recent and distant evolution of *C. difficile.* Many lineages appear to co-exist in pathogenic *C. difficile* suggesting that virulence evolved independently (He et al., 2010).

1.5.1 Virulence factors

The virulence factors are coded on a pathogenicity island called Pathogenicity Locus (PaLoc). Five genes are present in this region viz. *tcdA,B,C,R & E* responsible for the production and regulation of the virulence factors toxin A (TcdA) and toxin B (TcdB). The TcdC is a negative regulator of toxin production as inferred by its high expression during the early phase of growth when toxin production is minimal and decrease in the stationary phase when toxin production accelerates. TcdR bears considerable homology with other known toxin positive regulators TetR in *Clostridium tetanus* and BotR present in *Clostridium botulinum*. It is expressed co-ordinately with TcdA and TcdB and experimental studies suggest that it can enhance the expression of promoter-reporter fusions of the in *C. difficile* toxins (Moncrief et al., 1997). TcdE, is a protein resembling the holin proteins and may enable release of the toxins from the cells (Tan et al., 2001).

Although some pathogenic strains are known to lack TcdA, strain 630 produces both TcdA and TcdB. Some strains of *C. difficile* produce a third toxin called binary toxin

with two components: CdtA and CdtB. However, *cdtA* and *cdtB* are mere pseudogenes in strain 630. In non-toxigenic strains the PaLoc is replaced by a region of 115 bases (Rupnik et al., 1998).

Outside the cell wall of this organism, there is a complex paracrystalline array of proteins termed the S layer. This is composed of two proteins, which are coded by a single gene *slp*A. The higher molecular weight surface layer protein (SLP) is highly conserved among the strains, whereas the lower molecular weight SLP shows considerable sequence diversity. *C. difficile* is unique in that it produces two surface proteins distinct from each other but both are products of the same gene. The removal of peptides and a cleavage event occur in the post translational phase to produce the two separate proteins (Calabri et al, 2001) Since there are 28 paralogs of this gene and each contain multiple copies of Pfam (cell wall binding) motif PF04122, this property is being used to develop a separate typing scheme (Kato et al., 2005).

The whole genome sequence analysis also revealed several CDSs which resemble antibiotic resistance elements. The expression of these was tested by phenotypic means and it was found that of the predicted resistances, the organism was resistant to agents like tetracycline, erythromycin and daunorubicin, bacitracin, and nogalmycin but sensitive to vancomycin and teicoplanin.

1.5.1.1 Genes associated with toxin production and the pathogenicity locus (PaLoc)

Other genes present on the PaLoc are *tcdR, tcdE* and *tcdC* of which the last is a putative negative regulator of *tcdA* and *tcdB* (Rupnik et al., 2005). Since strains which do not produce either of these toxins are not associated with pathogenicity, diagnosis of CDI is based largely on the demonstration of toxins either directly form stool samples or by toxigenic culture. For the former traditional methods like ELISA have been used.

Studies have demonstrated various modifications of the PaLoc region which include

- deletions in the 3'-end of *tcdA* which result in non detectable levels of TcdA

- internal deletions and premature stop codons in *tcdC* with the potential to produce an inactive gene product
- polymorphisms in the PaLoc region which have been identified into 24 profile by PCR restriction fragment length polymorphism (RFLP)

1.6 Epidemiological typing

In an event of an increase in incidence of the disease it becomes important to ascertain whether there is a common source of the pathogen or if there is a loss of adequate infection control precautions enabling increased transmission. Since it is a coloniser in the gut, it is important to note whether the increased incidence is secondary to uncontrollable causes like patients' general condition and co-morbidities or whether it is due to increased transmission, a potentially controllable cause. In addition to this epidemiological typing can provide an indication as to whether an outbreak is associated with a strain associated with enhanced pathogenicity like the PCR ribotype 027. It can also be used to examine changes in circulating types over time.

1.6.1 Typing methods in use

In the past many methods of epidemiological typing have been employed germane to the molecular understanding at the given time. Antibiograms, bacteriocin and bacteriophage typing, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), serotyping, pyrolysis mass spectrometry, plasmid profiling, restriction endonuclease analysis, toxinotyping with restriction fragment length polymorphism, and arbitrary primed PCR have all been used with varying degrees of success (Tabaqchali et al., 1984, Delmee et al., 1985, Wren and Tabaqchali, 1987, Dei, 1989, McKay et al., 1989). However, over the years, practical application of each method has been concurred by its ease of use, inter laboratory comparability, cost and discrimination capacity. In the early 2000s, the methods employed in North America were pulsed field gel electrophoresis (PFGE) whereas most of Europe was using PCR ribotyping. More recently the reference laboratories in the UK are still using PCR ribotyping routinely with the addition of more discriminatory methods like multilocus variable number tandem repeat analysis (MLVA), which has been shown

to differentiate between ribotypes (van den Berg et al., 2007). The routine use of sequencers and specialized software has made the application of these techniques easier over time. More recently, with whole genome sequencing becoming an application with potential to provide real time epidemiological answers in the event of outbreaks, this may be the technique used more widely in the future (Singh et al., 2012)

1.6.1.1 PCR ribotyping

This technique uses variability in the intergenic spacer regions between the 16s and 23s RNA regions to differentiate between strain types. The method described by O'Neill et al (1998) was in use for a considerable length of time but had the disadvantages of needing a very stringent protocol for gel electrophoresis and a comprehensive database to compare strain types. In addition, comparison had a human visual component subject to user dependent error. This technique was further modified to enable more specific band discrimination using objective comparison by specialized software. Although the comparison still has a visual component and a database is required to compare the band patterns, the end result has the advantage of being more specific.

1.6.2 Subtyping methods

1.6.2.1 MLVA

This technique involves the amplification of multiple genomic loci where there are known VNTRs using PCR and establishing a MLVA type based on the number of repeat sequences found in each locus. Typically each repeat sequence was short, around 4-15 base pairs in length. It was first used as a typing method for *C. difficile* by March et al (2006) where they amplified seven loci to discriminate between (ribotypes/PFGE types). Subsequently this method also used by van den Berg et al (2007) with a slightly different combination of loci and primers although the total number of loci studied were still seven. It was found that ribotypes 027 and 001, could be subtyped into smaller groups by MLVA. This technique was further modified with the addition of eight loci to establish the extended MLVA scheme (eMLVA) (Manzoor et al., 2011). The additional loci were larger (up to 75 bp in length) than those previously used. Cluster analysis of the strains they studied

grouped all ribotypes together while enabling subtyping. Hence this method was advertised as a possible substitute to ribotyping. They simplified the technique by using capillary gel electrophoresis using four different fluorescent dyes in each well to enable four loci to be analysed simultaneously in each well of a 98 well microtitre plate. Analysis of the copy numbers was further automated by the formation of panels, which could be read by GeneMapper software. However since the number of ribotypes used in their study was limited, there was insufficient evidence to say whether it can be used as an alternative to ribotyping

1.6.2.2 Whole genome microarray analysis

This state-of-the-art whole genome analysis technique involves production of microarrays comprising DNA motifs from various genes of a fully sequenced strain. PCR amplified genes of test strains are compared by hybridisation with the DNA of the standard strain on glass chips. This technology has been recently applied to analyse 75 ecologically and clinically diverse *C. difficile* isolates. Using mathematical modelling and microarray data set the isolates could be grouped into 4 clades differentiating the toxin A deficient, hypervirulent and animal and human isolates. This technology is very demanding and expensive for routine studies but appears to be highly precise for advanced phylogenetic analysis.

1.6.2.3 MLST

MLST has been found to have similar discriminatory abilities as ribotyping indices of discrimination of 0.90 and 0.92, respectively. Some STs correspond to a single PCR ribotype (32 of the 40 studied isolates); other STs corresponded to multiple PCR ribotypes (8/40). Hence the PCR ribotype was not always predictive of the ST (Griffiths et al., 2010).

1.6.2.4 Whole genome sequencing (WGS)

This technology offers the greatest insight into the genomic structure of the organisms. Novel virulence factor encoding genes may be recognised by comparing different strains of the same species (Belkum, 2002). It also provides a model for the study of genetic variations (Spencer et al, 2003). However, the time and expense that used to be involved in applying whole genome sequencing as a routine

epidemiological procedure used to make it impractical technique for routine use. However advances in technology are changing that and whole genome sequencing has been used in a few studies to provide real time answers in with the right infrastructure could be used to identify outbreaks (Koser et al., 2012).

1.7 Molecular epidemiology

1.7.1 Geographical diversity and evolution

In 2005, a strain responsible for a large number of infections in hospitals across North America was identified as PCR ribotype 027 . This type was characterized as group BI by restriction endonuclease analysis (REA), as North American pulsed-field type NAP1 by pulsed-field gel electrophoresis (PFGE). Since then, this ribotype has been involved in a number of outbreaks in the US and Europe including the UK.

Rising rates of CDI in the past decade have been documented (Tan et al., 2007) and thought to be largely due to this strain although increased awareness, and a larger susceptible population no doubt play a role as well. Other strains of *C .difficile* are also found with varying prevalence depending on the geographical location. Outbreaks are not limited to PCR ribotype 027 and have been associated with many others including ribotype 106, 078, and 017 (Ratnayake et al., 2011, Goorhuis et al., 2011, Balassiano et al., 2011)

The prevalence of *C. difficile* ribotype 078 has increased recently from 3% to 13% in several countries in Europe (Goorhuis et al., 2008b, Burns et al., 2010, Goorhuis et al., 2008a). In the Netherlands, patients infected with ribotype 078 were younger (67.4 versus 73.5 years) and had community-associated disease more frequently (17.5% versus 6.7%; odds ratio = 2.98; 95% confidence interval = 2.11–8.02) than patients infected with ribotype 027 (Goorhuis et al., 2008a)

1.7.2 PCR Ribotypes in south-east Scotland

A collection of 179 isolates of *C. difficile* obtained from symptomatic adult patients in southern Scotland between 1979 and 2004 identified 56 different ribotypes of which ribotype 002 was the commonest, followed by ribotypes 014, 012, 015, 020

and 001. Ribotype 001 increased in prevalence from 1.5 to 12.2 % over the study years, whereas the prevalence of ribotype 012 decreased from 8.7 to 2 .0%. Ribotype 078 was also identified (Taori et al., 2010).

From all Scottish health boards PCR ribotypes106 (29.4%), ribotype 001 (22%) and ribotype 027 (12.6%) were the commonest between November 2007 and December 2009, followed by the less prevalent ribotypes including 002, 015, 014, 078, 005, 023 and 020. The predominant isolates varied between regions (Wiuff et al., 2011).

The most recent report by Health Protection Scotland suggests that ribotype 078 is the most common ribotype (28%) isolated among the 156 cases analysed in the clinical surveillance (severe cases and outbreaks) and 89 cases (21% of type 078) from the representative surveillance.

The proportion of ribotype 078 among isolates from severe cases/outbreaks has increased from 2.5% in 2008 to 3.2% in 2009, 4.3% in 2010 and 7.8% in 2011and almost comparably from the representative surveillance. Hence although it has been reported since the mid 1990s, its prevalence has increased substantially in the past 5 years. Whereas the other ribotypes 106, 001 and 027 have become comparatively low while many others have remained stable (HPS, 2012).

Aims

To determine the incidence and characteristics of CDI acquired from community sources

To study the incidence, clinical features and molecular characteristics of severe and recurrent CDI

To compare the emerging *C. difficile* ribotype 078 with other prevalent ribotypes and determine clinical and molecular differences between its endemic and outbreak-associated strains

To examine the incidence of *C.difficile* in asymptomatic children with a view to determining their role as a potential pool of pathogens in the community

Chapter 2
Materials and Methods

2.1 Caldicott approval, ethics clearance and consents

2.1.1 Approvals

Stool samples included in this study for aims 1-3 were sent to the diagnostic lab without interference by the researchers. After discussion with the University of Edinburgh and NHS Lothian Research and Development Department, Caldicott approval was sought for access to their names, dates of birth, CHI numbers, GP contact details and hospital notes.

For aim 4, ethics clearance was obtained from the NHS/RHSC Research and Development offices and Research Ethics Committee for collection of stool samples.

2.1.2 Consent

Information sheets were given and written informed consent was sought from parents (see appendix 3). Children of parents who provided consent were included in the study.

2.2 Patient inclusion

2.2.1 General study

Patients whose stool samples were sent to the Royal Infirmary of Edinburgh Microbiology laboratory and which tested positive for *C. difficile* toxin (A and/or B) by EIA and glutamate dehydrogenase (GDH) was included in study from the time the sample tested positive. These patients were prospectively followed up for one year after the first stool sample tested positive.

2.2.2 Paediatric colonisation study

Neonates admitted to the neonatal unit over a four-week period from 7th August to 3rd September 2010 were eligible for inclusion in the study. If consent was obtained from parents, those children were included in the study.

The children at the crèche at The Royal Hospital for Sick Children (RHSC) were sampled between 1st and 15th October 2011. Similar to the neonatal unit, those children whose parents provided consent were included.

2.3 Sample collection

2.3.1 General study

Attending clinicians based on the clinical symptoms and history of each patient requisitioned these samples. The staff collected them from the ward of admission. According to the Department of Heath guidelines repeat samples are generally not tested within 28d of a previous toxin positive stool. However they are often tested if a repeat sample is not identified, or if a clinician specially requests them for clinical reasons.

2.3.2 Paediatric colonisation study

Samples were collected by the attending nurses in the neonatal unit twice per week for four weeks and by the babysitters at the RHSC crèche as a one off sample per child. Parents collected samples at home if the child did not pass stool while at the crèche.

2.4 Confidentiality and storage

2.4.1 Confidentiality

All stool samples were linked with a confidential number made unidentifiable at the point of collection (either ward or clinical laboratory) before being brought to the research laboratory. A note was made of the patients' name, date of birth and sample number and this was stored confidentially. Each patient was given a new patient number and each sample was given a new sample number. All confidential information was stored on password protected NHS Lothian computers or confidential cupboard in the microbiology department.

2.4.2 Sample storage

Stool samples were cultured on CCEY medium and isolates stored in cooked meat broth. The remaining stool samples were stored at -20°C in the research lab. They were later used for DNA extraction and PCR for Norovirus.

2.5 Data collection

2.5.1 Follow up and data collection

Initial patient data was collected by personal visit to the ward of admission or by telephone contact with the attending ward doctor or the GP requesting the test. Follow up was performed at two weeks and on year by telephone contact with the ward doctors if the patient was still admitted or by reviewing written records on Lothian hospitals IT system (trak) and by telephone contact with the GP with whom the patient was registered. Recovery, recurrence, change of treatment, discharge and or death was recorded (see appendix 1) The Charlson Co-morbidity Index was calculated using a macro enabled Excel tool available online (Hall et al., 2004), see appendix 2.

Co-morbidities were determined by reviewing written records and by telephone conversation with GPs. Haematological and biochemical data was obtained from the Lothian hospitals IT system.

Mortality data and information on death certificates was obtained from the NHS Lothian Public Health Department.

2.6 Culture for *C. difficile*

All stool samples were cultured on pre-reduced CCEY agar plates after alcohol shock (see appendix 4). For the neonatal samples, an additional blood agar plate was also cultured without alcohol shock. The plates were incubated in an anaerobic cabinet for 48 hours after which they were examined for colonies resembling *Clostridium difficile.* Such colonies were subjected to fluorescent lamp to look for the characteristic green fluorescence. A single colony was then plated onto pre-reduced BA. After a further incubation of 24-48h, the colonies were examined for

characteristic smell and inoculated into Robertson's cooked meat broth for storage till further testing. All isolates were later subjected to toxin A and toxin B PCR to confirm identification. A known culture positive stool was included as a positive control with every batch of culture.

2.7 PCR ribotyping

2.7.1 Isolates

Stock cultures from cooked meat medium were sub-cultured onto CCEY and incubated anaerobically for 48h. A single colony was plated on BA and incubated for 24h-48h. All *C. difficile* isolates obtained were subjected to PCR ribotyping and toxin gene PCR as below.

2.7.2 DNA template preparation

All DNA templates were prepared by the Chelex DNA extraction method (Stubbs et al., 1999). A 1% Chelex suspension (0.1µg per 100µl) was prepared and dispensed in an Eppendorf tube in aliquots of 100µl. A 1µl loop was used to scrape colonies off the BA and this was suspended into the Chelex. This suspension was vortexed for a few min before placing in a 100°C heat block for 10 min. The heated suspension was again vortexed briefly and then centrifuged for 10 min. The supernatant was used as the template DNA. This was either used immediately in the PCR mix or aliquoted into fresh tubes and stored at 4°C for max of 3 days before use. A small aliquot of the template (20-30µl) was also stored at -20°C in case needed at a later stage.

2.7.3 Primers and reaction volumes

Table 1.1 Primer sequences used in ribotyping and toxin analysis

Gene segment	Primer sequence with fluorescent label used	Reference
Ribo	F 5'- PET - CTGGGGTGAAGTCGTAACAAGG	O'Neill et al 1996
	R 5' - GCGCCCTTTGTAGCTTGACC	O'Neill et al 1996
tcdA	F 5' - FAM - GCATGATAAGGCAACTTCAGTGGTA	Perssons et al 2011
	R 5' - AGTTCCTCCTGCTCCATCAAATG	Perssons et al 2011
tcdB	F 5' - FAM - CCAAARTGGAGTGTTACAAACAGGTG	Perssons et al 2011
	Ra 5' - GCATTTCTCCATTCTCAGCAAAGTA	Perssons et al 2011
	Rb 5' - GCATTTCTCCGTTTTCAGCAAAGTA	Perssons et al 2011
tcdC	F 5' - FAM - AAAAGGGAGATTGTATTATGTTTTC	Perssons et al 2011
	R 5' - CAATAACTTGAATAACCTTACCTTCA	Perssons et al 2011
cdtB	F 5' - FAM - TTGACCCAAAGTTGATGTCTGATTG	Perssons et al 2011
	R 5' - CGGATCTCTTGCTTCAGTCTTTATAG	Perssons et al 2011

Table 1.2 *C. difficile* ribotyping Mastermix/Fragment analysis

Component	Stock concentration	ml of X100	ml of X50
Nuclease-free water	-	251	125.5
BSA	2 mg/ml	100	50
Promega Flexi PCR Buffer X5	x5	200	100
dNTP mix	2 mM	100	50
$MgCl_2$	25 mM	140	70
Primer P3 (PET)	100 µM	2	1
Primer P5	100 µM	2	1
TAQ (Promega GoTaq)	5 U/µl	5	2.5
Total volume (ml)		800	400
Specimen added		200	100
Reaction volume		1000	500

Table 1.3 4-plex toxin PCR mastermix

Component	Stock concentration	ml of X100
Nuclease-free water	-	228.5
Bovine serum albumin	2mg/ml	100
PCR Buffer (Promega Flexi)	X5	200
NTP	2mM	100
$MgCl_2$	25mM	140
Primer *tcdA* F (FAM)	100µM	8
Primer *tcdA* R	100µM	8
Primer *tcdB* F (FAM)	100µM	3.5
Primer *tcdB* Ra	100µM	1.5
Primer *tcdB* Rb	100µM	1.5
Primer *tcdC* F (FAM)	100µM	1
Primer tcdC R	100µM	1
Primer *cdtB* F (FAM)	100µM	1
Primer *cdtB* R	100µM	1
TAQ (Go Taq Promega)	5U/µl	5
Total volume of mastermix		800
Specimen to be added		200
Reaction volume		1000

HiDi formamide mix was prepared (8µl HiDi Liz 600 in 500 µl formamide buffer) and stored in aliquots of 1ml at -20ºC till needed.

2.7.4 PCR thermo cycling protocol

The toxin multiplex and PCR ribotyping protocols were initially run on the same thermocycling protocol:

- Initial denaturation at 95ºC for 5 min
- 25 cycles of 95 ºC for 60 sec, 55 ºC for 60 sec and 72 ºC for 90 sec
- Final extension at 72 ºC for 5 min.

However since the initial reactions for ribotyping were rather weak, the cycle for ribotyping was later increased to 27 cycles.

Products were stored at 4 ºC for a maximum of 24h before post PCR dilutions.

2.7.5 Post PCR dilutions

After PCR, the products of the ribotyping and toxin gene multiplex were mixed as follows

- First dilution: 18 µl nuclease free water

10 µl ribotyping PCR products

2 µl toxin gene PCR product

This was mixed well by pipetting.

- Final dilution, 1 µl of the diluted products were mixed with 9 µl of HiDi formamide mix.

The final dilution was stored at -20 ºC till it could be sent to the sequencer for capillary gel electrophoresis.

2.7.6 Gel electrophoresis

Capillary gel electrophoresis was performed at the GenePool Sanger sequencing facility located at Ashworth Laboratories in the Kings Buildings, University of Edinburgh. Results were sent back from the sequencer in the form of FSA files,

which were then analysed as follows to assign ribotypes by comparing with a set of known strains.

2.7.7 Determination of ribotypes

This was performed by GeneMarker®software version 1.95(Softgenetics, PA, USA). Known strains of ribotype 001, ribotype 106 and ribotype 027 were included with each run of ribotyping and toxin multiplex PCR for quality control. A panel of known FSA images for ribotypes 001, 002, 003, 005, 012, 014, 015, 017, 018, 019, 020,023, 026, 027, 029, 046, 053, 056, 070, 075, 077, 078, 081, 087, 095, 103, 106, 117, 126, 131, 174, 193, 216 and 258 was obtained from Dr Derek Fairley (Belfast). Another panel of strains with known ribotypes (confirmed by the anaerobe Reference Laboratory, Cardiff by agarose gel electrophoresis) was created for ribotypes 001, 002, 009, 010, 011, 012, 013, 014, 015, 018, 020, 023, 026, 027, 029, 030, 032, 035, 039, 042, 046, 050, 053, 054, 056, 057, 059, 064, 069, 070, 072, 081, 087, 092, 100, 103, 104, 106, 111, 016, 119, 120, 125, 137, 142, 153, 154, 165, 170, 173, 176, 179, 184, 186, 189, 192 and 210. Each unknown image was visually compared with the known panel to determine PCR ribotypes.

The toxin gene images were compared against the PaLoc indicator panel (kindly provided by Dr Derek Fairley), which was loaded onto the software before analysis and automatically labelled the toxin gene products when they were loaded for analysis.

Toxin gene fragments were analysed on the blue channel and ribotyping fragments in the red channel.

2.8 PCR for Norovirus

Dr Alison Hardie, clinical scientist at the Royal Infirmary of Edinburgh, performed the Norovirus PCR. The protocol for the PCR has been adapted from a previously published study (Kageyama et al., 2003).

2.8.1 DNA extraction

Stool samples stored at -20°C were thawed and 10% (wt/vol) stool suspension was prepared with distilled sterile water and clarified by centrifugation at 3,000 × *g* for 20 min. 200µL supernatant was extracted with the Biomerieux easyMag according to the manufacturer's instructions. RNAs were eluted with 100 µl of diethyl pyrocarbonate-treated water and kept at −80°C until used for PCR. Known positive (Genogroup 1 or 2) and negative controls were included.

2.8.2 Primers sequences used

COG1F CGYTGGATGCGNTTYCATGA

COG1R CTTAGACGCCATCATCATTYAC

COG2F CARGARBCNATGTTYAGRTGGATGAG

COG2R TCGACGCCATCTTCATTCACA

Ring 1a HEX-AGATYGCGATCYCCTGTCCA-BHQ-1

Ring 1b HEX-AGATCGCGGTCTCCTGTCCA-BHQ-1

Ring 2 FAM-TGGGAGGGCGATCGCAATCT-BHQ-1

2.8.3 Mastermix

Mastermix was prepared using Express One-Step SuperScript qRT-PCR Kit (Invitrogen) .

Per reaction:

qPCR Supermix	10µL
SuperScript Mix	2µL
Primer/Probe mix*	2µL

*4μM each primer (COGs) and 0.8μM each probe (Rings)

Final reaction volume 20 μL (14 μL mastermix +6 μL RNA.

2.8.4 Thermocycing protocol

50°C for 15 min,

95°C for 20s

45 cycles of 95°C for 3s, 60° for 30s (with fluorescence detection)

2.9 Multilocus VNTR analysis (MLVA)

MLVA was performed to subtype the isolates and look for strain relatedness.

2.9.1 Isolates

All isolates identified as ribotype 001, ribotype 002, ribotype 078 and multiple samples obtained from the same patient (if they were of the same ribotype).

2.9.2 Protocols used

Initially the 15 locus method described by Manzoor et al (2011) was attempted on all isolates including ribotypes 078. However after a few runs it became apparent that the protocol was inadequate to amplify all loci of ribotypes 078 In addition, some of the loci are likely to be different from the published sequence of the *C. difficile* strain 630 on which the primers are based. Hence the protocol was modified to include only 6 loci which were consistently amplifying in the 078 strains The PCR thermocycling conditions were modified to those published by Bakker et al (2010) and van den Berg et al (2007).

2.9.3 DNA template preparation

Same as section 1.7.2

2.9.4 PCR primers

Primers used for the loci amplified in the MLVA protocol are given in table 1.4 and the components of the mastermixes are given in table 1.5

Table 1.4 :Primers used for MLVA

Locus	Primer sequence and fluorescent label	Reference
A6Cd	F-FAM-TTAATTGAGGGAGAATGTTAAA	van den Berg et al 2007
	R-AAATACTTTTCCCACTTTCATAA	van den Berg et al 2007
B7Cd	F-VIC-CTTAATACTAAACTAACTCTAACCAGTAA	van den Berg et al 2007
	R-TTATATTTTATGGGCATGTTAAA	van den Berg et al 2007
C6Cd	F-NED-GTTTAGAATCTACAGCATTATTTGA	van den Berg et al 2007
	R-ATTGGAATTGAATGTAACAAAA	van den Berg et al 2007
E7Cd	F-VIC-TGGAGCTATGGAAATTGATAA	van den Berg et al 2007
	R-CAAATACATCTTGCATTAATTCTT	van den Berg et al 2007
G8Cd	F-FAM-TGTATGAAGCAAGCTTTTTATT	van den Berg et al 2007
	R-AATCTAATAATCCAGTAATTTAAATT	van den Berg et al 2007
CDR60	F-NED-AGTTTGTAGGGAAGTGTGTAAATAGAT	Marsh et al,2006
	R-CGCATTAAATTTCACTCCTCAT	Marsh et al,2006
CDR5	F-PET-AGCCATTTTTATCAATCCTTTCTAT	Marsh et al,2006
	R-AATTTTAAGTTAACGTTTTTCTACAT	Marsh et al,2006
CD9	F-FAM-AAATAGAGAAATTGTTGTAGCACAAAG	Manzoor et al, 2011
	R-GTAAGGTGAGAAGCGGACT	Manzoor et al, 2011
CD12	F-VIC-TGACCCTTACAATAGCCAATCA	Manzoor et al, 2011
	R-ATCTGGCAGTTGATTCAGCA	Manzoor et al, 2011
CD14	F-NED-TTTCATAAAAGATTCCTTTCCTGT	Manzoor et al, 2011
	R-TGTGGTTGTTCCTGAAGTTTT	Manzoor et al, 2011
CD19	F-PET-AATTGGTAAGCAATCTGGACTTT	Manzoor et al, 2011
	R-TGCAGCTGGATATGTATCAGTTTA	Manzoor et al, 2011

CD35	F-FAM-TTGGTGAAGCATTAATGGATGT	Manzoor et al, 2011
	R- CGGCAATGCTTTGAACTATG	Manzoor et al, 2011
CD44	F-PET-TGCTGCGATAAGCTCTGCTA	Manzoor et al, 2011
	R-TCATTTCCATTCAAATTATGTGACTAT	Manzoor et al, 2011
CD102	F-NED-GCGTACAAGAGGTCGGAGTC	Manzoor et al, 2011
	R-CCACCCTCACTTTATTCAAACC	Manzoor et al, 2011
CD105	F-PET-TCAGCAACAGCAGAGGAAAG	Manzoor et al, 2011
	R-CATTTGTCTCATACTCGGTTCAA	Manzoor et al, 2011

2.9.5 Mastermixes for MLVA

Table 1.5 MLVA PCR mastermix

Component	Stock concentration	ml of X1	ml of X30
Nuclease-free water	-	16.3 µl	489µl
PCR Buffer (GeneAmp PCR Gold buffer)	1X	2.5 µl	75µl
dNTP	10 mM	0.5 µl	15µl
$MgCl_2$	25mM	2.5 µl	75µl
Primer F	100 µM	0.05 µl	1.5µl
Primer R	100 µM	0.05 µl	1.5µl
TAQ (AmpliTaq Gold DNA polymerase (Applied Biosystems)	5U/µl	0.1 µl	3µl
Total volume of mastermix		22 µl	800
Specimen o be added		3 µl	90
Reaction volume		25 µl	1000

For the loci B7Cd, G8Cd and C6Cd of ribotypes 078 only, the amount of magnesium used was increased to 4mM .

2.9.6 Thermocycling protocol for MLVA

MLVA loci (for all isolates except ribotypes 078) were amplified using the protocol of Manzoor et al (Manzoor et al., 2011).

- Initial denaturation at 95ºC for 5 min
- 35 cycles of 95 ºC for 60 sec, 56 ºC for 60 sec and 72 ºC for 60 sec
- Final extension at 72 ºC for 5 min.

2.9.7 Modifications for ribotype 078

Since ribotypes 078 loci were not consistently amplifying at the protocol of (Manzoor et al., 2011) the protocol was modified to that used by Bakker et al. (Bakker et al., 2010).

- Initial denaturation at 95ºC for 15 min
- 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 51ºC (47 ºC for B7Cd and G8Cd and 46ºC for C6Cd) for 30 sec and elongation at 72 ºC for 30 sec.
- Final extension at 72 ºC for 10 min.

2.9.8 Post PCR dilutions

After PCR, the products of loci were mixed to form four panels each composed of four (or three) loci, designed to contain one each of the four different fluorescent dyes as given in table 1.6

Table 6 Details of the loci amplified in the MLVA scheme

Locus	Panel/label	Size of repeated sequence	No of alleles in strain 630
A6Cd	A/BLUE	6	34
B7Cd	A/GREEN	7	16
C6Cd	A/YELLOW	6	15
CDR5	A/RED	8	11
CDR60	B/YELLOW	17	15
E7Cd	B/GREEN	7	7
G8Cd	B/BLUE	8	6
CD44	C/RED	42	6
CD9	C/BLUE	42	11
CD12	C/GREEN	42	2
CD14	C/YELLOW	45	6
CD19	B/RED	76	3
CD35	D/BLUE	41	9
CD102	D/YELLOW	50	6
CD105	D/RED	45	4

- First dilution 1 µl of each PCR product according to the panel given in table 6 into 6µl nuclease free water. This was mixed well by pipetting.

- Second dilution was performed by adding 1µl of first dilution products (4 loci in each well) to 6µl nuclease free water (1:10 dilution).

- The final dilution, 1µl of the diluted products were mixed with 9µl of HiDi formamide mix.

The final dilution was stored at -20 ºC till it could be sent to the sequencer for capillary gel electrophoresis.

2.9.9 Modifications to post PCR dilutions

It was observed that using the four panels there were experiments where all the loci did not show products. If that happened the loci were run individually to avoid inhibition of the fluorescent signals by other stronger signals. When this was done the dilution was altered to be

- First dilution 25µl of each PCR product into 125µl nuclease free water (1:5 dilutions). This was mixed well by pipetting.
- The second dilution was performed by adding 1µl of the first dilution products to 9µl of nuclease free water.
- The final dilution, 1µl of the diluted products were mixed with 9µl of HiDi formamide mix.

The final dilution was stored at -20 ºC till it could be sent to the sequencer for capillary gel electrophoresis.

2.9.10 Analysis of MLVA

This was performed by GeneMarker®software version 1.95(Softgenetics, PA, USA). Control strain 630 was included in each run. The product size for each locus was determined and the number of alleles calculated by subtracting the flanking regions and dividing the number of remaining base pairs with the number of base pairs in the repeat motif . This information was kindly provided by Susan Manzoor (Manzoor et al., 2011).

2.9.11 Cluster analysis

This was performed using Bionumerics version 2.5(Applied Maths BVBA, Texas USA). Clustering was performed according to MLVA type to determine genetic relationships. The summed absolute distance was used as the coefficient for calculating the minimum spanning tree.

2.10 Clinical case definitions

These were adapted from SHEA and HPA guidelines (HPA, 2008, McDonald et al., 2007)

2.10.1 Case

For the purpose of inclusion into the study, a case was defined as a patient with diarrhoea where the stool takes the shape of the container (grade 5-7 as per Bristol stool chart) or toxic megacolon with stool positive for *C. difficile* toxin A and /or B without other known aetiology (McDonald et al., 2007).

2.10.2 Episode

An episode of CDI was defined as a case with the criteria above who remains continuously symptomatic with a break of less than 48h in symptoms attributable to CDI.

2.10.3 Repeat episode/recurrence

When following up the patients, a repeat episode was defined as diarrhoea without another demonstrated cause where the stool was positive for *C. difficile* by toxin EIA+GDH, toxic megacolon, pseudomembranous colitis demonstrated on colonoscopy, or symptoms recurring where the physician recorded it as another episode of CDI with at least 48h of an intervening asymptomatic period after completion of initial treatment. This definition was different from the initial case definition because the diagnostic lab does not test patients for *C. difficile* toxin within 28 days of a previous toxin positive stool as per Department of Health guidelines (HPA 2008).

2.10.4 Hospital-associated infection

This was defined as CDI, which developed after 48h of admission into a health care facility (HCF), see chapter 5 figure 5.1.

2.10.5 Community-associated infection

This was defined as CDI, which developed in a patient with no history of health care contact within 12 weeks of admission or within 48h or admission into a hospital

without previous health-care contact in the past 12 weeks. However for the purpose of some analyses this definition was altered to include patients who were

a. Admitted in nursing homes (without admission to a hospital in the past 12 weeks). These facilities are more similar to old age homes and do not have a doctor present in the premises

b. Had contact with a hospital in the past 4-12 weeks (indeterminate category by SHEA definitions).

Patients admitted to hospital similar to long-term care facilities were excluded from CA-CDI and were included in HA-CDI instead.

This definition varies slightly from the European definition European which include patients who have not had contact with health care in the past 30d as CA-CDI (Kuijper et al., 2007).

2.10.6 Health care associated infection

This was defined as CDI developing in a patient within four weeks of contact with a health care facility (including GPs and outpatient contact) but less than 48h after admission to a HCF. For the purpose of some analyses, this was included in the hospital–associated category (see figure 5.1).

2.10.7 Severe infection

The definition of severity varies from study to study. Various studies have used differing end points when evaluating patients for severe disease. Although the HPA guidelines (HPA, 2008) suggest calling a case as severe or life threatening if S associated with a WCC >15 $X10^9$/L, or an acute rising serum creatinine (i.e. >50% increase above baseline), or a temperature of >38.5°C, or evidence of severe colitis (abdominal or radiological signs), hypotension, partial or complete ileus or toxic megacolon, or CT evidence of severe disease , other studies have used hypoalbunemia and admission into intensive care or surgical intervention due to CDI related complications in the definition (Hensgens et al., 2011).

At the Royal Infirmary of Edinburgh a composite definition of severity is used to define severe infection for the purpose of treatment. All non-severe cases get metronidazole as the first line treatment whereas all severe; life threatening and complicated cases get Vancomycin as first line treatment. For the purpose of consistency with clinical practice, the definition of severity adopted in this study was the same as the definition used at the Royal Infirmary of Edinburgh. Hence, a severe case of CDI was defined as a case where the patient developed any of the following markers of severity anytime between the onset and cessation of the symptoms in an episode of CDI.

- History of admission to an intensive care unit for complications associated with CDI
- History of surgery (eg, colectomy) for toxic megacolon, perforation, or refractory colitis attributed to CDI
- Total leucocyte count ≥15 cells$X10^9$/L
- Acute rising serum creatinine (i.e. >50% increase above baseline)
- Temperature of >38.5°C
- Evidence of severe colitis (pseudomembranous colitis, abdominal or radiological signs)
- Raised lactate
- Albumin ≤ 25g/L

2.10.8 Cause of death

Mortality data was obtained from the NHS Lothian Public Health Department. Cause of death was determined by reviewing the ICD codes on the death certificates. ICD10 codes A04.7, A09, A41.4, A49.8 (on either primary or secondary (contributory) causes of death) were included in analysis (Anonymous, 2006).

2.10.9 Immunosuppression

This was defined as the presence of one or more of the following conditions

- Acquired Immunodeficiency Syndrome (AIDS)
- Organ transplant (solid organ or haematopoietic stem cell transplant)
- Neutropenia
- Immunosuppressive drug use or systemic corticosteroids for >1 month.
- Corticosteroid use >10 mg.
- Chemotherapy in last two months

2.11 Statistical analysis

This was performed using Minitab® statistical software version 15.1.0.0

Fisher's exact text and chi square were used to for categorical variables and two-sample t test for numerical variables. Multivariate analyses were performed using binary or nominal logistic regression analysis.

Chapter 3
General epidemiology

3.1 Source of samples

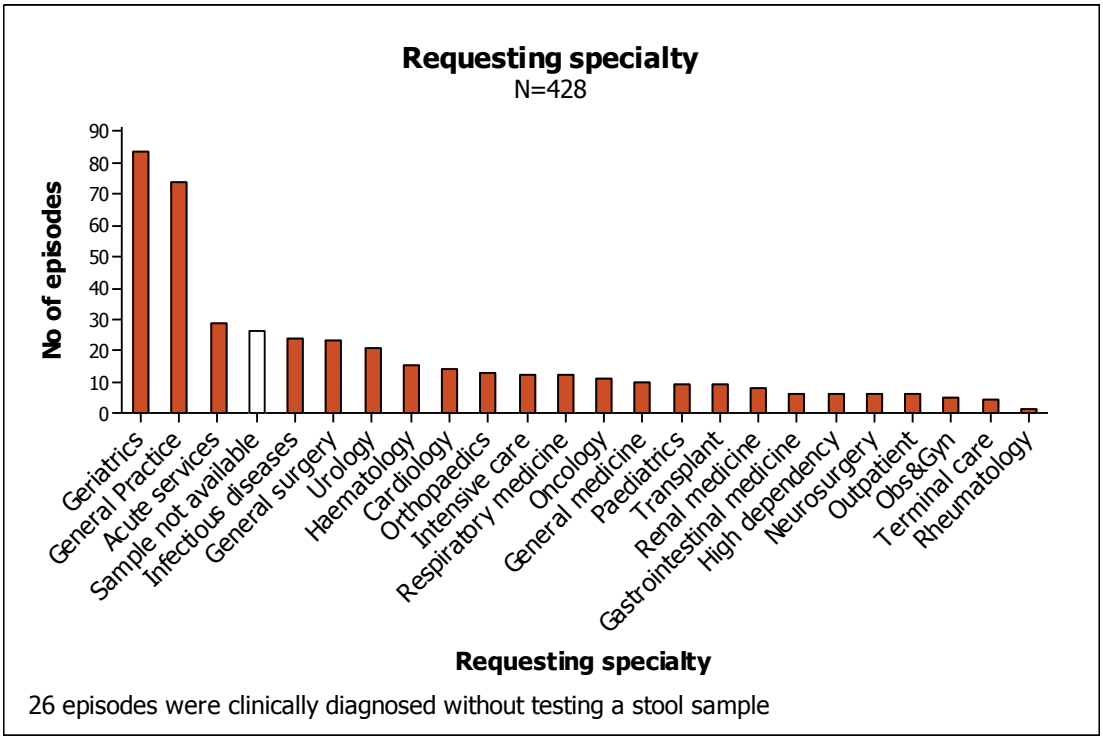
Four hundred and twelve samples from 345 patients (corresponding to 432 clinical episodes) which were positive with GDH and Toxin ELISA combination testing. Of these 10 samples were from patients under two years of age. Since the significance of toxin positivity from this age group is not clear, these were removed from further analysis. Hence 402 samples corresponding to 432 clinical episodes were analysed.

The clinical area these samples came from is given in figure 3.1. As expected, the majority of samples were obtained from geriatrics, which would be in keeping with the median age of 73 years (see figure 3.2). This reflects the trend in previous years (Reddy et al., 2010). Interestingly, the next common source was general practice, which contributed to 17.3% of all episodes. However, of 74 requests from GPs, only 23 (31.1%) did not have a previous contact with a health care setting in the preceding 12 weeks and hence were not community associated in the strictest sense.

In addition, acute services, including the accident and emergency department and the immediate care departments accounted for 29 (6.7%) requests, which included HA-CDI (recently discharged), as well as CA-CDI cases with an acute presentation.

As expected, the specialties of infectious diseases and general surgery (where complex CDI cases are managed) accounted for a large proportion as well (10.7% together).

Figure 3.1 Distribution of clinical specialty from where samples were obtained. Twenty-six episodes were repeat episodes, which were clinically diagnosed without testing a stool samples, were not obtained.

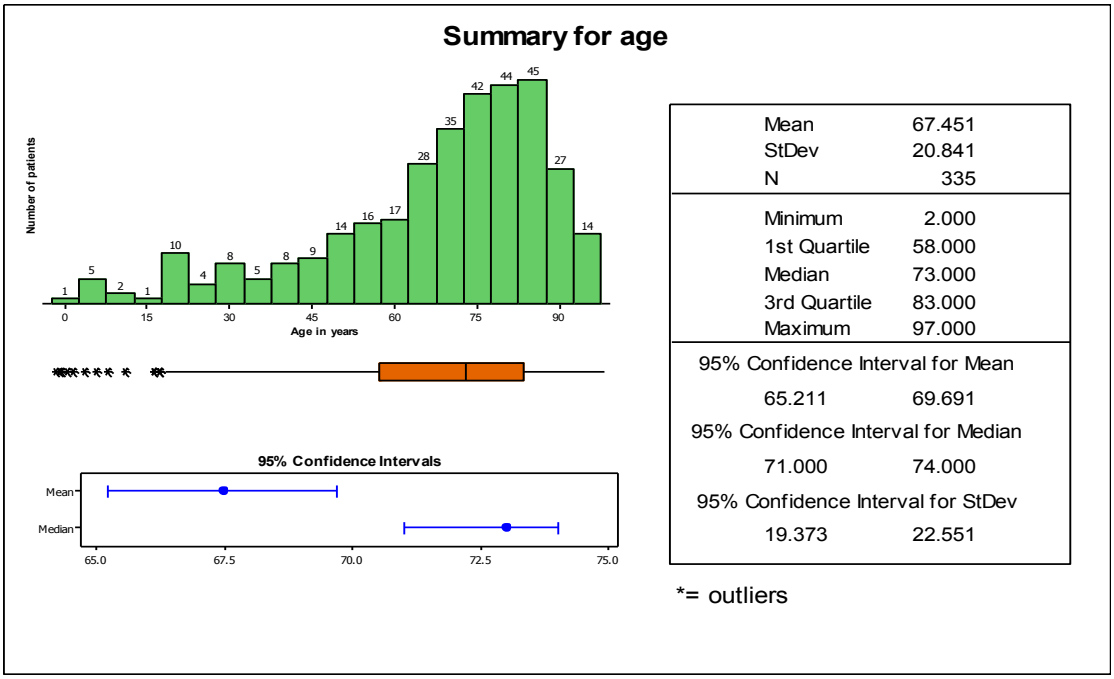


3.2 Patient demographics

3.2.1 Age and distribution

The age distribution of the patients ranged from 2 years to 97 years. Of the 335 patients 2.6% were under 18 years, 24.2 % from 18-60 years and the remainder 73.1% were over 60 years of age. A graphical and statistical summary is given in figure 3.2. The gender distribution of the entire population is given in figure 3.3 and the age stratified gender distribution is given in figure 3.4. Of note, the female prevalence in the overall population was 63.9%. No statistically significant difference in gender was found overall. However, the gender distribution after 80 years age was statistically significant when compared to the prevalence in <80 year olds (p =0.0213).

Figure 3.2 Graphical presentation and statistical summary of the age distribution of the patient population in the study.



Mean	67.451
StDev	20.841
N	335
Minimum	2.000
1st Quartile	58.000
Median	73.000
3rd Quartile	83.000
Maximum	97.000
95% Confidence Interval for Mean	
65.211	69.691
95% Confidence Interval for Median	
71.000	74.000
95% Confidence Interval for StDev	
19.373	22.551

Figure 3.3 Gender distribution of the patient population included in the study.

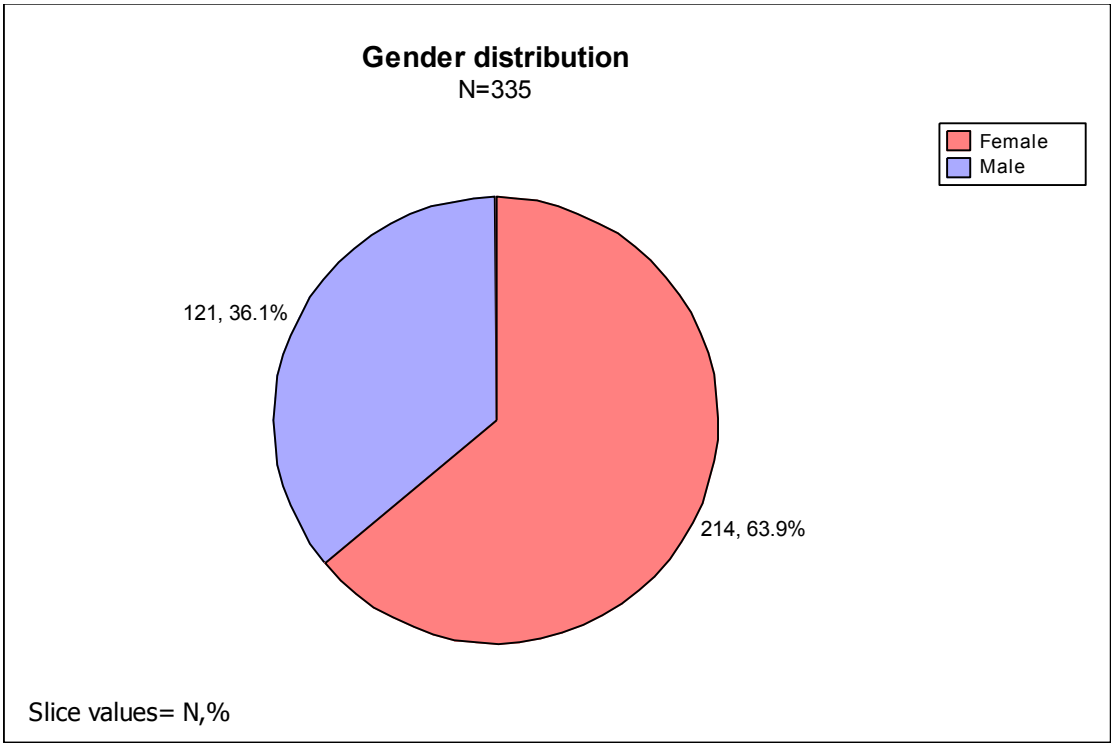
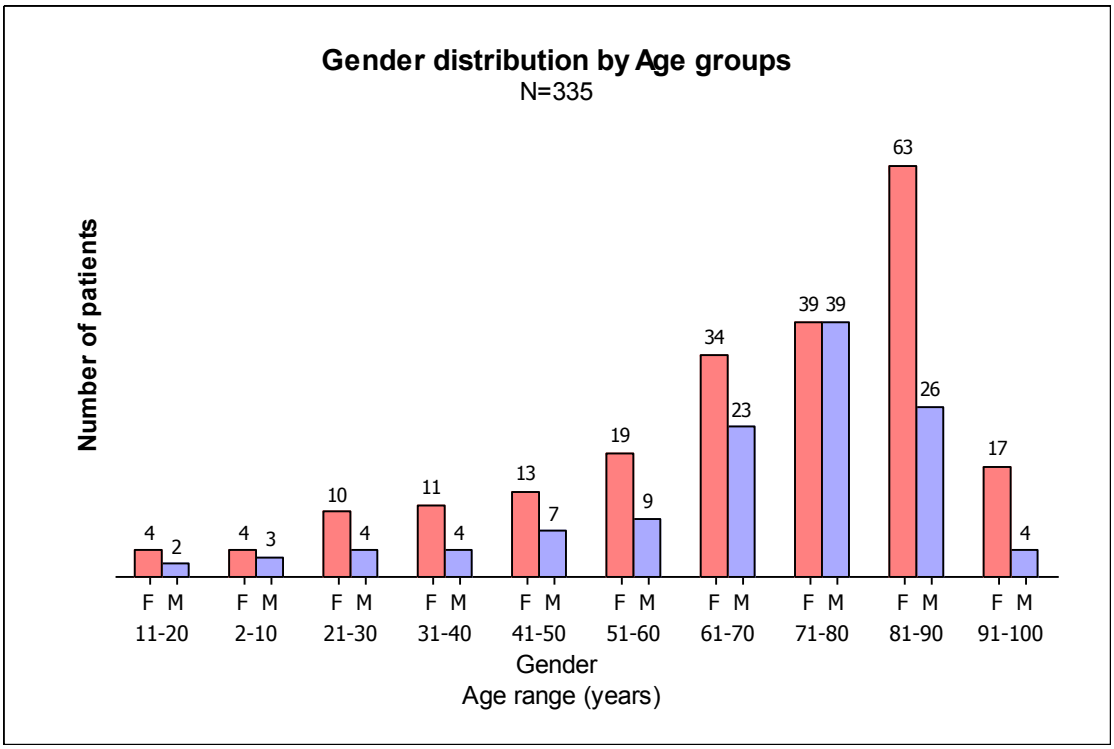


Figure 3.4 Gender distribution of the patient population stratified by age groups.



3.2.2 Proportion of patients with severe CDI

Severe and non-severe episodes were identified based on criteria set forth in Chapter 2. Patients from general practice who were not tested for severity markers and did not get admitted into hospital subsequently were included in the non-severe group. Patients who were from non-community sources but were not tested for severity markers are included in the indeterminate group (see figure 3.5: data not available). Further analysis of severe and non-severe episodes is in chapter 4.

Markers of severity for the entire study population (total leucocyte count and serum albumin levels) are given in figure 3.6 and 3.7. Further details of these are discussed in the subsequent chapters.

Figure 3.5 Proportion of episodes classified as severe and non- severe.

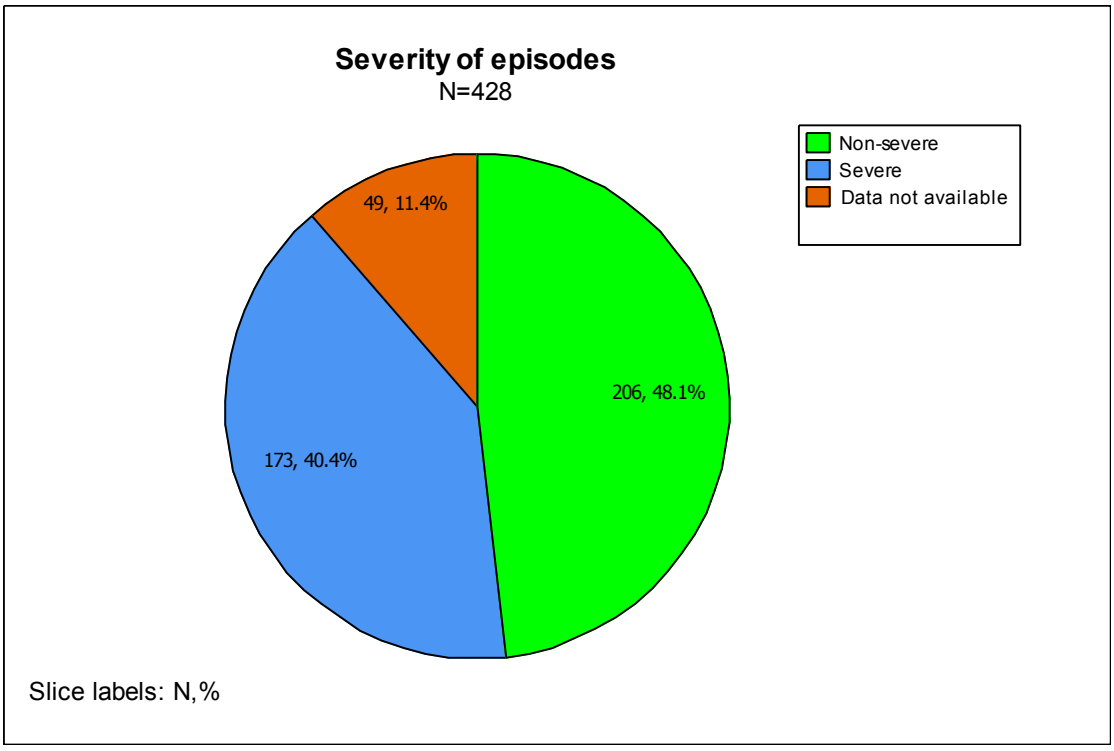


Figure 3.6 Graphical presentation and statistical summary of the total leucocyte count (cellsX10⁹/L) results of episodes from the study.

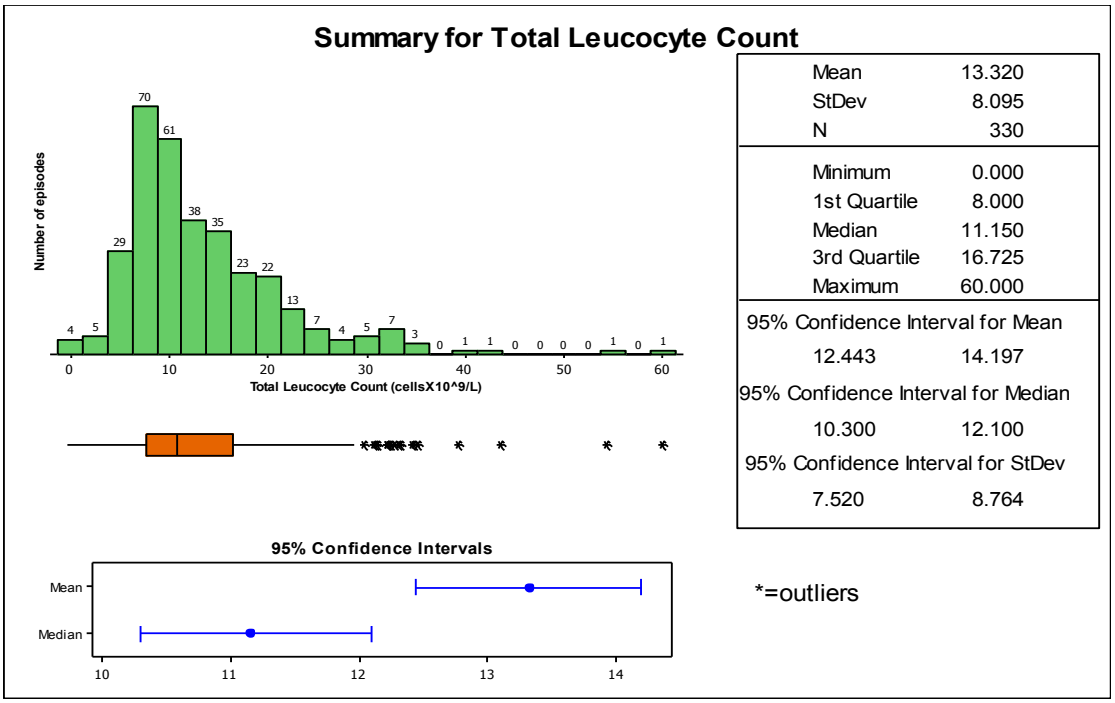
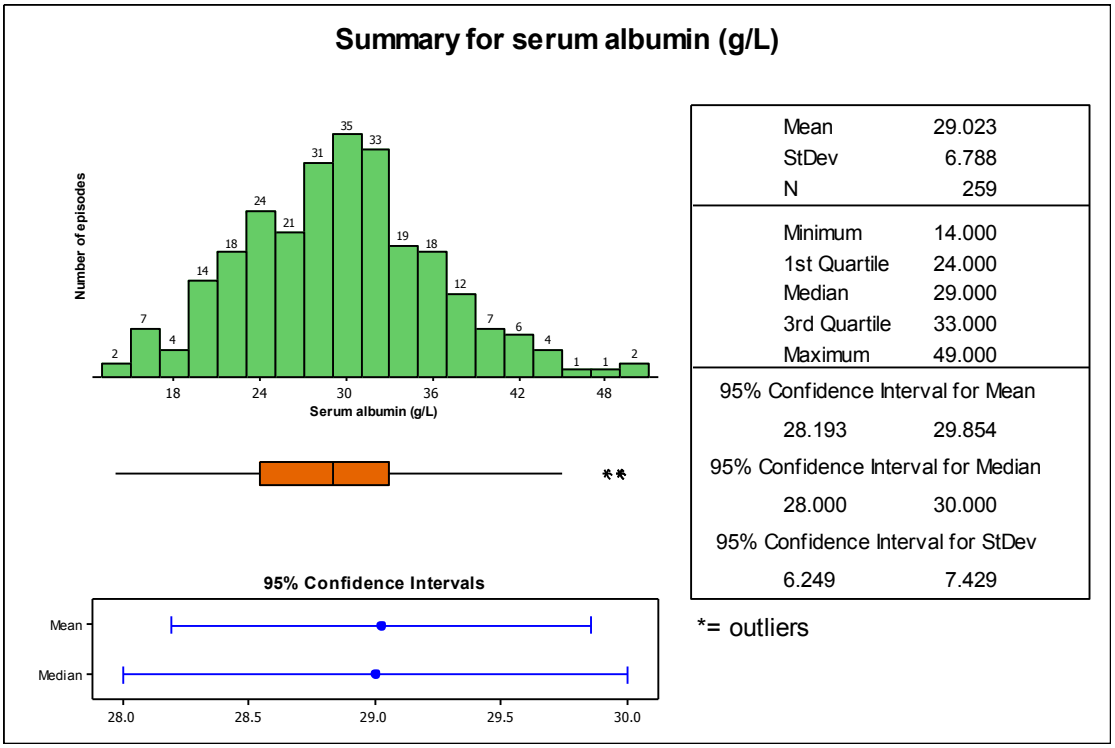


Figure 3.7 Graphical presentation and statistical summary of the serum albumin level (g/L) from patients taken from each episode.



3.2.3 Proportion of patients with repeat episodes of CDI

Figure 3.8 shows the summary of patients stratified according to the number of episodes on record. These include all the episodes on medical records including those prior to commencement of this study. Most patients (70.7%) suffered a single episode while the remainder (29.3%) had multiple episodes ranging from 2-10 episodes. Similar data limited to the duration of the present study is given in figure 3.9, where 19.9% patients suffered from relapsing or recurrent disease (classified as repeat episodes for the purpose of this study). These data corresponds well with a previous American study, which reported a 29% relapse rate over a 15-month period (Cadena et al., 2010) and from a recent study from Oxford which reported a cumulative recurrence rate of 22% (Eyre et al., 2012). Further analysis of recurrent cases is given in chapter six.

Figure 3.8 Summary of all episodes occurring per patient (cumulative data)

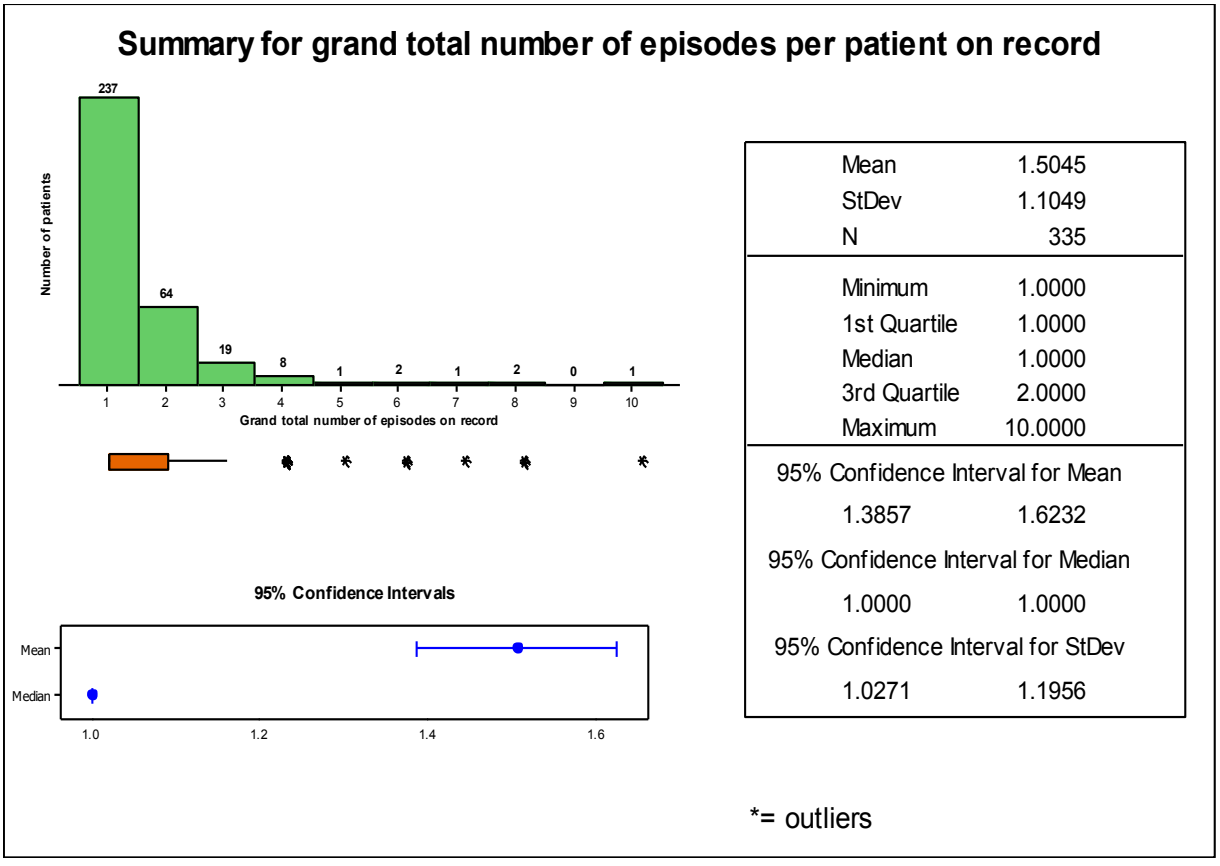
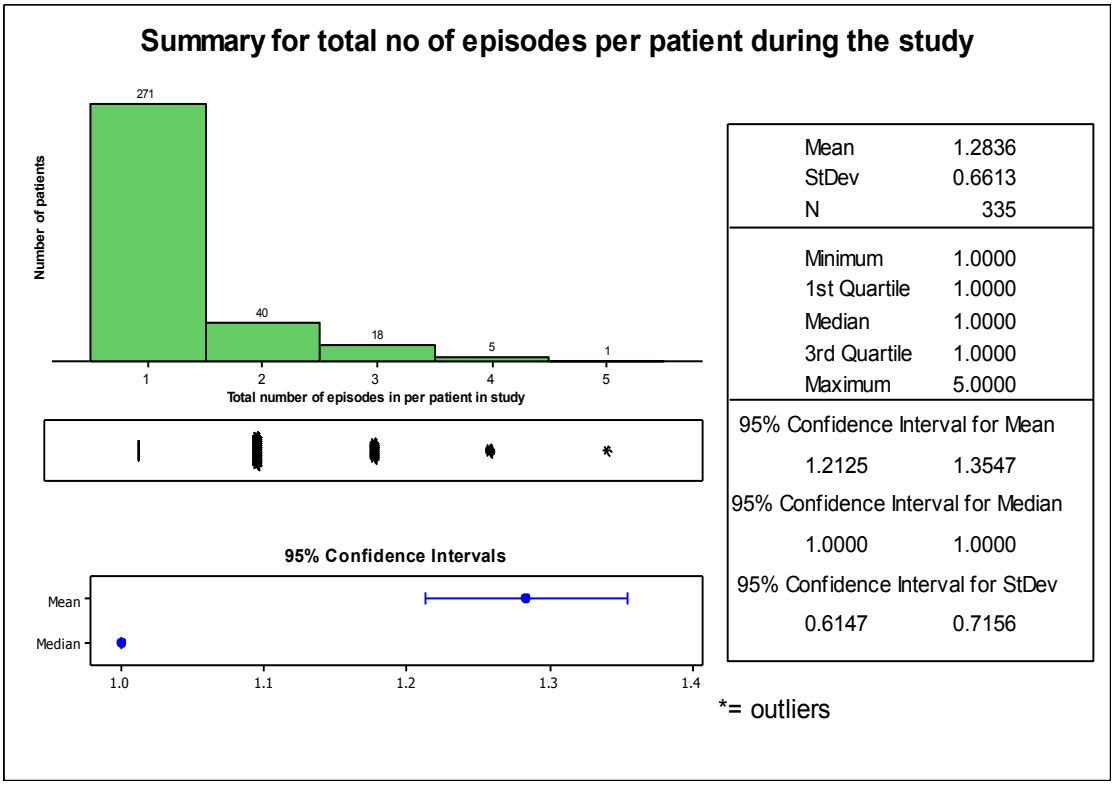


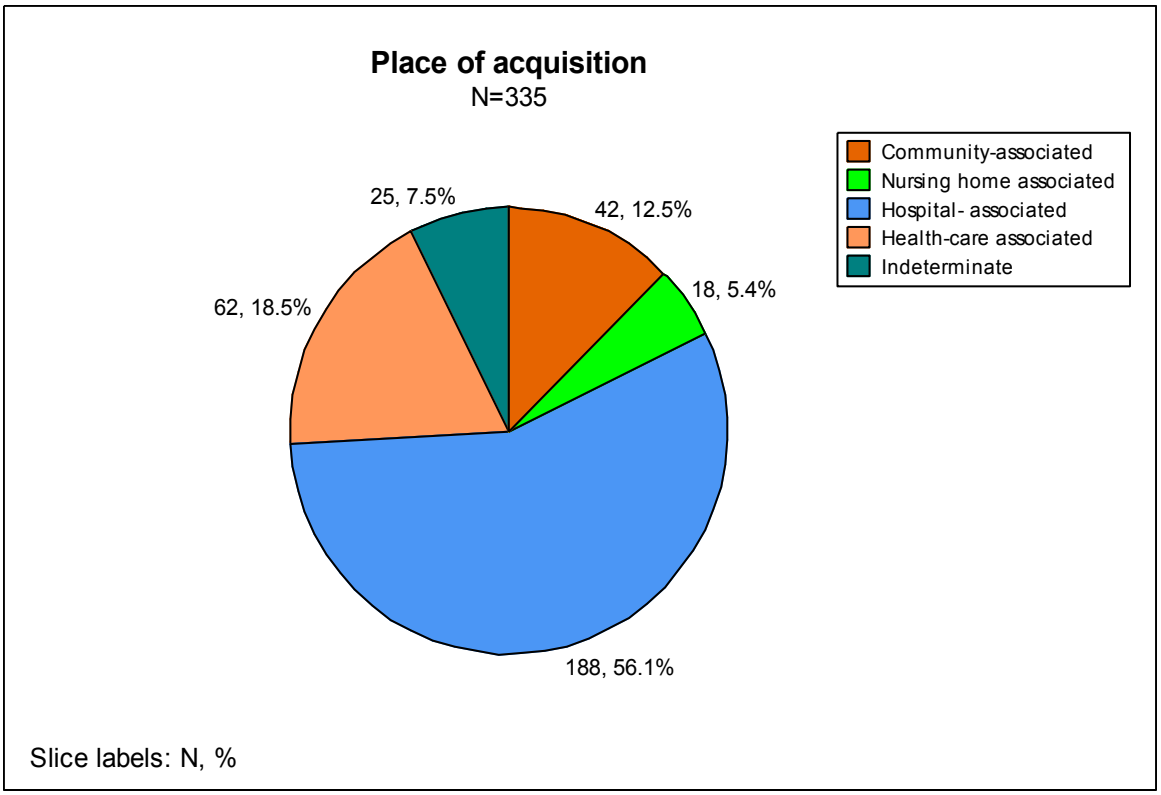
Figure 3.9 Summary of episodes per patient occurring in the duration of the present study (12-month follow up)



3.2.4 Place of acquisition of CDI

The place of acquisition of the first episode in the study from each patient was defined as given in chapter two. Since all previous available data were taken into account, if a patient had a previous episode in the past 12 weeks (even though it was not part of the study), such a patient was considered to have acquired it from a health-care setting. More details of community and health-care associated CDI are given in chapter five.

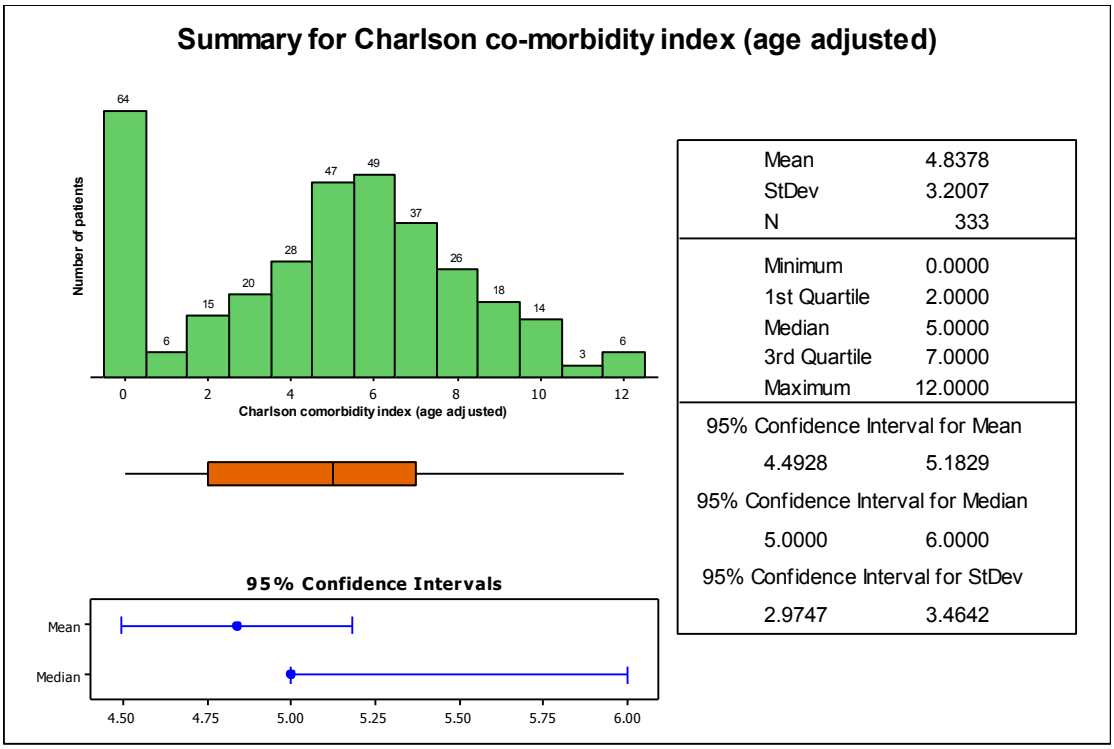
Figure 3.10 Place of acquisition of CDI



3.2.5 Charlson Comorbidity index

Charlson comorbidity index (CCI) was available for 333 patients. Although the unadjusted version was also recorded, the age-adjusted version was used in the analyses. Graphical and statistical summary of the distribution of the CCI of the patients in the study is given in figure 3.11. In this study 19.2% patients had a Charlson score of 0. More details among the subgroups of patients with CDI are given in the relevant chapters.

Figure 3.11 Graphical presentation and statistical summary of the age-adjusted Charlson score of patients included in the study



3.3 Predisposing factors

Predisposing factors analysed in the study are depicted graphically in figure 3.12 to figure 3.17

3.3.1 Antibiotics

Antibiotic information was retrospectively recorded for the eight weeks preceding CDI diagnosis. The different antibiotics recorded for the patients along with the proportion who received each are given in figure 3.13. The number of different antibiotics given per patient according to this classification is given in figure 3.12.

A very well-known predisposing factor for development of CDI, the various classes of antibiotics likely to predispose to CDI have been studied in many previous studies(Gorbach, 1999). The recent focus on reduction in the use of "high risk" antibiotics to reduce the overall incidence of CDI has also been studied (Aldeyab et al., 2012, Valiquette et al., 2007). Further analysis of antibiotics as a risk factor is presented in the following chapters.

Figure 3.12 Graphical and statistical summary of antibiotics administered per patient in the eight weeks preceding CDI

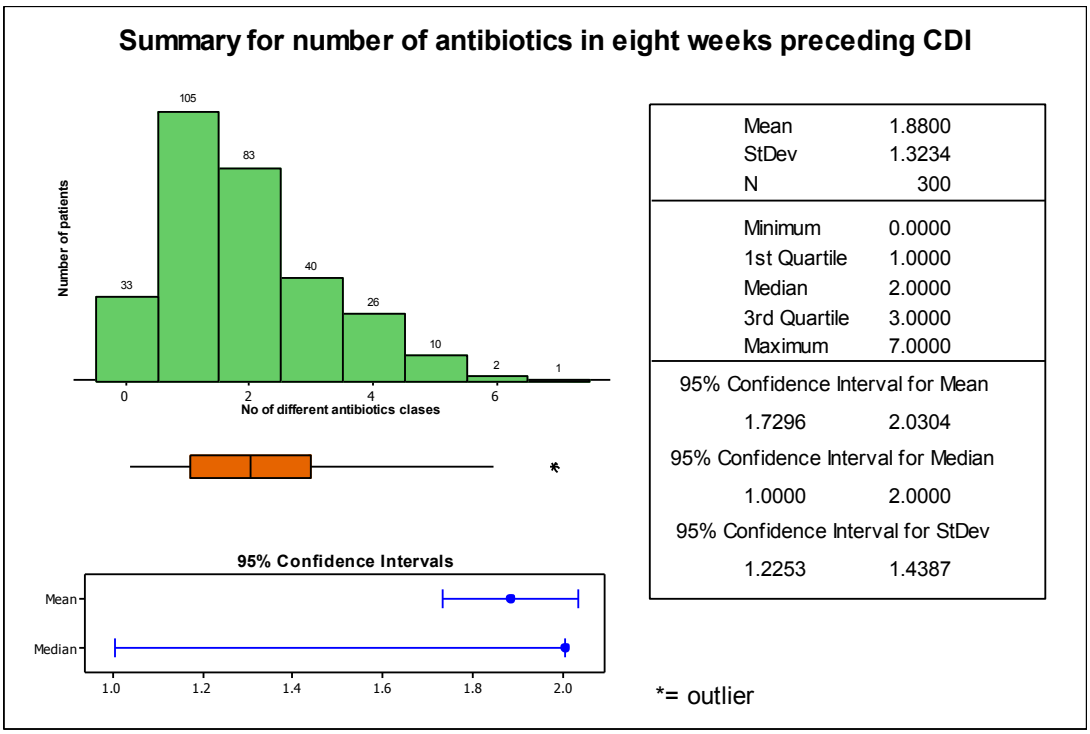
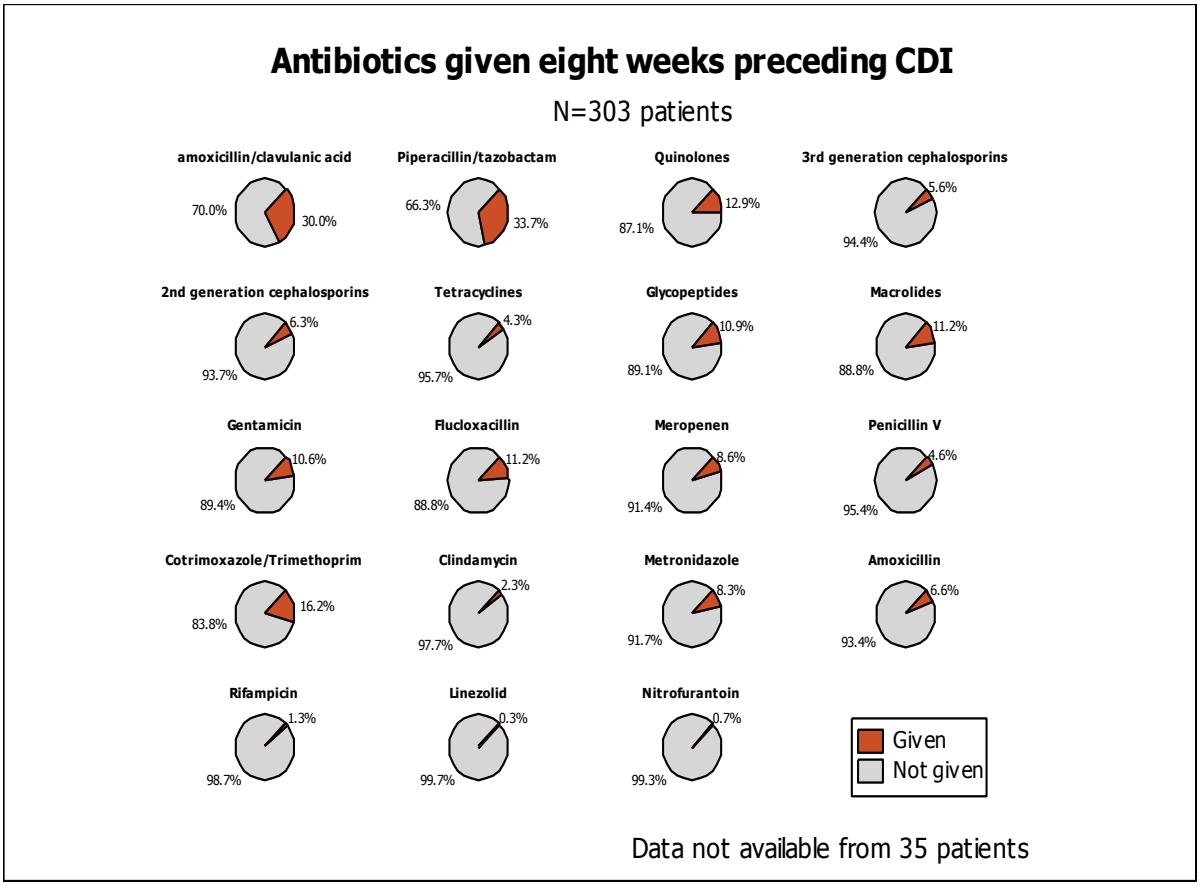


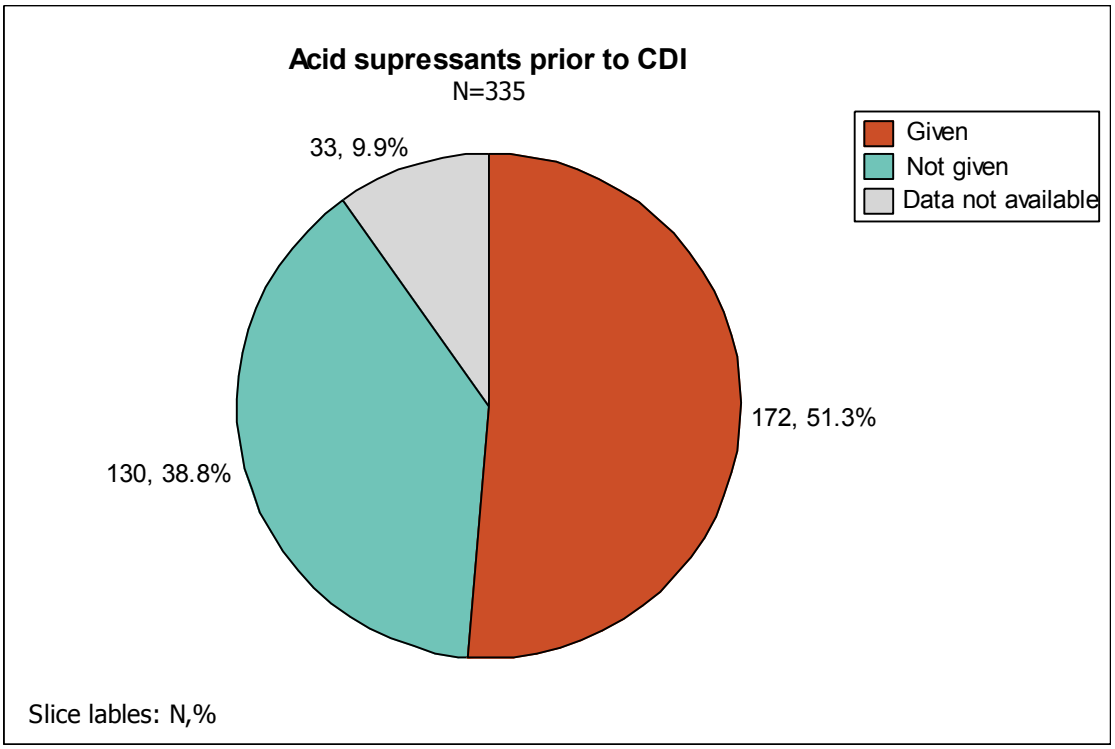
Figure 3.13 Antibiotics given to patients in eight weeks preceding CDI



3.3.2 Acid suppressive agents

The use of acid suppressing medication was found in 51.3% patients. Studies have suggested that the use of antacids can be a predisposing factor for *C. difficile* colonisation (McFarland et al., 1990). The proportion of patients on acid suppression medication up to two weeks before development of CDI is given in figure 3.14.

Figure 3.14 Proportion of patients on acid suppressive at the time of developing CDI

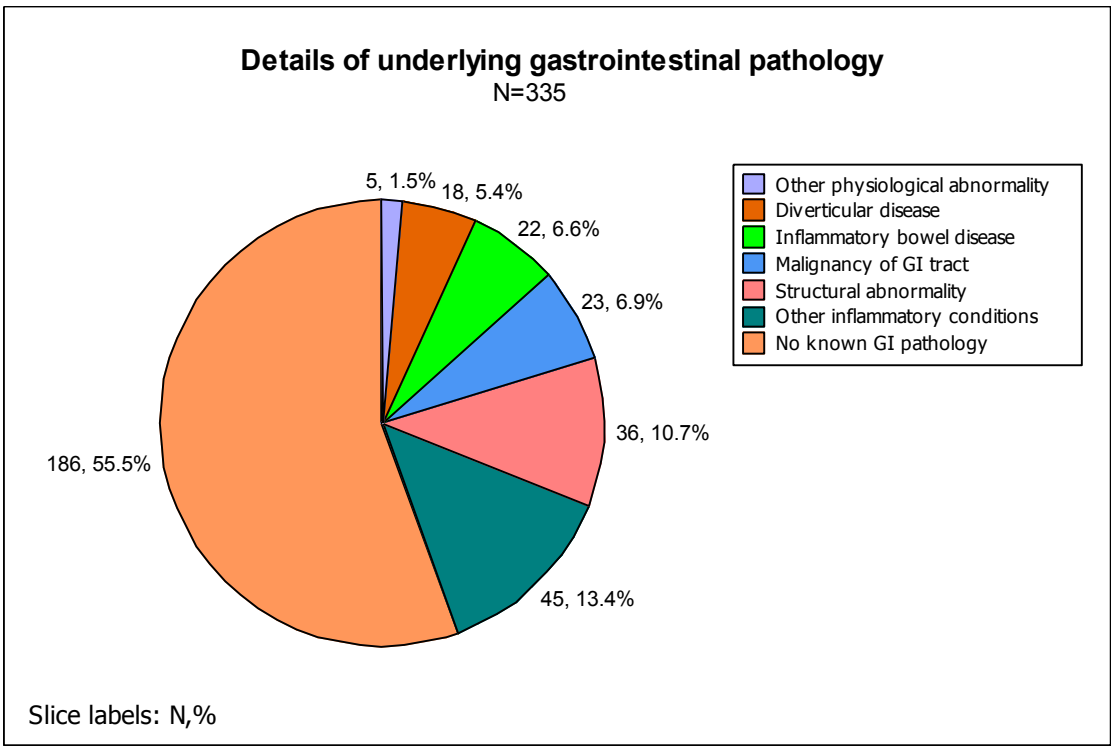


Of the 172 patients that were given antacids, Omeprazole was administered to 105 patients (61%), Lanzoprazole to 43 patients (25%), Ranitidine to 24 patients (14%, Pantoprazole to 5 patients (2.9%) and Esomeprazole to 3 patients (1.7%). Of the above, eight patients were given more than one antacid (2 Lanzoprazole and Omeprazole, 5 Omeprazole and Ranitidine, 1 Ranitidine and Lansoprazole) in the eight weeks preceding CDI.

3.3.3 Underlying gastrointestinal pathology

In this study, gastrointestinal pathology was found in 45.5% patients. The distribution of the various types of GI pathology seen in the study population is given in figure 3.15. Inflammatory bowel disease was seen in 6.6.% of the population

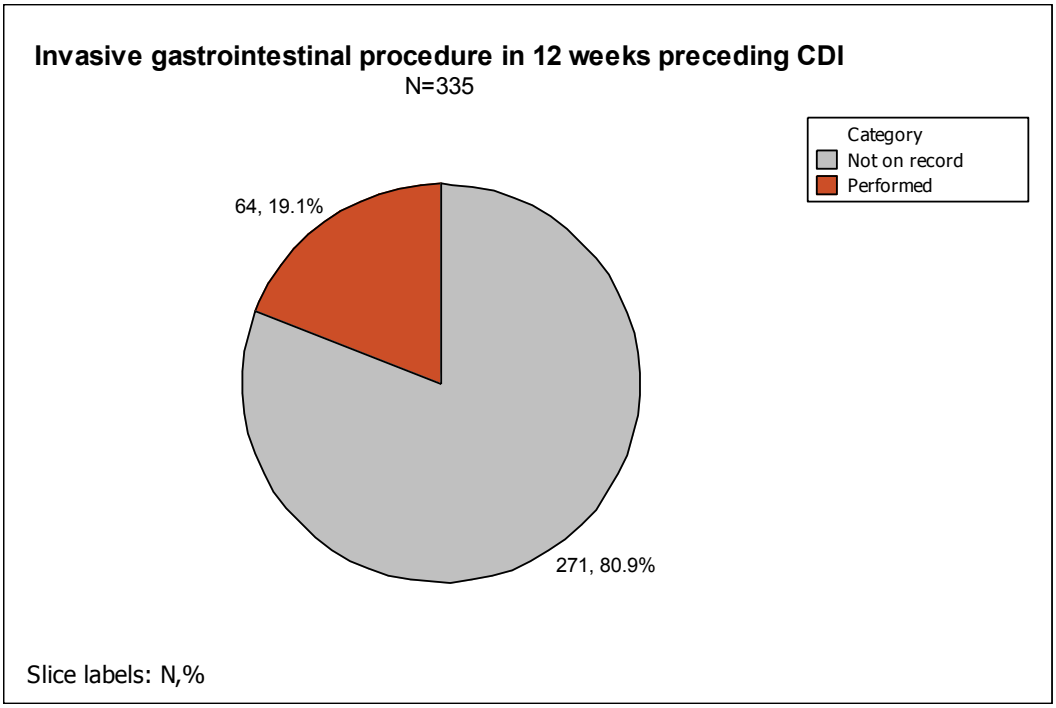
Figure 3.15 Distribution of underlying gastrointestinal pathology in patients with CDI



3.3.4 Gastrointestinal invasive procedure

A total of 19.1 % patients underwent a gastro-intestinal invasive procedure in the 12 weeks preceding development of CDI. The distribution is depicted in figure 3.16

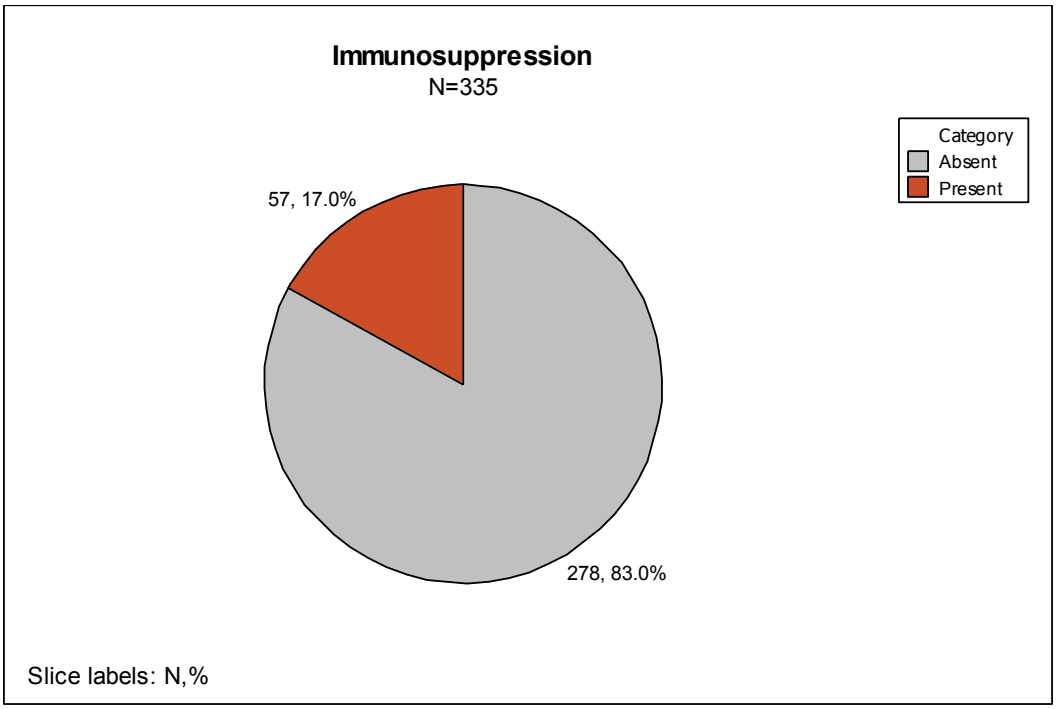
Figure 3.16 Proportion of patients who underwent an invasive gastrointestinal procedure in 12 weeks preceding CDI



3.3.5 Immunosuppression

Fifty-seven patients in the study were deemed to be immunosuppressed as defined in section 1.10.8. This corresponds to 17% of the total population as seen in figure 3.17

Figure 3.17 Proportion of patients who were immunosuppressed at the time of developing CDI



3.3.6 Mortality

One-year mortality among patients with CDI including cause of death is given in figure 3.18. Time to death is given in figure 3.19 and cause of death in the 30d time range is given in figure 3.20. It is apparent that death due to CDI may occur even after 30d of developing CDI (13 patients in the present study). Conversely death within 30d of CDI may not be due to CDI (69.8% in the present study).

Figure 3.18 One-year mortality and cause of death among patients included in the study

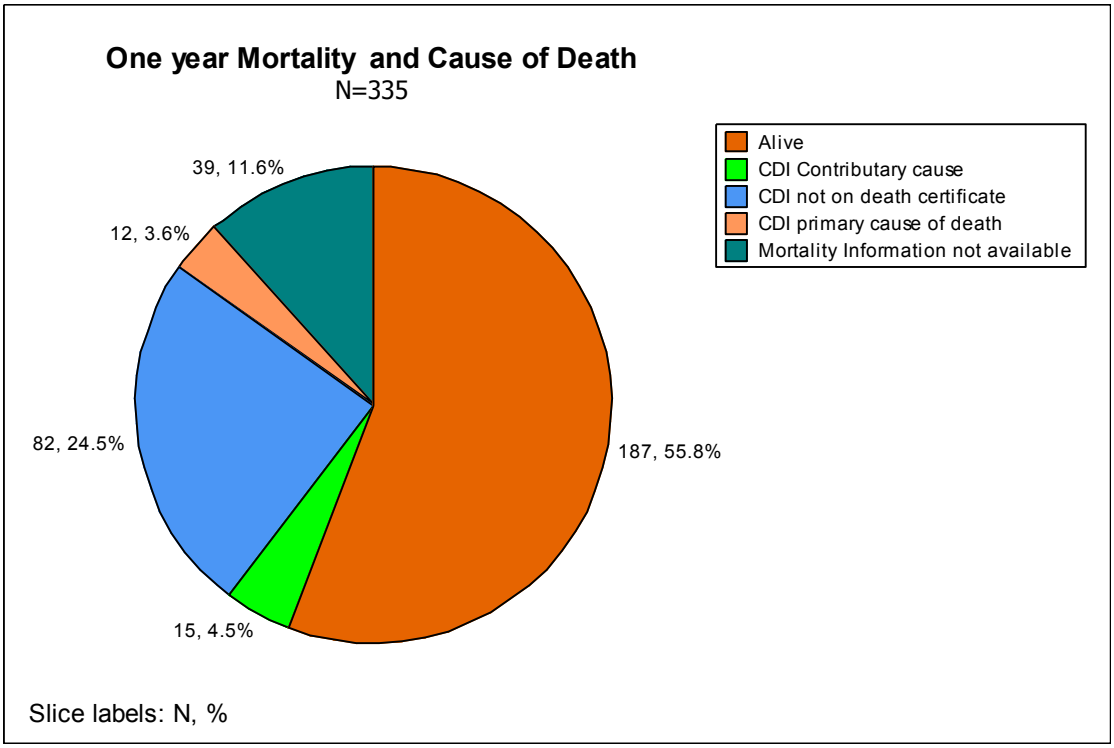


Figure 3.19 Mortality and time to death among patients included in the study

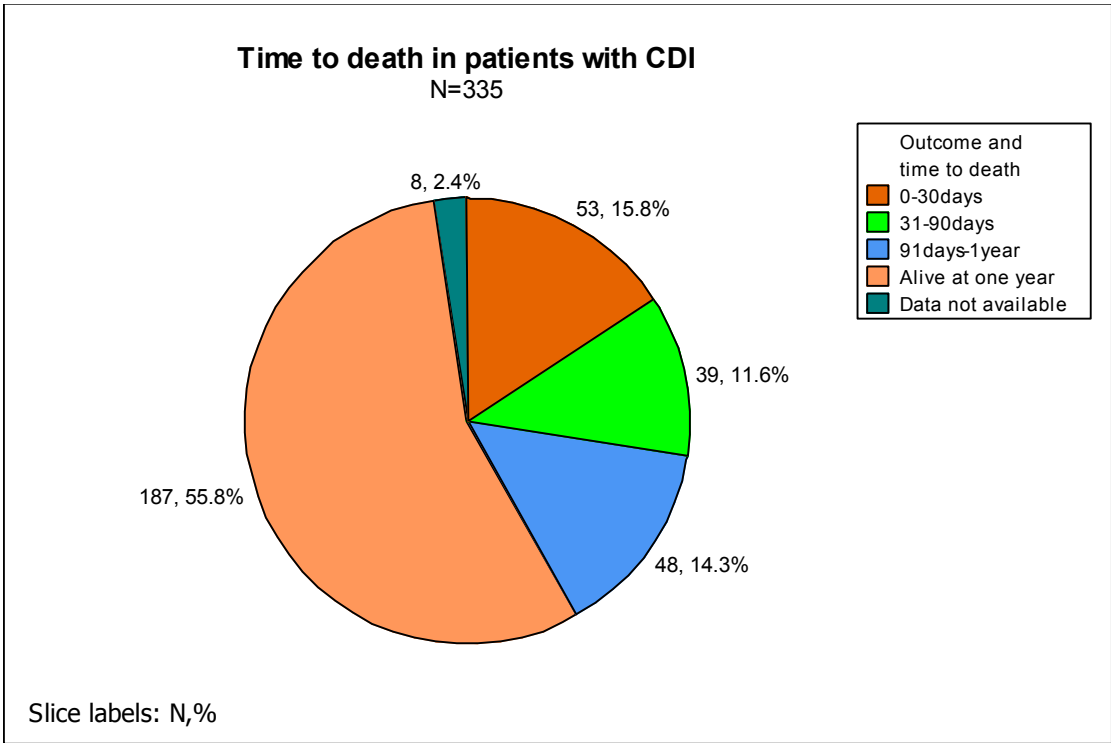
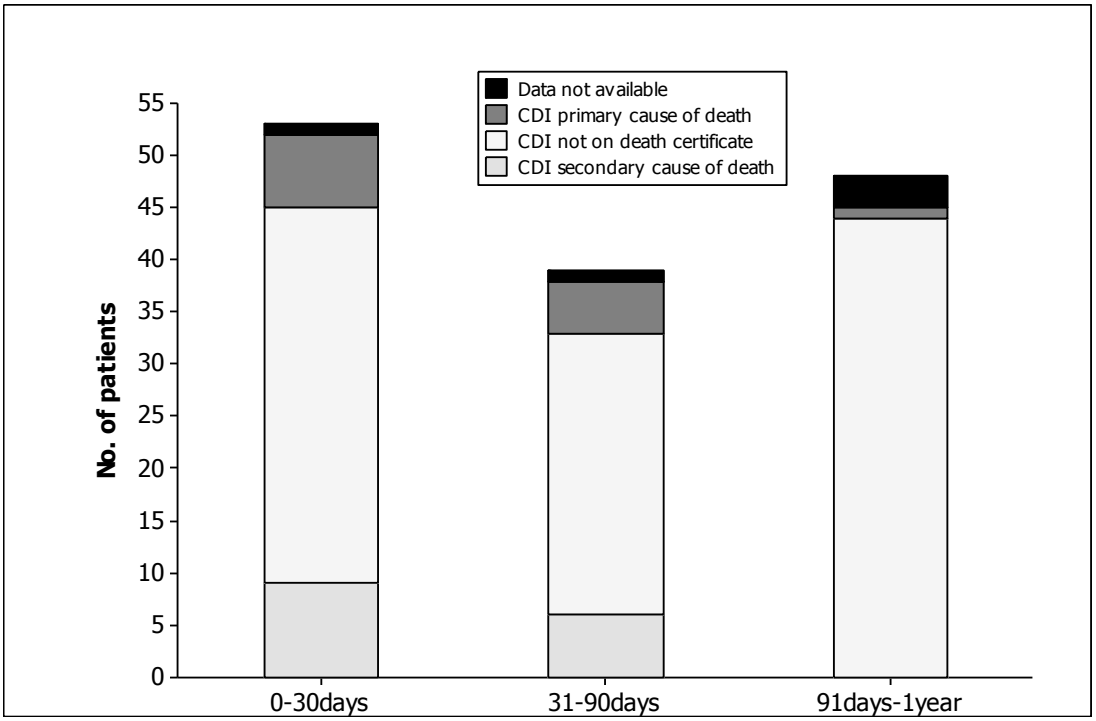


Figure 3.20 Cause of death and time range among people who died within 1 year of diagnosis

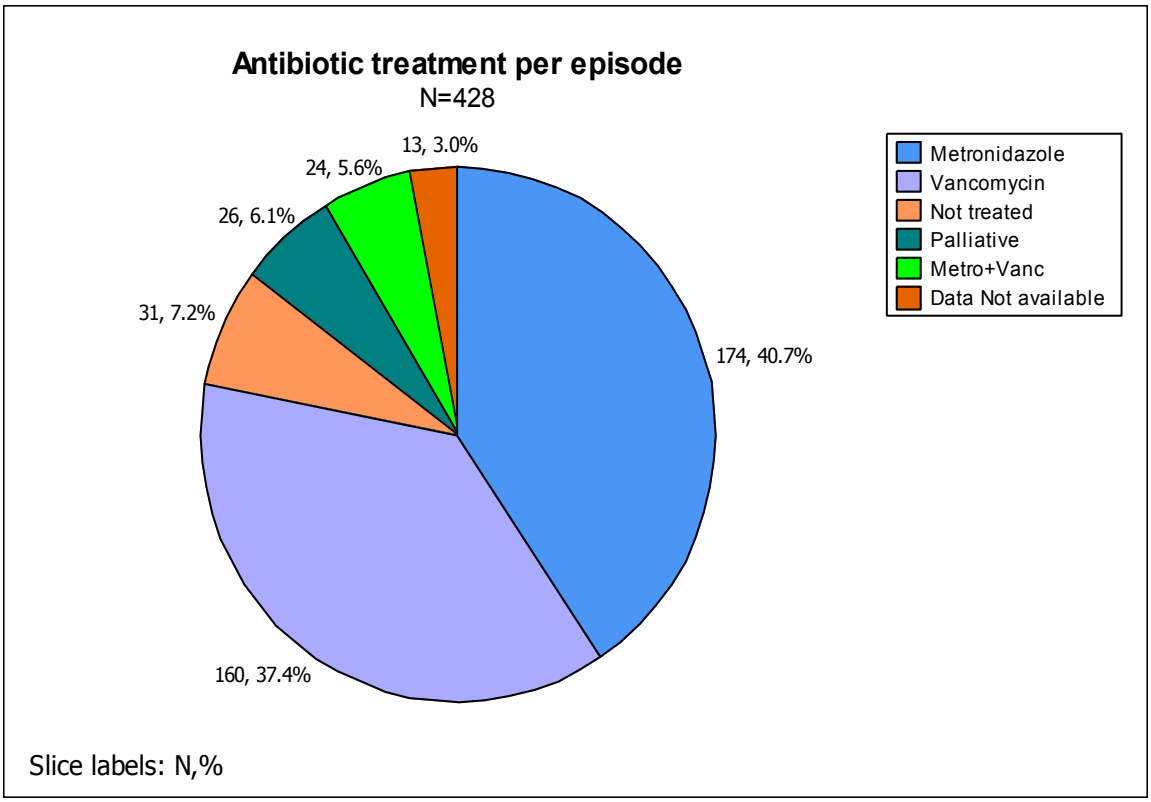


Note: The time to death has been calculated from the last recorded episode in the study.

3.3.7 Treatment given for CDI

The antibiotic combinations given to patients in the study are given in figure 3.21. Two patients received rifamycin in addition to vancomyin and metronidazole. No patient was given faecal transplant therapy though it was considered as an option for two patients with recurrent CDI. One patient was given IV immunoglobulin in addition to vancomycin and rifamycin. Treatment given has been further analysed in the following chapters.

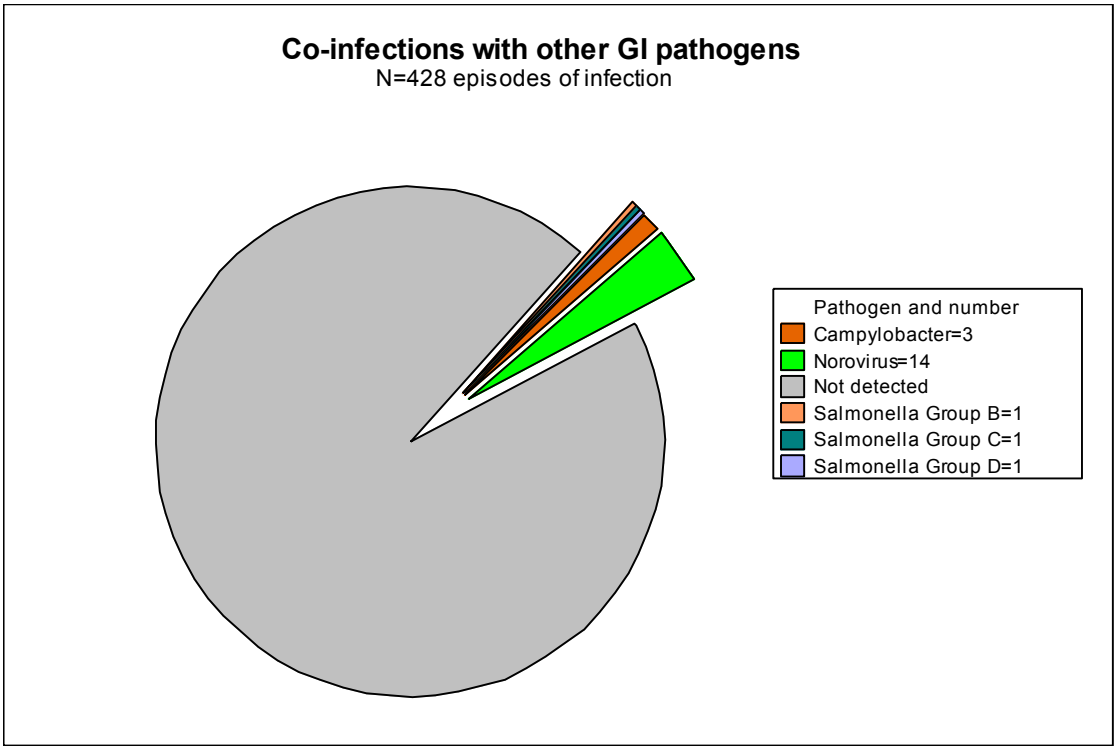
Figure 3.21 Non surgical treatment of CDI episodes



3.3.8 Co-infection with other GI pathogens

All stool samples sent to the enteric laboratory at the Royal Infirmary of Edinburgh are tested for the presence of *C. difficile* (if requested) and cultured for other bacterial pathogens (with special media added as per the travel history). Norovirus PCR is done if an outbreak is suspected. In the present study, additional Norovirus tests were done on stool samples thought to be representing CA-CDI. The proportion of patients in whom other diarrhoeal pathogens were found is given in figure 3.22. Of the six patients with co-infections (three *Campylobacter* and three *Salmonella* spp), five were treated with metronidazole by the attending clinician, in spite of the presence of another pathogen. Similarly 12 of the 14 episodes (85.7%) with Norovirus co-infection were treated for the *C. difficile* (eight received metronidazole, one metronidazole with vancomycin and three vancomycin alone). These samples have not been excluded from the study since it could not be determined whether their symptoms were solely due to the other pathogen or due to a co-infection.

Figure 3.22 Co-infecting gastro-intestinal pathogens detected from stool samples with C.difficile

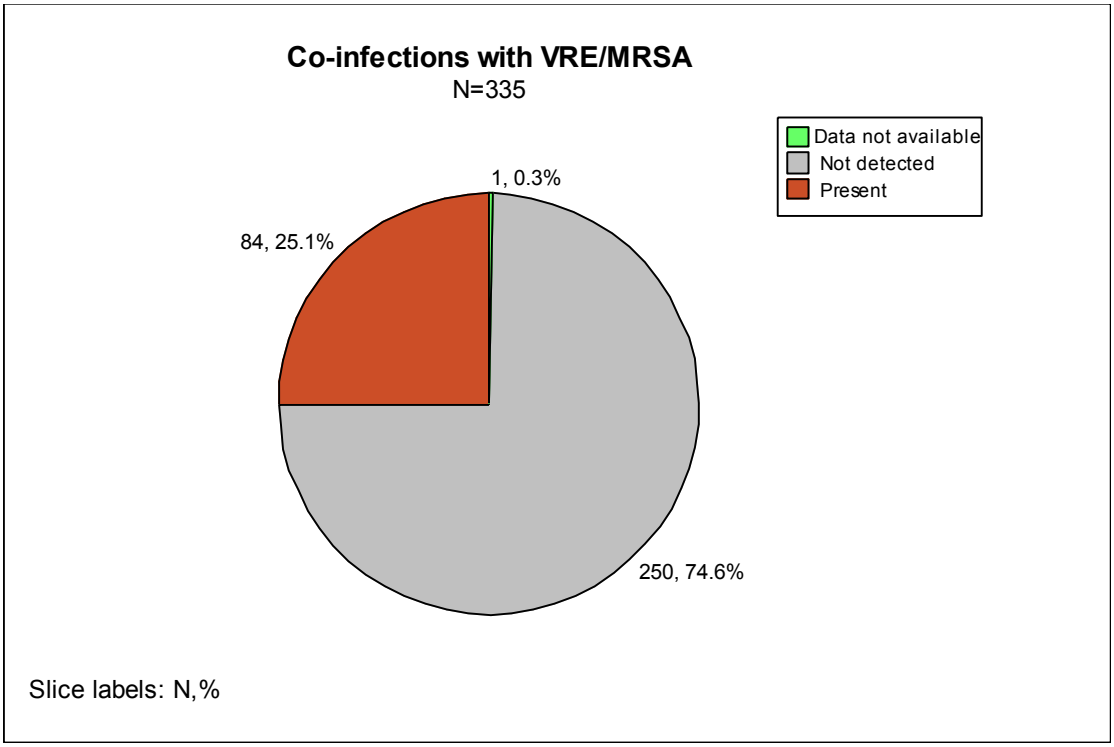


3.3.9 Co-infection with MRSA and VRE

Methicillin resistant *Staphylococcus aureus* (MRSA) and Vancomcin resistant Enterococci (VRE) are largely hospital-acquired organisms (HAI) (though community acquired strains exist, the proportion is relatively small). At the time of inclusion into this study, the previous acquisition of either of these pathogens was determined and during the one-year follow up period the acquisition of either of these pathogens was also recorded. Among the 335 patients included in this study, 25.1% were also colonised with either VRE or MRSA. 56.4% of these had pre-existing colonisation before they developed the first episode of CDI whereas in 43.6% colonisation was detected simultaneously or within a year of developing CDI.

Although not part of the original planned study, this additional data suggests that at least 25% of patients with CDI are at greater risk of developing multiple HAI-s (see figure 3.).The *C. difficile* maybe a consequence of antimicrobial treatment for MRSA/VRE, which is likely to increases the susceptibility to CDI (which would explain the later acquisition of *C. difficile*). Conversely it could be a result of multiple hospital admissions or breakdown in infection control precautions.

Figure 3.23 Co-infection with MRSA and/or VRE prior to or within 1 year of developing CDI



3.4 Molecular epidemiology

3.4.1 Prevalence of PCR ribotypes

Results of PCR ribotyping are given in figure 3.24. Samples from 54 episodes (19.2%) were unavailable for typing since they were repeat episodes which were either diagnosed solely on the basis of clinical criteria or the sample sent was insufficient in quantity for culture (but sufficient for toxin and GDH tests), or the culture did not yield *C. difficile*. At least one sample was available from 94.6% patients. Hence isolates from 94.6% patients and 80.8% episodes were available for typing. Of these, isolates from 28 episodes were typed but a ribotype number could not be assigned since the image did not exactly resemble any in the reference collection. These isolates were compared with each other and save two isolates from the same patient; all others possessed patterns different from each other.

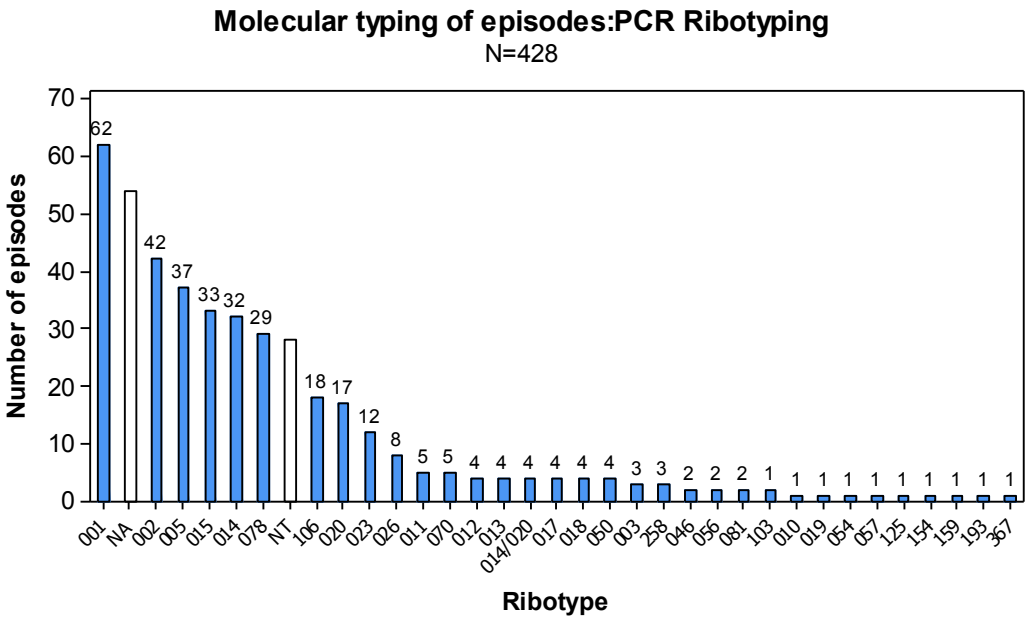
PCR ribotype 001 was the commonest in the entire population followed by ribotype 002, 005 and 014. It was not possible to reliably distinguish between ribotypes 014 and 020 in the case of four isolates, and hence they have been clubbed together in the chart. Previous studies have also reported the difficulty in distinguishing between

these two ribotypes since they cluster very closely in the minimum spanning tree even when typed using the 15 locus MLVA (Manzoor et al., 2011). On agarose gel, these two ribotypes are very similar (differing only in the intensity of a single band). Although capillary gel electrophoresis is known to be more accurate than agarose gel (Indra et al., 2008, Xiao et al., 2012), the patterns for these two ribotypes are difficult to distinguish in some cases. Indra et al (2008), found 7 different patterns for ribotype 014 when comparing agarose and capillary gels with MLVA. Hence there appears to be diversity within this group, which is reflected in the present study. Figure 3.25 shows the capillary gel electrophoresis images of known ribotype 014 and 020.

In Scotland, the Scottish *Salmonella, Shigella* and *C. difficile* reference lab (SSSCDRL) reported a change in the most prevalent ribotypes from July 2010-June 2011. This data is from the snapshot study conducted by the SSSCDRL, which includes approximately 15% of all isolates from each region of Scotland. There was a changing trend among the commonest ribotypes from ribotype 015 (11%), 001, 002 and 106 (all at 10%), 014 (8%), 027 (8%), 078 (7%), 020 (6%) in the third quarter of 2010 (HPS, 2011d) to ribotype 106 (12%), 015 (10%), 001 (10%), 005 (8%), 002 (8%), 078 (8%), 020 (8%), 014 (7%) and 027 (4%) in the last quarter of 2010 (HPS, 2011e). Further changes were noted in early 2011: ribotype 106 was the most common strain (15% of isolates typed) followed by types 002 and 020 (both at 12%) and type 001 (6%). Ribotype 027 was absent from this quarter. (HPS, 2011c). The changing trend continued with a decline in types 106, 001 and 027 whereas types 005, 023 and 078 emerged in greater numbers (HPS, 2011b). In spite of this changing trend, the 10 most common ribotypes in these reports are also the commonest ribotypes in the present study, hence reflecting the overall prevalence in Scotland. Interestingly, though ribotype 027 has been reported in Scotland in areas very close to Edinburgh (above data), only one case of this ribotype has ever been reported within Edinburgh itself. In keeping with this trend, the present study also did not find any of this so called "hypervirulent" strain. A recent study demonstrated that due to slight differences in banding patterns, ribotypes 176, 198 and 244 resemble PCR ribotype 027 (Valiente et al., 2012). Although PCR 176 was

present in the library, we did not find any isolate resembling it. The latter two patterns were absent from the library, hence there is a small chance that they may be identified in the strains currently called untypeable.

Figure 3.24 PCR ribotype distribution of each episode of CDI recorded in the study



NA= 54 episodes were either only clinically dignosed or sample insufficient for culture
NT=28 isolates were not typeable since they did not exist in the database

3.4.2 Presence of toxin A, toxin B, the binary toxin and deletions in the negative toxin regulator *tcdC*

All isolates in the study were tested for the presence of toxin A and toxin B genes *tcdA* and *tcdB*. These were found to be present in all strains. In addition, the binding component of the binary toxin *cdtB* and known deletions in the anti-sigma factor *tcdC* was also investigated. The latter was found in 11.1% episodes. The results of these are given in table 3.1.

Table 3.1 Deletions in *tcdC* found in the study.

Deletion	Number found	Percent of total episodes	Found in study ribotypes
53bp	13	3.1	023
19bp	14	3.47	015
39bp	30	7.43	078, 193

Chapter 4
Factors associated with severe infections

In this chapter comparisons have been made between three groups as follows:

- Severe and non-severe episodes where each episode has been taken as an individual unit. Patients with multiple, distinctly separate episodes have been included more than once. This was to analyse separately the episodes from which a patient has recovered and completed treatment. These episodes are sometimes due to a separate ribotype as seen in chapter 6.
- Patients who developed an episode of severe CDI at presentation or within one year of the presenting episode with those whose episodes were never classified as severe.
- Patients who died within one year of the presenting (index) episode and CDI was listed as a cause of death on the death certificate with those who did not die or died to non CDI related causes.

4.1 Molecular aetiology

4.1.1 Ribotypes associated with severe and non-severe episodes

Severe episodes in this study were compared to non-severe episodes. Figure 4.1 depicts the ribotype distribution between these two groups. Among the severe episodes, ribotype 001 was present in the maximum proportion (15.8%) and ribotype 002 was the second commonest (11.4%). Ribotypes 005 and 078 accounted for 9.5 and 9.4% each. Figure 4.2 gives the relative proportion of ribotypes among the severe episodes. As is apparent from these two graphs, there is heterogeneity within this population and no particular ribotype is over-represented in the severe episodes as compared to the non-severe episodes. Comparing the severe and non-severe episodes of the commonest ribotype 001, there was no significant difference

(p=0.667). Comparisons of other common ribotypes did not yield any statistically significant differences either. Although it may appear that representing each episode is likely to over-represent the prevalence of CDI due to a particular ribotype, it does correlate the workload of the hospital staff and the morbidity faced by the patient. The 28-day rule to call an episode separate from the previous has not been followed strictly to enable a uniform strategy to analyse the molecular aetiology.

As discussed earlier, there have been suggestions that the ribotype of a *C. difficile* isolate may determine its potential to cause severe disease (Arvand et al., 2009, Baldan et al., 2010). There have been reports of PCR-ribotype related differences in susceptibility to antimicrobials and disinfectants (Dawson et al., 2011, Huang et al., 2009a), which may be responsible for differences in pathogenicity. However a number of multivariate analyses now appear to be suggesting that severity is not related to ribotype whereas there is a positive correlation with haematological markers of severity like total leucocyte count and serum albumin (Walk et al., 2012, Wilson et al., 2010).

Ribotyping does however, provide a method for classifying *C. difficile* isolates, which is often required for epidemiological purposes and is a good starting point for comparative studies.

Figure 4.1 Distribution of ribotypes between severe and non severe episodes

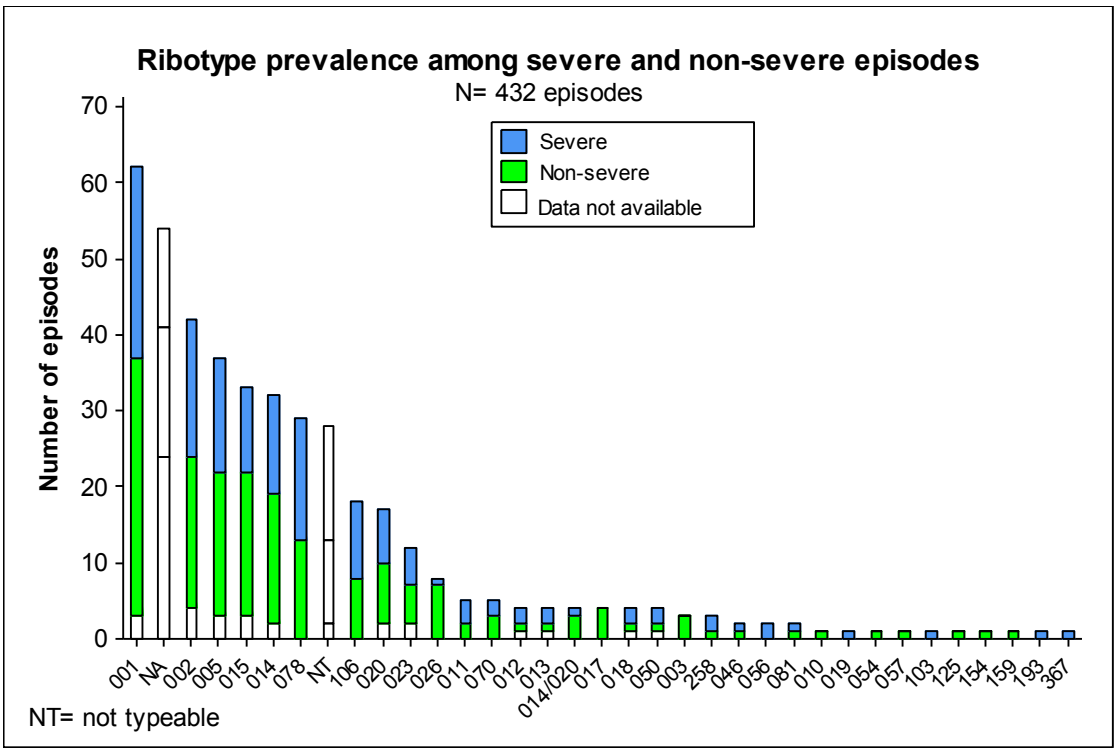
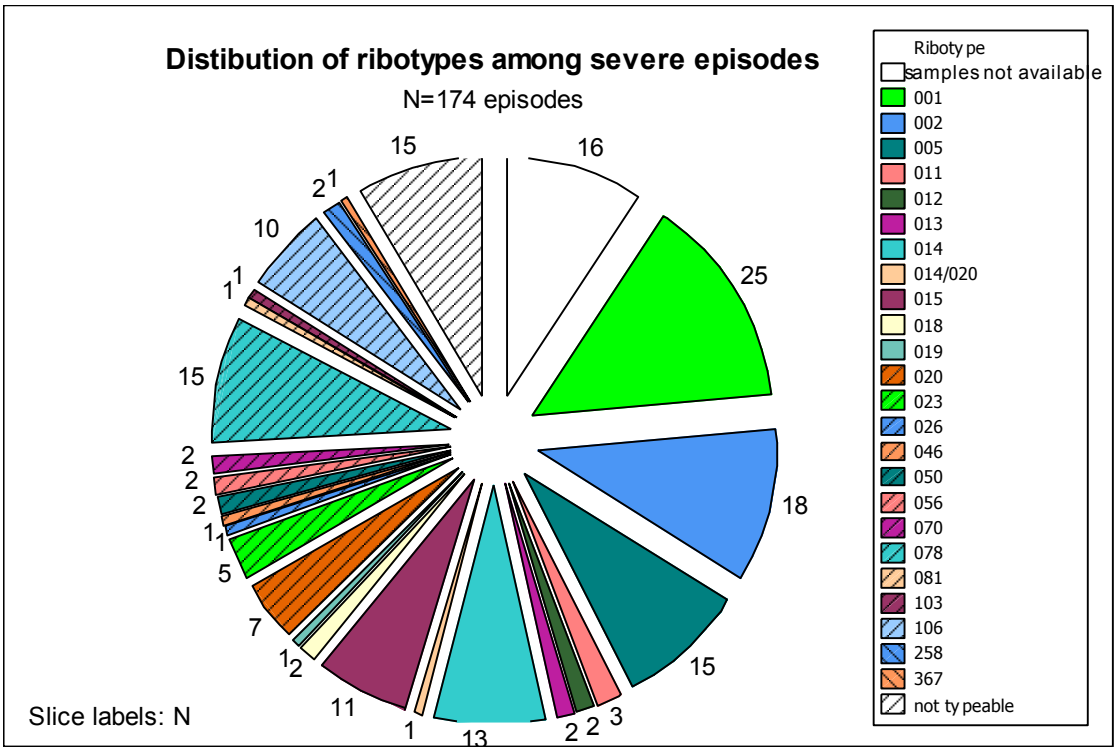


Figure 4.2 Ribotypes found among severe episodes



4.1.2 Differences in toxin genes *tcdC* and *cdtB*

The presence of the binary toxin and deletions in the toxin suppressor gene (anti-sigma factor) was studied. The results were compared between the groups mentioned above (see table 4.2, 4.5, 4.8 and 4.9). Neither of these was statistically different between the severe and non-severe episodes (p values 0.418 and 0.779 respectively), nor between patients who developed a severe episode at presentation (or within one year of the presenting episode), see table 4.5. However when the patients who died due to CDI (with CDI listed on the death certificate) within one year of the presenting (index) episode were compared with those did not die due to CDI (either alive at one year, or dead due to other causes), a significant difference was found between the presence of a deleted *tcdC* (p=0.013, see table 4.8) and presence of the binary toxin (p=0.014). However this difference was not found in the regression model (table 4.9) and the confidence interval was very large suggesting that the numbers included in the analysis (28 patients who died) was probably too low to make a strong conclusion.

Since ribotype 027 and ribotype 078 have been identified with the binary toxin and deletions in the *tcdC* (strains which have frequently been associated with outbreaks and reported increase in mortality), there was a suspicion that their deletion in the *tcdC* or presence of binary toxin may have influence of their virulence. An overall genetic relationship between strains which harbour mutations in the toxin regulatory genes has been suggested (Bouvet and Popoff, 2008) raising the question that increased virulence may be a feature associated with these mutations (Carter et al., 2011, Knetsch et al., 2011). However doubts had been raised earlier regarding the significance of gene deletions in gene functionality (Curry et al., 2007) and clinical studies performed so far have also not confirmed this suspicion. Analysis of 207 clinical isolates with and without the presence of *tcdC* truncation and binary toxin for severity markers and outcome revealed that there was no direct correlation between these parameters and disease outcome (Goldenberg and French, 2011). However the study suggested that the presence of binary toxin may be associated with increased total leucocyte count and all cause mortality at 30 days. Another study

compared 141 patients of whom 42 of had deletion-positive *C. difficile*. They concluded that the deletion-carrying *C. difficile* was commonly present but did not cause more severe disease (Verdoorn et al., 2010).

Another study compared the 30-day mortality rates for patients infected with *Clostridium difficile* strains with and without binary. Patients with binary toxin had higher case-fatality rates than patients without binary toxin, in univariate and multivariate analysis after adjustment for age, sex, and geographic region (RR 1.6, 95% CI 1.0-2.4) (Bacci et al., 2011)

From these studies and from the present study it appears that any correlation between gene mutation and clinical severity if present is unlikely to be due to the deletions detected in these studies. However there may be a factor indirectly related to these mutations, which may still not be known. The presence of binary toxin appears to be linked to severity but the reason is unclear. Further molecular work with clinical correlation is needed before such an association can be established confidently. Advances in molecular technology are already leading to more detailed work on this subject. Point mutation in the *tcdC* leading to a truncation in the protein of PCR ribotype 027 have suggested that this mutation is more likely to be associated with increase in toxin production. Similar work on other ribotypes is at lacking at present.

4.2 Clinical parameters

The factors compared between the severe and non-severe episodes are given in table 4.1 and 4.2. Table 4.3 summarises the multivariate analysis of the factors, which were found to be significantly different, by the bivariate analysis.

Table 4.1 Bivariate analysis of continuous clinical variables comparing all severe episodes with all non-severe episodes

Variable	Severe episodes				Non-severe episodes				p value	p value
	N	Mean	SD	SE Mean	N	Mean	SD	SE Mean	2-sample t test	Mann Whitney U test
Age (years)	174	69.9	17.7	1.3	210	66.9	22.7	1.6	0.138	
Charlson comorbidity index (age adjusted)	174	5.46	2.65	0.20	206	4.44	3.43	0.24	0.001	0.0054
Total number of different antibiotics given eight weeks preceding onset	158	2.13	1.41	0.11	187	1.76	1.33	0.097	0.015	0.0098
Total number of episodes on record	174	1.557	0.896	0.068	210	1.529	0.892	0.062	0.753	0.0018
Total leucocyte count (cells $X10^9$/L)	169	16.96	9.42	0.72	158	9.49	3.60	0.29	0.000	0.000
Serum albumin level at onset (g/L)	151	26.74	6.71	0.55	107	32.26	5.52	0.53	0.000	0.000

Table 4.2 Bivariate analysis of categorical variables comparing all severe and non-severe episodes

Variable		Severe episodes N (%)	Non-severe episodes N (%)	Total N	p value (Fisher's exact test)
Gender	Male	61	74	135	1.0
	Female	113	137	250	
Immunosuppression	Absent	131	186	317	
	Present	43	23	66	0.000
Place of acquisition	CA	26	56	82	0.007
	HA	117	122	239	
Antacids (PPI and H2 receptor antagonists)	Given	94	96	190	0.099
	Not given	60	89	149	
Underlying GI pathology	Not known to be present	76	130	206	0.000
	Present	98	80	178	
Mutation detected in tcdC	Absent	5	8	13	0.779
	Present	131	162	293	
Colonisation with MRSA or VRE prior to CDI	No	135	165	300	0.901
	Yes	39	45	84	
Invasive GI procedure in 12 weeks prior to CDI	No	118	167	285	0.003
	Yes	55	37	92	
Use of tazocin in eight weeks preceding illness	No	86	142	228	0.000
	Yes	75	48	123	
Use of clindamycin in eight weeks preceding CDI	No	160	182	342	0.042
	Yes	1	8	9	
Antibiotics given 8 weeks prior to CDI	No	12	21	33	0.28
	Yes	151	174	325	
Presence of cdtB	No	136	170	306	0.418
	Yes	22	21	43	
Norovirus co-infection	No	50	96	146	0.390
	Yes	3	11	14	

Table 4.3: Multivariate analysis by binary logistic regression of factors significantly associated on bivariate analysis for development of a severe episode compared to non-severe episodes

Variable	p value	Odds Ratio	95% CI	
			Lower limit	Upper limit
Charlson comorbidity index (age adjusted)	0.005	1.13	1.04	1.23
Immunosuppression	0.005	1.13	1.04	7.75
Presence of underlying GI pathology	0.063	1.65	0.97	2.80
Hospital-associated acquisition	0.632	1.17	0.61	2.25
Invasive GI procedure in 12 weeks preceding episode	0.086	1.72	0.93	3.19
Piperacillin-tazobactam 8 weeks prior to episode	0.016	1.98	1.13	3.46
Clindamycin 8 weeks prior to episode	0.268	0.29	0.03	2.59

Table 4.4 Bivariate analysis of continuous variables analysed for differences between patients who developed a severe episode at presentation or within the one-year follow up period to those who did not develop any severe episode

Variable	Development of any severe episode				No severe episodes				p value
	N	Mean	SD	SE Mean	N	Mean	SD	SE Mean	
Age (years)	153	69.1	18.3	1.5	170	65.7	23.1	1.8	0.142
Charlson comorbidity index (age adjusted)	153	5.38	2.75	0.22	168	4.28	3.47	0.27	0.002
Total number of different antibiotics given eight weeks preceding onset	138	2.07	1.33	0.11	153	1.71	1.28	0.10	0.018
Total number of episodes on record	153	1.68	1.34	0.11	170	1.341	0.697	0.053	0.006
Total leucocyte count (cells $X10^9$/L)	149	16.67	9.35	0.77	129	9.43	3.83	0.34	0.000
Serum albumin level at onset (g/L)	130	27.24	6.91	0.61	89	32.29	5.59	0.59	0.000

Table 4.5 Bivariate analysis of categorical variables analysed for differences between patients who developed a severe episode at presentation or within the one-year follow up period to those who did not develop any severe episode

Variable		Development of any severe episode N	No severe episodes N	Total N	p value (Fisher's exact test)
Gender	Male	55	61	116	1.0
	Female	98	109	207	
Immunosuppression	Absent	116	153	269	0.001
	Present	37	17	54	
Place of acquisition	CA	30	52	82	0.029
	HA	123	118	241	
Antacids (PPI and H2 receptor antagonists)	Given	84	82	166	0.078
	Not given	51	76	127	
Underlying GI pathology	Not known to be present	70	107	177	0.002
	Present	83	63	146	
Deletion detected in *tcdC*	Absent	115	134	249	0.353
	Present	27	23	50	
Colonisation with MRSA or VRE prior to CDI	No	107	132	239	0.098
	Yes	46	37	83	
Invasive GI procedure in 12 weeks preceding CDI	No	113	146	259	0.008
	Yes	40	24	64	
Use of tazocin in eight weeks preceding illness	No	78	116	194	0.000
	Yes	62	37	99	
Use of clindamycin in eight weeks preceding CDI	No	139	147	286	0.123
	Yes	1	6	7	
Treatment of first episode	Metro	44	101	145	0.000
	Vancomycin	76	31	107	
	Metronidazole &vancomycin	16	3	19	
	No treatment	3	20	23	
	Palliative	10	9	19	
Antibiotics given 8 weeks prior to CDI	No	11	20	31	0.184
	Yes	132	137	259	
Presence of *cdtB*	No	119	140	259	0.179
	Yes	23	17	40	
30-day mortality	No	124	143	267	0.531
	Yes	25	23	48	
1-year mortality due to CDI	No	129	159	288	0.004
	Yes	20	7	27	
Norovirus co-infection	No	48	83	136	0.770
	Yes	4	9	13	

Table 4.6 Binary logistic regression analysis of factors related to development of severe infection at presentation or within one year of index episode

Variable	p value	Odds Ratio	95% CI	
			Lower limit	Upper limit
Charlson co-morbidity index (age adjusted)	0.001	1.15	1.06	1.25
Presence of immunosuppression	0.001	3.27	1.58	6.76
Total no. of antibiotics administered 8 weeks before index episode	0.239	1.14	0.92	1.40
Presence of underlying GI pathology	0.119	1.52	0.90	2.56
Hospital-associated acquisition	0.790	0.92	0.48	1.74
Invasive GI procedure in 12 weeks preceding episode	0.023	2.21	1.12	4.37
Piperacillin-tazobactam 8 weeks preceding episode	0.103	1.62	0.91	2.90

Table 4.7 Bivariate analysis of continuous variables associated with death due to CDI (as listed on the death certificate) within one year of the index episode

Variable	One-year CDI related mortality				Absence of CDI related mortality				p value
	N	Mean	SD	SE Mean	N	Mean	SD	SE Mean	
Age (years)	28	77.5	12.9	2.4	299	66.5	21.4	1.2	0.000
Charlson comorbidity index (age adjusted)	28	5.46	2.65	0.50	298	4.76	3.24	0.19	0.195
Total number of different antibiotics given eight weeks preceding onset	25	1.60	1.29	0.26	270	1.91	1.33	0.081	0.226
Total number of episodes on record	28	1.750	0.928	0.18	299	1.49	1.13	0.065	0.176
Total number of episodes in one year of study	28	1.429	0.634	0.12	299	1.274	0.669	0.039	0.229
Total leucocyte count (cells $X10^9$/L)	26	19.9	11.6	2.3	246	12.70	7.45	0.48	0.005
Serum albumin level at onset (g/L)	23	25.57	5.96	1.2	189	29.78	6.86	0.50	0.004

Table 4.8 Bivariate analysis of categorical variables associated with death due to CDI (as listed on the death certificate) within one year of the index episode

Variable		Death due to CDI within 1 year of index episode N	No CDI related mortality within 1 year of index episode N	Total N	p value (Fisher's exact test)
Gender	Male	9	109	118	0.837
	Female	19	190	209	
Immunosuppression	Absent	23	250	273	0.793
	Present	5	49	54	
Place of acquisition	CA	11	72	83	0.109
	HA	17	227	244	
Acid suppressants (PPI and H2 receptor antagonists)	Given	10	159	169	0.193
	Not given	13	115	128	
Underlying GI pathology	Not known to be present	15	165	180	1.000
	Present	13	135	147	
Deletion detected in *tcdC*	Absent	18	235	253	0.013
	Present	10	40	50	
Colonisation with MRSA or VRE prior to CDI	No	19	226	245	0.367
	Yes	9	73	82	
Invasive GI procedure in 12 weeks prior to CDI	No	27	238	265	0.039
	Yes	1	61	62	
Use of tazocin in eight weeks preceding illness	No	18	180	198	0.661
	Yes	7	92	99	
Use of clindamycin in eight weeks preceding CDI	No	25	265	290	1.000
	Yes	0	7	7	
Treatment of first episode	Metro	11	136	147	Likelihood ratio chi square p 0.121 Pearson's chi-square p 0.169
	Vancomycin	8	99	107	
	Metronidazole & vancomycin	3	14	17	
	No treatment	0	22	22	
	Palliative	4	20	24	
Antibiotics given 8 weeks prior to CDI	No	4	29	33	0.329
	Yes	21	251	272	
Presence of *cdtB*	No	20	145	165	0.014
	Yes	8	30	38	
Ribotype	078	5	22	27	0.088
	Non-078	23	253	276	
Norovirus co-infection	No	15	124	139	1.000
	Yes	1	12	13	

Table 4.9 Binary regression model for multivariable analysis of factors associated with CDI related mortality within one year of index episode

Variable	p value	Odds Ratio	95% CI	
			Lower limit	Upper limit
Age	0.098	1.03	0.99	1.07
Presence of *cdtB*	0.351	2.52	0.36	17.68
Presence *tcdC* deletion	0.408	0.46	0.07	2.89
GI procedure in 12 weeks preceding index episode	0.022	0.06	0.00	0.65
Total leucocyte count (cells $X10^9$/L)	0.022	1.06	1.01	1.12
Serum albumin level at onset (g/L)	0.024	0.91	0.83	0.99

4.2.1 Age and Gender

Patients compared in the severe and non-severe episodes did not differ significantly in age and gender. These factors were both different between patients who did or did not develop a severe episode of CDI (tables 4.1- 4.6). However, age was significantly associated with mortality due to CDI within one year of presentation on bivariate analysis (table 4.7). Advancing age has been found to be significantly associated with development of CDI, and as a risk factor for mortality. Some ambiguity does remain as conflicting results have been reported. A study compared 82 patients who survived and 46 patients who died within 30d of CDI and did not find a significant difference in age (>75 years) or gender (p value 0.11 for age and 0.93 for gender). They did however classify their patients by age stratification >75 years and hence if a difference existed in the overall age (including those under 75 years), this would not be detected. Conversely an American study compared the 30d mortality due to CDI and found age >80years significantly associated with mortality (OR 7.91 95% CI 3.31–18.89, p value 0.001), however there was no such association with gender. These differences are likely to be due to variations in methodology and the way age is analysed. Stratification of age is more likely to yield a significant result if end

points are determined where the difference exists in the populations, whereas studies using age as a continuous variable may miss the correlation (Bloomfield et al., 2012).

4.2.2 Charlson Comorbidity index

The Charlson comorbidity index was significantly greater in severe vs. non-severe episodes as well as in the patients who developed severe disease vs. those who did not (table 4.1, 4.3, 4.4, 4.6). This association was statistically significant in the multivariable analyses. However there was no difference observed between the CCI of patients who died due to CDI compared to those whose death certificates did not include CDI (table 4.7). A German study analysed factors likely to predict severe CDI (defined as profuse diarrhoea associated with a heart rate beats pm/systolic blood pressure mmHg >1.5 at initial diagnosis) and concluded that CCI was independently associated with the risk ($p<0.05$, OR 1.29; 95% CI 1.02-1.61) along with C-reactive protein (Hardt et al., 2008). Other studies, which have used the CCI, have also concluded that it is significantly different between the two groups, although the end point used to define severity and the cut-off score used vary between the studies (Das et al., 2010, Labbe et al., 2008, Cadena et al., 2010).

The Charlson score includes various co-morbidities and is generally used as an indicator to assess the risk of dying within one year. Since it includes various chronic conditions, it is useful as a combination indicator negating the need to assess each co-morbidity independently. However, it involves the analysis of 19 different parameters and hence its practical application as a routine marker of severity is unlikely.

4.2.3 Antibiotics

Antibiotics disrupt the normal flora of the gut allowing *C. difficile* to proliferate and cause CDI. In the past, analysis of colonisation factors associated with CDI (S-layer proteins, adhesins) has been performed and an up-regulation of these factors observed along with increased adhesion to Caco-2/TC7 cells under sub-inhibitory concentrations of antibiotics (Deneve et al., 2008). Other studies have reported a complex strain dependent relationship between antibiotics, effects of growth and

toxin production (Drummond et al., 2003). However clinical implications of these studies are not completely clear.

In the present study we compared the administration of antibiotics in the 8 weeks preceding CDI onset. This factor was found to be statistically significant in the bivariate comparison of severe and non-severe episodes and in patients who developed severe CDI at onset or within the follow up period compared to those who did not (table 4.1, 4.4). However the significance was not continued in the multivariable models. In addition, there was no statistical difference between those who dies due to CDI compared to those who did not die due to CDI. The use of antibiotics has been studied in the past but an American study (Morrison et al., 2011) also did not find a statistical correlation between its use and 30d mortality.

Each individual antibiotic was also analysed for risk associated with severity but only piperacillin-tazobactam (PT) and clindamycin were statistically associated with a difference between the severe and non-severe episodes. This association did not hold in the multivariable model.

In addition piperacillin-tazobactam was also different between those who developed a severe infection compared to those who did not, its use being more common among those who did not develop severe CDI as opposed to those who did (p value 0.000). However this association was lost on multivariable analysis (p value 0.239, OR 1.14, 95% CI 0.92-1.40). Analysis of other antibiotics was difficult due to the small numbers involved. Piperacillin-tazobactam has been widely thought of as a relatively safe antibiotic with reference to CDI, since compared to third generation cephalosporins, it is associated with reduced rates of CDI (Wilcox et al., 2004). Though it is known to reduce the number of gut commensals similar to any other antibiotic studies in the human gut model have shown that in the presence of piperacillin-tazobactam the *C. difficile* population tended to remain in spores and there was no appreciable increase in cytotoxin production (Baines et al., 2005). These findings are in keeping with the piperacillin-tazobactam results of the present study.

4.2.4 Predisposing factors

4.2.4.1 Total leucocyte count and serum albumin

These factors were compared between all three compared groups (table 4.1, 4.4 and 4.7) and were found to be statistically significant in all three. However since they were used to define severe disease in this study, they were not entered in the first two comparison groups' multivariable models to avoid creation of bias. They were analysed in the multivariable model of death due to CDI within one year and a high total leucocyte count and low albumin were correlated with CDI related mortality. Previous studies have examined the association of these markers using cut off values varying from >15cells X 10^9/L to >30cells X 10^9/L for total leucocyte count and from <20g/L to <30g/L for albumin, some also having used mean values (Dharmarajan et al., 2000, Gujja and Friedenberg, 2009, Andrews et al., 2003, Pepin et al., 2007). Although the criteria for outcome varied between the studies, a recent meta-analysis found that both these parameters were useful markers of severity to determine outcome (Bloomfield et al., 2012).

4.2.4.2 Invasive GI procedures and underlying GI pathology

The performance of an invasive GI procedure was significantly associated with the development of a severe CDI within the follow up period in the bivariate and multivariate models (table 4.5 and 4.6). However there was a negative correlation with death due to CDI related causes (table 4.8 and 4.9). The reason for this paradoxical phenomenon is not clear but may be due to patients being generally more unfit to undergo a GI procedure and hence more likely to die than those who are considered fit to undergo GI invasion. It is also possible that this may be a statistical artefact due to the low number of patients who died due to CDI.

4.2.4.3 Immunosuppression

In the present study, immunosuppression varied significantly between severe and non-severe episodes as well as between patients who developed severe infection as opposed to those who did not in the bivariate and multivariate analyses (tables 4.2,

4.3, 4.5 and 4.6). The significance was however not present in the one-year CDI related mortality (table 4.8), possibly due to paucity of numbers.

The definition of immunosuppression is not standardised and hence varies from study to study. For this reason it is difficult to compare directly the results of this study with those of others. This may also be a reason why there is heterogeneity in the reported significance of immunosuppression as a predictor of severity.

Liver transplantation has been reported to be associated with a higher odds of developing CDI (OR 2.88, 95% CI 2.68-3.10) (Ali et al., 2012). A study examining the outcome of CDI in peripheral stem cell transplant recipients did not have enough power to define predictive variables (Bilgrami et al., 1999). However, a large study comparing three groups of patients with CDI (without steroids), CDI with steroids and a non-CDI control arm suggested that the use of any glucocorticoid resulted in an increased mortality with a hazard ratio of 2.1+/-0.19 ($p<0.001$) comparing all patients who had CDI. Mortality at 30d was higher in the CDI with steroids group as compared to those without steroids (19.3 vs. 9.6%) (Das et al., 2010). Hence it is likely that immunosuppression is related to adverse outcome in CDI. This was however only partially detected in the present study as presence of immunosuppression (which included the use of glucocorticoids in the definition) predicted the severity of illness but was not associated with CDI related death.

4.2.4.4 Hospital associated acquisition and prior colonisation with MRSA/VRE

These factors were not found to be significantly associated in any of the above multivariate models. Since the definition of hospital acquisition has only recently been standardised, the studies which have examined this variable, have used a variety of definitions. This parameter has been discussed in greater detail in chapter five.

4.2.4.5 Antacids (including PPIs)

A statistically significant correlation was not found in the use of acid suppressant medication and the development of severe CDI in any of the above analyses.

4.2.5 Mortality

The mortality due to CDI was compared between patients who developed a severe episode and those who did not. Two outcome measure were used, 30d all cause mortality and death due to CDI within one year of CDI. Time to death from first episode in the study to death due to CDI ranged from 2-156 days after toxin positive result. Although the 30d mortality was not significantly different between the group that developed severe CDI and those who did (p value 0.531), there was a statistically significant difference between the CDI specific mortality within one year (p value 0.004). However, since 30d mortality by itself is an outcome and not a predictor of severe disease, it was not included in the multivariable model. This observation is interesting since most authors use 30d all cause mortality as an end point for severe CDI rather than CDI specific mortality. It is suggested that CDI certification on death is poor, hence the justification in sing 30d all cause mortality. The CDI specific mortality was studied further to compare the different variables associated with this outcome (table 4.7-4.9). As discussed earlier, the total leucocyte count and a low albumin were associated with a statistically significant correlation, whereas a GI procedure in 12 weeks preceding CDI was negatively correlated with the risk of death due to CDI.

It may perhaps be of use to pool 30d all cause mortality and all other CDI confirmed mortality into one comparator group and perform the above analyses to extract a stronger correlation. The figures 4.3 and 4.4 give the distribution of one-year mortality and 30d all cause mortality in the present study group.

Figure 4.3 One-year outcome of patients who developed a severe episode during the study

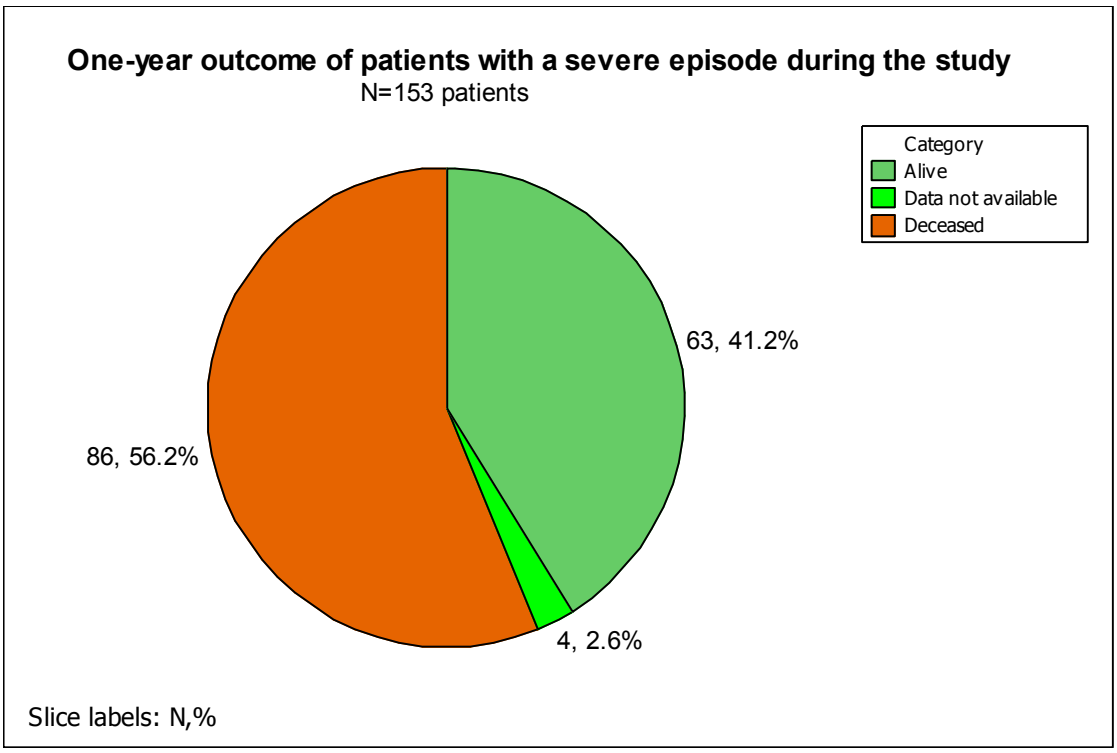
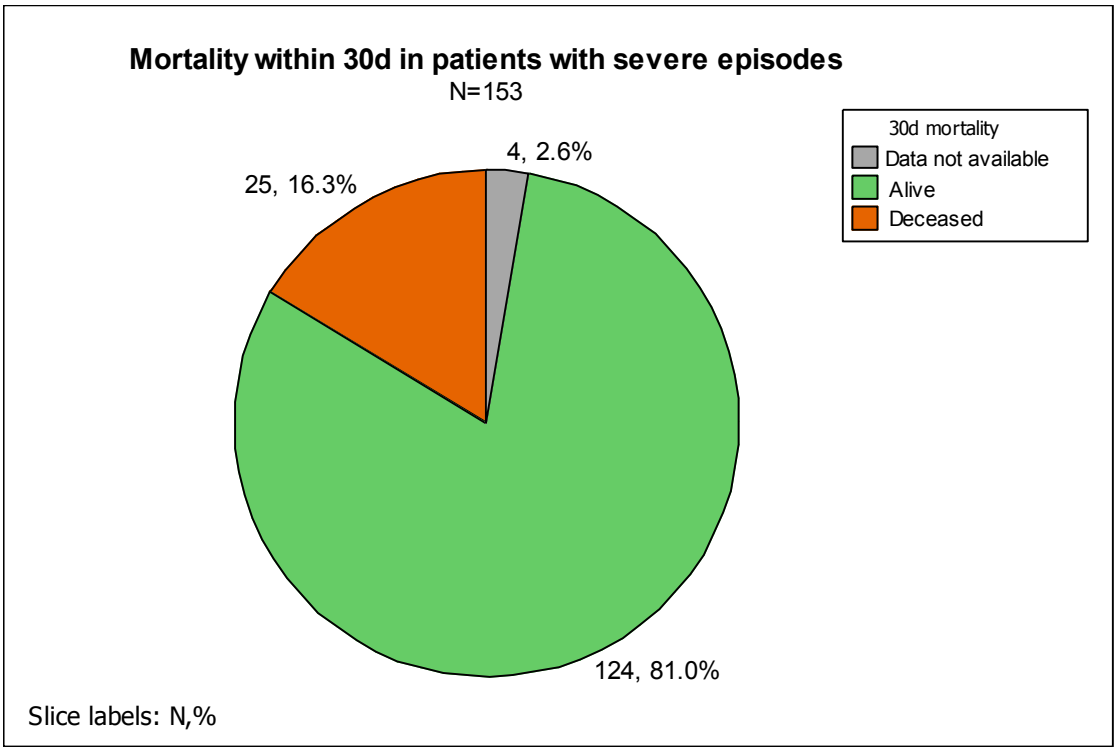


Figure 4.4 Thirty-day mortality in patients who developed a severe episode during the study



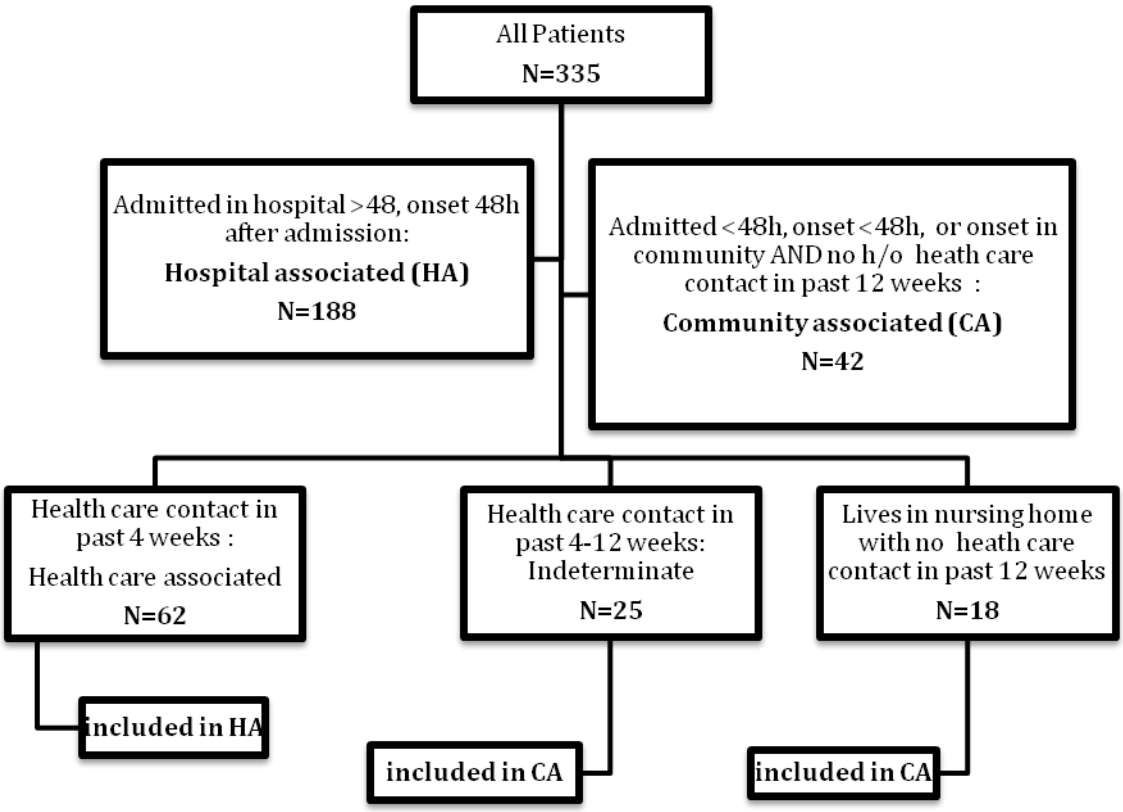
Chapter 5
Community associated CDI

5.1 Definitions

There is considerable variation in the definitions of CDI based on acquisition. However, to compare incidence rates and to gauge the efficacy of intervention strategies, (McDonald et al., 2007) proposed definitions to enable classification of CDI based on acquisition in the hospital and community setting, from which our definitions of hospital associated and community associated are derived. However, their definitions include an indeterminate classification where the symptoms develop in the community but there is a past history of admission in a heath care setting 4-12 weeks prior to the onset of symptoms. These definitions have been used in many subsequent studies (Khanna et al., 2012c, Kamboj et al., 2011), albeit with modifications. In the present study, the classification used by Khanna et al (2012b) was adopted and the following flowchart (figure 5.1) used to determine whether the onset was community associated or hospital associated.

A total of 250 patients was identified as developing hospital-associated acquisition of CDI and 85 patients were identified as community associated.

Figure 5.1 Flowchart showing the division of patients based on place of acquisition



h/o = history of

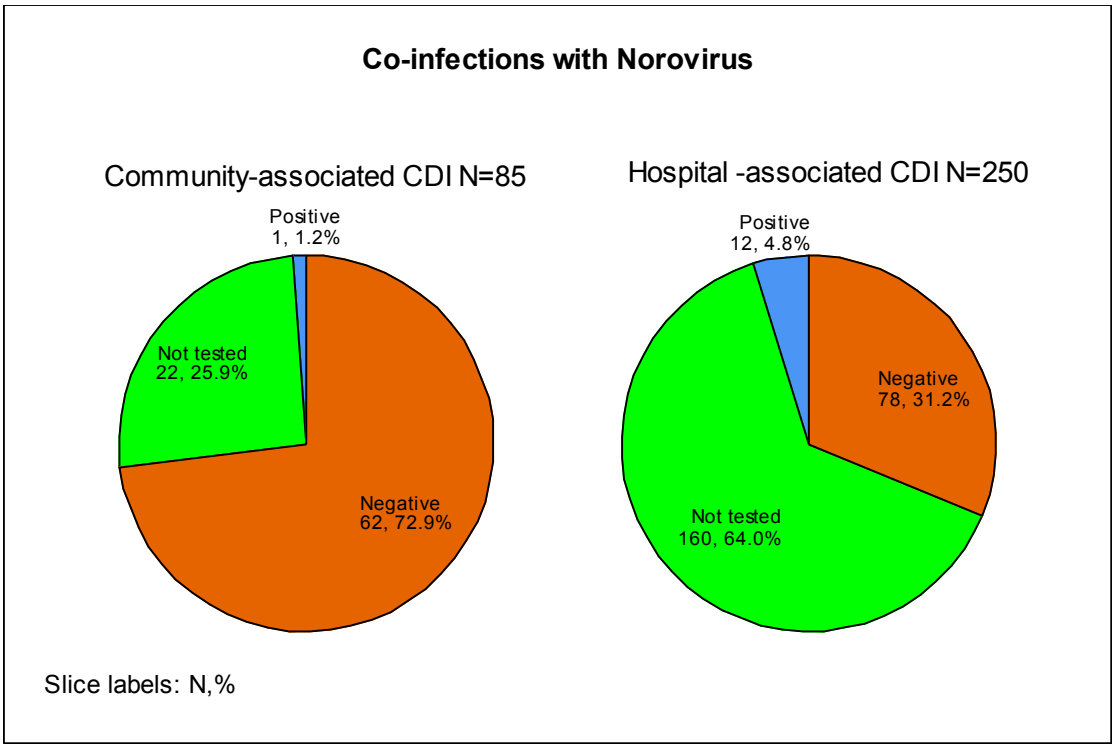
5.2 Association with co-infections

There is a possibility that diarrhoeal disease presenting in the community could have an aetiology different from CDI, although *C. difficile* toxin was detected in the stool. In such a situation it may be difficult to determine the actual cause of the disease. The incidence of community associated CDI could potentially be over-reported in case there is a substantial number of patients classified as CDI but also possessing other pathogens which could be the real cause of the diarrhoea. This is likely since *C. difficile* can be carried asymptomatically in the community whereas other pathogens like *Salmonella* and Norovirus would be less likely to be innocent bystanders.

5.2.1 Co-infection with Norovirus

In the present study 62 patients deemed to have CA-CDI were tested for Norovirus, however only one was found to be positive, giving an overall Norovirus co-infection rate of 1.2% among all patients with CA-CDI and 1.6% (1/63) among those which were tested (see figure 5.2). Comparatively, HA-CDI had a co-infection rate of 4.8% among all HA-CDI patients and 13.3% (12/90) among those which were tested and the difference was found to be statistically significant (p=0.015)when including the whole CA/HA-CDI population or only the tested population as the denominator. Although the numbers tested in the hospital were fewer, there was a significant proportion, which was found to be positive. This could be explained by the more frequent occurrence of Norovirus in the hospital setting as compared to the community. However, there are increasing reports of Norovirus occurring in the community. In Scotland, in 2010, there were at east two outbreaks of Norovirus, which were linked to food, and one was associated with contaminated oysters (HPS, 2011f). In the first quarter of 2012, there were five outbreaks of Norovirus, which occurred in the community (HPS, 2012). Hence community-associated Norovirus, though rare, does occur and has been consistently reported. However in this data set, there was only one patient who tested positive for Norovirus. This could be because patients were included in the study on the basis of *C. difficile* positivity. If *C. difficile* toxin was not requested on a sample, it may still have had Norovirus present in it, which in turn could have had a self limited course and not been tested. On the other it may have been tested for Norovirus , but not for CDI. Hence it is possible that a careful history taken from the patient can lead to good clinical suspicion of Norovirus as a potential cause of diarrhoea.

Figure 5.2 Patients co-infected with Norovirus.



5.2.2 Co-infections with other diarrhoeal pathogens

All stool samples were tested for bacterial causes of diarrhoea using selective media appropriate to travel history. The results are shown in table 5.1 One *Campylobacter* and two gastrointestinal *Salmonella* infections were found (3.6% bacterial co-infection rate). In the past, a study from Edinburgh evaluated diarrhoeic patients who presented to the infectious disease outpatients department and found that of the 19 patients from whom *C. difficile* was cultured, 4 had co-infections with non- typhoid *Salmonella* spp.(Brettle et al., 1982). It would appear that since asymptomatic carriage of non-typhoid *Salmonella* spp in the community is rare, the *C. difficile* could either be a true co-pathogen or asymptomatically carried. More recently, a questionnaire-based study of 58 CA-CDI cases reported 8.6% co-infection with *Campylobacter* and *Salmonella* (three *Campylobacter* spp. and one *Salmonella* spp.) (Fellmeth et al., 2010).

In the present study, patients with co-infections have been included in the statistical analysis. In addition, since these were a small proportion of the total sample (3.6% of all CA-CDI), the population defined as CA-CDI can be inferred to represents true CDI derived from the community

Table: 5.1 Co-infecting bacteria found in the study samples

Co-infecting bacteria	Community-associated N (%)	Hospital- associated N (%)	Total N (%)
Campylobacter spp	1(1.2)	3(1.2)	4(1.2)
Salmonella group B	0	1(0.4)	1(0.3)
Salmonella group C	1(1.2)	0	1(0.3)
Salmonella group D	1(1.2)	0	1(0.3)
Total samples	85	250	335

5.3 Comparison of CA-CDI and HA-CDI

The variables studied in this project were compared between CA and HA-CDI. The comparison of continuous and categorical variables is given in table 5.2 and 5.3. Table 5.4 contains the results of multivariate analysis of causative variable found to be significant on bivariate analysis.

Table 5.2 Bivariate analysis of continuous variables

Analysis done by paired t test

Variable	Community -associated				Hospital-associated				p value
	N	Mean	SD	SE Mean	N	Mean	SD	SE Mean	
Age (years)	85	62.7	23.8	2.6	250	69.1	19.5	1.2	0.029
Charlson Comorbidity index (age adjusted)	83	3.70	3.38	0.37	250	5.22	3.05	0.19	0.000
Total number of different antibiotics given in the eight weeks preceding onset	74	1.24	1.07	0.12	226	2.09	1.33	0.089	0.000
Total number of episodes on record	85	1.518	0.868	0.094	250	1.50	1.18	0.074	0.883
Total leucocyte count (cells $x10^9$/L)	53	14.80	9.47	1.3	226	12.93	7.79	0.52	0.186
Serum albumin level at onset (g/L)	45	31.20	7.39	1.1	174	28.80	6.65	0.50	0.052

Table 5.3 Bivariate analysis of categorical variables

Variable		Community-associated N(%)	Hospital-associated N(%)	Total N	p value (Fisher's exact test unless specified)
PCR Ribotype 078	Ribotype 078	11 (39.3)	17(60.71)	28	0.109
	Non Ribotype 078	74(24.1)	233(75.9)	307	
Severity of first episode	Severe	27(18.7)	117(81.3)	144	0.0141
	Non severe	55(31.3)	121(68.7)	176	
Antacids (PPI and H2 receptor antagonists)	Given	38(22.1)	134(77.9)	172	0.417
	Not given	34(26.2)	96(73.8)	130	
Treatment of first episode	Metro	45(30.2)	104(69.8)	149	0.047 (Pearson Chi square) 0.007 (Likelihood ratio Chi-square)
	Metro and vanc	0	19(100)	19	
	No abx given	7(29.2)	17(70.8)	24	
	Palliative	7(29.2)	17(70.8)	24	
	Vanc	23(21.3)	85(78.8)	108	
Underlying GI pathology	Not known to be present	51(27.6)	134(72.4)	185	0.315
	Present	24(22.7)	116(77.3)	150	
Inflammatory bowel disease	No	79(25.2)	234(74.8)	313	0.803
	Yes	6(27.4)	16(72.7)	22	
Immunosuppression	Absent	77(20.7)	201(72.3)	278	0.031
	Present	8(14.0)	49(86))	57	
Colonisation with MRSA or VRE prior to CDI or within follow up period	No	71(28.4)	1179(71.6)	250	0.019
	Yes	13(15.5)	71(84.5)	84	
Major GI procedure in 12 weeks prior to CDI	No	81(29.9)	190(70.1)	271	0.000
	Yes	4(6.3)	60(93.7)	64	
Urban Rural Index	Non rural	77(25.8)	221(74.2)	298	0.677
	Rural	7(20.6)	27(79.4)	34	
Death within 30d of CDI	No	69(24.5)	213(75.5)	282	0.392
	Yes	16(30.2)	37(69.8)	53	
Antibiotics given 8 weeks prior to CDI	No	20(60.6)	13(39.4)	33	0.000
	Yes	55(19.8)	223(80.2)	278	
Norovirus co-infection	No	62(44.3)	78(55.1)	140	0.015
	Yes	1(7.7))	12(92.3)	13	

Table 5.4 Multivariate analysis of causative factors found to be significantly related on bivariate analysis

Predictor variable	p value	Odds ratio	95% CI	
			Lower	Upper
Charlson Comorbidity Index (age adjusted)	0.072	1.11	0.99	1.24
Age	0.109	1.01	1.00	1.03
Major GI procedure in 12 weeks prior to CDI	0.000	7.39	2.44	22.35
Immunosuppression	0.103	2.39	0.84	6.80
Colonisation with MRSA or VRE prior to CDI or within follow up period	0.201	1.65	0.77	3.57
Total number of different antibiotics given in the eight weeks preceding onset	0.000	1.74	1.32	2.29

***MRSA/VRE colonisation was said to be present if either of these were detected any time prior to the development of CDI which could range from 2d to 10 years prior to CDI.**

5.3.1 Age

Average age (in years) was found to be significantly different between CA- and HA–CDI (62.7 years vs 67.1 years) on bivariate analysis. Although this difference remained apparent on multivariate analysis (OR >1, 95% CI 1, 1.03), the p value was no longer significant. This could suggest that the difference in age was small. Other studies have suggested that the average age of people presenting with CA-CDI is less than those with HA-CDI (Khanna et al., 2012c, Naggie et al., 2010, Naggie et al., 2011). This could reflect that fact that people admitted in hospital are generally older and have more comorbidities than people in the community.

5.3.2 Charlson Comorbidity index (CCI)

On bivariate analysis the mean age adjusted Charlson comorbidity index was found to be statistically significant (3.7 vs 5.2 p=0.000) but this difference too appears to diminish on multivariate analysis. Similar studies, which have evaluated the CCI, have also reported a significant difference (Khanna et al., 2012c, Khanna et al., 2012b) reflecting the fact that people with more comorbidities are likely to be admitted into a health care setting and acquire infection from there whereas healthier patients are more likely to acquire infection from the community.

5.3.3 Antibiotic usage 8 weeks prior to CDI

The administration of antibiotics in the eight weeks prior to CDI was recorded. In patients who were given antibiotics, the total number of different antibiotics given was also noted. Both these variables were found to be significantly different in the bivariate analysis and retained significance in the multivariate model. A number of studies have evaluated the association of antibiotics with CDI (Birgand et al., 2010, Wilcox et al., 2004, Wren et al., 2005, Starr et al., 2003). Interestingly, although CDI is often thought to be a disease almost exclusively associated with antibiotics use (Wilcox et al., 2004), this assumption has not been consistently proven. In Edinburgh 5 out of 18 patients who presented with CA-CDI did not have any history of antibiotics exposure (Brettle and Wallace, 1984, Brettle et al., 1982). More recently, systematic studies of CA CDI have also been reported CDI even in the absence of antibiotic exposure. In a Veteran's Affairs Medical Centre only 48% of CA-CDI patients had received antibiotics in the preceding 60d prior to symptoms (Naggie et al., 2010). Khanna et al (2012) reported that 22% of patients with CA-CDI did not have known exposure to antibiotics.

In this study, details of 20 patients who did not receive antibiotics 8 weeks prior to CDI were available. An equal number (six (30%) each) presented with severe and non-severe infection and seven were from GP practices and did not have severity markers evaluated). Hence it appears that there is a small proportion of patients who

present with CDI without any history of antibiotics exposure in the past 8 weeks, although they are also at risk of developing severe disease.

It is also worth considering the contribution of antibiotics given more than 8 weeks before CDI. Although this was not evaluated in the present study, recent evidence suggests that the effect of antibiotics on the normal flora of the gut may last for considerably longer than 8 weeks (Jernberg et al., 2010).

5.3.4 Association with relapsing and recurrent disease

In this study, no significant difference was found in the association of community-associated disease with the occurrence of multiple episodes. The study from Minnesota (Khanna et al., 2012c) did not find a significant difference between these two groups either (p=0.66). Hence it appears that both CA and HA CDI are equally at risk of developing future episodes of CDI.

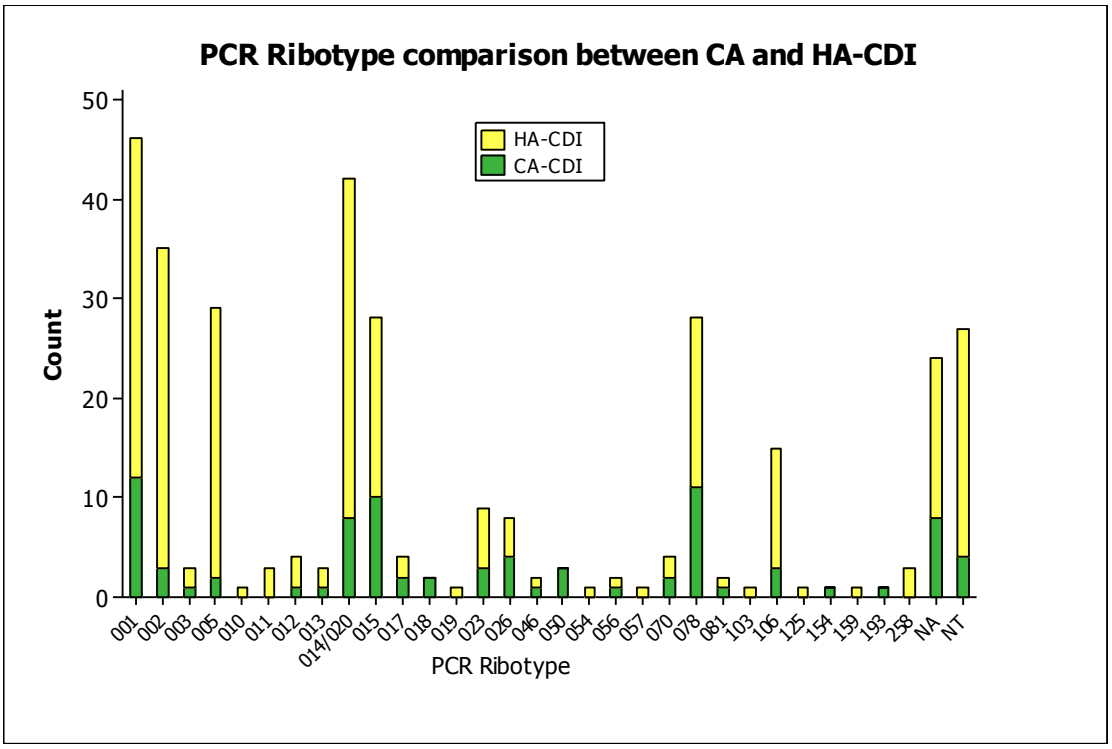
5.3.5 Total leucocyte count, serum albumin and severity of infection

In the present study, total leucocyte counts (TLC) and albumin levels were compared between the two group but neither difference was found to be statistically significant. However, only 63% and 53% patients of CA-CDI were tested for these markers, respectively. Other studies comparing these two categories are lacking, hence useful comparisons cannot be made. However, in our study there was a higher proportion of severe disease in HA-CDI as opposed to CA-CDI. This may explain why most patients presenting from the community were not tested for biochemical and haematological markers of severity. In a comparable study, there was no association between community acquisition and lack of severity if patients without haematological markers of severity were excluded from analysis. However, on including the untested patients (assuming they were not suffering from severe disease), there was a significant correlation. More studies with a greater sample size may be able to evaluate this association better.

5.3.6 PCR ribotype 078 and Urban Rural index

In the Netherlands, several studies have reported the association of PCR ribotype 078 with close contact with animals (Hensgens et al., 2012b, Goorhuis et al., 2008b, Debast et al., 2009). Other studies have found *C. difficile* of PCR ribotypes other than 078 in the stools of animals (Avbersek et al., 2011, Baverud et al., 1997). Hence it is a possibility that disease acquired in the community could be ribotype 078 or indeed some other ribotype. We examined the association between community acquisition and this ribotype (and ribotype 001) but there was no statistical association. The Urban – rural index differentiates postcodes as belonging to regions classified as rural, urban or intermediate. We examined the association of rural and non-rural residence with community acquisition with the hypothesis that rural areas are more likely to have farm animals (if farm animals predispose to CA-CDI). However no significant association was found. In a recent study, the rurality index was compared between cases of PCR ribotype 027, 078 and others and no significant association was found (Patterson et al., 2012). The distribution of ribotypes from CA-CDI and HA-CDI cases is given in figure 5.3

Figure 5.3 PCR ribotype distribution of CA and HA-CDI



NT: Not typeable NA: Sample not available

5.3.7 Antacids, underlying gastrointestinal pathology and inflammatory bowel disease (IBD)

The difference between CA and HA-CDI was evaluated for use of antacids (including proton pump inhibitors and H2 receptor antagonists), pre-existing gastrointestinal pathology (including inflammatory bowel disease functional and structural abnormalities of the GI tract, bowel carcinoma, other inflammatory conditions of the GI tract) and specifically inflammatory bowel disease. None of these were significantly different between the two populations. Most recent studies on CA-CDI have examined these factors, and two have found a significant difference between acid suppressants given to patients who develop CA-CDI vs those who develop HA-CDI (Khanna et al., 2012c, Naggie et al., 2010). However, a later case-control study by one of these two groups found no significant difference (Naggie et al., 2011). Hence there appears to be no consistent difference in acid suppressive use between CA and HA-CDI. This would suggest that patients residing in the community are just as likely to develop CDI secondary to acid suppressants as are patients admitted in hospitals. Further studies with a careful follow up with adequate sample size may be able to consolidate the hypothesis

5.3.8 GI procedure 3 months prior to CDI and co-infection with MRSA/ VRE

In this study, we compared whether a GI procedure was performed on the patients within 3 months of CDI and found a significant difference in both bivariate and multivariate models. It is possible that this difference was significant because of the underlying association with the definition of CA-CDI, wherein patients with a major GI procedure 4 weeks prior to CDI would automatically not be included in the CA category. However in spite of this bias, it appears that a GI procedure is likely to predispose a patient to develop HA-CDI. This would seem logical since, outpatients are less likely to have a GI procedure. Also patients who are admitted in hospital with a breach in the structural immunity of the gut in an atmosphere where *C. difficile* is present are more likely to be colonised and subsequently be symptomatic.

5.3.9 Treatment of first episode

The treatment given to patients who develop CA-CDI differed significantly in the bivariate analysis. This could be due to the difference in severity between the two groups, as Lothian *C. difficile* treatment protocols advise that non severe infections be treated with oral metronidazole and severe or recurrent infections with oral vancomycin with or without metronidazole.

5.3.10 Immunosuppression

In the present study, a significant difference was found between the two groups with reference to immunosuppressed status and administration of immunosupressants. Other studies have not evaluated this factor frequently, however a 2.7% incidence of immunosuppression was reported from the South of England (Fellmeth et al., 2010). The present study had 14% patients on immunosuppression, which although higher than the quoted study is significantly different from the hospitalised population who are in general likely to have a sicker population.

5.3.11 Death within 30d of CDI

There was no significant difference found between the likelihood of 30d mortality within the two groups in the present study, suggesting that the risk of death within 30d of CDI is equally likely whether CDI is acquired within the hospital or the community. The death certificates of the deceased patients were examined to look for the mention of *C. difficile* as a primary or contributory cause of death. CDI was listed on 43.7% death certificates of CA-CDI deaths and 21.6% HA- CDI related deaths. The difference was not found to be significant (p=0.182).

Chapter 6
Recurrent disease and analysis of sequential isolates

6.1 Recurrent disease

6.1.1 Selection of patients

In this section, an analysis of recurrent disease in the study population is presented. A total of 100 patients (29.9%) had more than one episode on record (see figure 6.1) and 19.4% experienced more than one episode (recurrence or relapse) in the duration of this study (see figure 6.2). Patients who had a documented episode in the past (N=35) before the commencement of this study were removed from analysis. In addition, since there is a mortality related bias among patients who do not develop recurrent disease (they may die before they get a chance to develop recurrence), all patients who died within 30d of development of CDI (N=53) were also removed from this analysis. An analysis based on removal of patients who died within 14d of first CDI was also considered (as used by Eyre et al, 2012), but this would not make a difference to the overall statistical inference from the present study as only one patient who died between 14 and 30d had developed a recurrent disease. Hence a total of 247 patients were included in the statistical analysis of this section (56 patients who developed more than one episode and 191 patients who had only one documented episode). Using this method, we had a recurrence rate of 22.9%.

Figure 6.3 graphically represents the proportion of patients with recurrent and non-recurrent CDI who died within 30d of the first episode included in the study (patients removed from statistical analysis).

Figure 6.1 Patients with multiple episodes on record (including those episodes before the commencement of the study)

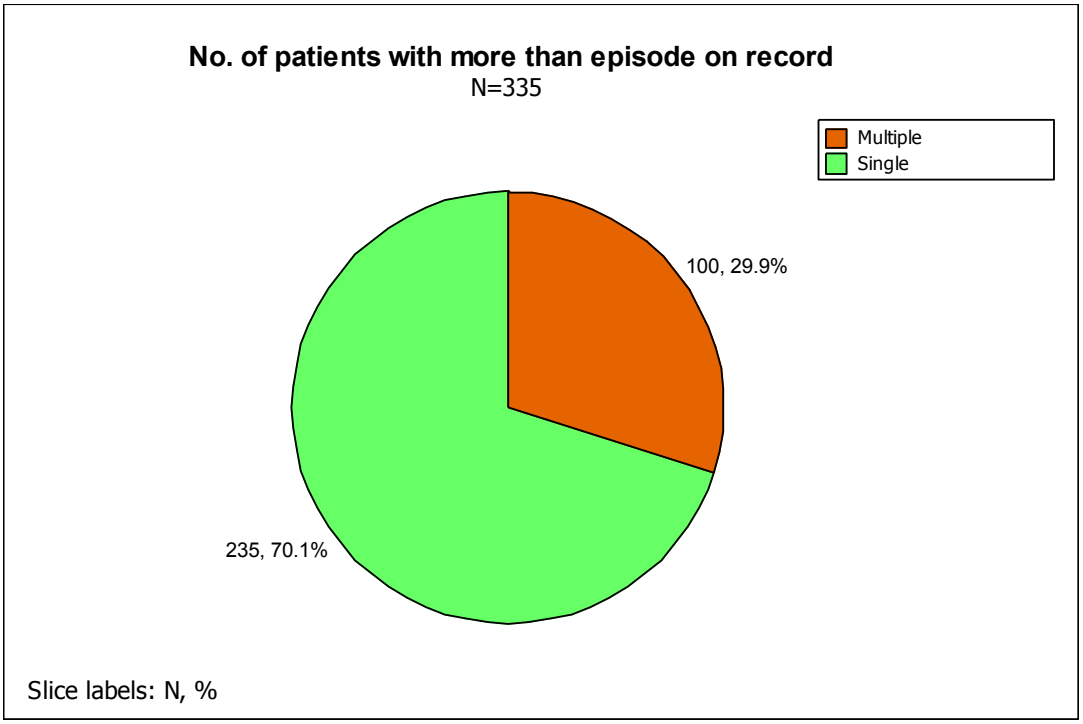


Figure 6.2 Patients who presented with multiple episodes in the duration of this study

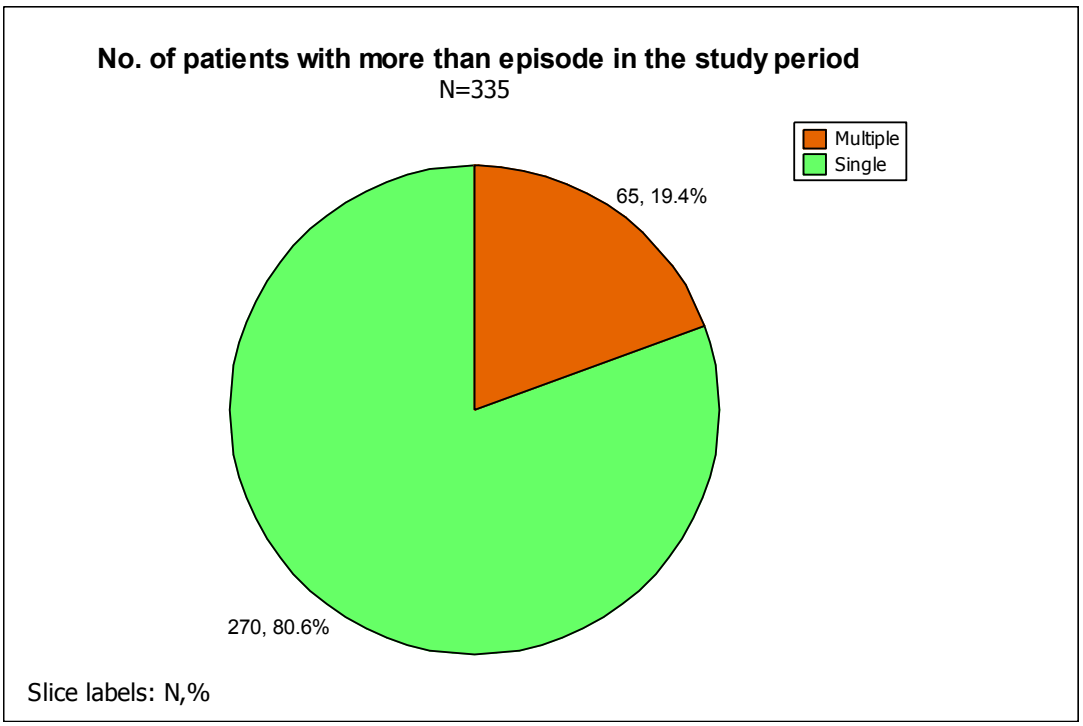
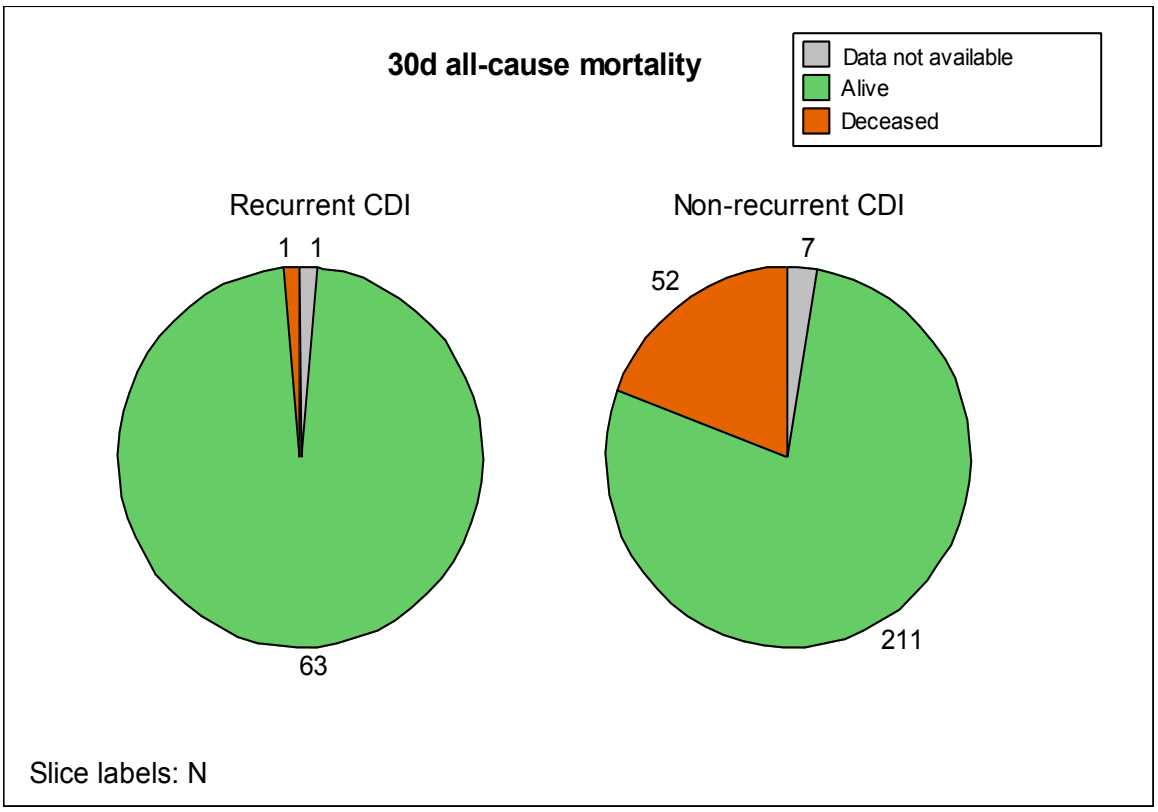


Figure 6. 3 All-cause mortality at 30d between patients with recurrent and non-recurrent CDI



These patients have been removed from statistical analysis

6.1.2 Statistical analysis of variables associated with recurrent and non-recurrent CDI

Tables 6.1 and 6.2 contain the bivariate analysis of continuous and categorical variables studied in this section. Multivariable analysis was attempted but there were insufficient statistically significant variables by bivariate analysis to establish a multivariate model.

6.1.2.1 Summary of results

Age and serum albumin at presentation were the continuous variables found to be statistically different between the groups that developed recurrent and non-recurrent disease (p values 0.02 and 0.045 respectively). Charlson co-morbidity index, administration of antibiotics eight weeks prior to the first episode and the total number of different antibiotics given prior to CDI were also trending towards a statistically significant value (p values 0.078, 0.089 and 0.092 respectively).

As also seen later in chapter 7, there appears to be a statistically significant difference between PCR ribotype 001 and endemic ribotype 078 in the occurrence of recurrent disease (p= 0.024). In addition, the one-year CDI related mortality among patients who survived the first 30d after CDI is greater in patients with recurrent disease than in those who did not develop a second episode. All other factors studied did not appear to have any statistically significant difference. Some of the factors found to be significantly associated in the present study and in similar analyses by other groups are discussed below.

6.1.2.2 Comparison with other published studies

A few studies in the past have analysed recurrent disease and some have tried to establish a predictive model for patients likely to develop a recurrent episode (Tal et al., 2002, Choi et al., 2011, Eyre et al., 2012, Alfa et al., 1999). These studies are discussed in the following sections.

6.1.2.3 Recurrence rates and risk score

One of the earlier studies on the topic, a prospective Canadian study by Alfa et al (1999), included 75 patients over two and half-year period. This study reported a 14.7% recurrence rate, which appears to be lower than that reported in the present study and others but they followed patients only up to 60d post cure. They did not find a statistically significant difference in age or co-morbidities between the recurrent and non-recurrent groups (probably due to the low power of the study to detect differences). However they did report a difference in duration of hospital admission before and after enrolment. Eyre et al (2012) reported a recurrence rate of 22%. They also examined past heath care exposure and found it significantly associated with the risk of developing recurrent CDI. They have included this variable in a proposed risk score for recurrent CDI. Unfortunately the present study did not evaluate the days before admission as a risk factor for recurrent CDI and may be recognised as a limitation of the study. These data are however available and can be evaluated at a later date. The effect of hospital or community-associated acquisition was however studied but not found to be statistically significant (p value 0.115 on bivariate analysis)

6.1.2.4 Total leucocyte count and serum albumin

The present study did not find an association between total leucocyte count and recurrence, however the albumin levels appeared to be statistically significant (see table 6.1). In the study of Choi et al (2012) the white blood cell count, the levels of serum albumin, and the levels of C-reactive protein at diagnosis were not significantly different between the recurrent and non-recurrent groups. Eyre et al (2012) studied 12 biomarkers in their study group but only higher C-reactive protein level and higher neutrophil count at first CDI independently appeared to increase the recurrence risk. Unfortunately neither neutrophil nor CRP were recorded in the present study. These markers are however available for many patients included in the study and can be evaluated at a later date.

6.1.2.5 Fever and gastric acid suppressing agents

An Israeli study published in 2002, followed up 43 patients who developed recurrent CDI and matched them with 38 patients who did not develop recurrent CDI (Tal et al., 2002). They found the use of H_2-receptor antagonists a statistically significant predictor of recurrent-CDI ($p \leq 0.02$), along with faecal incontinence and prolonged fever. In the present study, accurate documentation of fever was found to be difficult due to the multiple sites involved and hence has not been included in the analysis. The use of acid suppressing agents (proton pump inhibitors and H_2 receptor antagonists) although analysed in the present was not found to be statistically different between the two groups (p=0.334). The study of Choi et al (2012) examined gastric acid suppression but did not find it significantly associated with recurrence.

6.1.2.6 Immunosuppression

Although a study reported recurrent CDI in a patient with allogeneic stem cell transplant (Chang et al., 2012) and another has suggested that gastro-intestinal graft versus host disease is associated with an increased risk of recurrent CDI (AOR, 4.23; 95% CI 1.20-14.86; P = 0.02) (Alonso et al., 2012), the lower power in most studies does not allow this factor to be studied in details. A recent analysis of 1678 adults alive 14 days after their first episode of CDI did not find a statistically significant correlation between dialysis or chemotherapy and development of recurrent CDI (RR 1.15; 95% CI 0.46 – 2.82) (Eyre et al., 2012). Similar to this study, the present study

did not find a correlation between immunosuppression and risk of CDI. However since the definition of immunosuppression used in all of these studies is different from each other, there is still not enough evidence to support or refute the connection of immunosuppression with recurrence.

6.1.2.7 Other hospital-associated pathogens

Another group from Korea (Choi et al., 2011) evaluated a retrospective cohort of 84 patients with CDI and reported a recurrence rate of 13.1%, which is substantially lower than that reported in the present study and the others discussed earlier. Among the factors they studies, enteric colonization with VRE was found to be statistically significantly and correlated with the risk of developing recurrent CDI (odds ratio, 14.519; 95% confidence interval, 1.157-182.229; P = 0.038). The reason for this phenomenon is unclear though since VRE and *C. difficile* are both recognized hospital associated pathogens, the co-infection could be due to shared risk factors rather than a direct causative role. In the present study, the effect of co-infection with VRE and MRSA together was evaluated (table 6.2) but no statistically significant conclusion could be reached. Eyre et al (2012) also studied the effect of prior MRSA colonization and it appeared to be a possible factor in the uni-variable analysis but they did not find it a statistically significant predictor on the multivariable model (RR 0.7; 95% CI 0.31 – 1.59; p=0.39).

6.1.2.8 Association with antibiotics

In the same study above (Choi et al, 2011) patients who received more than three antibiotics were more common in the recurrent group as compared to the non-recurrent group (63.6% vs 22.4%, $p = 0.009$) but this association was not found after adjusting for confounders. In the present study administration of antibiotics eight weeks prior to CDI and the number of different antibiotics given were both trending towards significance but fell short (p values 0.078 and 0.092).

Table 6.1 Bivariate analysis of continuous variables compared between the patients with recurrent and non-recurrent CDI

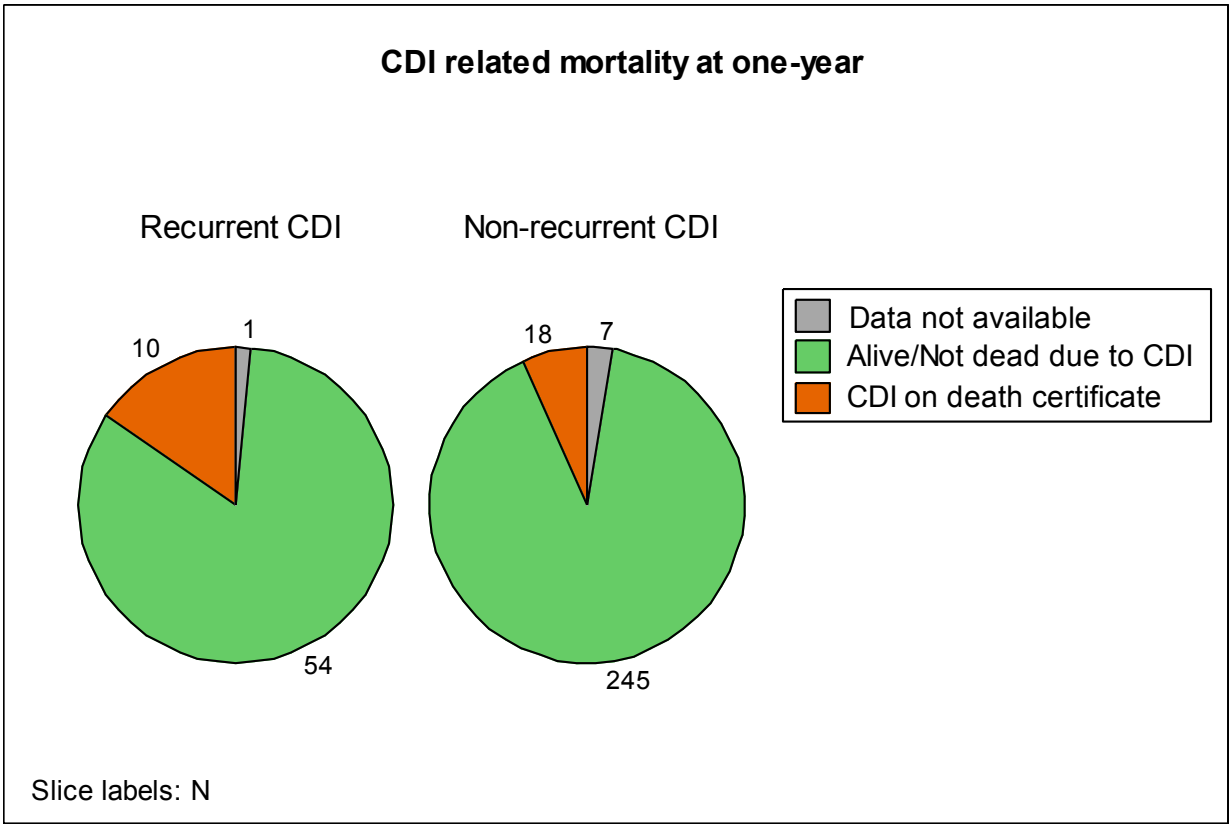
Variable	Recurrent CDI				Non- recurrent CDI				p value
	N	Mean	SD	SE Mean	N	Mean	SD	SE Mean	
Age (years)	56	69.9	18.8	2.5	191	62.8	22.5	1.6	0.020
Charlson co-morbidity index (age adjusted)	56	4.86	2.77	0.37	189	4.11	3.2	0.23	0.089
Total number of different antibiotics given eight weeks preceding onset	50	2.18	1.44	0.20	175	1.79	1.29	0.098	0.092
Total leucocyte count (cells $X10^9$/L)	49	13.8	7.47	1.1	155	12.21	7.33	0.59	0.198
Serum albumin level at onset (g/L)	35	28.11	5.7	0.97	123	30.49	7.13	0.64	0.045

Two-sample t-test without assuming equal variances was used for the above analysis

Table 6.2 Bivariate analysis of categorical patients between patients who developed recurrent and non-recurrent CDI

Variable		Recurrent CDI N	Non-recurrent CDI N	Total N	p value (Fisher's exact test)
Gender	Male	19	67	86	1.000
	Female	37	124	161	
Immunosuppression	Present	7	37	4	0.321
	Absent	49	154	203	
Place of acquisition	CA	18	42	60	0.155
	HA	38	149	187	
Antacids (PPI and H2 receptor antagonists)	Present	31	94	125	0.334
	Absent	19	82	101	
Underlying GI pathology	Present	28	80	108	0.288
	Absent	28	111	139	
Deletion detected in *tcdC*	Present	6	32	4	0.295
	Absent	48	144	192	
Colonisation with MRSA or VRE prior to CDI	Present	12	39	59	0.853
	Absent	44	151	195	
GI invasive procedure in 12 weeks prior to CDI	Performed	9	47	56	0.207
	Not performed	47	144	191	
Use of tazocin in eight weeks preceding illness	Yes	20	54	74	0.234
	No	30	122	152	
Severity of first episode	Non-severe	28	109	137	0.281
	Severe	27	75	102	
Treatment of first episode	Metronidazole	29	93	122	Sample size inadequate for statistical
	Vancomycin and Metronidazole	4	9	13	
	Vancomycin	20	61	81	
	No antibiotics given	3	17	20	
	Palliative	0	3	3	
Antibiotics given eight weeks prior to CDI	Yes	49	156	205	0.078
	No	2	24	26	
Presence of *cdtB*	Present	6	23	29	0.818
	Absent	48	153	201	
Ribotype	001	12	17	29	0.024
	078	2	18	20	
1-year mortality due to CDI	Yes	9	1	10	0.0000087
	No	46	183	229	

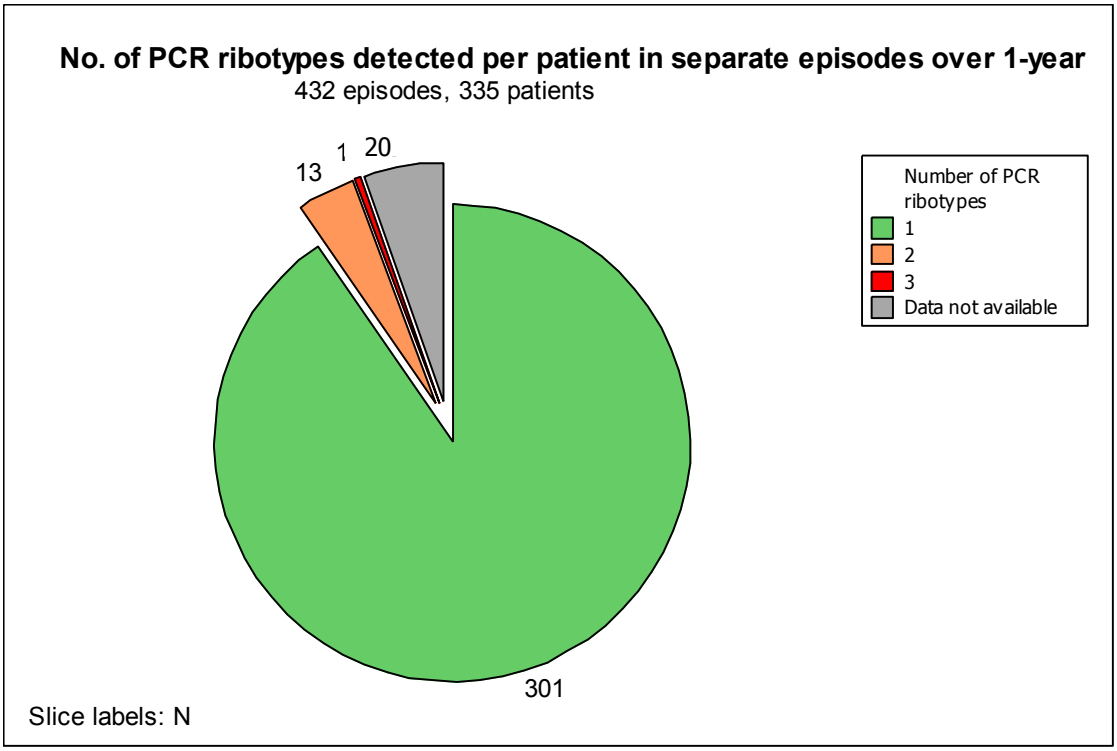
Figure 6.4 CDI related mortality: Comparison of patients with recurrent and non-recurrent CDI N=335



This figure includes patients who died within 30d of diagnosis. However this subgroup was not included in the statistical analysis.

6.2 Analysis of sequential isolates

Figure 6.5 Number of PCR ribotypes detected per patient



According to the Department of Health guidelines, a stool sample from an known case of CDI is not to be tested again within 28d of the original stool sample (HPA, 2008), it being considered a continuation of the same episode rather than a fresh one. This would however be an assumption that the strain infecting the patient within 28d would be the same as the one originally detected. The purpose of this part of the study was to investigate the changes in the strain type over time to determine whether the above hypothesis holds true.

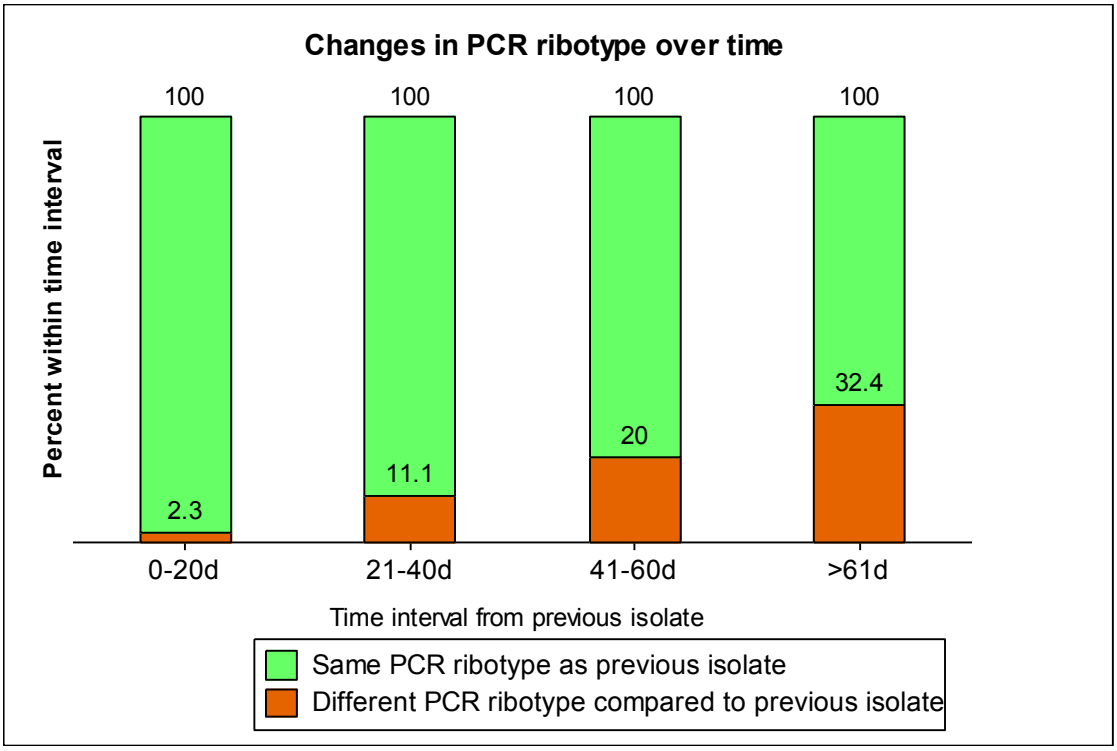
6.2.1 Isolates studied

One hundred and ninety four isolates from 79 patients were available for PCR ribotype analysis of changing epidemiological types. These isolates were stool samples obtained from patients collected prospectively during the course of the study, as well as those retrieved from stored samples from prior episodes (before the onset of this study). Of these, 167 isolates from 67 patients were available for MLVA analysis using the seven loci method (six loci for PCR ribotype 078).

6.2.2 Differences in PCR ribotype

Differences in PCR ribotype were observed in 2.3%, 11.11%, 20% and 32.4% isolates with time intervals between sampling of 0-20, 21-40, 41-60 and >60days respectively, see figure 6.1

Figure 6.6 Differences over time in PCR ribotype of isolates compared to previous isolates



This chart demonstrates a steady rise in the proportion of isolates, which were different as compared to the previous PCR ribotype isolated from the same patient when plotted against time. In the column 0-20d there was one isolate at 16d, in column 21-40d there were 3 isolates at 23, 26 and 27.

This result suggests that the arbitrary cut off of 28d to call a repeat infection a reinfection may be correct in the majority of cases where the PCR ribotype is the same, however there are some cases (4 in this study) where there is a different PCR ribotype isolated even before 28d. This must be kept in mind if a patient presents again with symptoms after a period of recrudescence or if the nature of illness changes from mild to severe, since this may be due to a reinfection.

On the other hand, there are a number of isolates obtained from the same patient even after 28d where the PCR ribotype is the same as the previous isolate. This

suggests that even though a patient may present with a fresh set of symptoms after 28d it may be the same episode and hospitals should not be considered liable for this new infection if it's the same strain being carried by the patient. If this new set of symptoms occurs in hospital, it is likely to be due to antibiotic usage or due to other predisposing factors, which may or may not be modifiable. However PCR ribotyping is not the most discriminatory method of epidemiological typing and more discriminatory techniques like MLVA or WGS may be needed to differentiate between strains.

An earlier study from 1997 (Wilcox et al., 1998) defined recurrence of *C. difficile* diarrhoea as the resumption of symptoms, after cessation for at least three days, with laboratory confirmed cytotoxin positive faeces. They analysed *C. difficile* isolates by RAPD analysis and found 56% of clinical recurrences of infection are in fact due to re-infection as opposed to relapse.

In a study comprising *C. difficile* isolates from 102 patients with repeat episodes of CDI (Kamboj et al., 2011), it was found that in those patients who had a second episode within 2-4 weeks of the original episode 90% had the same PCR ribotype, those who had a second episode 4-8 weeks apart had the same PCR ribotype in 86.5% cases and episodes separated by >8 weeks had the same PCR ribotype in 65% cases. Results from our study are roughly comparable with this recent study.

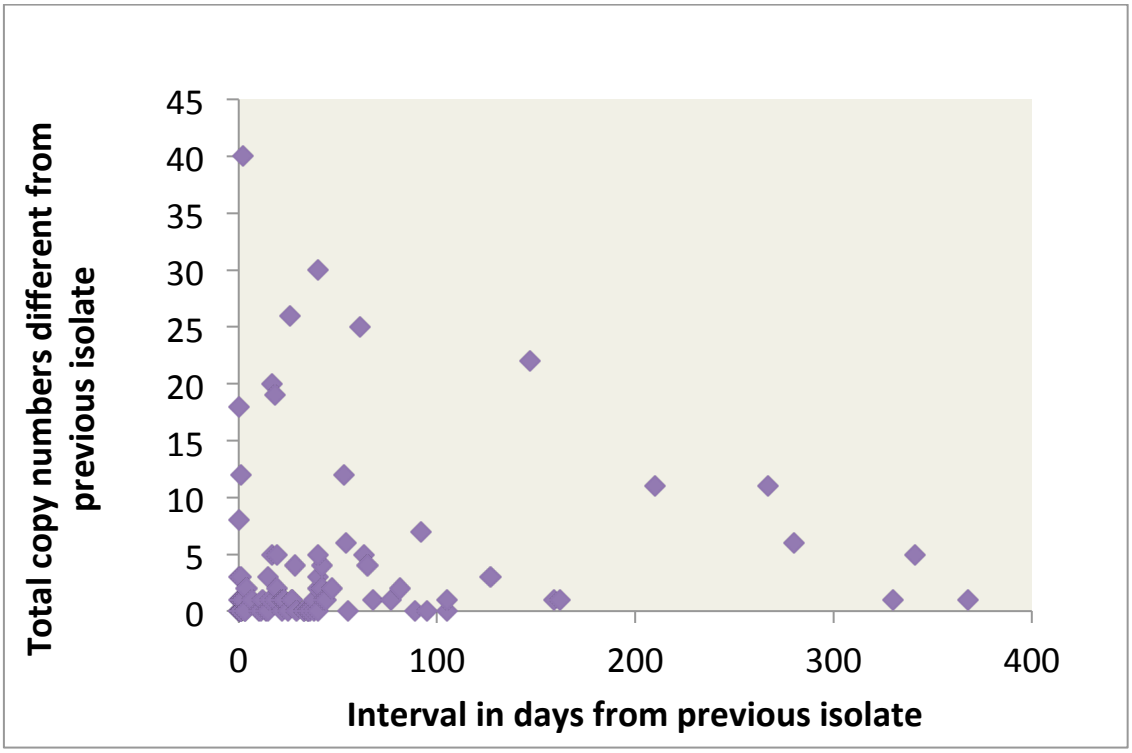
Another study which compared strains by serotyping from repeat episodes of CDI from patients found two different serogroups in 21.5% of patients and to the same serogroup in 78.5% of cases. PCR-ribotyping was used to discriminate the latter group showed a different pattern in 65.7% of cases. Their results suggest that 45 of 93 (48.4%) clinical recurrences were in fact due to reinfections with a different strain. Delay of relapse and reinfection were a median of 28 and 38 days (Barbut et al., 2000).

Keeping in mind that neither PCR ribotyping nor RAPD are the most discriminatory of typing methods (van den Berg et al., 2007); we performed MLVA using 7 loci (6

for ribotype 078), to see if there were further differences by using this more discriminatory method.

6.2.3 Differences in MLVA subtype

Figure 6.7: Temporal MLVA STRDs of isolates of the same PCR ribotype compared to previous isolates



This chart demonstrates differences in MLVA copy numbers of isolates obtained from patients with multiple isolates of the same ribotype when plotted against time. Each point on the graph represents the STRD difference between two consecutive isolates from the same patient. All isolates were obtained from symptomatic patients and in general those separated by more than 14d would be separate clinical episodes (recurrences) from the same patient. There appear to be large MLVA copy number differences in some isolates temporally close together, whereas isolates temporally far apart have fewer differences.

If each MLVA type were considered a different strain, then going by figure 6.6, it would be expected that there be a linear relationship between copy numbers different from previous isolates and time from previous isolate. However, this was not observed in the analysis (see figure 6.7). It was found that four isolates taken within 24h of the previous isolate had more than five copy number difference. There were

large copy number differences up to 147d from previous isolate. As time interval increased, STRD decreased (although the number of isolates studied was fewer as well).

There can be a number of explanations for this phenomenon. It could be that there were a number of different MLVA types infecting the patient and different isolates were picked up from different samples. A previous study has shown that 5 of 39 stool samples had more than one MLVA type (STRD of ≥5) of PCR ribotype 027 coexisting in one stool sample (Tanner et al., 2010). It could be an explanation for the variability seen in our study. However this would not explain why there was a larger STRD observed in isolates closer together in time as opposed to those further apart. It could be a possibility that since there were fewer isolates, which were further apart in time, the difference in STRD could be a sampling bias.

There is also a possibility that the MLVA technique is flawed and this variation was due to technical error. However the technique was validated on known isolates of PCR ribotype 001, PCR ribotype 106 and PCR ribotype 002. In addition, it is not a very likely possibility since the differing locus of most isolates showing discrepancy was repeated twice. MLVA has been shown to be a stable technique by a group, which studied serial, isolates from 20 patients and found STRD of ≤ 3 (Marsh et al., 2006). In another study *C. difficile* isolates were sub cultured over 10-30 passages and found a maximum of one STRD between the first and last isolate (van den Berg et al., 2007). Further analysis of these discrepant loci under differing PCR conditions may be needed. If the difference persists, whole genome sequencing may reveal clues on this phenomenon.

A later study (Eyre et al., 2012) analysed MLST data from two episodes each of 219 patients. Making comparisons on the basis of individual sequence types (ST), 169 (77%) recurrences had the same ST as the initial episode and 50 (23%) represented new STs, hence representing new infections. The risk for same-ST recurrence was highest at 14 days after the first CDT positive sample, but declined in the next 2–6 months. Conversely, the risk for new ST infections peaked 30 days after the first CDI and then decreased. However MLST has not been studied in the present study and

direct comparisons cannot be made. It would appear however, that these results are in keeping with the results from the present study where the risk of infection with the same PCR ribotype decreased after 60d from the previous isolate.

With the results of the present study, it appears that PCR ribotyping is a more robust method to study the changing epidemiology within a patient as compared to MLVA, since it is more stable and provides rapid answers. However, if there are only a few endemic types prevalent in a unit, PCR ribotyping would be unsuitable due to low discrimination. MLVA appears to be a useful technique to establish epidemiological linkages between strains suspected to be part of an outbreak as seen in chapter 7.

Chapter 7
Special features of PCR ribotype 078

7.1 General epidemiology of infections with PCR Ribotype 078 during the study

PCR Ribotype 078 has been identified in Scotland since 1996 (Taori et al., 2010). Subsequently, the incidence has been increasing steadily to 8% of all isolates in 2011 to the present time, where the Scottish reference laboratory reports a rate of 15% of all cases isolated from severe cases and outbreaks from January to March 2012 and 14% in the periodic surveillance (HPS, 2012).

In the present study, there were 28 patients (8.4% of total) who had PCR ribotype 078 identified as the first isolate. Only one of these patients suffered from two episodes, all others had a single episode in the one-year follow up period. However five other patients (included in the study) had previous episodes of infection, prior to the onset of this study. None of the cases patients were epidemiologically related to each other so they were considered endemic subtypes.

During the follow up of this study, an outbreak of PCR ribotype 078 occurred in Jan 2012. The MLVA subtype of these additional isolates was identical, so these were called the outbreak-related isolates. Initially four patients were affected, of who three died within four months of diagnosis. One patient survived but remained in hospital for a prolonged admission. In July 2012, an increased incidence of CDI was reported in a sister hospital where the surviving patient had been admitted for a short period. MLVA subtyping revealed three identical and two unrelated strains. Although identical by MLVA, these three patients were not directly related in time and space. However since they possessed an identical MLVA subtype, these additional cases were included in the analysis of the outbreak-related strains to determine whether this outbreak–related MLVA subtype was responsible for excessive mortality.

7.2 Comparison of cases infected with PCR ribotype 078, 001 and all other PCR ribotypes

To establish whether their features were similar to the other infecting PCR ribotypes, clinical features of patients infected with PCR ribotype 078 were compared to PCR ribotype 001 and all other PCR ribotypes (taken as a group). PCR ribotype 001 has been endemic in Lothian for many years (Mutlu et al., 2007, Taori et al., 2010) and has been consistently reported in Scotland even though its prevalence has gradually decreased from 17% to 10% over 2010 (HPS, 2011a). Their clinical and biochemical variables were compared and the results are given in table 7.1 and 7.2. Similarly a comparison of PCR Ribotype 078 with all other non 078, non 001 cases was made and the results are given in table 7.3 and 7.4. Multivariable multinominal regression analysis of variables which yielded a p value less than 0.05 is given in table 7.5.

Table: 7.1 Bivariate analysis of continuous variables associated with PCR ribotype 078 with PCR Ribotype 001

Variable	PCR ribotype 078				PCR ribotype 001				p value
	N	Mean	SD	SE Mean	N	Mean	SD	SE Mean	
Age (years)	28	65.7	21.2	4.0	46	77.1	15.9	2.3	0.017
Charlson Comorbidity index (age adjusted)	28	4.29	2.71	0.51	44	5.34	2.65	0.40	0.110
Total number of different antibiotics given in the eight weeks preceding onset	26	1.77	1.48	0.29	41	2.05	1.41	0.22	0.446
Grand total number of episodes	28	1.250	0.518	0.098	46	2.07	1.90	0.28	0.008
Total leucocyte count (cellsX10^9/L)	22	18.6	14.3	3.1	40	13.63	7.75	1.2	0.141
Serum albumin level at onset (g/L)	17	27.18	8.43	2.0	31	28.48	5.70	1.0	0.573

Table: 7.2 Bivariate analysis of continuous variables associated with PCR ribotype 078 with PCR ribotypes non 078/non 001

Variable	PCR ribotype 078				PCR ribotype non 001/ non 078				p value
	N	Mean	SD	SE Mean	N	Mean	SD	SE Mean	
Age (years)	28	65.7	21.2	4.0	237	65.4	21.3	1.4	0.950
Charlson Comorbidity index (age adjusted)	28	4.29	2.71	0.51	237	4.78	3.39	0.22	0.384
Total number of different antibiotics given in the eight weeks preceding onset	26	1.77	1.48	0.29	209	1.89	1.29	0.089	0.705
Grand total number of episodes	28	1.250	0.518	0.098	237	1.422	0.892	0.058	0.137
Total leucocyte count (cells$X10^9$/L)	22	18.6	14.3	3.1	198	12.63	7.22	0.51	0.066
Serum albumin level at onset (g/L)	17	27.18	8.43	2.0	152	29.89	6.93	0.56	0.216

Table: 7.3 Bivariate analysis of categorical variables associated with PCR ribotype 078 with PCR Ribotype 001

Variable		PCR ribotype 078 N (%)	PCR ribotype 001 N (%)	Total N	p value (Fisher's exact test unless specified)
Gender	Male	10 (41.7)	14 (58.3)	24	0.798
	Female	18 (36)	32 (64)	50	
HA/HCA/CA	HA	7 (17.5)	33 (82.5)	40	0.000 by Pearson Chi-Square & 0.000 by Likelihood Ratio Chi-Square tests
	HCA	10 (90.9)	1 (9.1)	11	
	CA	11 (47.8)	12 (52.2)	23	
Severity of first episode	Severe	15 (44.1)	9 (55.9)	34	0.343
	Non severe	13 (32.5)	27 (67.5)	40	
Antacids (PPI and H2 receptor antagonists)	Given	14 (42.4)	19 (57.6)	33	0.454
	Not given	11 (32.4)	23 (67.5)	34	
Treatment of first episode	Metronidazole	14 (41.2)	20 (58.8)	34	Pearson Chi-Square p=n/a Likelihood Ratio Chi-Square p=n/a
	Metronidazole &Vancomycin	3 (37.5)	5 (62.5)	8	
	No antibiotics given	1 (550)	1 (50)	2	
	Palliative	1 (16.7)	5 (83.3)	6	
	Vancomycin	9 (40.9)	13 (59.1)	22	
Underlying GI pathology	Not known to be present	10 (26.3)	28 (73.7)	38	0.054
	Present	18 (50)	18 (50)	36	
Inflammatory bowel disease	No	26 (36.6)	45 (63.4)	71	0.553
	Yes	2 (66.7)	1 (33.3)	3	
Immunosuppression	Absent	26 (40)	39 (60)	65	0.468
	Present	2 (22.2)	7 (77.8)	9	
Colonisation with MRSA or VRE prior to CDI or within follow up period	No	23 (43.4)	30 (56.6)	53	0.184
	Yes	5 (25)	15 (75)	20	
GI procedure in 12 weeks prior to CDI	No	20 (31.8)	43 (68.3)	63	0.016
	Yes	8 72.7)	3 (27.3)	11	
Urban Rural Index	Non rural	23 (35.9)	41 (64.1)	64	0.248
	Rural	5 (62.5)	3 (37.5)	8	
Death within 30d of CDI	No	21 (38.2)	34 (61.8)	55	1.0
	Yes	6 (37.5)	10 (62.5)	16	
Antibiotics given 8 weeks prior to CDI	No	3 (33.3)	6 (66.7)	9	1.0
	Yes	23 (37.7)	38 (62.3)	61	

Table: 7.4 Bivariate analysis of categorical variables associated with PCR ribotype 078 with non 001/non 078 PCR Ribotypes

Variable		PCR ribotype 078 N (%)	PCR ribotype non 001/ non 078 N (%)	Total N	p value (Fisher's exact test unless specified)
Gender	Male	10 (10)	90	(90)	1.0
	Female	18 (10.9)	147 (89.1)	165	
HA/HCA/CA	HA	7 (4.9)	135 (95.1)	142	0.006 by Pearson Chi-Square & 0.005 by Likelihood Ratio Chi-Square tests
	HCA	10 (17.2)	48 (82.8)	58	
	CA	11 (16.9)	54 (83.1)	65	
Severity of first episode	Severe	15 (13.2)	99 (86.8)	114	0.423
	Non severe	13 (9.6)	123 (90.4)	136	
Antacids (PPI and H2 receptor antagonists)	Given	14 (10)	126 (90)	140	0.831
	Not given	11 (11.1)	88 (88.9)	99	
Treatment of first episode	Metronidazole	14 (11.4)	109 (88.6)	123	Pearson Chi-Square p= 0.594 Likelihood Ratio Chi-Square p= 0.606
	Metronidazole and Vancomycin	3 (21.4)	11 (78.6)	14	
	No antibiotics given	1 (5)	19 (95)	20	
	Palliative	1 (5.9)	16 (94.1)	17	
	Vancomycin	9 (10.8)	74 (89.2)	83	
Underlying GI pathology	Not known to be present	10 (7.0)	132 (93)	142	0.07
	Present	18 (14.6)	105 (85.4)	123	
Inflammatory bowel disease	No	26 (10.6)	219 (89.4)	245	1.0
	Yes	2 (10)	18 (90)	20	
Immunosuppression	Absent	26 (12.0)	190 (88.0)	216	0.125
	Present	2 (4.1)	47 (95.9)	49	
Colonisation with MRSA or VRE prior to CDI or within follow up period	No	23 (11.3)	180 (88.7)	203	0.637
	Yes	5 (8.1)	57 (91.9)	62	
GI procedure in 12 weeks prior to CDI	No	20 (9.6)	189 (90.4)	209	0.329
	Yes	8 (14.3)	48 (85.7)	56	
Urban Rural Index	Non rural	23 (9.8)	212 (90.2)	235	0.209
	Rural	5 (17.2)	24 (82.8)	29	
Death within 30d of CDI	No	21 (9.6)	198 (90.4)	219	0.395
	Yes	6 (15)	34 (85)	40	
Antibiotics given eight weeks prior to CDI	No	3 (12.5)	21 (87.5)	24	0.729

Table 7.5 Multivariable multinominal regression analysis of variables with P values <0.05 in the bivariate analysis. PCR Ribotype 078 set as baseline

Variable		PCR Ribotype 078 vs Others				PCR Ribotype 078 vs 001			
		p value	OR	95% CI		p value	OR	95% CI	
				Lower limit	Upper limit			Lower limit	Upper limit
Grand total number of episodes		0.097	2.27	0.86	5.97	0.013	3.70	1.31	10.42
Presence of Underlying GI Pathology		0.986	0.99	0.36	2.71	0.124	0.35	0.09	1.33
Healthcare-associated acquisition	HA	0.002	9.80	2.34	41.10	0.005	10.91	2.03	58.69
	HCA	0.826	1.14	0.35	3.74	0.085	0.11	0.01	1.35
GI procedure in preceding 12 weeks		0.402	0.61	0.19	1.93	0.064	0.16	0.02	1.11
Age		0.434	0.99	0.96	1.02	0.103	1.03	0.99	1.08

7.2.1 Age

The comparison between PCR ribotype 078 cases and those with PCR ribotype 001 revealed a significant difference in the age of the patients between these groups (p=0.017), although this difference did not exist when PCR ribotype 078 (median 71.5 years, range 11-91 years) cases were compared with the non 001/non078 PCR ribotypes (median 72 years, range 2-96 years). On multivariable analysis the difference in the former remained but the association was weaker (OR 1.03, 95% CI 0.99-1.08) perhaps due to the limited numbers in the study. A similar study from Ireland compared PCR ribotype 078 with PCR ribotype 001 and PCR ribotype 027 and did not find any significant differences between either of the two groups (p=0.96)(Patterson et al., 2012). However another study from the Netherlands had earlier found that the population affected by PCR ribotype 078 (N=58) was significantly less likely to be older than that infected by PCR ribotype 027 (OR 0.57, 95% CI 0.33–0.98 for age >85 on multivariate analysis)(Goorhuis et al., 2008a). From their study it appears that patients with PCR ribotype 078 are likely to be

younger but still among the elderly group (>65years). The results from the present study also appear to suggest a similar result, however the comparator group is a different PCR ribotype. The present study and the Irish study did not have enough numbers to stratify according to age.

7.2.2 Recurrent/relapsing disease

When compared to disease caused by PCR ribotype 078, PCR ribotype 001 appeared to cause more number of episodes (p=0.013, OR 3.70 95% CI 1.31-10.42 on multivariable analysis). However this phenomenon does not exist between PCR ribotype 078 and non-078/non 001 PCR ribotypes. Goorhuis et al (2008) found that PCR ribotype 027 is likely to cause recurrent disease compared to PCR ribotype 078, but their analysis did not include comparison with PCR ribotype 001. Similar to the present study, they found no difference in the recurrent disease caused between PCR ribotype 078 and other PCR ribotypes taken together. When the first report of an Irish outbreak from 2008 came out (Burns et al., 2010), descriptive analysis of the outbreak suggested that PCR ribotype 078 in the outbreak caused many more recurrences (46% patients developed recurrent disease) than that suggested in the Dutch study (15% recurrence) (Goorhuis et al., 2008a). However, the latter study described the characters of the disease in an endemic setting whereas the former were observations during an outbreak.

There could be an alternative reason for the observed difference in the present study. It could be that PCR ribotype 078 causes very severe disease and leads to mortality; hence the bacterium does not have an opportunity to cause recurrent infection. There was no significant difference in the 30d mortality between PCR ribotype 078 and PCR ribotype 001 in this study. However further comparing the 30d mortality, 3/10 (30%) patients who died within 30d with PCR ribotype 001 had CDI listed on the death certificate and these had an average Charlson comorbidity index of 2.8. Whereas 5/6(83.3%) patients who died within this time with PCR ribotype 078 had CDI listed on the death certificate. cause (p=0.12). This latter group had a mean CCI of 8.7. Hence, although the numbers are too small for strong statistical correlation, it appears that the patients who died from PCR ribotype 078 within 30d had a lesser risk of dying from co-morbidities than the counterparts with PCR ribotype 001.

Hence at least in those patients, PCR ribotype 078 caused more severe disease than PCR ribotype 001.

7.2.3 Association with severity and mortality

Since *C. difficile* was observed to cause increased mortality in the early 2000s, there was a simultaneous increase observed in the presence of PCR ribotype 027. Studies have looked into the virulence factors of this type and found that it produces more toxin than others in the exponential and stationary phase of growth (Vohra and Poxton, 2011a).

Due to the 38-nucleotide deletion in the *tcdC* gene of PCR ribotype 078, earlier researchers hypothesised that this strain had the potential to cause disease similar to PCR ribotype 027 which had an 18bp deletion in *tcdC* (Goorhuis et al., 2008a, Curry et al., 2007, Jhung et al., 2008).

In this study the severity or 30d mortality caused by endemic PCR ribotype 078 strains was not found to be significantly different from the disease caused by PCR ribotype 001 or other non-078/non 001 PCR ribotypes. There have been mixed reports from various groups regarding the association of this PCR ribotype with severity. Comparison between studies was difficult because each study used different criteria for grading severity.

In 2008, a hospital-associated outbreak of PCR ribotype 078 occurred in Ireland where 15 patients developed CDI due to this PCR ribotype. Of these, four were classified as severe using criteria very similar to this study. Eight of these patients died of which five (62%) were due to causes directly related to CDI (Burns et al., 2010). A subsequent systematic case-case study, also from Ireland compared the features of PCR ribotype 078 (N=29) with PCR ribotype 001(N=43) and PCR ribotype 027(N=42) and found no significant differences in the 30-day mortality caused by the former as compared to either of the two latter PCR ribotypes (RRR 2.94, 95% CI 0.84-10.30 and RRR 1.88, 95% CI 0.49-7.17) respectively (Patterson et al., 2012).

A group from the Netherlands reported a case series of 13 patients with CDI due to PCR ribotype 078. Of these 69% had severe diarrhoea (though the criteria for defining severity was not disclosed). Of these two patients died within 30d of developing CDI and one death (8%) was attributable to CDI (Goorhuis et al., 2008b). The same group in a later publication compared 1687 patients (of which 9% were PCR ribotype 078) for severity, recurrence, hospital acquisition, mortality and antibiotic use compared to PCR ribotype 027 and all other PCR ribotypes. No significant difference was found in the severity of CDI neither between PCR ribotype 078 and other PCR ribotypes nor between PCR ribotype 027 and PCR ribotype 078 (Goorhuis et al., 2008a).

An American study compared the effect of PCR ribotype 027/078 on severe disease and found that although on the initial analysis there appeared to be a correlation (OR 2.33, CI 1.03, 5.02, p=0.035), this relationship disappeared on multivariate analysis when the other 11 covariates were accounted for (OR 0.82, CI 0.07, 10.0, p=0.874)(Walk et al., 2012). However their definition of severity was different from that used in this study, and included only three criteria (intensive care unit admission, interventional surgery, or death within 30 days of diagnosis). In addition, they compared PCR ribotypes 078 and PCR ribotype 027 together and there is an additional bias, which makes direct comparison with the results of the present study difficult.

Hence although there is suspicion that PCR ribotype 078 may cause severe disease, this association has not been proven by any published study so far.

In addition, in a recent study, the 18-nucleotide deletion in the *tcdC* of PCR ribotype 027 was restored and the naturally occurring intact *tcdC* of strain 630 was deleted and then subsequently restored using reverse genetics to determine changes in toxin production. However no difference was observed suggesting that a deletion in the negative regulator may not affect the production of toxin (Cartman et al., 2012). Also observed was the phenomenon that the expression of *tcdC* does not decrease in the stationary phase of growth, hence its functional contribution to toxin suppression is doubtful (Vohra and Poxton, 2011a).

In whole genome analysis experiments of virulent strains of PCR ribotype 027 and PCR ribotype 078, gene inserts have been revealed in addition to virulence factors which can also be found in related PCR ribotypes (Knetsch et al., 2011). Analysis of the clinical features related to these genetically distinct strains might reveal genetic markers of severity.

7.2.4 Association with community acquisition

In the present study, PCR ribotype 001 and other non-078/non 001 PCR ribotypes were more likely to be hospital associated than the endemic cases of PCR ribotype 078 on multivariable analysis (p=0.002 OR 9.80 95% CI 2.34-41.10 for PCR ribotype 078 vs others and p=0.005 OR 10.9 955 CI 2.03-58.69 for PCR ribotype 078 vs PCR ribotype 001; see table 7.2, 7.4 and 7.5). In the previously quoted study from Ireland (Patterson et al., 2012) a higher risk of community acquisition was reported for patients infected with PCR ribotype 078 compared to PCR ribotype 001 or PCR ribotype 027 (risk was 96% and 62% higher respectively for community compared with inpatient specimens : RRR 1.62; 95% CI: 0.53-4.97; and RRR 1.96; 95% CI: 0.62-6.18, respectively).

It is interesting to note that all studies that have compared the acquisition of PCR ribotype 078 have linked it to community sources. The question remains where CA-CDI is acquired. A suggested hypothesis is that humans acquire it from animals and animal products in addition to other vegetable products like salads. The association with animals was suggested by the isolation of PCR Ribotype 027 from a dog following a visit to the hospital (Lefebvre et al., 2006). In addition, MLVA analysis of 11 porcine and 21 human isolates of PCR Ribotype 078 isolates from the Netherlands suggested they were genetically related and revealed a clonal complex which contains both porcine and human isolates (Debast et al., 2009). This suggests that there are sources in the community, which may serve as a reservoir from which humans can acquire infection. However, till date no study has been able to irrefutably define the relationship between CDI due to PCR ribotype 078 and its acquisition from community sources like animals or animal products (Bakri et al., 2009b, Hopman et al., 2011a). In addition since other PCR ribotypes like PCR ribotype 027 and PCR ribotype 015 (Rodriguez-Palacios et al., 2006, Songer et al., 2009, Hopman

et al., 2011b) have also been found in animals and meat products, the significance of its presence in animals appears to be of zoonotic acquisition as an additional mode of infection in general rather than specifically related to PCR ribotype 078.

Clinical epidemiological analysis of the 2008 Irish outbreak of PCR ribotype 078 suggested that most patients had contact with either a hospital or a nursing home, however on molecular analysis a few clones were discovered where the origin could not be established (Burns et al., 2010). This finding suggests that molecular analysis along with careful history taking is needed to define the relationship with community acquisition.

7.2.5 GI surgical procedure in 12 weeks prior to CDI and underlying GI pathology

Although there appears to be a link between handling of the GI tract (GI surgical procedure) in 12 weeks prior to the development of CDI, this association reached statistical significance only in the bivariate analysis suggesting that PCR Ribotype 078 infections have a greater chance of being preceded by a GI procedure. This correlation was lost in the multivariable analysis suggesting that it could be a confounded by other factors or the sample size was too small to make conclusions. However it would be interesting to study further whether this PCR ribotype has an affinity for damaged GI tract. No other study appears to have analysed this factor although comparisons with digestive diseases and malignancy in general are available (Goorhuis et al., 2008a). Similarly, the presence of an underlying GI disease was trending towards significance (p=0.06) but this trend further diminished in the multivariate analysis.

7.3 Endemic and outbreak-related isolates: Variation within isolates of PCR ribotype 078

As reported in section 2.1, there were 28 endemic isolates from the original study and six outbreak-related strains. The latter appeared to have characteristics suggestive of more severe and recurrent disease as opposed to the PCR ribotype 078 seen in the endemic setting.

Since there were only six outbreak-related strains, there was insufficient numbers to perform statistical tests of significance. These were however attempted, but none of the variables tested reached statistical significance. Hence a descriptive analysis of the endemic and outbreak-related strains is given in the following figures (figures 7.1-7.12).

Figure 7.1 Box plot of age distribution of patients infected with endemic and outbreak-related strains of PCR ribotype 078.

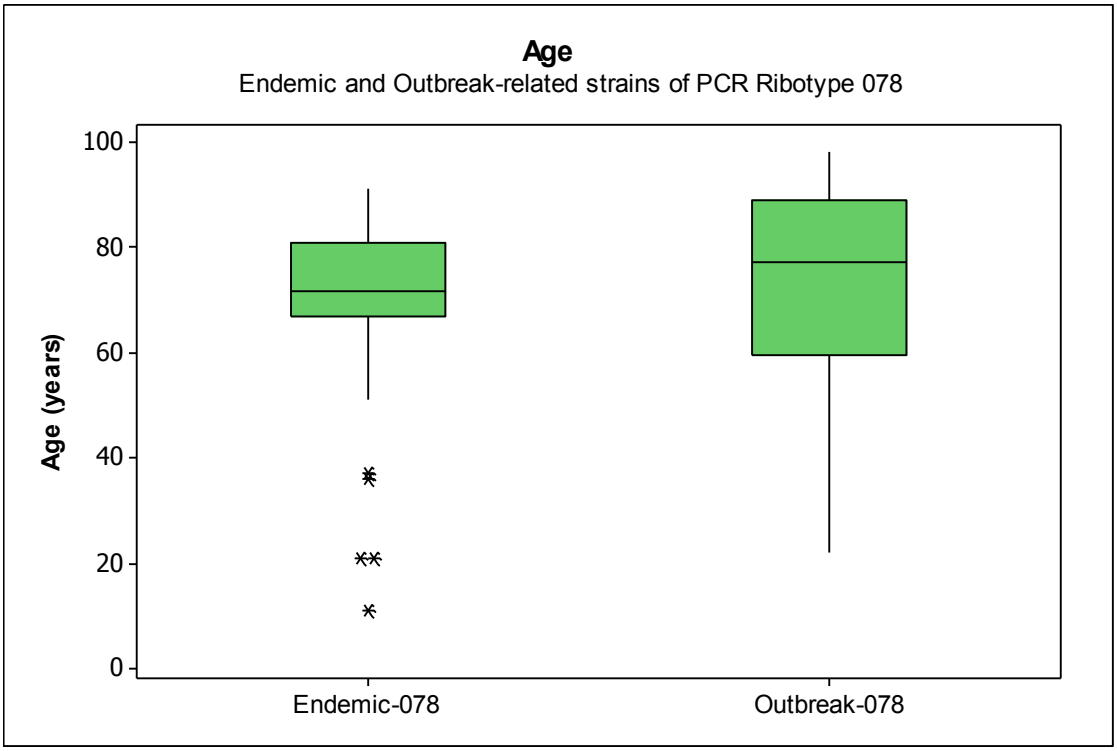


Figure 7.2 Gender distribution of endemic and outbreak-related strains of PCR ribotype 078.

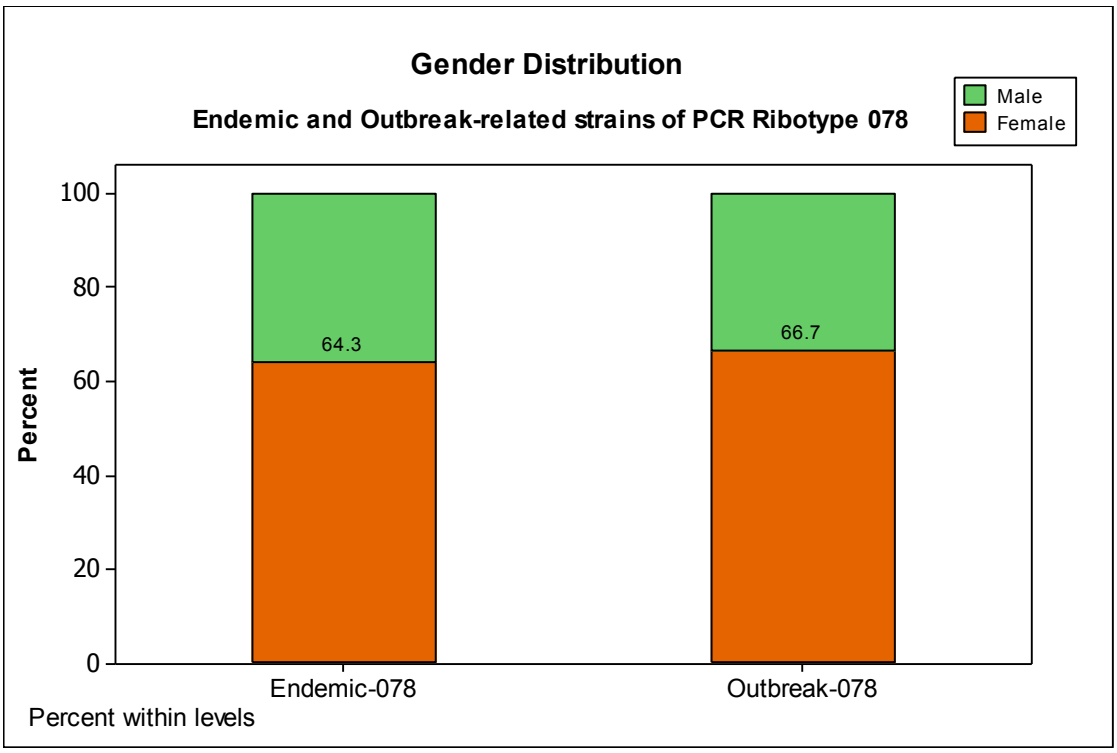


Figure 7.3 Box plot of total leucocyte count and serum albumin observed among patients infected with endemic and outbreak-related strains of PCR ribotype 078.

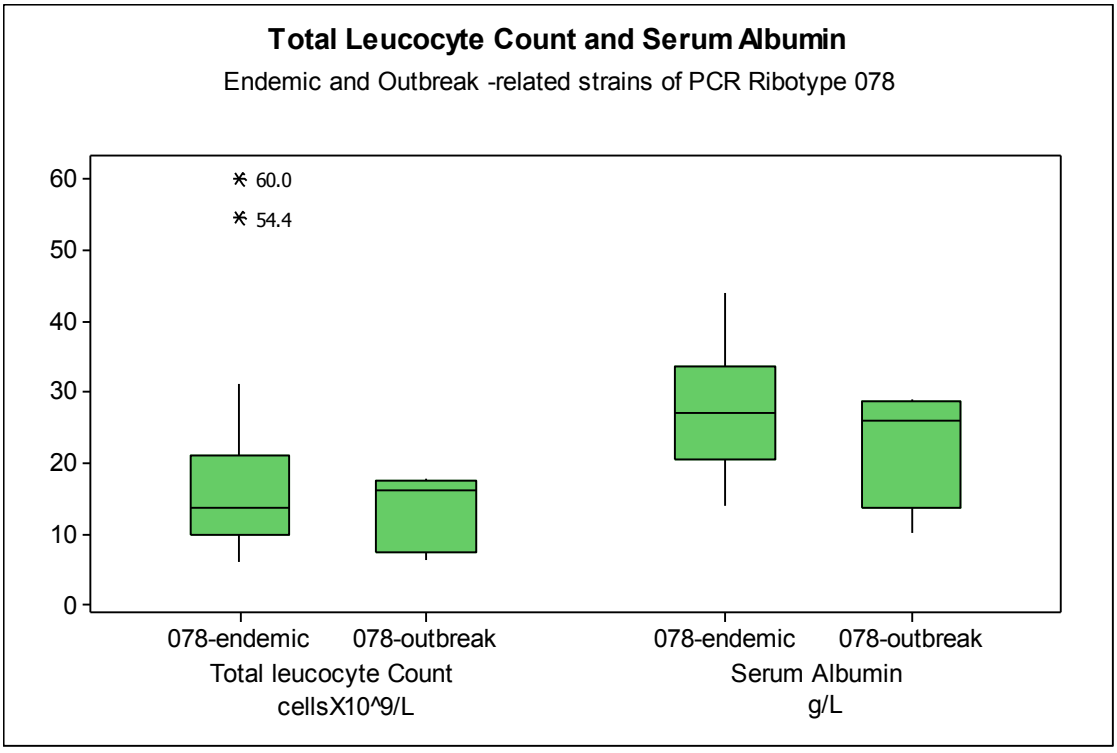


Figure 7.4 Box plot of number of episodes per patient and the age adjusted Charlson comorbidity index of endemic and outbreak-related strains of PCR ribotype 078.

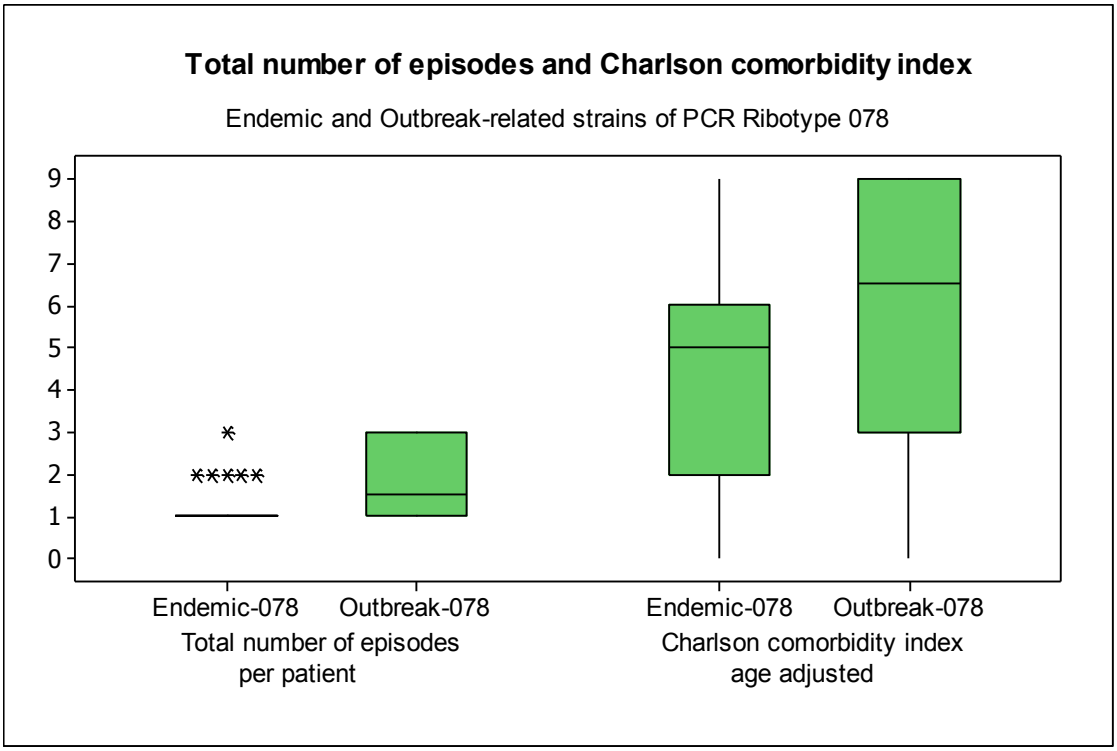


Figure 7.5 Severity of endemic and outbreak-related strains of PCR ribotype 078.

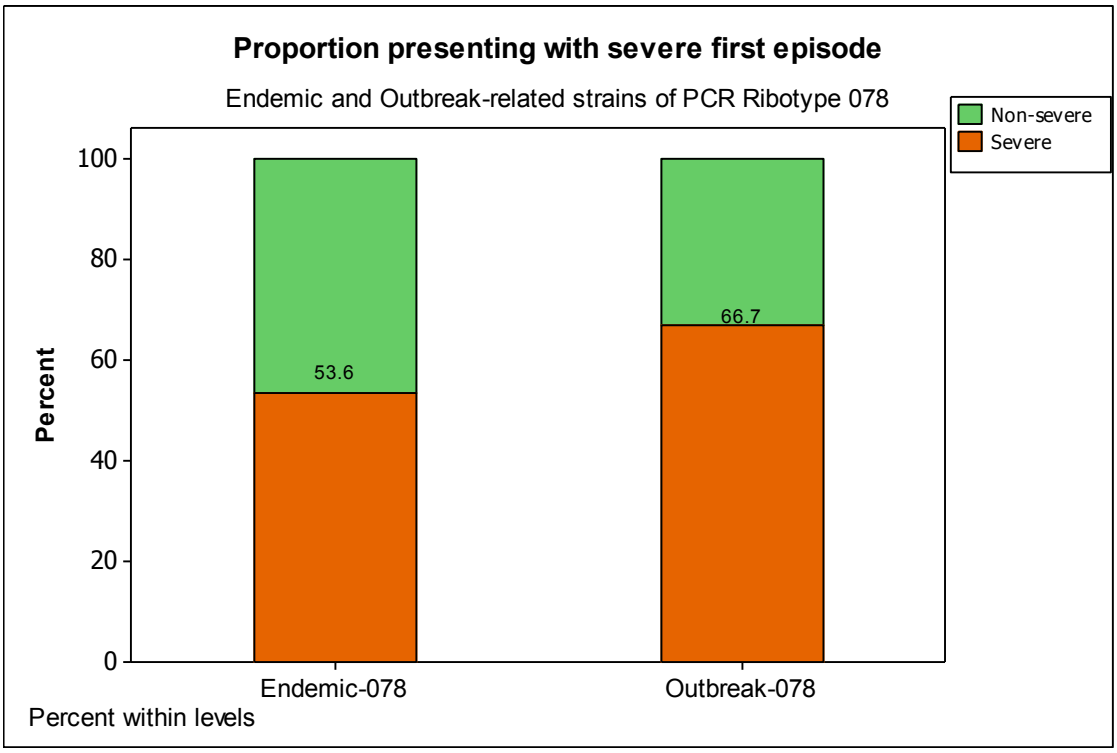


Figure 7.6 Comparison of location of acquisition between endemic and outbreak-related strains of PCR ribotype 078.

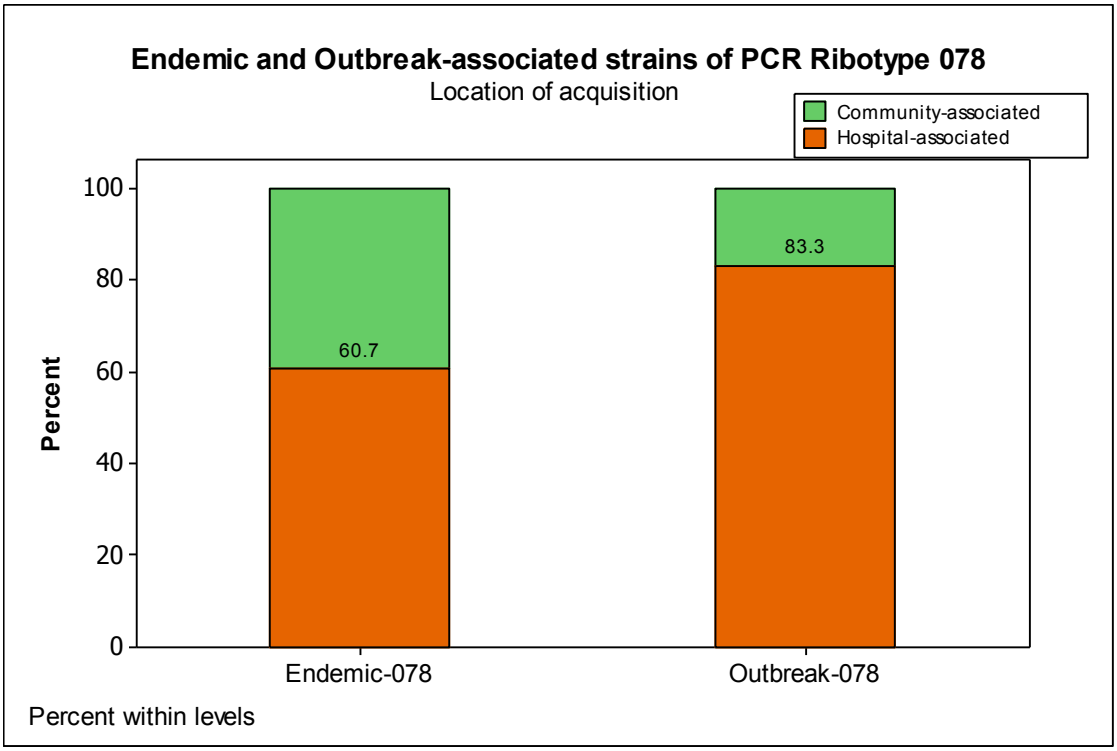


Figure 7.7 Underlying GI pathology of endemic and outbreak-related strains of PCR ribotype 078

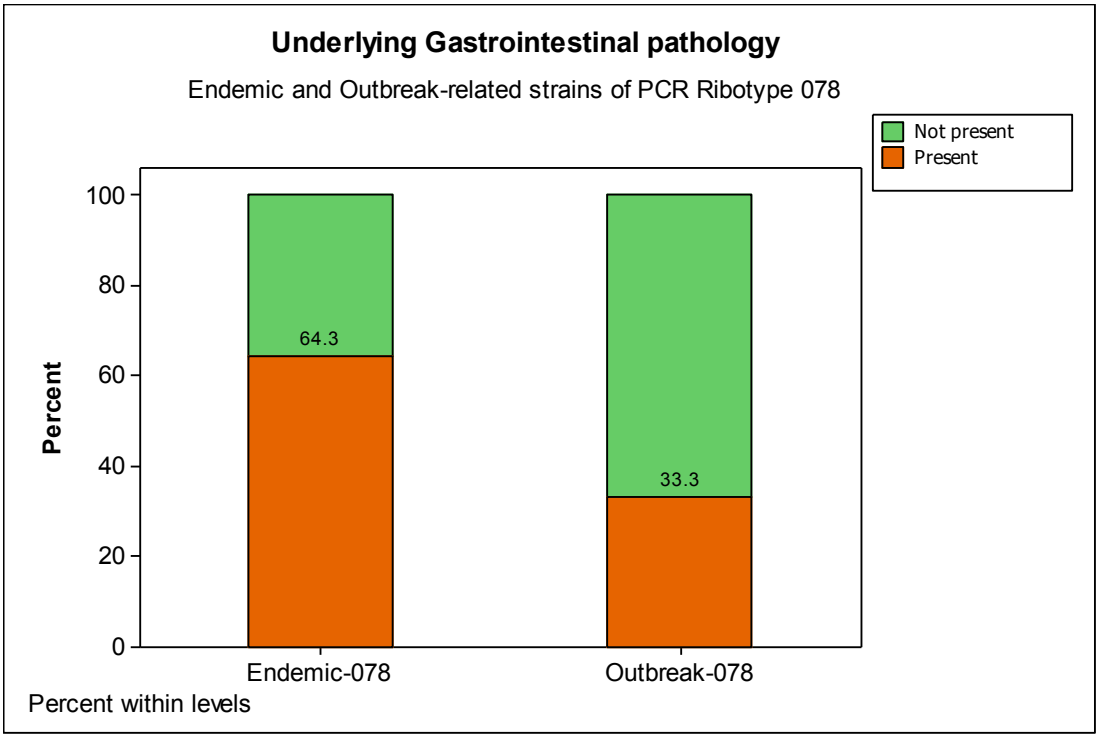


Figure 7.8 GI procedures performed on patients infected with of endemic and outbreak-related strains of PCR ribotype 078

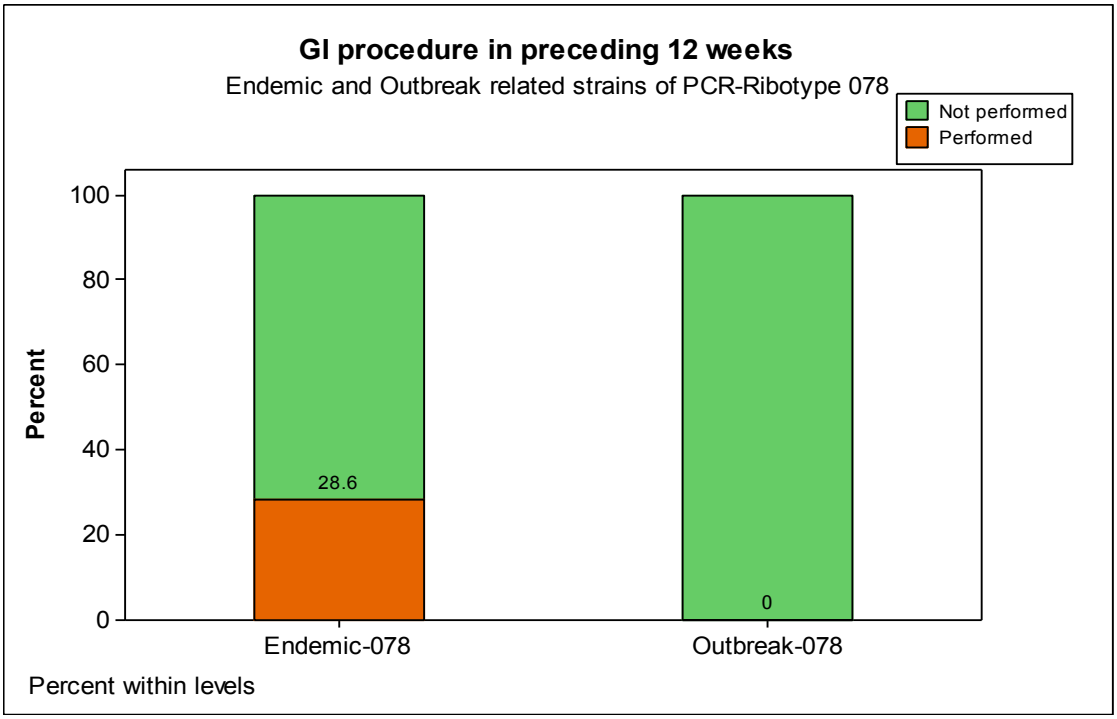


Figure 7.9 Immunosuppression in patients infected with of endemic and outbreak-related strains of PCR ribotype 078.

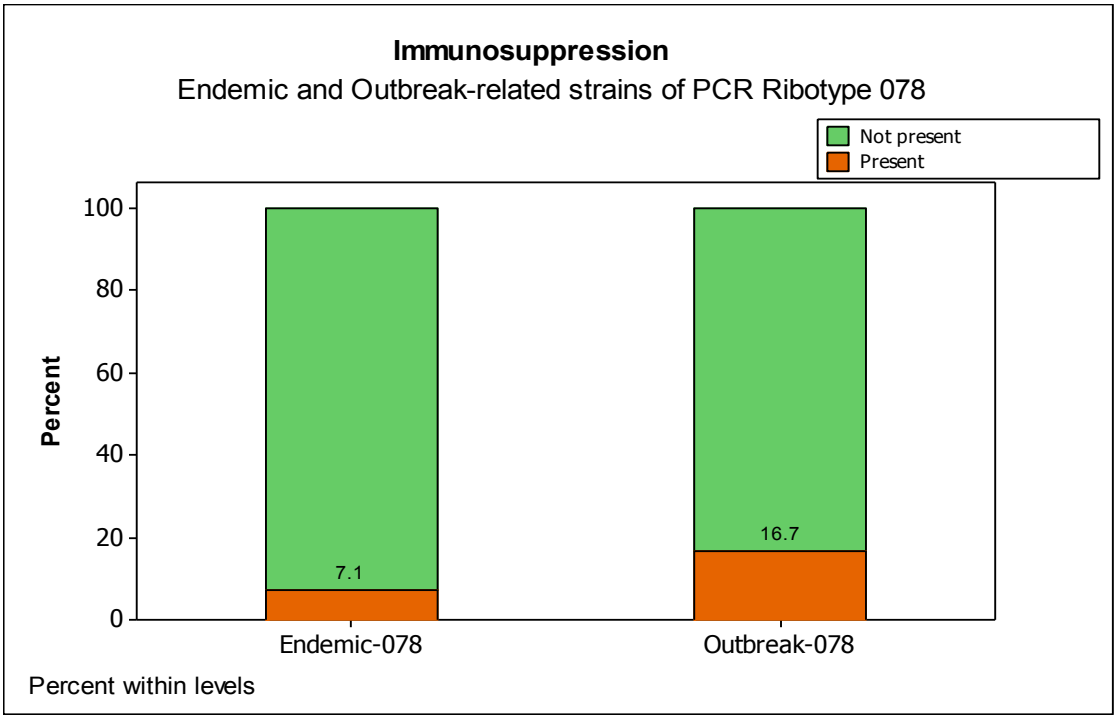


Figure 7.10 Death within 30 d of diagnosis in patients infected with of endemic and outbreak-related strains of PCR ribotype 078.

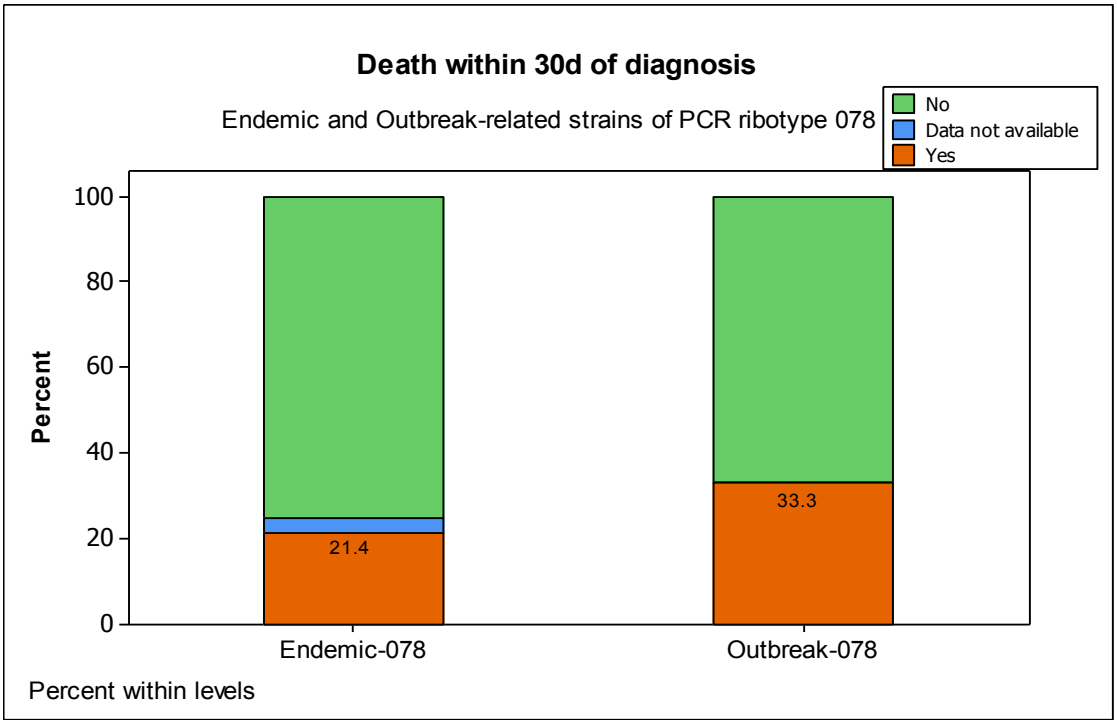
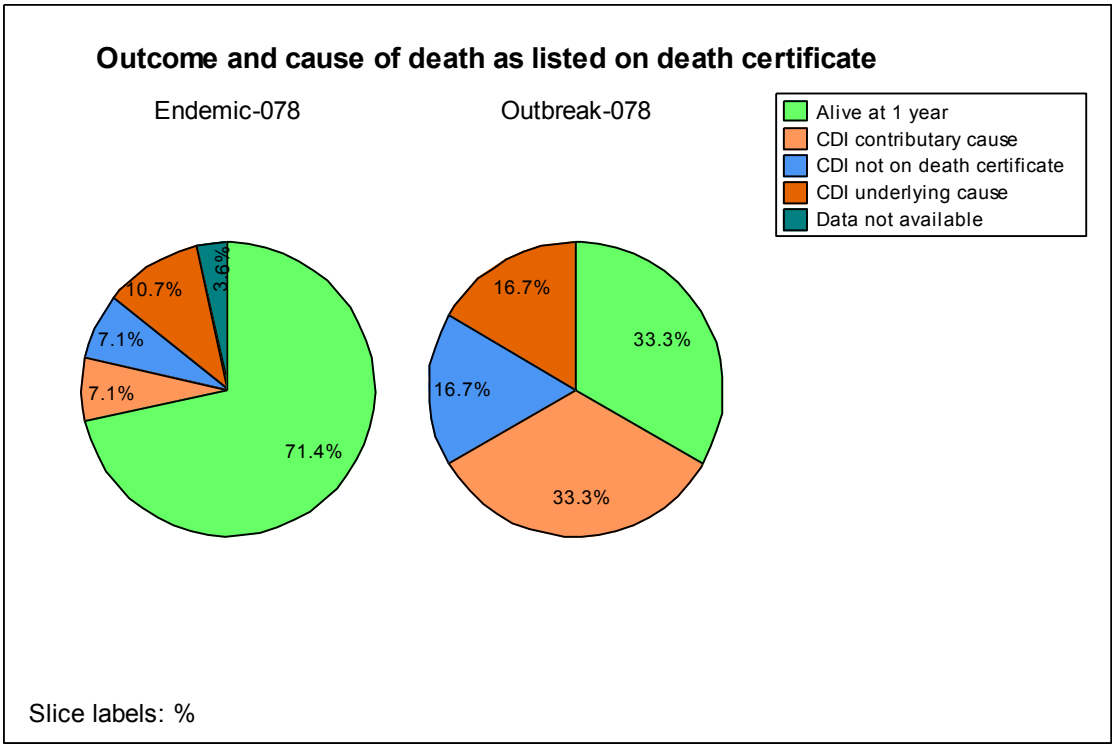


Figure 7.11 Comparison of outcome and cause of death in patients infected with of endemic and outbreak-related strains of PCR ribotype 078



7.3.1 Severity, recurrence and mortality associated with endemic and outbreak-related strains of PCR ribotype 078

In this study the comparison between the endemic and outbreak-related strains of PCR ribotype 078 suggests that the outbreak-related strains may cause more severe disease compared to the endemic PCR ribotype 078 isolates (66.7% vs 53.6% see figure 7.5). In addition, analysis of the mortality data suggests that 33.3% patients in the former group died within 30d of CDI whereas 21.4% of the latter suffered early mortality. A further detailed examination of all the patients among the former group suggests that 71% of infected were still alive at the end of the one year follow up period. This is in direct contrast to the outbreak group among which only 33.3% were alive even before a one-year follow up was completed. However, the patients in the outbreak group did have a higher Charlson co-morbidity index and hence were more likely to die due to co-morbidities. In addition, the death certificate information revealed that that CDI had contributed to the death of 50% of all patients infected with the outbreak-related strain whereas 17.8% of those infected with the endemic strains died of CDI. Figure 7.4 also suggests that the outbreak-related strains cause more recurrent disease (1.5 episodes /patients) than the endemic strains (only 6/28 patients developed recurrent disease).

Comparing the location of acquisition, by definition the outbreak related strains are more likely to be hospital associated (83.3%) as opposed to the endemic strains (60.7%). It is interesting to note that one of the patients who acquired the outbreak associated strain resided in a nursing home and did not have any apparent contact with the hospital where these outbreak-related cases occurred. This would suggest that the particular strain may be found in the community, however this particular MLVA type was not found in any of the other 28 isolates, nor in the four isolates from the historical collection stored at the MPRL laboratory.

From the predisposing factors, it appears that the endemic isolates are more likely to infect patients who have had a previous GI surgical procedure or have an underlying GI pathology. This would suggest that they infect patients whose GI tracts are

already compromised in some way whereas the outbreak strains, perhaps more virulent are likely to infect those even without this GI compromise.

The above information suggests that there may be differences in virulence factors between the endemic strains and the outbreak-related strain, which could account for the observed difference in mortality. However the numbers in the present study were too small to make a definitive conclusion.

Although the factors responsible for this observation are not clear, a recent study (Corver et al., 2012) that analysed the genome of 173 human isolates and 58 porcine isolates of PCR ribotype 078 for the presence of Tn6164 (a transposable genetic element associated with tetracycline resistance) and found that 18 of these strains contained this element. However, clinical details of many patients were unavailable, hence association with clinical severity could not be established. In the present study, the presence of Tn6164 has not been tested but since the clinical details of all 078 isolates are available, if this is done in the future valuable information may be obtained.

The above study (Corver et al., 2012) also examined the genomes of 67 other PCR ribotypes for this Tn6164 insertion element and did not find it in any of them. This suggests that the genome of PCR ribotype 078 is different from others and this may have implications in the difference in clinical presentation.

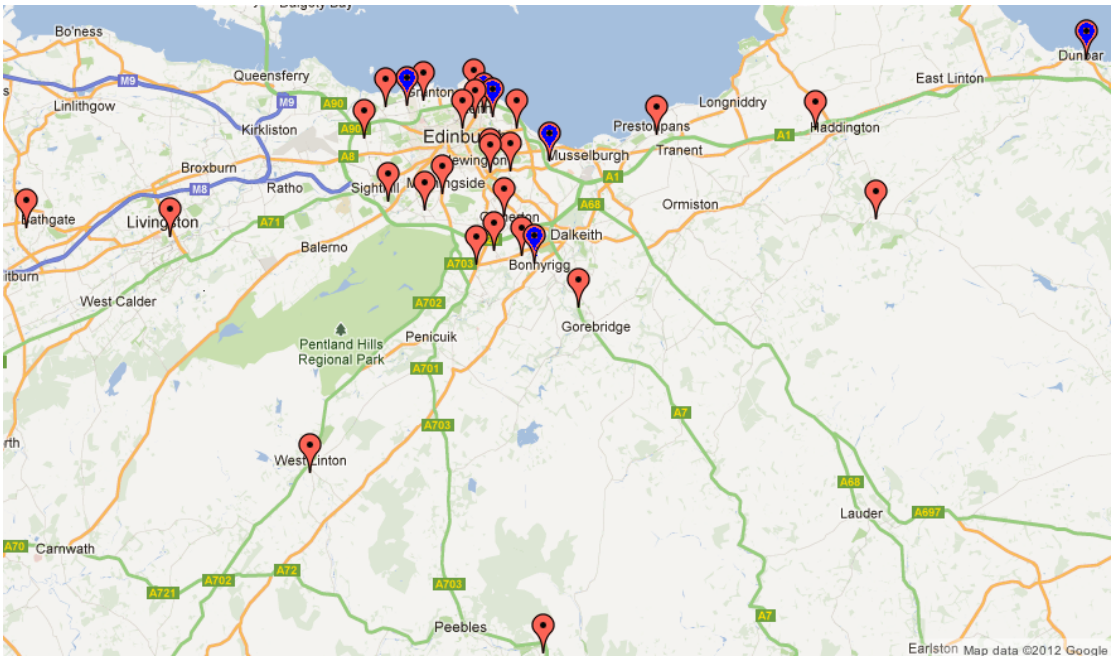
Till the time of writing, literature search of the English language did not reveal any study comparing endemic strains with outbreak related strains of PCR ribotype 078.

In a non-epidemic setting, comparing a set of 21 CDI patients with varying degrees of severity, a study found that although PCR ribotype 027 produced more toxin than other PCR ribotypes, there was no association between severity and clinical outcome between PCR ribotype 027 and non-027 PCR ribotypes (Sirard et al., 2011). Another study from Brighton (UK) analysed 128 patients with CDI in a non-epidemic setting. They compared detailed predisposing factors and epidemiological types with outcome and found that there does not seem to be an association between PCR ribotype and severity. However they did find a correlation between mortality due to

CDI and co-morbid conditions like ischaemic heart disease (OR 3.88, 95% CI, 1.61, 9.37; P=0.003) hypoalbuminemia (OR 3.13, 95% CI, 1.26, 7.75; P=0.014) and renal impairment (Wilson et al., 2010).

In addition, comparing CDI cases in an endemic setting, a study found that the risk factors in anoutbreak situation are different from the endemic risk factors (Hensgens et al., 2011). Molecular comparison of the virulence factors between the endemic and outbreak-related strains may give an insight on these perceived differences.

Figure 7.12 Distribution of postcodes of patients with CDI due to PCR ribotype 078 from the present study



The above two maps show the geographical location of the residence of patients infected with PCR ribotype 078 in the study. Included are the outbreak–related strains (shaded in blue). The second picture is a zoomed view of the first, focussing on Edinburgh.

7.4 Molecular features

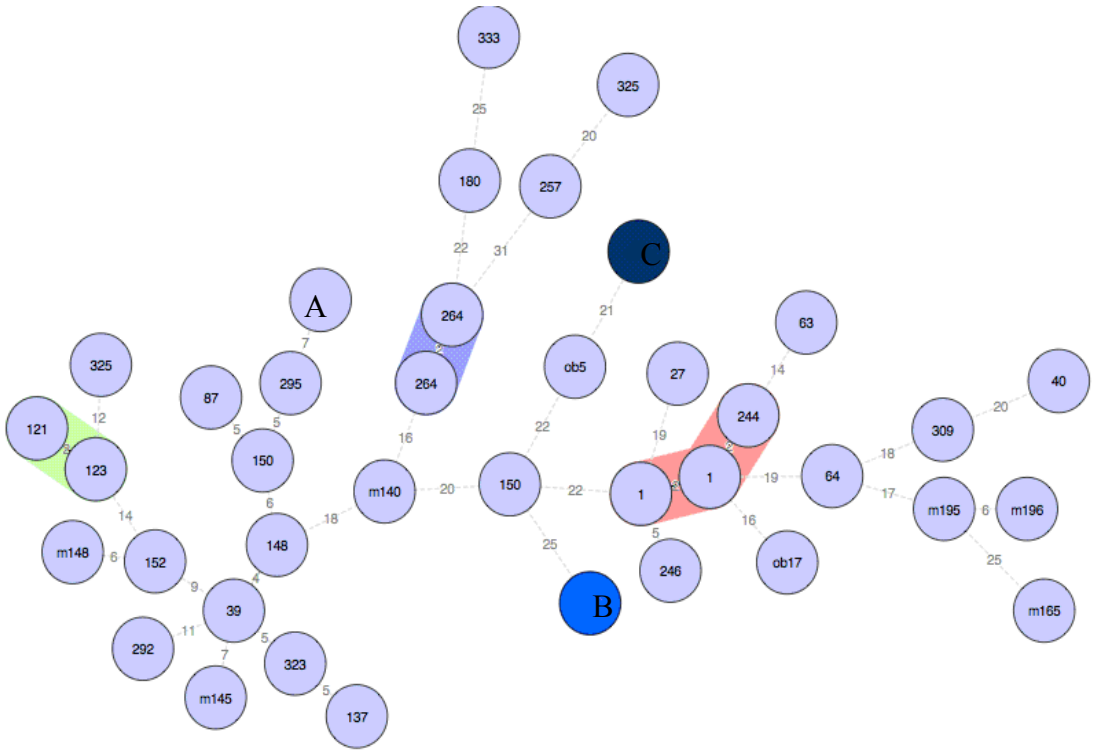
All isolates of PCR ribotype 078 had toxin genes *tcdA, tcdB, cdtA* (binary toxin) and possessed a 39–base pair deletion in the binding component of toxin regulator gene *tcdC.*

When the January outbreak of PCR ribotype 078 was first discovered, the patients were located in different wards. Since the time period between the cases ranged from 3-12 days, a question was raised whether the patients could have carried these isolates asymptomatically from the community and developed disease within the health care setting as a consequence of antimicrobial therapy and other comorbidity factors. This factor may be more relevant since PCR ribotype 078 is known to be community associated (Goorhuis et al., 2008a, Patterson et al., 2012) and no other outbreak with this PCR ribotype had ever been reported in the past. If true, this would absolve the heath care institute from inflicting a hospital acquired infection to the affected patients.

PCR-ribotyping is comparatively a simpler procedure compared to subtyping and the genome of this PCR ribotype is known to be difficult to subtype (Bakker et al., 2010) because of variations in annealing regions to primers, which will otherwise anneal well to other PCR ribotypes. In addition there are some MLVA loci, which are absent in this PCR ribotype (though present in other PCR ribotypes). Hence it has been difficult to standardize a PCR protocol for MLVA, which will subtype PCR ribotype 078 along with other PCR ribotypes. Although some groups have performed MLVA for this PCR ribotype successfully (Manzoor et al., 2011), in many labs it is still not routine procedure. Among the 34 isolates from the present study, 8.8% of G8*Cd* and 11.8% of B7*Cd* and 38.2% of C6*Cd* could not be amplified and the lack of this data is reflected in the STRDs between strains. However, all loci of the outbreak-related strains could be amplified.

The maximum variability was seen in the loci B7*Cd* (6-28 copy numbers) and C6*Cd* (11-39 copy numbers). This study also highlights the importance of subtyping for *C. difficile* isolates. In spite of the limitations mentioned above, the outbreak-related strain could be easily differentiated from the other endemic strains and hence the technique proved useful in the present study.

Figure 7.13 Cluster analysis of all strains of PCR ribotype 078 analysed in this study including all 6 loci tested



Minimum spanning tree (Manhattan) cluster analysis of strains of PCR ribotype 078, by 6 locus MLVA. Each node represents a single MLVA profile. The circles are labelled with patient numbers (those with the prefix m are historical isolates from 1990s and those with prefix ob are outbreak-related strains). The numbers between circles are summed tandem repeat differences (STRD). Shaded zones indicate related isolates. Circles numbered A, B and C represent more than one isolate.

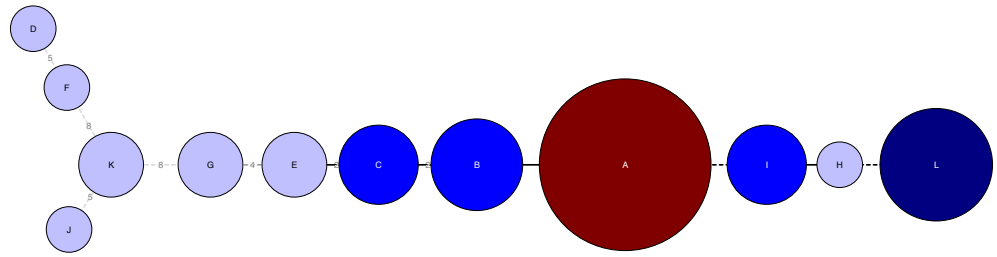
No alleles were detected at B7Cd in 5 isolates, at C6Cd in 16 isolates and at G8Cd in 4 isolates.

A= Two isolates from different patients

B= Two isolates from the same patient and one from an unrelated patient

C=Five outbreak related isolates

Figure 7.14: Cluster analysis of all strains of PCR ribotype 078 analysed in this study including only 3 loci (E7Cd , CD60 and CD105) in which alleles were detected in all isolate



Minimum spanning tree (Manhattan) cluster analysis of strains of PCR ribotype 078, by 3 locus MLVA. Each node represents a single MLVA profile. The circles are labelled with letters (index given below). Those with the prefix m are historical isolates from 1990s and those with prefix ob are outbreak-related strains). The numbers between circles are summed tandem repeat differences (STRD).

A= patient no. 1, 121, 123, 137, 215, 228, 246, 264, 292, 295, 323, 325, 64, M140

B= patient no. 148, 152, 244, 39

C= patient no. 150, 63, 87

D= patient no. 257

E= patient no. 309, 333

F= patient no. 40

G= patient number 27, 180
H= patient no. M145
I= patient no. M148, M195, M196

J= patient no. M165

K= patient no. 80, 95

L= patient no. OB1, OB2, OB3, OB5, OB13, OB18

Chapter 8
Paediatric and neonatal colonisation with *C.difficile*

8.1 Paediatric nursery (crèche) colonisation

Parents of 13 children gave consent, of which only two children were found to be colonised giving a carriage rate of 15.4%.

A previous study found *C. difficile* in only one of 40 neonates during the normal 1-week stay in the hospital after delivery. Since the mother also carried the same strain, as determined by PCR ribotyping, pulsed-field gel electrophoresis analysis, and toxin gene type it is assumed that the baby acquired it from the mother. In addition, the same study found a higher (84,4%) carriage rate in infants < 2 years of age. They also found that several children were colonised with the same type of *C. difficile* which was similar to that found on the floors of the nurseries suggesting cross-infection among children and their environments.(Matsuki et al., 2005).

However, the carriage rate in our study was too small to make any significant deduction.

8.2 Neonatal colonisation

One hundred and thirty-one stool samples were obtained over a 28-day period from 30 neonates admitted to the neonatal care ward at the Royal Infirmary of Edinburgh. The total number of samples obtained per baby ranged from 1-9. Enrichment cultures were initially used to allow growth of scanty *C.difficile*, if present. However this did not give results any different from direct cultures on selective medium, hence subsequently only non-selective medium (blood agar) and selective medium (CCEY) were used. Only one positive culture (PCR ribotype 159) was obtained from the last sampled stool of a baby who had been in hospital for all the four weeks of the study. It is possible that culture is less sensitive than PCR/GDH combination testing and

hence the latter would have given a higher positivity rate. Although found in symptomatic adults, PCR ribotype 159 is a relatively rare type in the adult population (commonest being PCR ribotypes 002, 001, 14, 20 and 15). It was found in one adult hospitalized patient in the same period as the neonatal study although no other epidemiological link could be established between the two.

The stool culture results are given in table 8.1

Table 8.1 Results of stool *C. difficile* carriage among 30 neonates sampled over a four weeks

Sampling period	No of babies sampled	No of babies carrying C .difficile	Carriage rate	PCR ribotype
Aug 2010	30	1	3.3%	PCR ribotype 159

This study showed a 3.3% carriage rate in hospitalised neonates. Similar studies have demonstrated a carriage rate of up to 71% (Al-Jumaili et al., 1984) .

Among the early studies, *C. difficile* was isolated from 28.9% healthy neonates who had never received antimicrobials (Viscidi et al., 1981) and *C. difficile* toxin was found in 10.5% of normal newborn infants and 55% of neonates in the intensive care unit (Donta and Myers, 1982).

Our own unit has previously had carriage rates of up to 57% in 1995/96 (see table 8.2), which was shown to decrease after implementation of infection control precautions in the ward. This raises the question whether the implementation of modern infection control practices like strict implementation of hand washing, one to one nursing and strict control of visitors before entering a ward have contributed to the decline in *C. difficile* acquisition in the neonatal unit of the hospital and whether this decline in carriage rate among hospitalized neonates is likely to reduce immunity to the disease in adulthood (see bullet-point list below).

Table 8.2 Results of previous studies in the same hospital which have investigated asymptomatic colonisation

Sampling period	No of babies sampled	No of babies carrying C .difficile	Carriage rate	No of different S layer protein patterns
Apr-May 1995	28	16	57%	1
Aug 1995	19	2	10%	2
Nov 1995	22	6	27%	5
Apr-May 1996	30	4	13%	4

Taffinder, Beal, Shepherd, Laurenson , Brown and Poxton, 1997 see table 8.2

- Restricted entry to the neonatal unit
- Any person (including staff) entering or leaving the unit are obliged to wash their hands with soap and water
- Any objects brought in from outside are kept away from the babies in a separate room.
- Each baby has a separate cubicle on which only sterilized or objects amenable to disinfection are permitted.

However, the parents are encouraged to room in with the babies and mothers are encouraged to feed. In spite of this, the measures used to prevent the spread of infection are stricter than they would be in a general ward, at a nursery or at home. This may be the reason for the low incidence of colonisation. We could not sample

the parents and hence transmission to babies from their parents could not be determined.

The sampling method was however biased since the patients studied were all neonates who had not had contact with the general population. Most were delivered by Caesarian section and hence had no contact with the maternal vaginorectal flora at the time of birth. Since the mothers were not studied for *C. difficile* colonisation, the association between maternal and neonatal colonisation cannot be commented upon.

Implications of early carriage to the immunological development of neonates should be an area for further study

Conclusions

This study has investigated all patients diagnosed with CDI over a period of one year. Hence the epidemiology of the entire population served by the Microbiology Department of the Royal Infirmary of Edinburgh has been represented. For this reason, it provides a complete picture, which may be missed if only a selected population is evaluated.

The unique characteristics of severe and recurrent disease seen in this region have been documented and the effects of potential predisposing factors analysed. This study adds to the existing knowledge of these complications of CDI.

The study of community-associated CDI is very often limited by lack of follow up information from community patients. In this study, careful follow up has allowed the availability of a complete data set to compare against hospital-associated disease.

This region of Scotland was unusual in its absence of PCR ribotype 027, but doubts persisted whether this PCR ribotype has been missed for lack of ribotyping information. This study confirms that this PCR ribotype is not prevalent in this region.

The recent increase in the incidence of PCR ribotype 078 in Scotland has been a cause for concern especially since there is gap in the knowledge of the characteristics of this PCR ribotype in this region. This study establishes its link with community associated disease and documents the characteristics of the disease caused by it in an endemic as well as outbreak situation. Larger studies documenting the characteristics of PCR ribotype 078 are needed to determine its potential as a "hypervirulent" strain.

MLVA as a subtyping technique in research and clinical practice has been studied and found useful for establishing epidemiological connections in the presence of an outbreak related to PCR ribotype 078.

Finally, low rates of neonatal and paediatric colonisation with *C. difficile* as compared to previous studies suggest better infection control measures. However

they raise concerns on the development of an immune response in these children, which is recommended as an area for further study.

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Appendix 1

Data collected for patients included in the general arm of the study for each episode

Demographics
Age
Gender
Date stool sample collected
Ward/GP requesting the sample
Date of admission and discharge
Healthcare contact in past 4 weeks/8 weeks/12 weeks

Present history
Reason for admission
Presenting symptoms
Diarrhoea onset date
Diarrhoea stopped on
Fever
Co-infection with other GI pathogens

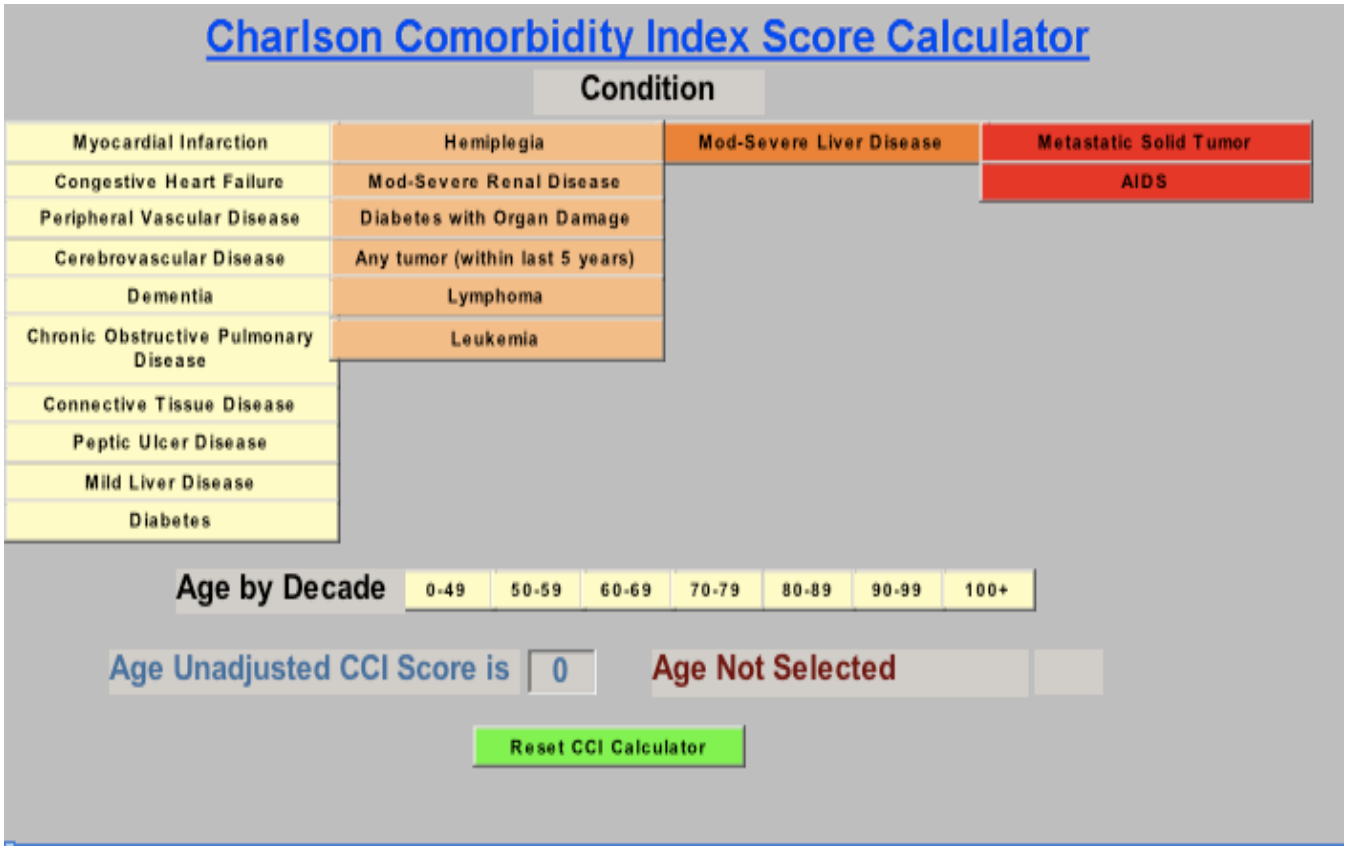
Previous history
Previous CDT positive in past 2 weeks/ 4 weeks/ 8 weeks or longer
Overnight hospital admission in past 12 weeks
Antibiotics given in past 8 weeks
PPIs H2 blockers in past 2 weeks
Underlying GI pathology, if any
Previous GI surgery/ endoscopy in past 12 weeks
Recent travel history
Recent chemo/radiotherapy
Prior MRSA/ VRE colonisation on record

Assessment of severity
Total leucocyte count
Serum albumin
Creatinine
Lactate
Suspected/confirmed pseudomembranous colitis
Abdominal X-ray results/ colonic dilatation on CT
Immunosuppression
ITU admission
Co-morbidities as per appendix II

Treatment given
Metronidazole/vancomycin (dose and duration)/ surgery
Any other: Immunoglobulin etc.

Appendix 2

Charlson Comorbidity Index calculator



Appendix 3

INFORMATION SHEET (under 12s)

Title of the project: *Clostridium difficile* in the neonatal populations: pool of potential pathogens and implications for immunity in adulthood

Your child is being invited to take part in a research study. Please take your time to read this information sheet before you agree for him/her take part. Please feel free to ask questions if anything is not clear or if you require more information.

1. WHAT IS THE PURPOSE OF THE RESEARCH PROJECT?

Clostridium difficile is a bacterium that can be found in the bowel without causing any harm; however, it can cause diarrhoea in adults, especially after taking antibiotics for any reason. However neonates (babies less than 4 weeks old) do not develop this disease.

It is normal for the body to fight infections by producing antibodies and immune system cells. Sometimes the body does not produce these antibodies and cells after infection by *Clostridium difficile* and then people can get diarrhoea.

We are trying to understand why babies are protected against the disease whereas adults and older children develop disease. We also want to know which type of *C. difficile* is found in babies.

2. WHY HAVE WE BEEN CHOSEN?

We need a stool sample and if possible a blood sample (see 4 below) to determine which factor(s) protect them from developing disease and to determine if the type of *C. difficile* which is present in the child is the same or different from those in adults.

3. DOES MY CHILD HAVE TO TAKE PART?

It is up to you to decide whether or not to allow your child to take part.

If you decide to consent for your child to take part, you will be given a copy of this information sheet and will be asked to sign a consent form. You may withdraw at any time without giving a reason. A decision to take part or not will not affect the standard of care that your child receives. If you decide to withdraw at any time, this will not affect the standard of care that your child receives.

4. WHAT WILL HAPPEN IF I AGREE TO TAKE PART?

If you consent to agree that your child takes part we will take a stool sample and if there is any blood left over from routine testing we will use some of this. We will analyse your child's stool for the presence of *Clostridium difficile* and we will check for antibodies to *C. difficile* in the blood specimen.

5. WILL MY CHILD NEED TO TAKE ANY DRUGS IF I AGREE FOR HIM TO TAKE PART?

This study does not involve taking any drugs. We will only take a sample of blood and stool.

6. WHAT ARE THE POSSIBLE DISADVANTAGES OF TAKING PART?

There are no risks to your child if you consent for them to take part in this study.

7. WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

There are no benefits for your child if you decide to take part; however, the results of our study could be beneficial for other patients in the future.

8. WHAT HAPPENS WHEN THE RESEARCH STOPS?

We will analyse the data and we will investigate if there is a way of applying our results to other patients like your child. After the study is finished, all the samples will be destroyed.

9. WILL ANY GENETIC TESTS BE DONE?

No genetic tests will be done.

10. WILL MY CHILD'S PARTICIPATION IN THE RESEARCH PROJECT BE KEPT CONFIDENTIAL?

We will note your child's age, gender and the reason he/she is in hospital. We will keep those records anonymous. We will also keep all records confidential. (We will inform your GP if you agree to take part in the study).

11. WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

The results of the study will be published in scientific journals and presented at scientific meetings. You will not be informed of individual test results but can access the results of the study by contacting Professor Ian R Poxton (i.r.poxton@ed.ac.uk)

12. WHO IS ORGANISING AND FUNDING THE RESEARCH?

The study has been organised by the University of Edinburgh Centre for Infectious Diseases in collaboration with the Royal Hospital for Sick Children and the Microbiology department of Royal Infirmary of Edinburgh. The costs of the research will be met from a grant from the Scottish Infection Research Network.

Thank you for taking the time to read this information sheet.

June 2010, version 4.

Appendix 4

Stool culture on CCEY after alcohol shock

Take 200 microliter absolute alcohol in a 1.5ml Eppendorf tube

Mix 200 microlitre or one large loopful stool sample with the alcohol

Briefly vortex

Let the alcohol mix with the stool at room temperature for 10min

Plate one loopful of diluted stool onto pre-reduced CCEY agar