# Evaluating patient susceptibility in *Clostridium difficile* infection

Jasmin Islam

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Department of Clinical Medicine

Pathogen-Host-Interactions Group

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# Abstract

*Clostridium difficile* infection (CDI) is the leading cause of nosocomial diarrhoea and causes substantial morbidity and mortality. Efforts to reduce the impact of CDI have succeeded in reducing rates through antibiotic stewardship, improved diagnostic testing and optimisation of infection control measures. Further reductions in CDI could be achieved through a better understanding of what makes patients susceptible to CDI. Such knowledge would support interventions targeting patients most at risk and help develop treatments to reduce susceptibility. The aim of this thesis was to further our understanding of patient susceptibility to CDI by investigation of three specific areas.

The first study investigated the role of the probiotic *Lactobacillus casei* DN114001 in preventing antibiotic associated diarrhoea (AAD), including CDI, as part of a large multicentre, double-blind, randomised placebo-controlled trial. Probiotics are live microorganisms that may help restore antibiotic disruption to the host microflora and prevent *C. difficile* colonisation. The final results were not available at the time of writing this thesis and therefore a descriptive analysis of the first 650 blinded cases is provided. This is the largest probiotic study ever conducted and will contribute significantly to the existing literature in the field.

The humoral immune response has been implicated in determining outcome in CDI. Previous studies have focused on recurrence of CDI and toxin A (TcdA), which was originally thought to be the most important virulence factor in CDI. However, recent studies have suggested toxin B (TcdB) may be essential for CDI pathogenesis. Therefore, the second study tested the hypothesis that antibodies to TcdB determine patient susceptibility in CDI. A case-control laboratory based study was conducted using a novel antibody ELISA and antibody responses to both toxins were assessed in two cohorts recruited in Brighton, UK and Michigan, USA. Lower antibody levels to TcdB, but not TcdA, were found in cases of acute CDI compared to controls. These novel findings are in contrast to previous studies and confirm the importance of TcdB in CDI pathogenesis. In addition, the antibody response to TcdB could be used as a surrogate marker for the efficacy of novel therapeutic agents.

The third study sought to identify risk factors predicting recurrence of CDI. A longitudinal cohort study of 248 patients with confirmed CDI was conducted that confirmed the previously observed relationship between concomitant antibiotic treatment and risk of recurrence. The study also identified a novel risk factor namely that treatment on a cohort ward was associated with recurrence of CDI. This is likely to be a result of reinfection of patients who remain susceptible to CDI after treatment. This is the first study to demonstrate an association between cohorting of patients and recurrence of CDI and raises important questions about current infection control policies in hospitals.

Efforts to combat CDI have focused on reducing exposure of patients to infection. The data presented here contribute to a rapidly emerging understanding that patient susceptibility is a crucial factor in determining risk of infection, risk of severe disease and risk of recurrence following treatment. In the near future interventions targeting susceptibility including probiotics, specific antibiotics such as fidaxomicin and immunotherapies such as vaccines may all have a role to play in combatting this devastating disease.

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# Author Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. This thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed:

Date: 3.4.13

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# Abbreviations

| | |
|---|---|
| AAD | Antibiotic associated diarrhoea |
| ADP | Adenosine diphosphate |
| AE | Adverse event |
| bp | base pair |
| BSA | Bovine Serum Albumin |
| CaCo-2 | Colorectal adenocarcinoma cells |
| CamSA | Cholate *meta*-benzene sulfonic derivative |
| CCA | Cell cytotoxicity assay |
| C-Terminal | Carbon terminal |
| CDI | *Clostridium difficile* infection |
| CDRN | *C. difficile* Ribotyping Network |
| CDT | *C. difficile* transferase or binary toxin |
| *cdtA* and *cdtB* | genes encoding CDT production |
| *cdtR* | gene encoding a regulator of CDT expression |
| cfu | colony forming units |
| CRF | Case report form |
| CROPs | Clostridial repetitive oligopeptides |
| CT | Computer tomography |
| C-terminal | Carboxy terminal |
| DAB | 3,3'-diaminobenzidine |
| DH | Department of Health |
| DMC | Data Monitoring and Safety Committee |
| DNA | Deoxyribonucleic acid |
| EIA | Enzyme immunoassay |
| ELISA | Enzyme Linked Immunosorbent Assay |
| EPC | Endpoint Committee |
| FMT | Faecal microbiota transplantation |

| | |
|---|---|
| FOXP3 | Forkhead box P3 |
| FQR | Fluoroquinolone resistant |
| GDH | Glutamate dehydrogenase |
| GDP | Guanosine diphosphate |
| GTP | Guanosine triphosphate |
| HFF | Human Foreskin Fibroblast cells |
| HIV | Human immunodeficiency virus |
| HPA | Health Protection Agency |
| HRP | Horse Radish Peroxidase |
| HumAbs | Humanised monoclonal antibodies |
| IFNγ | Interferon-Gamma |
| Ig | Immunoglobulin |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IL | Interleukin |
| IQR | Interquartile range |
| IVIG | Intravenous immunoglobulin |
| kb | kilo base |
| kDa | kilo Dalton |
| MALDI-TOF | Matrix-assisted laser desorption time of flight mass spectrometry |
| mL | millilitre |
| MLST | Multilocus sequence typing |
| MLVA | Multilocus variable number tandem repeat analysis |
| mM | millimolar |
| mV | millivolts |
| NAP1 | North American pulse field gel electrophoresis (type 1) |
| NTCD | Non-toxigenic *C. difficile* |

| | |
|---|---|
| N-terminal | Amino-terminal |
| OPD | O-Phenylenediamine |
| OR | Odds ratio |
| PaLoc | Pathogenicity locus |
| PBS | Phosphate Buffered Saline |
| PBS-T | Phosphate Buffered Saline and Tween 20 |
| PCR | Polymerase chain reaction |
| PFGE | Pulse field gel electrophoresis |
| pg | picogram |
| PPI | Proton pump inhibitor |
| PVDF | Polyvinylidene Fluoride |
| REA | Restriction endonuclease analysis |
| RNA | Ribonucleic acid |
| RR | Relative risk |
| rRNA | ribosomal Ribonucleic acid |
| RSCH | Royal Sussex County Hospital |
| RT | Room temperature |
| SAE | Serious adverse event |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | SDS - polyacrylamide gel electrophoresis |
| SF-12 | Short form 12 |
| sIgA | Secretory Immunoglobulin A |
| SLP | Surface layer protein |
| 16S | 16 subunit |
| *tcdA* | gene encoding Toxin A |
| TcdA | Toxin A |
| *tcdB* | gene encoding Toxin B |

| | |
|---|---|
| TcdB | Toxin B |
| TcdC | negative regulator of TcdA and TcdB |
| TcdE | holing protein involved in cell wall pore formation |
| TcdR | positive regulator of TcdA and TcdB |
| TLR | Toll like receptor |
| TNFα | Tumour Necrosis Factor Alpha |
| $T_{Reg}$ | Regulatory T Cell |
| TSC | Trial Steering Committee |
| UK | United Kingdom |
| USA | Unites States of America |
| v/v | volume/volume |
| w/v | weight/volume |
| µg | microgram |
| µL | microlitre |

# Chapter 1: Introduction

## 1.1 An historical perspective

In 1935, Hall and O'Toole isolated the organism *Bacillus difficilis* from the stool of four healthy infants (1). *B. difficilis* produced a lethal toxin that caused marked tissue oedema and convulsions when given subcutaneously to rabbits and guinea pigs. The organism became known as *Clostridium difficile.* In 1978, Bartlett *et al.* established a link between antibiotic use and *Clostridium difficile* infection (CDI) after the bacteria was isolated from hamsters suffering from antibiotic-induced colitis (2). During the same year Larson *et al*. recovered toxigenic strains of *C. difficile* from the caecal contents of hamsters that were pre-treated with vancomycin. The hamsters had been inoculated with strains of *C. difficile* isolated from patients suffering from severe pseudomembranous colitis and all animals developed a fatal enterocolitis that was similar to the disease in humans (3). It is now well recognised that certain antibiotics are able to disrupt the intestinal microflora, commonly referred to as the microbiota, which can increase patient susceptibility to *C. difficile* colonisation and overgrowth.

Over the following years *C. difficile* remained relatively obscure; however, the past decade has been characterised by a dramatic increase in cases and CDI is now the commonest cause of nosocomial diarrhoea in the developed world (4). This might be explained by a combination of factors that included changes to antibiotic prescribing patterns in the United Kingdom (UK) and the emergence of a hypervirulent strain that was capable of increased toxin production (5)(6). Until now, efforts to reduce CDI have focused on improving infection control measures and optimising diagnostic testing, which have met with some success. However, targeting patient susceptibility may be a more effective approach that has previously been investigated using stochastic modelling (7). Starr *et al.* used a mathematical modelling system to demonstrate a 43% reduction in CDI could be achieved by halving patient susceptibility compared to only a 15% reduction in CDI by reducing transmission. The purpose of this thesis was to evaluate the role of the host immune response and the intestinal microbiota or microflora in determining patient susceptibility to CDI.

## 1.2 Epidemiology

During the 1990s, rates of CDI began to gradually climb in the UK and had risen from 18,354 cases in 1999 to 28,819 by 2002 (8). There followed a dramatic increase in cases that coincided with the emergence of a previously uncommon variant strain of *C. difficile* known as polymerase chain reaction (PCR) ribotype 027, North American pulse field gel electrophoresis type 1(NAP1) or restriction endonuclease analysis (REA) group BI, depending on the molecular typing technique used (see section *1.2.1.*). In subsequent years 027/BI/NAP1 would come to account for >40% of isolates in the UK (9). The strain was first identified in Canada and quickly became known as the 'hypervirulent' strain that produced increased levels of toxin and was resistant to fluoroquinolones, which were a commonly used group of antibiotics. The strain was responsible for several outbreaks that were characterised by increased disease severity and high mortality rates (5)(6).

In 2004, the Department of Health (DH) introduced mandatory reporting of all cases of *C. difficile* to the Health Protection Agency (HPA). Between 2004 and 2006, two large outbreaks took place at Stoke Mandeville Hospital and Maidstone Hospital that were later attributed to 027/BI/NAP1 (10)(11). A recent study used whole genome sequencing to confirm the epidemic strain originated from two distinct lineages known as fluoroquinolone resistant 1 and 2 (FQR1, FQR2). The strains responsible for the UK outbreaks both belonged to FQR2 (12). The rise in CDI was accompanied by a rise in mortality, reflected by an increase in the number of death certificates mentioning *C. difficile* from 2,238 in 2004 to 8,324 in 2007 across England and Wales (13). During 2007, over 55,000 cases were reported to the HPA and the DH were forced to take decisive measures to try and control the rising rates of CDI (Figure 1.1) (14).

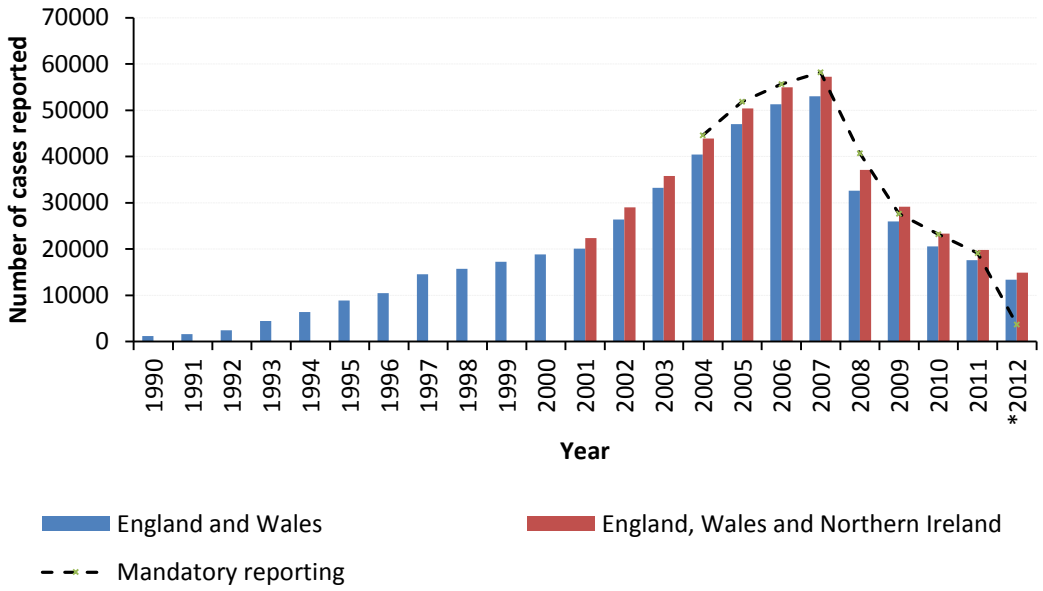



**Figure 1.1. Voluntary and mandatory reporting of *C. difficile*. Voluntary surveillance data for *C. difficile* positive faecal specimens in Northern Ireland was only available from 2001. Mandatory reporting to the HPA began in 2004 (15). * 2012 data only corresponds to the first quarter.**

The DH released a set of guidelines that focused on two key areas to reduce CDI. Firstly, they advocated improving infection control by rapid isolation of cases to limit transmission and use of 'root-cause analysis' to identify sources of transmission. Antibiotic stewardship policies that limited use of 'high-risk' antibiotics were also encouraged (13). Secondly, they addressed the diagnostic testing methods used. Previous diagnostic tests relied heavily on detection of toxin using commercial enzyme immunoassay (EIA) kits. These were easy and quick to use; however, they were limited by poor sensitivity and resulted in a large number of false positives that might have contributed to an over-reporting of cases (17). More recently, a two stage testing approach has been adopted that consists of an initial sensitive test for glutamate dehydrogenase (GDH), an ubiquitous enzyme found in all *C. difficile* strains, followed by an EIA test for confirmation (Figure 1.5) (18).

As a result of these interventions, UK rates of CDI have declined from 55,000 to 18,000 cases over the past five years (14). However, the epidemiology of CDI appears to be changing characterised by an increase in the diversity of strains and the emergence of different 'hypervirulent' strains. In addition, a rise in CDI cases has occurred in groups of patients that were previously perceived to be at low risk of infection.

### *1.2.1 Molecular typing methods used in CDI*

Molecular typing is useful in the setting of outbreaks to explain potential routes of transmission and to monitor the emergence of strains that may display novel antimicrobial resistance or virulence factors. Typing techniques can be divided into PCR-based methods, sequence based methods and those based on detection of polymorphisms after digestion of chromosomal DNA (19).

Globally, *C. difficile* isolates are named based on the molecular technique used for epidemiological typing. Commonly used techniques include Restriction endonuclease analysis (REA), Multilocus sequence typing (MLST), Multilocus variable number tandem repeat analysis (MLVA) and Pulse field gel electrophoresis (PFGE). A brief description of each is given in the glossary.

In the UK, an understanding of CDI epidemiology was greatly facilitated by the development of ribotyping in the 1990s that allowed isolates to be categorised. Ribotyping discriminates strains based on differences in spacer region between the 16S (subunit) and 23S ribosomes, followed by the amplification of DNA encoding these regions, which produces distinct bands that can be viewed by gel electrophoresis. Each distinct pattern corresponds to an individual ribotype (20).

#### *1.2.1.1 Toxinotypes*

Differences exist within the region of the *C. difficile* chromosome known as the Pathogenicity locus (PaLoc) that encodes *C. difficile* toxin A (TcdA) and toxin B (TcdB). Sequence variations, deletions and duplications within the PaLoc might account for differences in the toxins produced by individual strains (21)(22). These differences have allowed the development of a typing system that can be used to distinguish strains and a total of 31 toxinotypes (I to XXXI) have been discovered so far (23).

### *1.2.2 The emergence of novel strains*

The *C.difficile* Ribotyping Network (CDRN) was introduced by the HPA as part of an enhanced disease surveillance program across England. During the first three years of reporting, a decline in the proportion of cases attributed to ribotype 027 was accompanied by a significant increase in the prevalence of other strains that included ribotypes 002, 016 and 078. In the UK, the number of cases caused by ribotype 078

increased from 35 (2%) in 2007-2008 to 285 (5%) in 2009-2010, while in Europe ribotype 078 rose from eleventh to become the third most commonly isolated strain (24)(25). A study conducted in the Netherlands found patients infected with 078 tended to be younger, have fewer comorbidities and originated in the community. Furthermore, the degree of disease severity and the spectrum of microbiological activity reported was similar to 027/BI/NAP (26). Across Europe, previously underreported strains, such as ribotypes 018 and 056, have become increasingly associated with complicated disease and therefore clinicians should remain vigilant against the emergence of potentially virulent strains.

### *1.2.3 Increased prevalence in low risk groups*

*C. difficile* predominantly affects older hospitalised patients with multiple co-morbidities who often require recurrent courses of antibiotics. However, cases have been reported with increased frequency in groups of patients previously thought to be at low risk, which includes pregnant women and children (27)(28). A number of recent case studies also suggest a global increase in community onset CDI and those affected appear to be younger, healthier and less likely to have been exposed to antibiotics in the preceding weeks (29)(30). The lack of established risk factors suggests the possibility of an unknown selection mechanism that favours the emergence of strains leading to the onset of community CDI. One theory is the existence of new *C. difficile* animal and environmental reservoirs in the community that may provide a source of human *C. difficile* acquisition.

In a recent study Janezic *et al.* used PFGE to compare 786 ribotypes isolated from humans, animals and environmental sources that overlapped in geographical location and time (31). Ribotypes 014 and 001 were found in both humans and animals and clustered together on PFGE, with similar patterns of antibiotic sensitivities. Other studies have identified ribotype 078 strains in cattle and pigs that were indistinguishable to strains found in humans (32). In addition, strains capable of causing human disease have previously been isolated in raw and ready-to-eat meat products purchased at grocery stores in America. The majority of isolates recovered belonged to ribotype 078 and the remainder to ribotype 027 (33). *C. difficile* was also recovered from three ready-to-eat salads in Scotland (34). Taken together, this

supports the possibility of zoonotic transmission from one reservoir to another that may account for the emergence of new strains in the community.

### *1.2.4 Cost*

A recent systematic review explored the clinical and economic burden of CDI throughout the European Union (35). A total of 14 countries were included and data in relation to increased cost were available for Germany, Ireland and the UK. The incremental costs related to CDI were £6986 in the UK (based on historical data standardised to 2010 prices) and as high as £8843 in Germany. The study confirmed an association between CDI and increased length of stay, which can leave patients at increased risk of developing hospital acquired infections that require further treatment. Costs are further increased by recurrence of CDI that can be extremely difficult to treat and may require repeated or prolonged treatment.

## 1.3 The bacterium

*C. difficile* is a Gram-positive, anaerobic, spore forming organism, which consists of a highly dynamic genome that has undergone horizontal gene transfer of mobile elements and antibiotic resistance genes over millions of years. Amongst the clostridial species, approximately 15% of sequences are conserved and encode essential functions; however, almost half appear unique to *C. difficile* (36). The genome contains genes that make the organism well suited to the intestinal environment and include genes for carbohydrate metabolism (37). The bacteria exist as both inactive endospores that are essential for disease transmission and as vegetative cells that produce toxins. The majority of *C. difficile* strains are toxigenic and produce TcdA and TcdB in different combinations. Non-toxigenic strains also exist but do not appear to cause disease.

## 1.4 Virulence factors & pathophysiology

Pathogenesis begins with the ingestion of metabolically inactive endospores that are produced in response to nutrient deprivation and are shed in the faeces of infected patients (38). The spores can persist in the environment for prolonged periods of time due to their resistance to a variety of hospital disinfectants (39).

### *1.4.1 Colonisation*

Antibiotic disruption of the intestinal microbiota enables ingested spores to colonise the intestinal tract. Colonisation is facilitated by adhesin molecules that include cell wall proteins (Cwp66 and Cwp84) and the surface binding protein Fbp68, which facilitate adherence and binding of *C. difficile* to the intestinal mucosa (40)(41). Flagella proteins are involved in the penetration of the intestinal mucus layer and enterocyte attachment. *C. difficile* can produce both FliC and FliD flagellar proteins that have been shown to increase adherence to the caecal mucus layer in germ-free mice (42).

### *1.4.2 Sporulation*

The endospores germinate into vegetative cells that are capable of the production of toxins responsible for intestinal inflammation in CDI. Spore germination is defined as the irreversible loss of spore specific characteristics that results in vegetative cell growth (43). The process of germination has been extensively studied in *Bacillus* species and begins when nutrients bind to specific receptors located at the inner-spore membrane, which commit the spore to undergo germination (44). Bile salts combined with specific amino acids are now known to play a key role in germination, although the exact mechanism underlying germination of *C. difficile* spores has been limited by a lack of homologues for the spore receptor.

Howerton *et al.* were able to determine structural relationships for germinant binding and germination activation of *C. difficile* spores *in vitro* using chemical probes that consisted of the secondary bile salt taurocholate and glycine analogs. One probe in particular called cholate *meta*-benzene sulfonic derivative (CamSA) was shown to strongly inhibit spore germination (45). When given prophylactically to mice CamSA prevented subsequent CDI. In mice already infected with *C. difficile*, CamSA limited the extent of gastrointestinal tract inflammation and the rate of disease recurrence (46).

### *1.4.3 Toxins*

Toxin expression by vegetative cells occurs during late log and stationary growth phase in response to antibiotic exposure and catabolic repression (47). TcdA (308kDa) and TcdB (270kDa) belong to the family of large clostridial glucosylating toxins that inhibit the action of the proteins Rho and RAS. These proteins belong to a family of enzymes that can bind and hydrolyse guanosine triphosphate (GTP) and are known as GTPases. In the active state, they act as molecular switches and are involved in regulation of cell signalling pathways and cell cytoskeleton maintenance (48)(49). They remain inactive in the guanosine diphosphate (GDP) bound form.

Both toxins are encoded by genes that are closely located within the PaLoc, which consists of a 19.6kb single open reading frame (Figure 1.2). This is replaced by a 115 base pair (bp) non-coding sequence in non-toxigenic strains. Three additional genes are located within the PaLoc that encode proteins involved in toxin expression. TcdC is a negative regulator of toxin production, TcdR is an alternative sigma factor involved in the positive regulation of toxin genes and TcdE, is a putative holin that aids cell wall pore formation and toxin release into the cytosol (50).

#### *1.4.3.1 Toxin structure*

A theory exists that TcdA and TcdB may have arisen following a gene duplication event that is supported by the structural and functional similarities within their enzymatic and receptor-binding domains (51). The original structure proposed for TcdA and TcdB was a two domain ‘AB’ structure based on the biologically active and binding translocation domains seen in diphtheria toxin (52). This model was later extended to a four domain ‘ABCD’ model, where ‘A’ corresponded to biological activity, ‘B’ binding, ‘C’ cutting and ‘D’ delivery (Figure 1.2) (53).

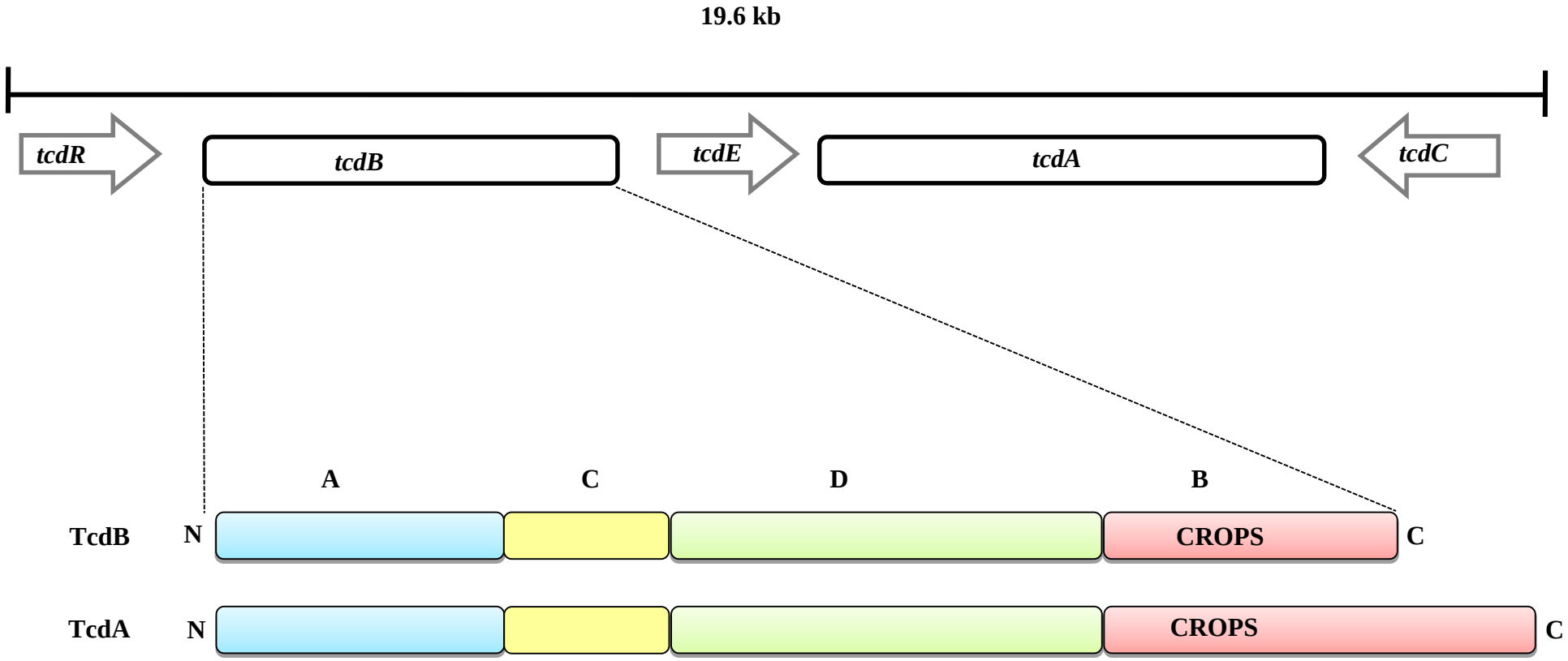



**Figure 1.2. *C. difficile* Pathogenicity Locus. Genes encoding toxins and proteins involved in toxin regulation are located here. TcdR is a positive regulator and TcdC is a negative regulator of toxin production. TcdE assists toxin cellular entry through pore formation. 'ABCD' toxin structure: A- biologically active glucosyltransferase domain, B- receptor binding domain, C- auto-catalytic protease cutting domain and D- hydrophobic translocation domain. Combined repetitive oligopeptides (CROPs) are highly repetitive structures that play a role in cell binding. Adapted from Jank and Aktories (53).**

#### *1.4.3.1 Processing of TcdA and TcdB*

The toxins bind to cell surface receptors using the carboxy-terminal (C-terminal), which consists of clostridial repetitive oligopeptides (CROPs) that may play a role in target cell recognition and binding. Studies have demonstrated that the C-terminal of TcdA folds into a solenoid structure that increases the surface area of the protein and facilitates interactions with other proteins (54). X-ray crystallography, homology modelling and small-angle X-ray scattering methods were recently used to develop the first three-dimensional model of TcdB. This showed that the C-terminal of TcdB was similar to TcdA but with fewer CROPS (55). Once bound, receptor-mediated-endocytosis and internalisation of the toxin-receptor complex occurs (Figure 1.3) (56). The acidic endosome environment induces a conformational change in toxin structure that exposes a hydrophobic region within the toxin, which enables pore formation and membrane insertion (57). Auto-proteolytic cleavage of toxins occurs before translocation of the glucosyltransferase domain into the cytosol (58). The amino-terminal (N-terminal) of TcdA and TcdB contains the region that modifies Rho GTPases, by glucosylation of the amino acid threonine at residue 35 that blocks signal transduction pathways (59). In this state, the proteins can no longer switch to the active form and disruption to the actin cytoskeleton occurs, characterised by cell rounding, detachment and cell death. TcdA-intoxicated cells show a distinct cell retraction phenotype that is linked to changes in the microfilament system and similar effects have been observed with TcdB (60). Both toxins cause disruption to epithelial tight junctions with resultant fluid accumulation and migration of neutrophils into the colonic lumen that causes intestinal inflammation and oedema (61).

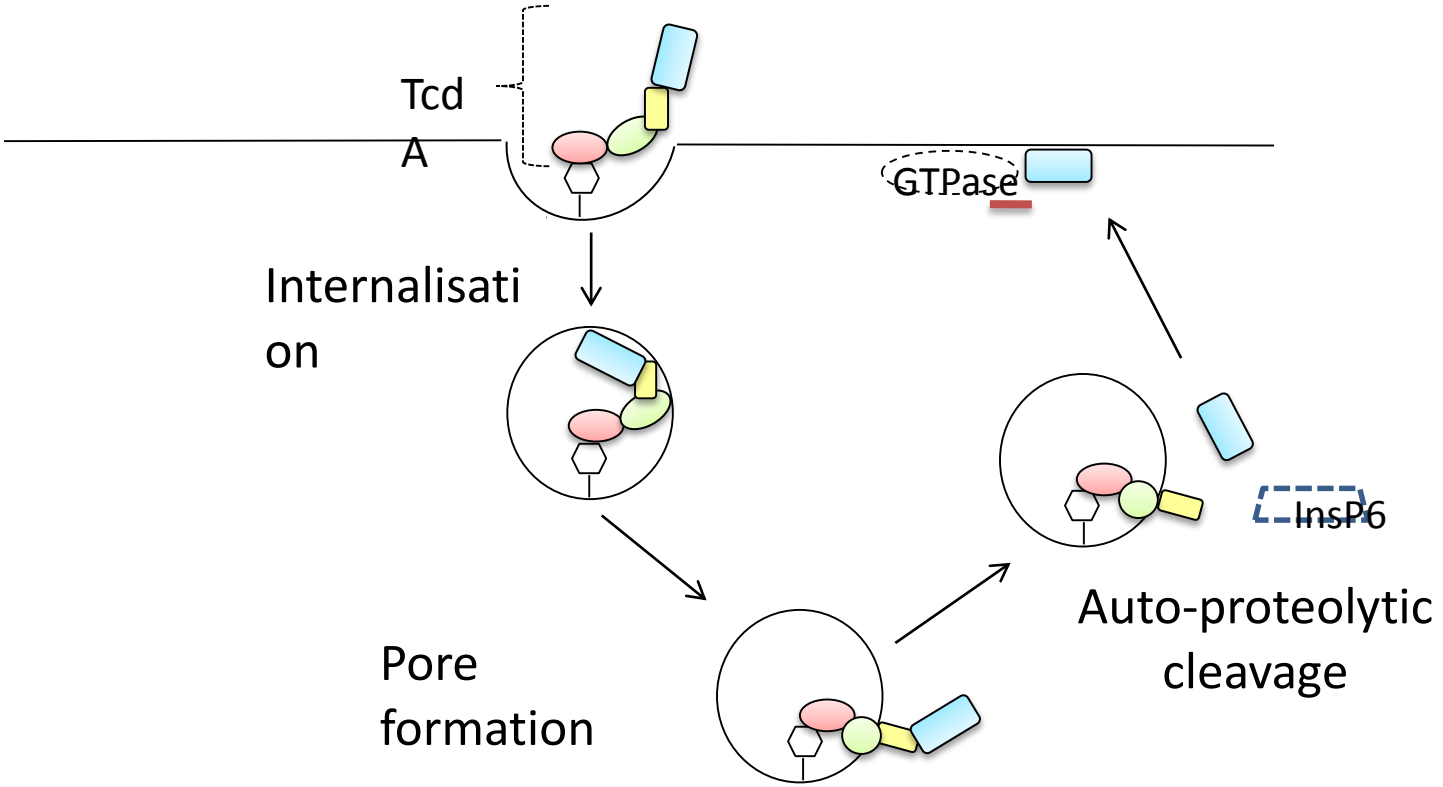



**Figure 1.3. Mechanism of TcdA and TcdB entry into cells. Toxin bind to the cell membrane via the C-terminal and are internalised by receptor-mediated-endocytosis. Once internalised, the acidic environment results in a conformational change to the toxin that allows insertion into the endosomal membrane and pore formation. Auto-proteolytic processing of toxins occurs and the glucosyltransferase domain is released into the cytosol in the presence of the inositol phosphate InsP6. Glucosylation of Rho GTPase interferes with its function that results in damage to the cytoskeletal function and cell integrity. Adapted from Pruitt and Lacy (62).**

#### *1.4.3.2 Role of toxins in CDI*

The relative contributions of TcdA and TcdB to the pathogenesis of CDI are not fully understood. Earlier studies suggested TcdA was the most important virulence factor with TcdB unable to cause intestinal effects in animal models. An influential early study demonstrated that TcdA alone could trigger intestinal inflammation in hamsters and mice but TcdB could not unless co-administered with TcdA or after epithelial injury had already occured (63). However, this may be explained by the absence of TcdB receptor binding sites in the rodent intestine that was demonstrated using radiolabelled iodine (64). Studies have shown a correlation exists between the concentration of membrane receptors for TcdA and TcdB and the biological responsiveness to these toxins. Eglow *et al.* demonstrated TcdA receptor binding in rabbits was age-dependent with an absence of TcdA-specific brush border receptors in new born rabbits that gradually increased from birth to age 24 days (65). Despite failure to demonstrate an enterotoxic effect in earlier experimental animal models, TcdB is extremely cytotoxic to cultured mammalian cell lines and 10 times more potent than TcdA when applied to human colonic epithelium *in vitro* (66)(67). In a zebrafish embryo model, TcdB localised to the pericardium and caused cardiotoxicity and reduced ventricular contractility, which suggests this toxin may play a role in the systemic effects seen in severe CDI (68).

Historically, difficulties associated with manipulation of the bacterial genome of *C. difficile* may have limited our understanding of the contribution of each toxin to CDI. However, recently isogenic *tcdA* and *tcdB* mutants were created and evaluated in a hamster model of infection. Lyras *et al*. demonstrated reduced virulence of isogenic *C. difficile* strains that lacked TcdB, but not TcdA (69). A second study by Kuehne *et al*. showed that complete abrogation of virulence was only achieved following knockout of both toxin genes (70). This apparent discrepancy in evidence for the roles of TcdA and TcdB may be partly explained by the different strains used by each group that resulted in a three-fold difference in toxin production or the use of different endpoints in the respective animal models. However, in both studies the TcdA mutant strains were as virulent as wild type strains, which suggest that TcdA is not essential for disease. These finding demonstrate the important role of TcdB in CDI pathogenesis. This is further supported by the presence of virulent *tcdA-/tcdB+* strains

in clinical practice, with *tcdA+/tcdB-* strains yet to been identified in human disease (71).

#### *1.4.3.3 Binary toxin*

A third of isolates produce another toxin known as *C. difficle* transferase (CDT) or binary toxin, which belongs to the family of binary actin adenosine diphosphate (ADP) ribosylating toxins. These toxins consist of a biologically active actin-modifying ADP ribosyltransferase and a separate binding component, which is involved in the binding and transport of the enzyme component into the cytosol of target cells (72). The genes *cdtA* and *cdtB* encode toxin production and are located with a regulator of toxin expression, *cdtR*, at a separate locus to the PaLoc in the bacterial genome (73). Binary toxin can induce microtubule production that may aid adherence at the intestinal epithelial cell surface (74). However, despite detection in epidemic 027/NAP1/BI strains the contribution of this toxin to CDI pathogenicity remains to be determined.

### *1.4.4 Surface layer proteins*

*C. difficile* is surrounded by a para-crystalline surface array known as the S-Layer that is composed of identical glycoprotein subunits arranged to form a regular lattice on the bacterial cell surface that acts as an interface between the bacteria and host cells (75). The structure is encoded by a single *slpA* gene that is co-located with genes encoding the adhesin Cwp66 and a secretory enyme in a 10kb S-layer cassette. The S-layer consists of two surface layer proteins (SLP); a high molecular weight protein that is relatively conserved between strains and a low molecular weight protein that demonstrates considerable sequence diversity (76)(77). Recent work has demonstrated that exchange of Deoxyribonucleic acid (DNA) fragments occurs between isolates within the S-layer cassette, which is similar to the polysaccharide capsular switching that occurs in *Neisseria meningitides* that facilitates bacterial immune evasion (78). The exchange of DNA by homologous recombination may contribute to differences in antigenicity and virulence between strains.

## 1.5 Determining outcome in CDI

CDI occurs across a wide clinical spectrum that includes asymptomatic carriage, mild diarrhoea and fulminant severe life threatening disease. Recurrence of CDI can occur and poses a therapeutic challenge for which limited evidence based treatments exist (Figure 1.4). It is still unclear what determines patient susceptibility and disease outcome in CDI; however, increasing evidence suggests a role for both pathogen and host factors.

### *1.5.1 Bacterial factors*

#### *1.5.1.1 027/NAP1/BI*

The epidemic 027/NAP1/BI strain coincided with a change in disease phenotype that was characterised by an increase in the severity of CDI and higher mortality rates (5)(79). This strain was capable of producing 16 times more TcdA and 23 times more TcdB than other strains (80). A frame shift mutation and 118 bp deletion in the gene encoding the TcdC toxin regulator protein was initially thought to be responsible for the increase in toxin production. However, this theory was recently contested by Cartman *et al*. who used an allele exchange system to systematically restore the mutation and deletion in the *tcdC* gene, which did not effect the level of toxin production (81).

Earlier *in vitro* studies suggested epidemic hypervirulent 027/NAP1/BI strains were capable of increased sporulation that contributed to their increased virulence (82)(83). However, Burns *et al*. compared 14 different clinical isolates and found sporulation rates to be lower in some 027/NAP1/BI strains compared to other *C. difficile* strains. Therefore currently, insufficient evidence exists to support increased sporulation in all hypervirulent 027/NAP1/BI strains (84).

Differences in the toxins themselves may be responsible for the altered virulence. A comparison between the hypervirulent 027/NAP1/BI and a historic ribotype 027 strain revealed a wider variation in the C-terminal region of TcdB in the epidemic strain that might facilitate increased binding across a broader cell tropism (85). Studies have also demonstrated variations in flagellar genes, metabolic genes and phage islands within the hypervirulent 027/NAP1/BI chromosome that may account for the difference in virulence and clinical phenotype associated with this strain (86)(87).

The impact of strain type on mortality and biomarkers of disease severity was recently evaluated by Walker *et al.* who compared over 2000 cases of *C. difficile* (confirmed on toxin EIA) to a similar number of negative controls (88). All isolates underwent ribotyping and MLST. The 14 day attributable-mortality differed between strain types and was highest in patients infected with ribotype 078 (25%), followed by ribotype 027 (20%). Strain-specific changes in inflammatory biomarkers that included higher neutrophil and white cell counts were also highest in those infected with ribotypes 027 and 078. This is one of the first studies to describe this association and is in contrast to earlier studies; however, previous studies tested small numbers and were therefore underpowered to detect a difference between strains (89)(90).

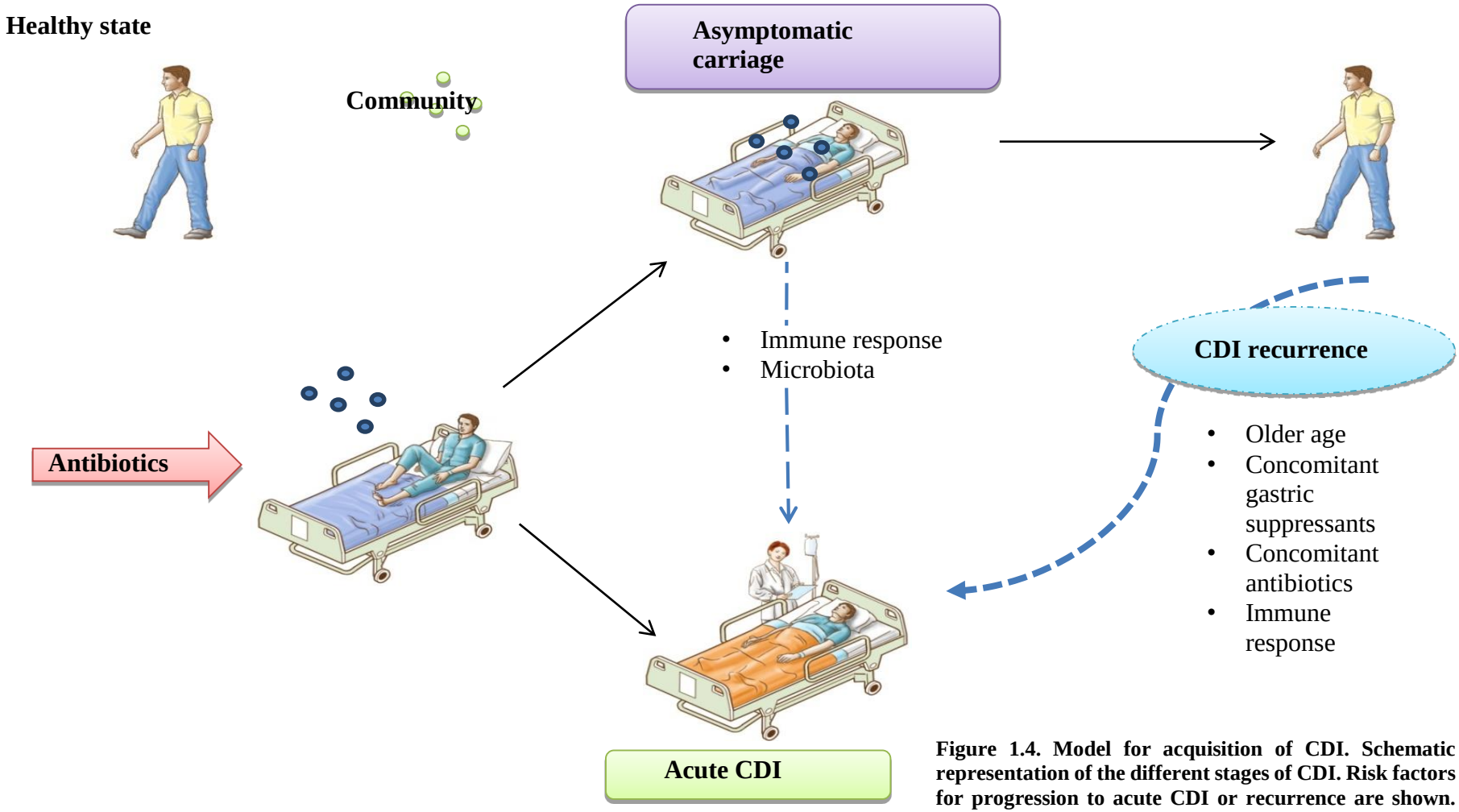



**Figure 1.4. Model for acquisition of CDI. Schematic representation of the different stages of CDI. Risk factors for progression to acute CDI or recurrence are shown. Figure adapted from Rupnik *et al* (37) .**

### *1.5.2 Patient susceptibility factors*

Host factors implicated in determining outcome in CDI include increased age, degree of underlying co-morbidity, the immune response to TcdA and TcdB and antibiotic disruption to the intestinal microbiota. The immune response and the microbiota have emerged as key factors in determining patient susceptibility and are discussed in more detail below.

## 1.6 Microbiota

At birth the human intestinal tract is sterile but becomes rapidly colonised by a variety of organisms to form the microbiota. During the first few days of life, a great deal of temporal and inter-individual variation occurs, which is influenced by the mode of delivery, breast-feeding and the environment (91)(92). By the second year, a highly diverse microbiota has been established that remains stable until older age when diversity is known to decrease.

### *1.6.1 Metagenomics and the molecular era*

Between 60-80% of the microbiota remains uncultivable and until recently scientific knowledge about the structure and function of the intestinal microbiota was limited by a reliance on culture-dependent techniques (93). However, recent advances in molecular sequencing have allowed the identification of previously undetected microorganisms, through the isolation and sequencing of cloned small sub-unit ribosomal ribonucleic acid (rRNA) genes from human samples that are identified by comparison with existing DNA sequences stored in publically available databases. The bacterial genomes detected are collectively known as the metagenome and the experimental process is referred to as metagenomics. Developments in high-throughput sequencing and bioinformatics have allowed the construction of large gene libraries that allow wide-scale comparisons of microorganisms to be made (94).

Microorganisms exist in site-specific communities and different groups predominate in the skin, vaginal tract and intestinal tract (95). Over 1,800 genera and between 15,000-36,000 individual bacterial species have been reported within the intestinal tract (96). A gradual increase in the number of microorganisms occurs with progression from the proximal to distal intestines; however, the degree of microbial diversity appears to remain consistent in different intestinal compartments. This was demonstrated by Eckburg *et al*. who compared multiple caecal and colorectal biopsies

and found the same major phyla were present at each site (93). The predominant intestinal bacterial phyla are Firmicutes and Bacteroidetes and remain consistent between individuals, although inter-individual variation does occur at the species level (97)(98). Recently, the metagenomes of 39 individuals from across Europe, America (USA) and Japan were compared that revealed the existence of clusters of microbial communities known as enterotypes, which differed in their composition and functional activities (99).The enterotypes did not correlate with differences in nationality, gender, age or body mass index in contrast to earlier work by Ley *et al.* that demonstrated the composition of microbiota in obese individuals was altered after a change to a low-calorie diet (100).

### *1.6.2 Ageing and the microbiota*

Older individuals experience a shift in the microbiota composition that is accompanied by changes to the immune system and intestinal physiology. These changes include increased mucosal permeability and a decline in secretory immunoglobulin A (sIgA), defensins and gastric acid that may contribute to increased disease susceptibility (101). The intestinal microbiota in the older population is known to be dominated by Bacteroidetes in contrast to younger individuals where Firmicutes dominate (101)(102).

### *1.6.3 Functions*

The intestinal microbiota has co-evolved with humans and serves a mutually beneficial role. In addition to providing nutrients and helping with carbohydrate metabolism, the microbiota plays an important role in the development of the immune system. The body of evidence supporting the role of the microbiota in development and maintenance of the immune system has rapidly expanded over the past five years (103). Studies have demonstrated germ-free mice that lack a commensal microbiota fail to develop isolated lymphoid follicles and produce lower levels of sIgA (104). In addition, the microbiota has been implicated in determining the shape of T cell subsets. This was demonstrated by transplantation of faecal flora, containing 46 clostridial species, from wild-type mice into germ-free mice that caused an expansion in T regulatory cells and expression of the interleukin IL-10 (105).

One of the earliest recognised functions of the intestinal microbiota was the ability to prevent pathogen colonisation through direct competition for receptor binding sites

and nutrients by a process known as colonisation resistance (106). Antibiotics can destroy members of the indigenous microbiota that results in antibiotic associated diarrhoea (AAD) by one of two main mechanisms. Firstly, they can alter the intestinal metabolic profile resulting in a build-up of unfermented small carbohydrate molecules and short chain fatty acids in the intestinal lumen results, which can cause an osmotic diarrhoea (107). Secondly, antibiotics can prevent colonisation resistance and provide an opportunity for colonisation and overgrowth of pathogens that include *C. difficile.*

### *1.6.4 The effect of antibiotics on the microbiota*

Colonisation resistance was first described in the 1970's by van der Waaij *et al.* who investigated the ability of antibiotic treated germ-free mice to resist colonisation by the pathogens *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* (108). The number of pathogens recovered on faecal culture was used as a measure of the extent of colonisation resistance. During the period immediately after antibiotic treatment all three pathogens were undetectable, which suggested a loss of colonisation resistance amongst the normal intestinal microbiota. However, following antibiotic cessation all three pathogens were subsequently detected in mice faeces that indicated re-population of the resident microbiota had occurred. This process has also been demonstrated in human studies using molecular techniques that showed a gradual recovery in the microbiota after cessation of antibiotic treatment (109). However, the microbiota may not always completely recover. This was highlighted by the failure to recover previously detected *Bifidobacterium,* at 14 days post-cessation of antibiotic treatment in a patient that developed AAD (110). The long-term effects of antibiotics on the microbiota are not fully known; however, in a study by Janberg *et al.* healthy individuals treated with clindamycin demonstrated a continued reduction in detectable numbers of *Bacteroides* up-to two years after discontinuation of treatment (111).

### *1.6.5 The microbiota and CDI*

A recent community based study conducted in an older population in Ireland used molecular sequencing to demonstrate the microbiota composition of asymptomatic carriers of *C. difficile* was similar to culture-negative individuals (112). In the two

individuals colonised with ribotype 027 who developed acute CDI, lower numbers of *Faecalibacterium spp*. and *Bifidobacterium* were detected; this is of interest given that both species are reduced in patients with chronic gut inflammation (113).

Chang *et al*. used 16S rRNA–encoding gene sequence analysis to compare the microbial stool profile of four patients with an initial CDI episode and three patients with a recurrence of CDI to healthy controls (114). Similarities were observed between healthy controls and patients with an initial CDI episode with a predominance of organisms belonging to the phyla Bacteroidetes and Firmicutes. However, the composition of the microbiota was more variable in patients with a recurrence of CDI and included a marked decline in Bacteroidetes and an associated increase in 16S phylotypes related to *C. difficile*. This suggests that the size of the *C. difficile* population may be directly controlled by the composition of the existing intestinal microbiota and highlights the important role of the microbiota in determining patient susceptibility.

## 1.7 CDI and the immune response

A considerable body of evidence exists describing the role of the immune response during each stage of CDI. Early in the disease process, toxin-mediated intestinal inflammation results in the induction of the innate immune response, which produces a milieu of inflammatory and regulatory cytokines to try and limit the extent of inflammation. The adaptive immune response is also important and has been implicated in determining outcome in CDI.

### *1.7.1 Innate immunity*

Paneth cells are specialised epithelial cells that reside in intestinal crypts and secrete antimicrobial peptides, such as defensins and cathelicidin, which have been implicated in reducing intestinal inflammation in CDI. In *C. difficile* infected mice, intracolonic cathelicidin treatment resulted in a reduction in colonic inflammation and *in vitro* studies have demonstrated that human alpha-defensins applied to human epithelial colorectal adenocarcinoma cells (CaCo-2 cells) caused inhibition of TcdB-mediated glucosylation of cells and reduced cell cytotoxicity (115)(116). A novel mechanism recently described by Savidge *et al.* used S-nitrosylation, which involves the addition of a nitric oxide molecule to a thiol group, to prevent the auto-catalytic cleavage of toxins that resulted in attenuation of toxin effect (117).

Once the epithelial barrier is breached, direct interaction between TcdA or TcdB and host macrophages and monocytes results in the rapid release of pro-inflammatory cytokines that include interleukins (IL-1, IL-6, IL-8) and tissue necrosis factor alpha (TNFα) (118)(119). Toll like receptors (TLR) are specialised immune receptors that can detect conserved molecular patterns on pathogens. TLR4 has been shown to be important in generating T helper cells and Ryan *et al.* demonstrated mice deficient in TLR4 developed severe CDI compared to wild-type mice (120).

A notable feature of CDI is the magnitude of the systemic blood neutrophil response and patients with CDI often mount a 'leukaemoid' neutrophil response. In contrast to other examples of bacterial sepsis the magnitude of the neutrophil response in CDI has been shown to correlate with adverse outcome (121). Early experiments demonstrated that monocytes exposed to TcdA and TcdB released increased levels of the neutrophil chemotactic molecule IL-8 and the leukocyte adhesion molecule CD18 integrin that suggests both toxins contribute to neutrophil recruitment and tissue infiltration (122). It is interesting that both toxins can also inhibit the phagocytic response in CDI. TcdA stimulation of neutrophils *in vitro* resulted in impaired migration and decreased oxidative activity, while TcdB stimulation of macrophages resulted in impaired phagocytosis (123)(124). Anti-inflammatory cytokines initiate cellular repair processes and minimise the harmful systemic effects associated with a severe inflammatory response. In piglets exposed to *C. difficile*, raised levels of the anti-inflammatory cytokine IL-4 were detected in animals that failed to develop severe CDI and *in vitro* work has demonstrated dendritic cells can produce increased levels of IL-10 in response to the SLP from different C. *difficile* isolates (125)(126).

### *1.7.2 Adaptive immunity*

Studies have demonstrated that over 60% of the adult population possess both serum and mucosal antibodies to TcdA and TcdB in the absence of colonisation or active CDI (127)(128). Antibodies to both toxins are generated following transient *C. difficile* colonisation during infancy but may result from exposure to other clostridial species that includes *Clostridium sordelli,* which possess cross-reacting antigens. The observational case-studies below have provided the scientific rationale for developing passive and active immune based therapeutic interventions for CDI.

#### *1.7.2.1 Antibody response to TcdA and TcdB*

Generation of an effective antibody response to TcdA and TcdB is important in determining patient susceptibility at each stage of CDI, with the exception of colonisation (129). Asymptomatic carriers have raised serum IgA, Immunoglobulin M (IgM) and polyvalent immunoglobulin levels to somatic-cell antigens of *C. difficile* compared to symptomatic patients that suggests an effective antibody response may prevent progression to active disease (130). This early observation was confirmed in a prospective study of 271 patients that showed asymptomatic carriers had significantly higher serum Immunoglobulin G (IgG) antibody levels to TcdA compared to those that subsequently developed acute CDI (129). Failure to mount an effective antibody response to TcdA has been associated with prolonged diarrhoea and can leave patients susceptible to recurrence (131). Kyne *et al*. found patients with raised IgM antibody levels to TcdA on day three and raised IgG levels to TcdA on day 12 of CDI were less likely to experience a recurrence (132). Lower antibody titres to TcdB have been found in convalescent serum and in patients that experienced a recurrence of CDI (133)(134).

#### *1.7.2.2 Antibody response to SLP and surface cell proteins*

The outermost layer of *C. difficile* is the S-Layer, which consists of two separate proteins of differing molecular mass and is known to be highly immunogenic. Drudy *et al.* measured antibody responses to the low molecular SLP, high molecular SLP and combined SLPs in four patients; two were asymptomatic carriers and two had active CDI (135). The study found IgM titres to all SLPs were significantly higher in asymptomatic carriers compared to patients with active disease. The same study measured antibodies in 34 patients with active disease and found IgM titres to SLPs on day three of diarrhoea were higher in patients with a single episode of CDI compared to those who developed a recurrence.

In a separate study, the median antibody titres to four surface proteins FliC, FliD, Cwp66 and Fbp68 were significantly lower in patients compared to controls (40). These studies highlight that antibodies to virulence factors involved in colonisation and adherence of *C. difficile* are also important in patient susceptibility and could be used as future therapeutic targets.

#### *1.7.2.3 Passive immunotherapy*

Colostrum produced from cows immunised with *C. difficile* culture filtrate toxoid, prevented *C. difficile* associated diarrhoea when given orally to hamsters (136). The anti-bovine immunoglobulin contained high levels of neutralising IgG antibodies to both TcdA and TcdB and inhibited the enterotoxic effects of *C. difficile* in a rat ileal-loop model of disease (137). However, despite promising results, bovine colostrum has not been evaluated further in human studies. A similar product called Mucomilk was developed that consisted of 40% immune whey protein concentrate. The product was well-tolerated in an uncontrolled pilot study of 16 CDI patients and nopatients experienced a recurrence of CDI (138). A randomised controlled trial of Mucomilk demonstrated equivalent efficacy to metronidazole in preventing recurrence but unfortunately the study was terminated prematurely due to limited funding (139).

Parenteral passive immunotherapy with pooled human immunoglobulin was first used to treat five paediatric cases suffering from recurrent disease. Intravenous immunoglobulin (IVIG) resulted in resolution of symptoms in all five cases and increased antibody titres to toxins were detected following IVIG use (140). Subsequent studies that have used IVIG to treat severe refractory disease have shown mixed results and currently no standardised algorithms exist for IVIG use in clinical practice (19)(141).

Monoclonal antibodies directed against the repeating binding unit of TcdA were administered to *C. difficile* inoculated gnotobiotic mice that resulted in lower detectable levels of TcdA and reduced mortality compared to control mice (142). Babock *et al.* developed fully humanised monoclonal antibodies (HumAbs) against both TcdA and TcdB by immunising mice that were transgenic for human immunoglobulin genes with inactivated toxins (143). Both HumAbs demonstrated neutralising ability *in vitro* and reduced mortality from 100% to 45% in hamsters when administered as a combined preparation (143). The HumAbs were subsequently evaluated in a large randomised, placebo-controlled trial to establish their role in preventing recurrence of CDI (144)(145). Patients with symptomatic CDI were randomised to receive either a single infusion containing both monoclonal antibodies or a placebo infusion of normal saline, in addition to standard antimicrobial treatment. The recurrence rate was significantly lower in the active group compared to the placebo group (7% vs. 25%, 95% confidence interval [CI] 7-29, $p<0.001$). The

combined HumAb infusion also proved effective in reducing recurrence in patients with multiple episodes of CDI.

#### *1.7.2.4 Active immunotherapy*

Active immunisation has been demonstrated using different vaccination methods and a variety of animal models. A toxoid based vaccine prevented lethal CDI in hamsters and a recombinant vaccine, expressed in *Vibrio cholerae,* generated immunity to CDI in a rabbit model of disease (146)(147). Recently, a DNA vaccine was created that contained a synthetic gene encoding the receptor-binding domain of TcdA (148). After optimisation for expression in human cells, the vaccine was tested in CDI infected mice who developed raised titres of neutralising antibodies and were protected against death. The latest 'chimera' vaccines combined the binding domains of both toxins and were able to elicit protection in mice and hamsters, including animals infected with 027/NAP1/B1 (149)(150).

Despite the success in animal models an effective human vaccine has remained elusive. A toxoid vaccine containing both toxins was well-tolerated in healthy individuals and used to effectively treat three patients suffering from recurrent refractory CDI but further large scale studies are needed (151)(152). Future vaccination strategies may combine both recombinant toxins and surface proteins to target different stages of *C. difficile* pathogenesis.

### *1.7.3 Ageing and the immune response*

The immune system undergoes age-related change that is known as immunosenescence. Early observations suggested that a decline in immunity affected all components of the immune system in an indiscriminate way; however, it is now thought a remodelling affect occurs and the greatest changes take place in the adaptive immune response (153). An overall reduction in the T cell repertoire occurs and chronic antigenic stimulation by cytomegalovirus has been implicated (154). Animal models have demonstrated a decline in antibody diversity due to the clonal expansion of memory B cell subsets, combined with the generation of antibodies of lower affinity due to a switch from IgG to IgM isotypes (155)(156). These changes are likely to contribute to increased susceptibility to CDI and may pose an important challenge to the use of immunotherapies in this age group.

## 1.8 Clinical presentation of CDI

### *1.8.1 Asymptomatic carriage*

Asymptomatic carriage is the term used to describe patients colonised with *C. difficile* that do not develop the features of active CDI. Between 1-3% of healthy adults are thought to be colonised with either non-toxigenic or toxigenic strains with increased rates of 7-26% reported in hospitalised patients (157)(158)(159). Studies have shown that patients who remain asymptomatically colonised for prolonged periods of time are less likely to develop active disease (129)(160). A recent study conducted over a two year period used MLST to demonstrate that only a quarter of the 1276 isolates tested were linked to a ward-based inpatient source. The authors concluded that the large number of unlinked cases detected might be explained by transmission from asymptomatic carriers that contribute to environmental loads through continued shedding of spores (161).

In the community, asymptomatic carriage has been reported in 10- 21% of long-term care residents, with rates as high as 52% reported in this group during an outbreak (112)(162)(163). When combined with transient *C. difficile* colonisation of infants, which can be as high as 60%, these groups may act as potential reservoirs of disease that have contributed to the recent rise in community cases of CDI (164).

### *1.8.2 Acute CDI*

Definitions of CDI vary slightly between European and American guidelines but both include increased frequency of diarrhoea and a positive stool toxin test (13)(19) (165). The incubation period of *C. difficile* is estimated to be around three days and clinical features range from mild diarrhoea to severe disease which is characterised by fever, abdominal discomfort, leucocytosis and electrolyte disturbance (4)(158). In fulminant cases pseudomembranous colitis may develop and diagnosis is made endoscopically. The colon appears inflamed and is covered with small yellow plaques of varying diameter that consist of epithelial necrosis accompanied by an exudate of fibrin and neutrophils (166). The pseudomembrane itself consists of mucin, fibrin, leucocytes and cellular debris (167). Rapid progression to toxic dilatation of the bowel and subsequent bowel perforation can occur and carries a high mortality rate. Extra-intestinal manifestations of CDI are rare although cases of bacteraemia have been reported in the literature (168).

Current guidelines stratify disease severity based on leucocytosis, an increase in creatinine of more than one and a half times above baseline, development of features of septic shock and ileus (19)(165). Clinical prediction tools for predicting severity in CDI have included laboratory parameters, radiological markers and host factors such as underlying comorbidities and age (169)(170). However, such tools were not validated in separate independent cohorts and were developed using small sample sizes. Therefore, currently no clinical prediction tools are routinely used in CDI (171).

### *1.8.3 Recurrence of CDI*

Recurrence affects 10-30% of patients despite successful treatment of the initial infection and occurs either due to relapse caused by the original infecting strain or reinfection with a new strain (172). In clinical practice it is impossible to distinguish between the two mechanisms, although relapse due to the original strain has been shown to occur much sooner (173). Recurrence most commonly occurs in the first week after cessation of CDI treatment but can occur several months after the initial infection (174)(175). Risk factors for recurrence are discussed in Chapter 4 but include increased age, concomitant antibiotics, concomitant gastric suppressants, failure to mount an effective antibody response to TcdA and TcdB and the underlying degree of disease severity (176)(177).

## 1.9 Diagnosis

Accurate diagnosis of CDI is vital as false positive results can result in inappropriate treatment of patients; conversely, false negative results can cause treatment delays and increased risk of cross-infection through delayed patient isolation. Several different tests exist for the diagnosis of *C. difficile* and can be divided into three groups: tests for *C. difficile* products (GDH, TcdA and TcdB), culture methods and detection of *C. difficile* genes (toxin genes, PCR) (178).

*C. difficile* was originally named due to difficulties associated with culturing the organism, although culture remains the gold standard. When plated, colonies appear flat, yellow and ground-glass in appearance and can produce a distinctive 'horse-barn' odour (179). The cell cytotoxicity assay (CCA) is an alternative reference test to culture that involves the application of stool filtrate onto a monolayer of cells, before observing for characteristic cell rounding caused by the cytotoxic effect of TcdB. Specificity of this effect is confirmed by neutralisation with *C. difficile* antitoxin. However, both reference tests can be laborious and time consuming.

Until recently, diagnostic testing has relied on EIA tests that detect the presence of TcdA and TcdB in stool. However, a study of nine commercially available tests showed the overall sensitivity ranged from 66.7% to 91.7% with positive predictive values as low as 48.6% (180). Therefore, a two-step diagnostic algorithm was proposed that combined a highly specific test, such as GDH, with a toxin EIA test. This approach was validated in a large study that tested over 12,000 stool samples from four UK laboratories and has formed the basis of current UK testing guidelines (summarised in Figure 1.5) (18)(181).

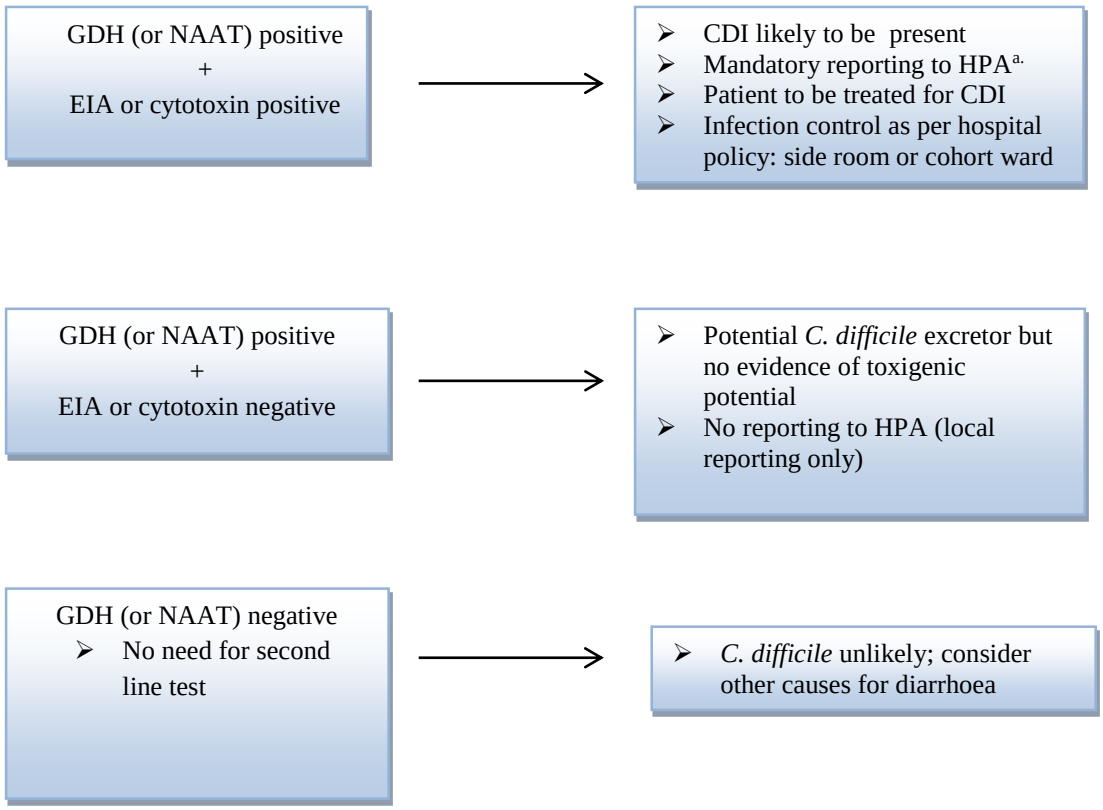



**Figure 1.5. Diagnostic algorithm for CDI. Abbreviations: Glutamate dehydrogenase (GDH), enzyme immunoassay (EIA), nucleic acid amplification test (NAAT), Health protection agency (HPA). a. The following cases of CDI should also be reported to the HPA: Pseudomembranous colitis, colonic histopathology characteristic of CDI (in the absence of diarrhoea or toxin detection) obtained during endoscopy or colectomy and a positive *C. difficile* toxin faecal test at post-mortem. Algorithm adapted from the Department of Health guidelines (18).**

## 1.10 Management of CDI

The effective management of CDI involves a combination of infection control measures and antimicrobials as first-line treatment. Infection control policies have focused on reducing horizontal transmission and limiting patient risk once exposure to CDI has occurred. Patients should be isolated in single rooms or on cohort wards and regular hand-washing with soap and water is encouraged as *C. difficile* spores are resistant to alcohol gel (182). In addition to reducing transmission between individuals, ward cleaning with a chlorine based agent is essential to limit environmental transmission of spores that survive as fomites on multiple surfaces for extended periods of time (183)(184).

Since 2007, antibiotic restriction policies have been in place across the UK to try and limit the use of antibiotics perceived to be strongly associated with CDI. Recent figures published by the UK CDRN reported a significant decline in both fluroquinolone and cephalosporin use over a three year period, which are both associated with an increased risk of CDI. This was accompanied by an increase in co-amoxiclav and piperacillin-tazobactam use (24). The successful implementation of antibiotic restriction policies might explain why the UK has seen a greater decline in rates of CDI compared to the USA where rates have remained at almost epidemic proportions (185).

### *1.10.1 Acute CDI*

Early cases of CDI were successfully treated with vancomycin but reports of effective treatment with metronidazole quickly followed in the same year (186)(187). The first randomised controlled trial was conducted in 1983 and demonstrated metronidazole was similar to vancomycin in terms of efficacy and much better in terms of cost (188). However, as metronidazole is almost completely absorbed in the small intestines it is unclear how much reaches the colon. In addition, increased colonic inflammation occurs in severe disease that may further reduce the drugs effect. Vancomycin has more favourable pharmacodynamics and persistently high faecal levels have been detected throughout the course of treatment (189).

A recent systematic review of treatment efficacy in CDI included three studies that directly compared metronidazole and vancomycin. No significant difference was found in efficacy between the two antibiotics (190). However, one of the studies by

Zar *et al*. pre-stratified patients into mild and severe disease based on a clinical scoring system, before patients were randomly assigned to receive metronidazole or vancomycin (191). Although rates of clinical cure were similar in patients with mild disease, significantly higher cure rates were achieved with vancomycin in patients with severe disease (97% vs. 76%, p=0.02). Therefore, current guidelines recommend metronidazole for mild CDI and vancomycin for moderate to severe disease (19)(165). In severe disease, if ileus is suspected vancomycin can be given intra-colonically by retention enema. Surgical colectomy is reserved for life-threatening cases that are associated with a rapid deterioration in clinical condition, and surgery should be performed before the serum lactate exceeds 5mmol/L (165).

#### *1.10.1.1 Fidaxomicin*

Recently, the antibiotic fidaxomicin was licenced for CDI treatment in adults and may be useful for preventing CDI recurrence (192). Fidaxomicin is a novel macrocyclic antibiotic that inhibits transcription by the enzyme RNA polymerase. The drug has a narrow spectrum of activity and is minimally absorbed from the intestinal tract (193). The drug was evaluated in two large phase three non-inferiority randomised controlled trials conducted in Europe and North America (194)(195). In the first study performed in North America, fidaxomicin was shown to be non-inferior to vancomycin in terms of clinical cure and was associated with lower rates of recurrence in the modified intention-to-treat analysis (15.4% vs. 25.3%, p=0.005) and the per-protocol analysis (13.3% vs. 24.0%, p=0.004). These results were replicated in the European study with significantly lower recurrence rates in the fidaxomicin group (12.7% vs. 26.9%, p=0.0002). Fidaxomicin causes less perturbation to the host intestinal microbiota than vancomycin, which may explain the lower rates of recurrence associated with this drug (196). However, currently a single course of fidaxomicin costs twenty times that of vancomycin and therefore at present, the drug is likely to only be used on a named patient basis (192).

### *1.10.2 Recurrence of CDI*

For a first recurrence, the same antimicrobial regimen used to treat the index episode is recommended (13). However, in the case of multiple recurrences there is no consensus or algorithm for the correct order of interventions. A prolonged course of tapered or pulsed vancomycin is often used in patients with multiple episodes of

recurrence based on the theory that spores may still germinate long after resolution of clinical symptoms has occurred. A retrospective study compared standard antimicrobial treatment, tapered vancomycin (gradual reduction in dose by 125-750mg per day from varying starting doses) and pulsed vancomycin (125-500mg vancomycin given every three days over a three week period) (197). Recurrence rates were lower in the pulsed (14%) and tapered (31%) groups compared to a standard vancomycin regimen (54%). However, the grade of evidence is weak and further studies are needed. The antibiotic rifaximin was evaluated in an uncontrolled case series and prevented recurrence in seven out of the eight patients included when given after standard vancomycin treatment (198). However, this drug has been associated with an increased risk of resistance that was detected in a third of isolates and 81.5% of 027/NAP1/B1 strains in one study (199) .

#### *1.10.2.1 IVIG*

IVIG has been used to treat refractory cases with mixed results and the overall grade of evidence remains weak due to a lack of large randomised controlled clinical trials (see section *1.7.2.3*).

### *1.10.3 Novel treatments*

Several different novel treatments for the prevention and treatment of CDI are currently being developed. Therapies that target the immune system include monoclonal antibodies and vaccines and have previously been described (section *1.7.2.).*

The antibiotic Surotomycin (previously known as CB 183,315) is a bacteriocidal lipopeptide that causes minimal disruption to the intestinal microbiota. Pre-clinical studies have shown the drug to be equivalent to vancomycin in a hamster model of disease and well-tolerated in humans (200)(201). The drug is currently being evaluated in a phase three study (Clinical trials.gov identifier NCT01598311).

The intra-luminal toxin binding agent tolevamer was evaluated *in vitro* and demonstrated effective toxin neutralisation in different isolates (202). Despite suggesting great promise, tolevamer was found to be markedly inferior to both metronidazole and vancomycin in a phase three study and was therefore abandoned as a therapeutic option for CDI (203).

Restoration of the microbiota remains one of the most active fields of research and includes the use of faecal transplants, non-toxigenic *C. difficile* and probiotics. Non-toxigenic *C. difficile* (NTCD) strains were able to prevent CDI in hamsters by restoring colonisation resistance (204). Advantages of this strategy include ease of administration and speed of effect, with orally administered spores able to rapidly pass through the gastric acid barrier and provide protection within a couple of days. NTCD spores are well-tolerated in healthy volunteers and are currently undergoing evaluation in a phase two clinical trial (205).

Faecal transplantation or faecal microbiota transplantation (FMT) was first described in the 1950s and involves instillation of a liquid suspension of stool from a healthy donor into an infected patient's gastrointestinal tract by nasogastric tube, colonoscopy or rectal enema (206). Advantages include low cost and the sustainable effects produced, with one study demonstrating the persistence of donor flora at 24 weeks post-transplantation (207). FMT has been used to treat severe refractory disease and has resulted in resolution of symptoms in over 80% of cases that have previously been described in individual reports and case series (208).

The first randomised controlled clinical trial of FMT was conducted recently and compared infusion of donor faeces following standard vancomycin treatment and bowel lavage to vancomycin therapy alone or with bowel lavage (209). A total of 43 patients were recruited and a significantly higher cure rate was observed in patients treated with FMT compared to those that received vancomycin or vancomycin and bowel lavage (81% vs. 31% vs. 23%, $p<0.001$). However, the trial was terminated early after an interim analysis demonstrated almost all patients in the control groups experienced a recurrence of CDI.

Limitations of FMT include the logistical challenges of harvesting and processing stool and the aesthetics involved with using donor faeces. In the future, these may be overcome through the storage of pre-screened anonymous donor material (210). All donors should be screened for blood borne viruses and enteric pathogens, have no history of antibiotic use in the previous six months or any history of gastrointestinal disorders such as inflammatory bowel disease.

A recent study by Lawley *et al.* isolated a mix of six distinct species from donor stool that were capable of restoring a healthy and diverse microbiota in mice infected with

*C. difficile* (211). In the future, this may be feasible in humans given the rapid expansion in the field of microbiome research.

Probiotics have been defined as 'live microorganisms, which when administered in adequate amounts, confer a health benefit to the host' (212). The following section outlines the growing body of evidence that suggests a role for probiotics in the prevention of AAD.

## 1.11 Probiotics

The beneficial effect of live microorganisms to human health was first suggested at the turn of the twentieth century by the Russian Nobel Laureate Elie Metchnikoff based on his observations that Bulgarian peasant farmers who consumed vast amounts of soured yoghurt were more likely to live longer (213). Probiotics are frequently used in the food and veterinary industry and are now being evaluated for use in human health and disease. They exist in a variety of different formulations that include yoghurt drinks, capsule and dietary supplements. In addition to probiotics, prebiotics are non-digestible food ingredients that are consumed with the aim of stimulating the growth or activity of bacteria in the gastrointestinal tract. Some products are marketed as a combination of the two and are known as synbiotics (214).

In 2002, joint guidelines were released by the Food and Agricultural Organisation and World Health Organisation that outlined the necessary requirements for an organism to be classified as a probiotic (Table 1.1). Probiotic strains are named based on the genus, species and a specific strain identifying name (e.g. *Lactobacillus rhamnosus GG*). One of the first strains described was *E. coli* Nissle 1917 that was identified in the stool of a soldier who survived an outbreak of dysentery in the First World War. The majority of probiotics that have been studied are species of *Lactobacillus* and *Bifidobacterium,* which both form part of the normal microbiota. The yeast *Saccharomyces boulardii* has also been extensively evaluated and although not part of the intestinal microbiota, is known to colonise the skin of fruit such as lychees.

**Table 1.1. Classification of probiotics. Adapted from joint guidelines released by the Food and Agricultural Organisation and World Health Organisation (215).**

| Category | Recommendations |
|---|---|
| **Strain Identification** | • Effects are strain specific<br>• Allow accurate surveillance and epidemiological studies<br>• Phenotypic (eg. PFGE) and genotypic (eg. 16S RNA) techniques should be used<br>• Screening potential strains: *in vitro* |
| **Mechanism of action** | • Resistance to bile and gastric acid<br>• Adherence to mucus and human epithelial cell lines<br>• Limit pathogen adherence |
| **Safety** | • Antibiotic resistance patterns of probiotic<br>• Assessment of toxin production |
| **Efficacy studies: *in vivo*** | • Animal studies should be used if appropriate prior to human trials<br>eg. Establish the level of infectivity in immunocompromised animal models<br>• In randomised placebo controlled trials the placebo should be the same as the test strain in terms of vehicle of delivery and starter cultures<br>• Phase two studies should incorporate a quality of life tool |

### *1.11.1 Probiotic mechanism of action in the gastrointestinal tract*

Probiotics exert their effects in the gastrointestinal tract through a variety of different mechanisms that have been assessed using both culture-dependent and metagenomic sequencing (Figure 1.6). Any effects seen are strain specific and may be explained by structural differences in the microorganism-associated molecular patterns found on bacterial cell surfaces that are recognised by epithelial cell receptors (216).

#### *1.11.1.1 Competitive exclusion of pathogens*

Direct inhibition of pathogens occurs through competitive exclusion for binding sites and surface receptors. Collado *et al.* investigated the ability of 12 commercially available probiotic strains to prevent the adhesion and binding of eight pathogens to mucus recovered from healthy sections of resected colonic tissue (217). All 12 strains tested were able to reduce pathogen adherence through competitive exclusion, although efficacy varied between probiotic strains. Probiotics can indirectly inhibit pathogen colonisation through secretion of enymes and bactericidal products. Bacteriocins are antimicrobial peptides synthesised by bacteria that enable the producers to compete within their own ecological niche (218). *Lactobacillus salivarius* was able to prevent *Listeria monocytogenes* infection in mice using the bacteriocin Abp118 (219). *S. boulardii* produced a serine protease that hydrolysed TcdA and inhibited TcdA binding to its brush border glycoprotein receptor in a rat ileal-loop model of disease (220) .

#### *1.11.1.2 Reinforcement of the intestinal barrier*

A more subtle mechanism involves the preservation of an existing host defence, such as the intestinal barrier, through direct strengthening of epithelial tight junctions or up-regulation of mucus production through signalling pathways. *E. coli* Nissle 1917 conferred protection against colitis in a murine model of disease through induction and up-regulation of specific scaffolding proteins (zonula occludens 1 and 2) that led to a reduction in mucosal permeability and strengthening of epithelial tight junctions (221). Mucins are the proteins responsible for mucus production and are encoded by at least nine different MUC genes (222). Cabellero-Franco *et al.* demonstrated an increase in MUC2 gene expression in Wisteria rats treated with a multi-species probiotic that was accompanied by an accumulation of mucin in the colonic lumen

(223). A secondary effect of thickening the mucus layer is the reduced translocation of bacteria across the intestinal lumen.

#### *1.11.1.3 Immunomodulation*

Probiotics modulate the immune system through production of secreted factors and metabolites that affect the growth and function of intestinal epithelial and immune cells. FOXP3 (forkhead box P3) is a cell marker expressed on T regulator cells that is involved in suppression of the immune response. *Lactobacillus* free mice treated with the probiotic *Lactobacillus reuteri* 100-23 demonstrated higher numbers of cells expressing FOXP3 in their spleen and mesenteric lymph nodes when compared to control mice (224). This suggests this strain may influence the development and recruitment of T cells within the intestinal epithelium. Yang *et al.* studied *L. rhamnosus GG* and found the strain prevented cytokine induced apoptosis *in vitro* through activation of the protein kinase Akt, which promotes cell survival (225). They subsequently isolated and purified two proteins (p75 and p40) from activated *L. rhamnosus GG* culture supernatant that reduced damage to intestinal epithelial cells by TNFα (226). Following the incubation of intestinal epithelial cells with the pathogen *Salmonella typhimurium*, two separate probiotic strains, *L. salivarius* UC118 and *Bifidobacterium infantis* 35624, caused attenuated secretion of the pro-inflammatory cytokine IL-8 and increased the production of the anti-inflammatory cytokine IL-10, which resulted in an overall reduction in inflammation (227).

Although the effects discussed above provide proof of concept, they have all been demonstrated *in vitro* or using animal models of disease. Future work should focus on evaluating probiotic effects in humans to assess if the observed mechanisms are upheld when faced with the complexities of the human intestinal microbiota.

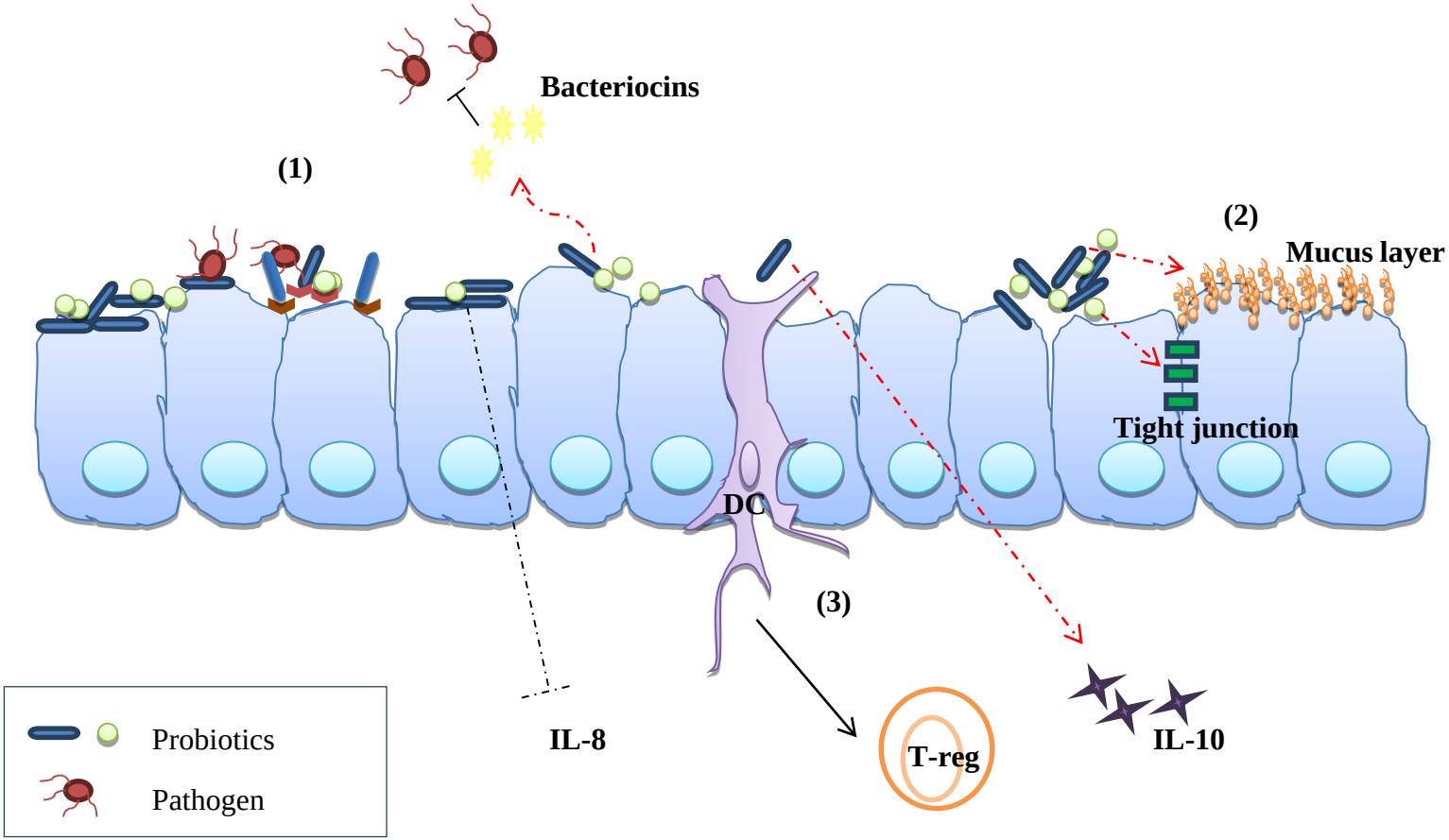



**Figure 1.6. Probiotic mechanisms of action in the intestinal tract. The three main mechanisms of action are 1. Competitive inhibition of pathogens (direct or indirect) 2. Reinforcement of the epithelial barrier and tight junctions 3. Immunomodulation. Abbreviations: Dendritic cell (DC), Tregulatory cell (T-reg). Adapted from O'Toole *et al.* (228).**

### *1.11.2 Probiotic use in clinical practice*

Probiotics have been used in a wide range of conditions that includes atopy, sinusitis and bacterial vaginosis. However, the great majority of experience relates to their use in gastrointestinal disease. The best evidence for the clinical efficacy of probiotics exists for the prevention of necrotising enterocolitis in preterm infants. Preterm infants often receive courses of broad-spectrum antibiotics, have protracted lengths of stay in hospital and have different patterns of intestinal colonisation that all contribute to the pathogenesis of necrotising enterocolitis. A Cochrane review recommended the use of certain probiotics for the treatment of necrotising enterocolitis but not all strains were of equivalent efficacy (229). In adults, different probiotic preparations have been studied in inflammatory bowel disease (230). Notably, three randomised placebo controlled trials have shown equivalence of *E. coli* Nissle 1917 to mesalazine in maintaining remission of ulcerative colitis and the combination probiotic preparation VSL3# has shown benefit in pouchitis (231). Over the past decade, a number of studies have investigated the role of probiotics in AAD and can be divided into those looking at prevention and treatment.

### *1.11.3 Primary prevention of AAD and CDI*

Multiple published studies describe the use of probiotics to prevent AAD and several relevant meta-analyses have been performed. D'Souza *et al*. identified nine placebo controlled studies; seven were in adults and two in children, with a total of 1,214 subjects. The probiotic strains tested varied by study, with four using *S. boulardii*, four *Lactobacillus* species and one strain of *Enterococcus* species. All but one study of *S. boulardii* indicated a protective effect and the combined odds ratio (OR) in favour of active treatment was 0.37 (95% CI 0.26–0.53) (232). In a separate analysis, Sazawal *et al.* identified 18 studies that described the prevention of AAD and one the prevention of CDI specifically. All showed a positive effect and the authors concluded that probiotics significantly reduce AAD by 52% (95% CI 35%-65%) (233). Both analyses suggested bias away from publication of negative results but Sazawal *et al.* estimated that 330 unpublished negative trials would have to exist to overturn their findings.

Previous probiotic studies have shown inconsistencies in study design that has resulted in heterogeneity between studies. Differences include the definition of AAD, duration of follow-up, age of patients recruited and strain of probiotic used.

A recent meta-analysis by Hempel *et al.* identified 63 randomised controlled trials and included 11,811 patients (255). The authors concluded that there was a statistically significant pooled relative risk (RR) in favour of probiotic reduction of AAD (RR 0.58, 95% CI 0.50-0.68, $p<0.001$, number needed to treat 13). The result was relatively unaffected on sub-group analysis; however, poor documentation of probiotic strain type, adverse event reporting and the antibiotics used meant a moderate degree of heterogeneity remained.

Since 2006, there have been ten randomised controlled trials investigating the role of probiotics in the prevention of AAD and seven have suggested a benefit (234-240) (Table 1.2). The three remaining studies failed to demonstrate any difference in the incidence of AAD in patients treated with a probiotic compared to those treated with a placebo. The first study was a pragmatic study conducted in a community General Practice surgery with three intervention arms; a commercially available 'Bio yoghurt' containing two probiotic strains, a standard yoghurt preparation and no treatment (241). Only 9/131(7%) patients in the Bio-yoghurt group developed AAD compared to 13/118 (11%) in the standard yoghurt group and 17/120 (14%) in the untreated group. Although, the lowest rate of AAD occurred in the probiotic group, the difference between the groups did not reach statistical significance. The second study was conducted in Sweden and found lower rates of loose stool and nausea in the active group, despite similar rates of AAD in both groups (242). The third study was conducted in Chile and reported in Spanish so full details are not available; however, as only 81 patients were recruited, the study was underpowered to detect any difference in the rate of AAD (243).

There have been fewer studies specifically addressing the effect of probiotics on CDI, which is usually measured as a secondary outcome. In a study by Hickson *et al.* 135 patients were randomised to receive a probiotic drink (*Lactobacillus casei* DN-114001, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) or placebo milkshake drink twice daily for the duration of antibiotic use and the following seven days. An absolute risk reduction of 17% (7% to 27%) occurred with no cases of CDI

in the probiotic group (235). Recently, the first dose-response effect study was conducted at a single centre in China. Patients were randomised to two probiotic capsules containing *Lactobacillus acidophilus* CL1285® and *L. casei* LBC80R® (Pro-2), single probiotic capsule and placebo capsule (Pro-1) or two placebo capsules. The incidence of CDI was lowest in the Pro-2 group (1.2%) with higher rates seen in the Pro-1 (9.4%) and placebo groups (23.8%). The overall rates of CDI were greater in this study compared to rates observed in Europe and North America and might be explained by the exclusive inclusion of patients prescribed antibiotics associated with an increased risk of CDI (234).

### *1.11.4 Treatment of CDI*

There has never been a trial investigating probiotics as the sole treatment for CDI. A Cochrane systematic review looked at four studies investigating the use of probiotics as an adjunct to first line conventional antibiotic treatment (244). However, the authors concluded that there is insufficient evidence to support the use of probiotics for the treatment of CDI.

### *1.11.5 Secondary prevention of CDI*

Five studies have investigated the use of probiotics for the prevention of recurrence of CDI; only two showed a significant effect. The first study established that in patients who had experienced a recurrence, *S. boulardii* in combination with standard CDI treatment prevented further episodes (RR 0.43, 95% CI 0.2-0.97) (245). The second study, by the same group, replicated these results and demonstrated the combination of *S. boulardii* with high dose vancomycin (2g/day) to be most effective. A lower dose of vancomycin (500mg/day) also reduced recurrence of CDI (21% versus 62% placebo) but at the expense of a longer mean duration of treatment (246). The remaining studies are limited by small sample sizes and are underpowered, which makes it difficult to draw a meaningful conclusion (247)(248)(249).

### *1.11.6 Safety*

At least three cases of endocarditis have been described in the literature following consumption of probiotics and all have occurred in patients with underlying cardiac pathology that includes valvular defects (250). No cases of systemic bacteraemia have been reported in trials using a probiotic test strain in otherwise healthy adults, including trials involving older people. Concerns remain about the use of probiotics in severely immunocompromised patients but the significance of this is not clear. In a multicentre study carried out in intensive care, 298 patients with severe pancreatitis were randomised to receive a multispecies probiotic preparation or placebo. Rates of intestinal ischaemia and mortality were higher in the active group compared to placebo (16% vs. 6%, RR 2.53 95% CI 1.22-5.25). The authors suggested that the introduction of billions of probiotic organisms may have further compromised an already metabolically unstable environment that occurs in severe pancreatitis (251). A separate study in mechanically ventilated patients in intensive care, reported similar 28 day crude mortality rates in the active multi-species probiotic group (25.3%) and placebo group (23.7%) (252). Therefore, it is likely that the exact nature of immune compromise is an important factor as probiotics have been used in other immunosuppressed patients, which includes preterm neonates and HIV (Human immunodeficiency virus) patients, with no reported serious adverse events (253).

In summary, although probiotics have demonstrated a beneficial effect in the prevention of AAD several previous studies have been subject to varying levels of publication bias due to missing participant data or omission of allocation concealment (254). Furthermore, the majority of studies have been small and underpowered. In order to demonstrate the effect of an intervention on a relatively rare event such as AAD, sample sizes should include 200 cases in each study arm to achieve a power of 80% (255). However, only 10% of studies conducted to date fulfil this criterion. Therefore, a need remains for a large well-designed, multicentre randomised controlled clinical trial in order to definitively address the role of probiotics in preventing AAD.

**Table 1.2. Probiotics used for the primary prevention of AAD. Only studies that demonstrated a beneficial effect are shown. The remaining negative studies are discussed in the text. Abbreviations: Standard deviation (S.D.). *Rate in group receiving two probiotic capsules**

| Author | n | Probiotic strain | Study design | CDI rate Probiotic (%) | CDI rate Placebo (%) | Comments |
|---|---|---|---|---|---|---|
| **Gao, X W *et al.* (2010)** | 255 | *L. acidophilus* CL1285® and *L.casei* LBC80R® | • 50-70 years of age<br>• Product given for the duration of antibiotics plus five days<br>• Follow-up: 21days post cessation of product | 1.2 * | 23.8 | Probiotic blend showed dose-response effect (100 billion colony forming units versus 50 billion). Limitations: Single-centred study and the sole inclusion of patients receiving high risk antibiotics. |
| **Sampalis, J *et al.* (2010)** | 437 | *L. acidophilus* CL1285® and *L. casei* LBC80R® | • >18 years of age<br>• Product given for the duration of antibiotics plus five days<br>• Follow-up: 21days post cessation of product | 6.2 | 13.3 | Multi-centre study in Canada. Patients in the active group had a shorter duration of AAD. |
| **Safdar, N *et al.* (2008)** | 40 | *L. acidophilus* | • >18 years of age<br>• Product given for the duration of antibiotics plus 14 days<br>• Follow-up: not defined | 0(0/3) | 25 (1/4) | The study was a small pilot study and therefore ultimately underpowered. Only seven cases were tested for *C. difficile* toxin. |
| **Beausoleil, M *et al.* (2007)** | 89 | *L. acidophilus* CL1285® and *L. casei* | • Age range not defined; mean age in active group 68.8 years (S.D.14.5)<br>• Product given for the duration of antibiotics only<br>• Follow-up: 21 days post cessation of product | 2.3 | 15.6 | A significant difference occurred in the rate of AAD between the groups; this was not seen for CDI due to the small numbers of patients with CDI. |

**Table 1.2. Continued.**

| Author | n | Probiotic strain | Study design | CDI rate Probiotic (%) | CDI rate Placebo (%) | Comments |
|---|---|---|---|---|---|---|
| **Hickson, M *et al.* (2007)** | 135 | *L. casei* DN114001, *L. bulgaricus, S. thermophilus* | • ≥50 years of age<br>• Product given for the duration of antibiotics plus seven days<br>• Follow-up: four weeks post-discharge | 0 | 17 | Only 8% of the screened population were recruited; therefore it is difficult to generalise findings to the wider hospital population. |
| **Plummer, S *et al*. (2004)** | 138 | *L. acidophilus, B. bifidum* | • Elderly patients<br>• Primary outcome was the rate of CDI<br>• Product given for 20 days<br>• Follow-up: nil | 3 | 7 | Poor recruitment resulted in reduced statistical power. |
| **Surawicz, M *et al*. (1989)** | 180 | *S. boulardii* | • Mean age 47.8 years (S.D. 21)<br>• Product given for the duration of antibiotics plus two weeks<br>• Follow-up: mean duration of 17.3 days (S.D. 8.6) | 3 | 5 | The intervention was tested in much younger population. |

## 1.12 Aims and original contribution

The overarching theme of this project was to explore various aspects of patient susceptibility to CDI. Until now, efforts to control CDI have focused on preventing transmission of infection through infection control measures and optimising diagnostics. However, the importance of patient susceptibility has been illustrated previously using stochastic mathematical modelling (7). Particularly in settings where, as in the UK, infection prevention has already yielded substantial reductions in the incidence of CDI, further reductions may only be achievable through targeting patient susceptibility.

The work described in this thesis addresses three specific aims. The first was to explore the role of the probiotic *L. casei* DN114001 in preventing AAD including CDI. An earlier study demonstrated this strain was able to reduce rates of both AAD and CDI; however, only a small proportion of the total patients screened were recruited and this has been considered a limitation on the generalisability of the study's results (235). This thesis describes a multicentre, double-blind, randomised placebo-controlled trial that is the largest probiotic study ever conducted. The second aim was to investigate the role of humoral immune responses to *C. difficile* in patient susceptibility to CDI. This was investigated in a case-control laboratory based study using an antibody ELISA, which was tested in two cohorts of patients recruited in Brighton, UK and Michigan, USA. The third aim was to identify patient risk factors that could be used to predict recurrence of CDI. This was achieved through a longitudinal cohort study of patients who were managed for CDI in Brighton over a three year period.

## 1.13 Hypotheses

1) The probiotic *L. casei* DN114001 will reduce the incidence of AAD, including CDI, when given prophylactically with antibiotics as a primary prevention.

2) Antibodies to TcdB determine CDI susceptibility in patients and more specifically lower antibody levels predispose individuals to CDI in the acute setting.

3) Risk factors can be used to predict recurrence of CDI and more specifically treatment on a cohort ward is associated with an increased risk of recurrence.

# Chapter 2: The role of *L. casei* DN114001 in preventing antibiotic associated diarrhoea

## 2.1 Introduction

Probiotics have been defined as live microorganisms that confer a health benefit to the host when given in sufficient amounts (212). Over the past decade there have been an increasing number of clinical trials carried out in response to the growing interest in the role of probiotics in human health and disease. The largest body of work has focused on their role in preventing gastrointestinal disease that includes necrotising enterocolitis, inflammatory bowel disease and AAD (229)(255)(256).

Certain antibiotics are now known to disrupt the intestinal microbiota causing a reduction in organisms that have specific metabolic functions. This can result in the build-up of waste metabolites in the intestinal lumen that manifests as functional osmotic diarrhoea (107). Alternatively, pathogens take advantage of the extra space and nutrients that are made available. Probiotics may ameliorate these effects by helping restore colonisation resistance through a series of mechanisms that are described in the main introduction (see section *1.11.1*).

The role of probiotics in the prevention and treatment of AAD has been investigated in at least 82 randomised controlled trials and recently in three separate meta-analyses (254)(255)(257). Historically, a comparison of probiotic studies in this area has revealed a degree of ambiguity due to differences in the primary end-point, strain used, duration of treatment and population tested.

The largest meta-analysis addressed this problem using meta-regression and sub-group analysis (255). The authors included a total of 63 trials and found probiotics were significantly associated with a reduction in AAD (RR 0.58, 95% CI 0.50-0.68, $p<0.001$). However, despite the large number of studies included a moderate degree of heterogeneity remained on sub-group analysis that was attributed to differences in strain type and study population. The authors concluded that there remains a need for a large well-designed randomised controlled trial to determine which probiotics should be used in specific populations. This reinforces the aim of the current study which was to design and carry out a multicentre randomised controlled trial and address key areas

in relation to clinical trial design, in order to definitively address the role of probiotics in the prevention of AAD

### *2.1.1 Probiotic strain selection*

For a probiotic strain to be effective in the prevention of AAD it must withstand changes in pH and survive transition through the gastrointestinal tract. A genetically modified strain of *L. casei* DN114001 that contained the *lux* gene from *Photorhabdus luminescens* was assessed *in vivo* using mice (258). The strain was recovered from mice faeces, which indicated the strain survived transition through the intestinal tract. A change in light absorption corresponded to the synthesis of the enzyme luciferase that showed the strain was capable of metabolic activity *in situ.*

In an open label study involving 12 healthy volunteers, aged 23-44 years, the same strain was subsequently recovered in human faeces (259). Human colonic cells infected with enteropathogenic *E. coli* showed a reduction in permeability after incubation with *L. casei* DN114001 *in vitro* (260). This preservation of the intestinal barrier function may be of importance in severe colitis. A previous randomised controlled trial showed *L. casei* DN114001 was associated with fewer cases of AAD when compared to a placebo (235). *L. casei* DN114001 is delivered as a yoghurt based drink rather than in powdered form. No studies have compared probiotics given as capsules to yoghurt preparations and the degree to which this might impact on probiotic efficacy remains unknown.

### *2.1.2 Study design*

#### *2.1.2.1 Single centre vs. multicentre studies*

Since 2006, 10 randomised controlled trials have evaluated the role of probiotics in AAD with nine conducted at single centres (261). Single-centre studies have a number of advantages over multicentre trials: they are logistically easier, require data collection from only one site and are more economical as they require fewer resources in terms of staff and travel costs. However, the disadvantages of such studies include the potential for bias and that observations based on routine practice in one centre may not be indicative of other hospitals, which can limit the generalisability of results. This was highlighted by a study conducted in a single centre using the probiotic strain *L. acidophilus* CL1285® + *L. casei* LBC80R® (234). Patients were all of Asian descent, had an average age of 60 years and received only high risk antibiotics. These factors

reduce the applicability of the results to patients in the UK and Europe where the patient demographics are different and restrictive antibiotic policies are used. The main advantage of a multicentre study is to test the intervention in a more heterogeneous population; however, this is not always guaranteed. In the study by Hickson *et al*. although recruitment occurred at three separate sites, all centres were in close location to each other and the overall population demographics were likely to be very similar.

#### *2.1.2.2 Stratified randomisation*

The technique of stratified randomisation is often used in clinical trials to account for differences in the recruited patient population that could impact on the outcome. Stratified randomisation involves grouping individuals, on entry to a clinical trial, into separate groups (strata) on the basis of clinical features that may alter outcome risk. The intervention is then assigned to individuals based on separate randomisation schedules within each stratum (262). In a study investigating the prevention of AAD, subjects should be stratified by age as older patients are more likely to receive repeated courses of antibiotics and have multiple comorbidities that may influence the rate of AAD. The advantages of stratification include a reduction in type 1 errors and facilitation of subgroup and interim analyses (263). Stratification also ensures that if a site closes early, patients recruited at each site will be evenly distributed between interventions although this problem is reduced as the sample size increases. The randomisation lists should be generated in such a way that allocation concealment is ensured to reduce the potential for bias or unblinding.

#### *2.1.2.3 Placebo*

As there is no known standard treatment for AAD it is important to compare the probiotic with a carefully selected placebo. A placebo is an inactive agent designed to balance the perceived effect of an intervention, therefore allowing for an independent assessment of the treatment effect (264). It is important to select a placebo of the same consistency and texture, which should be delivered in identical packaging to the active product.

#### *2.1.2.4 Endpoint definition*

The definition of the primary endpoint of AAD is known to vary widely amongst probiotic studies and may account for the incidence of 5-25% that is frequently quoted in the literature (265). The definition of AAD chosen should be relevant to current clinical practice and address disease severity by incorporating a measure of stool frequency and consistency. A review of published probiotic and AAD studies highlighted a lack of well-defined secondary endpoints and CDI incidence and duration of AAD were the only secondary endpoints mentioned (235)(236). There is a lack of data in relation to the cost-benefit ratio of introducing probiotics into routine clinical care and a formal health economics analysis that includes the effect of probiotics on length of stay is needed.

#### *2.1.2.5 The aetiology of AAD*

A limited number of studies have examined the underlying aetiology of AAD (266). Some previous probiotic studies have focused exclusively on *C. difficile*; however, several other pathogens that include *Staphylococcus aureus* and *Klebsiella oxytoca* have been implicated in causing AAD (107). Non-infectious causes that include the number of comorbidities and medications on admission such as laxatives and anti-inflammatory agents should be taken into account during the final analysis (267).

## 2.2 Aim

The aim of this study was to determine the role of the probiotic *L. casei* DN114001 in the primary prevention of AAD as part of a multicentre, double-blind, randomised placebo-controlled trial (Probiotic NU278).

## 2.3 Methods

### *2.3.1 Statement of contribution to Probiotic NU278 trial*

The study protocol and Ethics approval was written and obtained by the Chief Investigator. I have been involved in all remaining aspects of the clinical trial with the exception of consenting patients into the study in order to maintain study integrity (Figure 2.1).

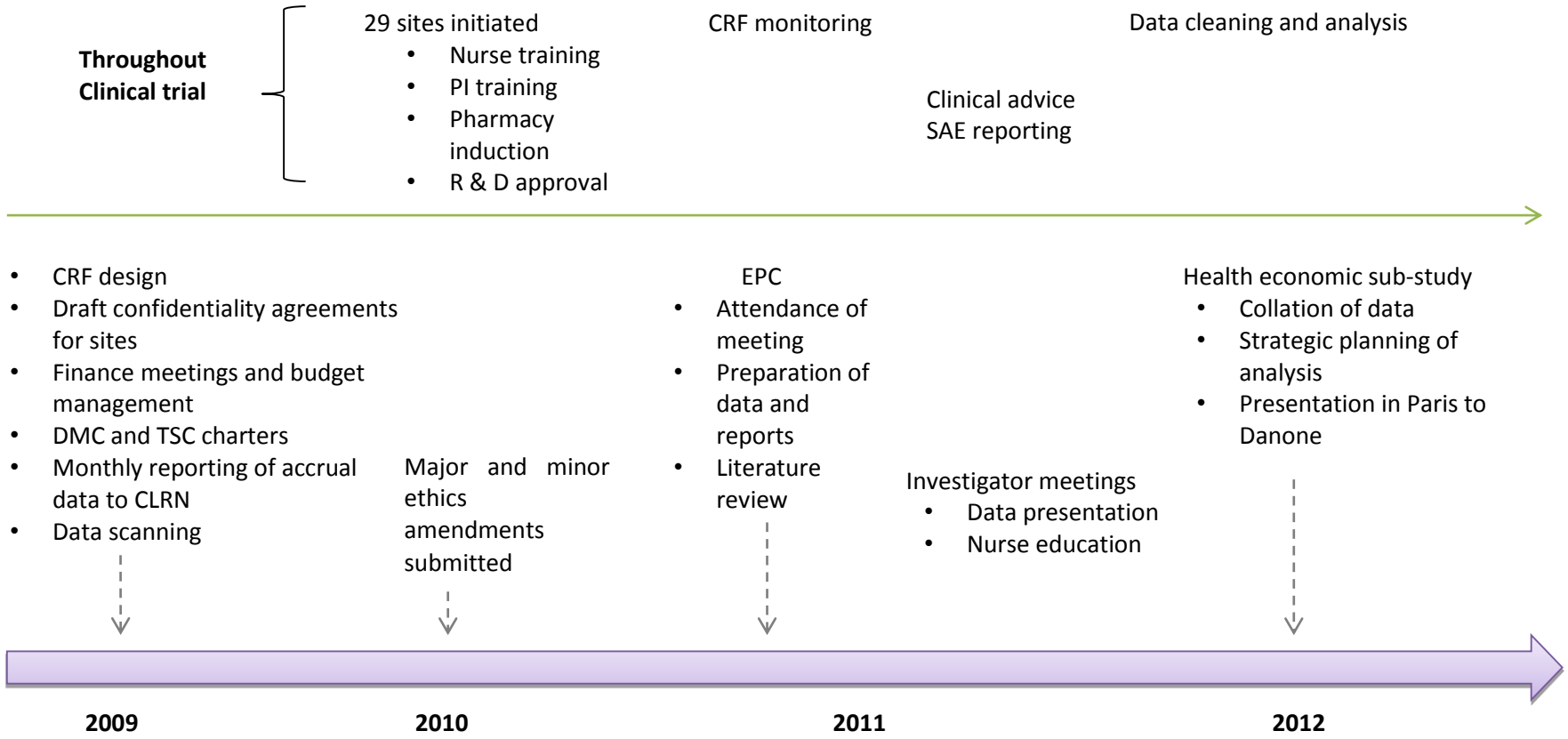



**Figure 2.1. Individual contribution to Probiotic NU278 Study. Outline of my personal involvement in the Probiotic NU278 study. Abbreviations: Case report form (CRF), Research & Development (R&D), Serious adverse event (SAE), Data Monitoring and Safety Committee (DMC), Trial Steering Committee (TSC), Endpoint Committee (EPC) and Comprehensive Local Research Network (CLRN).**

### *2.3.2 Study design*

The study was designed as a multicentre, double-blind, randomised placebo-controlled trial (Probiotic NU278) and was conducted in accordance with the Declaration of Helsinki and principles of Good Clinical Practice (268). Recruitment was planned over a two year period across 10 centres in the South of England. The study protocol was written by the Chief Investigator and was funded by an educational grant from Danone to the University of Sussex (the Sponsor). An independent Trial Steering Committee (TSC) was established to ensure adherence to the trial protocol. Patients were randomised in a 1:1 equal allocation, to the active product (100ml fermented drink containing *L. casei* DN114001 and two regular yoghurt cultures *L. delbrueckii* subspecies *bulgaricus* and *S. thermophillus*) or a matched non-fermented acidified placebo. The product was prescribed twice daily for the duration of antibiotic use and the following seven days. Compliance was verified by the study research nurse and recorded daily in the case report form (CRF). Serum and stool samples were collected within 48 hours of randomisation and further samples were obtained at the onset of any diarrhoea.

### *2.3.3 Data collection and monitoring*

Demographic and clinical data were recorded in the CRF and patients were followed for a further 14 days after completion of the therapeutic stage. The Barthel score was used to measure pre-morbid functional status and a validated and frequently used questionnaire called the Short Form 12 (SF-12) was used to assess quality of life (Appendix). Both forms were recorded at baseline and follow-up. All CRF's were monitored at the end of patient follow-up and data was scanned onto a central data-base (Formic Fusion software, UK).

### *2.3.4 Microbiology*

Stool samples were cultured for *Salmonella, Shigella, Campylobacter, E. coli* 0157 ova, cysts and parasites as part of the microbiological diagnostic testing service at the Royal London Hospital, UK. The methods used are outlined in the HPA standard operating procedure (BSOP 30) (269). The four pathogens: *S. aureus, K. oxytoca, C. difficile* and *Candida albicans* were tested using the method outlined below (see SOP in Appendix) and all work was carried out by Dr. Mark Wilks at the Royal London Hospital, UK.

#### *2.3.4.1 Method for the detection of organisms in stool samples*

All faecal samples were weighed and then diluted 1:10 (w/v) in a cryopreservative broth (Brain heart infusion broth [Oxoid, UK]). Samples were vortexed briefly and further tenfold serial dilutions made in Brain Heart Infusion broth (Oxoid, UK) The serial dilutions were then inoculated onto the surface of the following media: Saborauds Agar for *C. albicans*, 5% sodium chloride agar (Oxoid, UK) for staphylococci and MacConkey agar (Oxoid) for *K. oxytoca. C. difficile* was detected by mixing an aliquot of the 1:10 dilution of faeces with an equal volume of alcohol and left for 30 minutes before being sub-cultured.

Following incubation, colony types were enumerated. Colonies were Gram stained or identified directly using Matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF). Any *C. difficile* isolates were checked for toxin production using a QuickCheck *C. diff* toxin LFD card (Alere, UK) that combines an EIA toxin assay with a GDH test.

#### *2.3.4.2 Quality control testing of study product*

Samples from different batches were supplied from the main production plant in Paris and delivered to the microbiology laboratory at the Royal London Hospital, UK for testing by Dr. Mark Wilks. In addition, samples were collected at random from different centres at the end of a two week randomisation block. The bacterial content of the trial product was tested using methods used by Danone for their quality control programme and performed on all batches before release. This was to ensure that no great variation from the expected bacterial counts occured. The expected counts of organisms were: *S. thermophilus* $10^6$ cfu/ml, *L. bulgaricus* $10^6$ cfu/ml and *L. casei* DN114001 $10^8$ cfu/ml.

Serial dilutions of the product were performed to $10^{-6}$ and plated out onto the surface of M17 agar and MRS agar. In addition Maconkey agar and Blood agar were used to detect the presence of faecal organisms and other bacteria respectively (See appendix for the full method).

#### *2.3.4.3 Taste test*

A formal taste test was carried out by a panel that were independent of the trial personnel that scored the active and placebo products based on taste, texture, smell and consistency.

### *2.3.5 Study population*

Patients aged over 55 years were recruited within 48 hours of receiving inpatient antibiotics that were prescribed for a minimum of 72 hours. Individuals were required to provide written consent to take part in the study; Consultee assent was approved at a later stage to allow patients from Nursing and Residential Homes to be recruited. A full list of exclusion criteria are outlined in the Appendix but briefly, patients were excluded if they had a history of diarrhoea in the preceding week, serious active or evolving gastric pathology on admission, a history of endocarditis or history of immunosuppression.

### *2.3.6 Ethics approval*

Ethical approval was given by the Brighton East Research Ethics Committee and the study was registered with Clinical trials.Gov (trial identifier NCT01087892).

### *2.3.7 Outcomes*

The primary outcome was the incidence of AAD in the active and placebo groups by the end of the follow-up period. Secondary outcomes were the incidence and duration of *C. difficile* toxin positive diarrhoea, duration and recurrence of AAD and length of stay in the active and placebo groups.

### *2.3.8 Study definitions*

On the advice of an independent committee (the TSC), the original definition of diarrhoea was changed in December 2011 from ‘more than two liquid stools a day (type 6 or 7 on the Bristol Stool Chart) for three or more days in quantities in excess of normal for each patient’ to ‘at least two loose stools within 24 hours (types 5-7 on the Bristol Stool Chart)’ based on the European guidelines for CDI (165). The American definition of CDI was also considered, however as the study was conducted in the UK, a decision was taken by the TSC to use the European definition and was approved by an Ethics Committee (19). All reported cases of loose stool were discussed by an independent Endpoint Committee (EPC) every three months. Duration

was defined as the total number of days that met the trial definition of diarrhoea, in a given period, before a return to normal bowel habit (less than or equal to type 4). Recurrence was defined as diarrhoea after completion of antibiotics (for the index event); providing the initial diarrhoeal symptoms had resolved for a minimum of 72 hours. Compliance was defined as consumption of at least 50% of the study product.

### *2.3.9 Statistical analysis*

For each centre-age group stratum, a random allocation was carried out using random permuted blocks of length four. The randomisation allocation sequences were generated using Random Allocation Software version 1.0 (Saghaei M Random allocation software for parallel group randomized trials).

The original sample size assumed a prevalence of 20% AAD in the control group based on the meta-analysis of D'Souza *et al.* and that a 40% reduction in prevalence to 12% (based on an odds ratio of 0.55) would produce a clinically significant result (232). A sample size of 440 in each group (880 in total) would have 90% power to detect this reduction using a two group chi-square test with a 0.05 two-sided significance level. Therefore the initial sample size required would be 1000 patients.

During the trial, the incidence of AAD was found to be 18% and an increased withdrawal rate from 10% to 20% was noted. Therefore, in December 2011, the independent TSC decided to adjust the sample size and this decision was approved by an Ethics Committee. This meant a sample size of 1200 patients was needed to achieve a 90% power with a type 1 error of 0.05 (two sided). The sample size was increased using the method of Fleiss *et al*. (270). No interim analysis was planned. Statistical analysis was carried out using SPSS (version 20, IBM®, USA). Continuous variables were compared using t-tests or Mann Whitney U test (for non-parametric data) and categorical variables using chi-squared test. Pearson's correlation coefficient was used to compare the rate of AAD and antibiotic use by centre.

### *2.3.10 Safety*

An adverse event (AE) was defined as any unwanted effect that occurred in a subject during the clinical study, whether or not related to the study product. Details of all AE were recorded in the CRF and each observed AE was recorded separately. All AE were categorised as mild, moderate or severe. A serious adverse event (SAE) was defined as any event considered to be life-threatening, resulted in disability or permanent injury, led to hospital readmission, prolonged stay or resulted in death. The sponsor was notified by fax of any SAE within 24 hours. An independent Data Monitoring and Safety Committee (DMC) reviewed all SAEs throughout the course of the trial.

## 2.4 Results

Between October 2009 and January 2013, 1002 patients were recruited and randomised to receive the active probiotic or placebo (Figure 2.2).

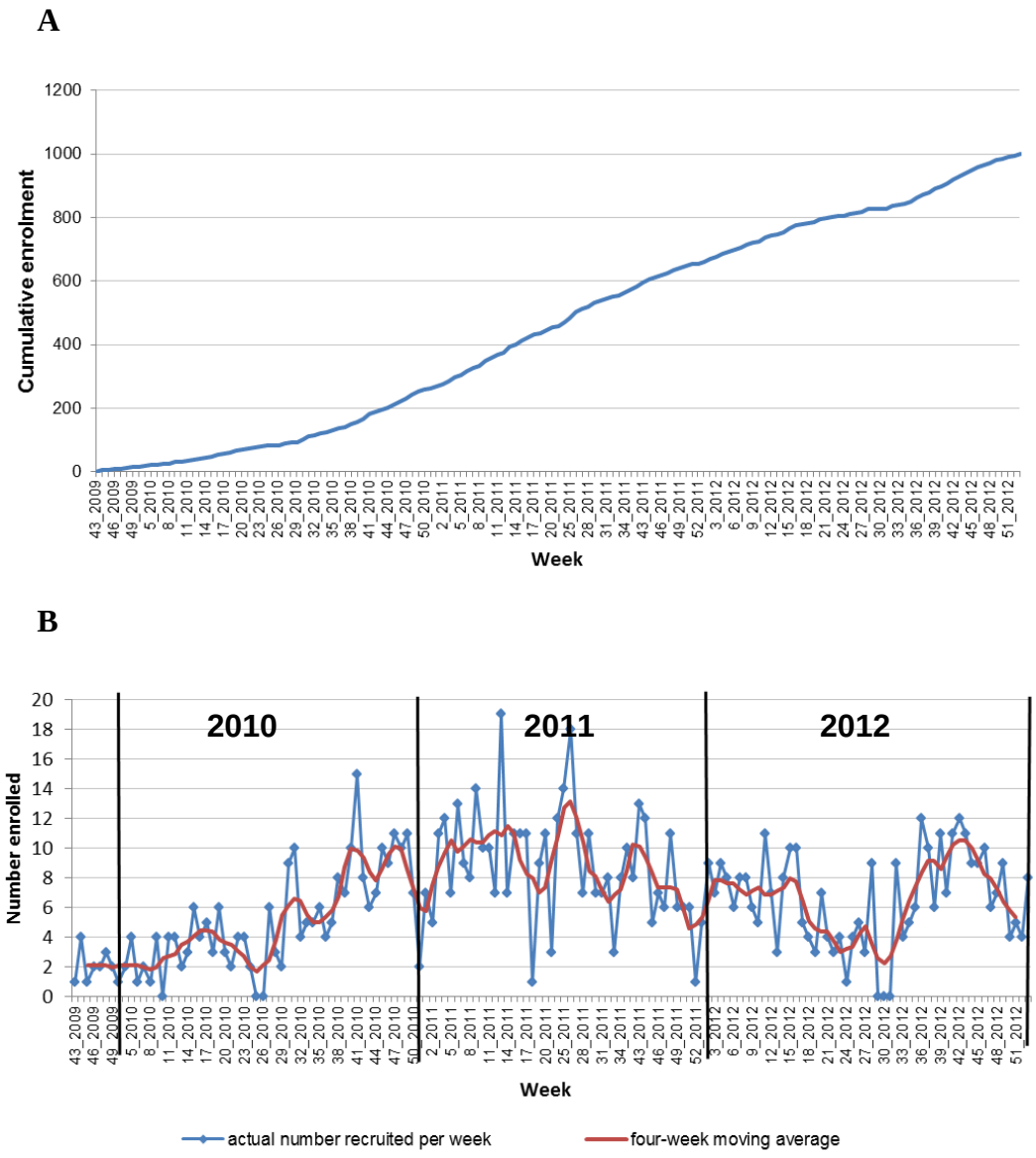



**Figure 2.2. Rate of recruitment to the Probiotic NU278 trial. Between October 2009 and January 2013 a total of 1002 patients were recruited to the study. A. Cumulative enrolment B. Enrolment per week. Recruitment was suspended for a period of 8 weeks due to problems with production of the study product.**

### *2.4.1 Rate of AAD*

A total of 118/650 cases (18.2%) met the trial definition of AAD. These patients were older (75 years IQR 66-82 vs. 73 years IQR 65-81) and remained in hospital for longer (7 days IQR 4-10 vs. 6 days IQR 4-11) than patients without AAD. The mean duration of AAD was 2.23 days (S.D. 1.84) and only two cases of recurrence occurred. On univariate analysis, patients suffering from AAD were similar to non-AAD cases in terms of underlying comorbidity, admission ward, medications on admission, baseline laboratory parameters and Barthel Index on admission (Tables 2.1 and 2.2). Compliance was similar between AAD and non-AAD patients (80.5% vs. 80.0%).

**Table 2.1. Descriptive demographic data and risk factors for AAD on univariate analysis. Count and percentage (%) or medians and interquartile range (IQR) are shown. Abbreviation: Proton pump inhibitor (PPI) a. Other: multiple sclerosis, depression, epilepsy, anaemia. b. Other: osteomyelitis, diabetic foot, pyelonephritis, chronic venous ulcers infection. c. Other: surgical, gastroenterology, endocrine, rehabilitation ward. d. Other: failed re-supply, incorrect product supplied, patient discharged without product, relative choice, commercially purchased active product given in addition to study product. The reason for withdrawal was unknown in six cases.**

| | | Overall | AAD | Non-AAD | Odds ratio | 95% Confidence Interval | p-value |
|---|---|---|---|---|---|---|---|
| | **Total patients (%)** | **650 (100)** | **118 (18.2)** | **532 (81.8)** | | | |
| | **Male (%)** | 325 (50) | 58 (17.8) | 267 (82.2) | 0.96 | (0.64-1.43) | 0.84 |
| | **Age in years (IQR)** | 73 (65-81) | 75 (66-82) | 73 (64-81) | 1.02 | (1.00-1.04) | 0.13 |
| **Age class** | **55-69 years (%)** | 247 (38) | 41 (16.6) | 206 (83.4) | **Reference category** | | |
| | **≥70 years (%)** | 403 (62) | 77 (19.1) | 326 (80.9) | 1.19 | (0.78-1.70) | 0.42 |
| **Comorbidity** | **Cardiovascular (%)** | 64 (9.8) | 13 (20.3) | 51 (79.7) | 1.17 | (0.31-2.22) | 0.64 |
| | **Dementia (%)** | 9 (1.4) | 2 (22.2) | 7 (77.8) | 1.29 | (0.27-6.31) | 0.75 |
| | **Diabetes (%)** | 28 (4.3) | 2 (7.1) | 26 (92.9) | 0.34 | (0.79-1.43) | 0.14 |
| | **Respiratory (%)** | 136 (20.9) | 23 (16.9) | 113 (83.1) | 0.90 | (0.54-1.48) | 0.67 |
| | **Musculoskeletal (%)** | 85 (13.1) | 11 (12.9) | 74 (87.1) | 0.64 | (0.33-1.24) | 0.18 |
| | **Gastrointestinal (%)** | 54 (8.3) | 8 (14.8) | 46 (85.2) | 0.77 | (0.35-1.67) | 0.51 |
| | **Stroke (%)** | 43 (6.6) | 11 (25.6) | 32 (74.4) | 1.61 | (0.79-3.29) | 0.20 |
| | **Malignancy (%)** | 11 (1.7) | 1 (9.1) | 10 (90.9) | 0.45 | (0.057-3.52) | 0.44 |
| | **Renal (%)** | 5 (0.8) | 2 (40) | 3 (60) | 3.04 | (0.50-18.4) | 0.23 |
| | **Other [a.] (%)** | 28 (4.3) | 5 (17.9) | 23 (82.1) | 0.98 | (0.36-2.63) | 0.97 |
| **Medication on admission** | **PPI (%)** | 203 (31.2) | 41 (20.2) | 162 (79.8) | - | - | 0.36 |
| | **Statin (%)** | 250 (38.5) | 54 (21.6) | 196 (78.4) | - | - | 0.072 |
| | **Laxatives (%)** | 51 (7.8) | 8 (15.7) | 43 (84.3) | - | - | 0.63 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Steroids (%)** | 73 (11.2) | 13 (17.8) | 60 (82.2) | - | - | 0.94 |

| | | **Overall** | **AAD** | **Non-AAD** | **Odds ratio** | **95% Confidence Interval** | **p-value** |
|---|---|---|---|---|---|---|---|
| **Infection on admission** | **Skin & soft tissue (%)** | 81 (12.5) | 16 (19.8) | 65 (80.2) | 1.27 | (0.57-2.85) | 0.56 |
| | **Respiratory (%)** | 373 (57.4) | 66 (17.7) | 307 (82.3) | 1.11 | (0.58-2.12) | 0.76 |
| | **Urinary (%)** | 102 (15.7) | 18 (17.6) | 84 (82.4) | 1.10 | (0.51-2.42) | 0.80 |
| | **Other [b.] (%)** | 80 (12.3) | 13 (16.2) | 67 (83.8) | **Reference category** | | |
| **Admission ward** | **Acute medical unit (%)** | 405 (62.3) | 81 (20) | 324 (80) | 1.90 | (0.73-4.98) | 0.19 |
| | **Elderly (%)** | 45 (6.9) | 5 (11.1) | 40 (88.9) | 0.95 | (0.26-3.55) | 0.94 |
| | **General medicine (%)** | 51 (7.8) | 12 (23.5) | 39 (76.5) | 2.34 | (0.75-7.28) | 0.14 |
| | **Respiratory (%)** | 100 (15.4) | 14 (14) | 86 (86) | 0.70 | (0.42-3.68) | 1.24 |
| | **Other [c.] (%)** | 43 (6.6) | 5 (11.6) | 38 (88.4) | **Reference category** | | |
| **Barthel Index** | **Baseline (IQR)** | 20 (18-20) | 20 (18-20) | 20 (18-20) | 0.98 | (0.92-1.03) | 0.42 |
| | **Follow-up (IQR)** | 20 (17.5-20) | 20 (18-20) | 20 (17-20) | 0.97 | (0.92-1.02) | 0.97 |
| | **Withdrawals (%)** | 152 (23.4) | 28 (18.4) | 124 (81.6) | 1.02 | (0.64-1.63) | 0.95 |
| **Reason for withdrawal** | **Patient choice (%)** | 72 (54.1) | 15 (20.8) | 57 (79.2) | 0.99 | 0.29-3.41 | 0.98 |
| | **Clinically unwell (%)** | 15 (11.3) | 2 (13.3) | 13 (86.7) | 0.58 | 0.09-3.68 | 0.56 |
| | **Loss to follow-up (%)** | 15 (11.3) | 2 (13.3) | 13 (86.7) | 0.58 | 0.09-3.68 | 0.56 |
| | **Protocol deviation (%)** | 12 (9.0) | 4 (33.3) | 8 (66.7) | 1.88 | 0.37-9.57 | 0.45 |
| | **Other [d.] (%)** | 19 (14.3) | 4 (21.1) | 15 (78.9) | **Reference category** | | |

**Table 2.1. Continued.**

**Table 2.2. Baseline laboratory parameters. Median values and interquartile range (IQR) are shown. Univariate analysis was used to calculate odds ratios, 95% CI and p-values. All blood results were analysed as continuous variables. Odds ratios are shown for each unit increase. Normal ranges: Haemoglobin (11.5-16.5), platelets (150-450), white cell count (4-11), neutrophils (2-7.5), C-reactive protein (<5), sodium (136-145), potassium (3.2-5.1), urea(1.7-8.3), creatinine (44-80), albumin (35-52), alanine transferase (0-33), alkaline phosphatase (35-104).**

| | Overall | AAD | Non-AAD | Odds ratio | 95% Confidence Interval | p-value |
|---|---|---|---|---|---|---|
| **Total patients (%)** | **650 (100)** | **118 (18.2)** | **532 (81.8)** | | | |
| **Haemoglobin g/dL (IQR)** | 12.9 (11.5-14.1) | 12.8 (10.9-13.7) | 12.8 (11.8-14.1) | 1.01 | (0.99-1.03) | 0.42 |
| **Platelets $10^9$/L (IQR)** | 251 (196-319.8) | 241.5 (194-316) | 252 (199-327) | 1.00 | (0.99-1.00) | 0.61 |
| **White cell count $10^9$/L (IQR)** | 11.4 (8.3-15.1) | 10.3 (8.1-13.9) | 11.6 (8.4-15.5) | 1.00 | (0.98-1.01) | 0.64 |
| **Neutrophils $10^9$/L (IQR)** | 8.9 (6.1-12.5) | 7.9 (6.2-11.3) | 9 (5.8-12.3) | 0.99 | (0.96-1.02) | 0.51 |
| **C- reactive protein mg/L (IQR)** | 71.1 (21.2-166) | 69 (21.3-170) | 77.5 (21-163) | 1.00 | (0.99-1.00) | 0.35 |
| **Sodium mmol/L (IQR)** | 137 (134-140) | 137 (134-140) | 138 (135-141) | 1.00 | (0.98-1.01) | 0.53 |
| **Potassium mmol/L (IQR)** | 4.1 (3.8-4.5) | 4.1 (3.7-4.3) | 4.1 (3.7-4.4) | 0.78 | (0.54-1.11) | 0.17 |
| **Urea mmol/L (IQR)** | 6.4 (4.7-8.9) | 6.5 (4.7-9.5) | 6.2 (4.7-8.6) | 1.00 | (0.98-1.01) | 0.64 |
| **Creatinine µmol/L (IQR)** | 86.5 (71-110) | 86.5 (70-114) | 85.5 (73-110) | 1.00 | (0.99-1.01) | 0.27 |
| **Albumin g/L (IQR)** | 36 (32-40) | 35.5 (32-41) | 37 (33-41) | 0.97 | (0.94-1.00) | 0.09 |
| **Alanine transferase iu/L (IQR)** | 20 (15-32) | 21.5 (16-33) | 20 (14-31) | 1.00 | (0.99-1.01) | 0.27 |
| **Alkaline phosphatase iu/L (IQR)** | 88 (69-115) | 86 (67-118) | 88 (69-122) | 1.00 | (0.99-1.00) | 0.93 |

### *2.4.2 Comparison between younger and older age groups*

Patients were stratified into two age groups (55-69 years and ≥ 70 years) and two thirds were recruited to the older age group. A comparison between age groups revealed the older group had significantly more comorbidity, with 76/403 (18.9%) admitted with multiple comorbidities compared to 28/247 (11.3%) in the younger group ($p<0.001$). A greater proportion of older patients had Barthel Indices <20 on admission (58.3% vs. 29.9%, $p<0.001$) and significantly worse renal function, lower albumin and lower haemoglobin at baseline (Table 2.3).

Older patients accounted for a greater proportion of total respiratory infection (64.3%) and urinary tract infection (69.6%) on admission compared to younger patients. Compliance was similar in both age groups (92.3% vs. 93.8%). Length of stay was significantly increased (7 days [IQR 4-11.5] vs. 5 days [IQR 4-9], $p<0.0001$) in the older group and a greater proportion of the total 152 withdrawals occurred in this group (96/152 [63.2%] vs. 56/152 [36.2%], $p=0.77$). Patient choice (65.3%) and clinical deterioration (66.7%) were the commonest reasons recorded in this age group. Out of a total of 15 patients lost to follow-up, only three patients were in the older age group (20%). A significantly higher number of deaths occurred in the older age group (21/22 [95.5%] vs. 1/22 [4.5%], $p<0.001$), although none were in relation to the study product.

**Table 2.3. Comparison between younger and older age groups. Patients were pre-stratified into two age groups, 55-69 years and ≥70 years. Count and percentage (%) or medians and interquartile range (IQR) are shown. Abbreviation: Proton pump inhibitor (PPI). Normal ranges: Haemoglobin (11.5-16.5), platelets (150-450), white cell count (4-11), neutrophils (2-7.5), C-reactive protein (<5), sodium (136-145), potassium (3.2-5.1), urea (1.7-8.3), creatinine (44-80), albumin (35-52), alanine transferase (0-33), alkaline phosphatase (35-104).**

| | | ≥ 70 years | 55–69 years | p-value |
|---|---|---|---|---|
| | **Total patients (%)** | **403 (62)** | **247 (38)** | |
| **Baseline laboratory markers** | **Haemoglobin g/dL (IQR)** | 12.5 (11.4-13.8) | 13.2 (12.2-14.6) | **<0.001*** |
| | **Platelets $10^9$/L (IQR)** | 245 (190.5-305.5) | 260 (212-347.5) | 0.064 |
| | **White cell count $10^9$/L (IQR)** | 11.2 (8.4-14.8) | 11.8 (8.3-14.9) | 0.88 |
| | **Neutrophils $10^9$/L (IQR)** | 9 (6.3-12.1) | 8.2 (5.4-12.3) | 0.14 |
| | **C- reactive protein mg/L (IQR)** | 77 (21.0-169.1) | 77 (21.2-159) | 0.87 |
| | **Sodium mmol/L (IQR)** | 137 (134-140) | 139 (135-141) | 0.21 |
| | **Potassium mmol/L (IQR)** | 4.1 (3.8-4.4) | 4.1 (3.7-4.3) | 0.43 |
| | **Urea mmol/L (IQR)** | 7.2 (5.4-9.9) | 5.4 (4.2-6.8) | **<0.001*** |
| | **Creatinine umol/L (IQR)** | 89.5 (73-119) | 81 (69.5-98.5) | **<0.001*** |
| | **Albumin g/L (IQR)** | 36 (31-40) | 38 (34-42) | **<0.001*** |
| | **Alanine transferase iu/L (IQR)** | 19 (14-28.5) | 23 (16-37) | **0.012*** |
| | **Alkaline phosphatase iu/L (IQR)** | 90 (69-121.5) | 84 (69-114.5) | 0.58 |
| **Medication on admission** | **PPI (%)** | 146 (71.9) | 57 (28.1) | **<0.001*** |
| | **Statins (%)** | 163 (65.2) | 87 (34.8) | 0.18 |
| | **Laxatives (%)** | 36 (70.6) | 15 (29.4) | 0.19 |
| | **Steroids (%)** | 42 (57.5) | 31 (42.5) | 0.40 |

### *2.4.3 Antibiotic use*

All randomised patients received in-patient antibiotics for a minimum of 72 hours and included 133/650 (20.5%) patients who had started on antibiotics in the community (for <7 days). Patients were prescribed an average of 2.83 (S.D.1.41) antibiotics each, with a maximum of eight prescribed in one case. Co-amoxiclav (46.3%) was most frequently used and the median duration of antibiotics was 8 days (IQR 7-11) (Table 2.4). On univariate analysis, clindamycin was the only antibiotic associated with an increased risk of AAD (OR 3.11, 95% CI 1.09-8.92, p=0.035), although clindamycin use was low overall (n=15). Antibiotic duration (OR 0.96, 95% CI 0.92-1.01, p=0.098) and use of multiple antibiotics (OR 1.34, 95% CI 0.78-2.32, p=0.29) were not statistically significantly associated with an increased risk of AAD.

**Table 2.4. Antibiotic use and risk of AAD on univariate analysis. Count and percentage (%) or medians and interquartile range (IQR) are shown.**

| | Overall | AAD | Non AAD | Odds ratio | 95% Confidence Interval | p-value |
|---|---|---|---|---|---|---|
| **Total patients (%)** | 650 (100) | 118 (18.2) | 532 (81.8) | | | |
| **Co-amoxiclav (%)** | 301 (46.3) | 61 (20.3) | 240 (79.7) | 1.30 | (0.87-1.94) | 0.20 |
| **Clarithromycin (%)** | 268 (41.2) | 51 (19) | 217 (81) | 1.11 | (0.74-1.65) | 0.63 |
| **Amoxicillin (%)** | 193 (29.7) | 31 (16.1) | 162 (83.9) | 0.81 | (0.52-1.28) | 0.37 |
| **Flucloxacillin (%)** | 77 (11.8) | 14 (18.2) | 63 (81.8) | 1.00 | (0.54-1.86) | 1.00 |
| **Penicillin (%)** | 98 (15.1) | 23 (23.5) | 75 (76.5) | 1.48 | (0.88-2.47) | 0.14 |
| **Doxycycline (%)** | 78 (12) | 12 (15.4) | 12 (15.4) | 0.80 | (0.42-1.53) | 0.50 |
| **Piperacillin-tazobactam (% )** | 56 (8.6) | 14 (25) | 42 (75) | 1.57 | (0.83-2.98) | 0.17 |
| **Trimethoprim (%)** | 57 (8.8) | 11 (19.3) | 46 (80.7) | 1.09 | (0.55-2.17) | 0.82 |
| **Gentamicin (%)** | 40 (6.2) | 7 (17.5) | 33 (82.5) | 0.95 | (0.41-2.21) | 0.91 |
| **Teicoplainin (%)** | 22 (3.4) | 2 (9.1) | 20 (90.9) | 0.44 | (0.10-1.92) | 0.28 |
| **Ciprofloxacin (%)** | 26 (4) | 5 (19.2) | 21 (80.8) | 1.08 | (0.40-2.92) | 0.88 |
| **Metronidazole (%)** | 22 (3.4) | 3 (13.6) | 19 (86.4) | 0.70 | (0.21-2.42) | 0.58 |
| **Clindamycin (%)** | 15 (2.3) | 6 (40) | 9 (60) | 3.11 | (1.09-8.92) | **0.035*** |
| **Levofloxacin (%)** | 13 (2) | 1 (7.7) | 12 (92.3) | 0.37 | (0.048-2.88) | 0.34 |
| **Vancomycin (%)** | 15 (2.3) | 3 (20) | 12 (80) | 1.13 | (0.31-4.07) | 0.85 |
| **Duration in days (IQR)** | 8 (7-11) | 8 (6-9) | 8.5 (7-11) | 0.96 | (0.92-1.01) | 0.098 |
| **Multiple antibiotics (%)** | 526 (81.3) | 100 (19) | 426 (81) | 1.34 | (0.78-2.32) | 0.29 |
| **Antibiotics started in the community for <7 days (%)** | 133 (20.5) | 24 (18) | 109 (82) | 0.99 | (0.60-1.63) | 0.97 |

### *2.4.4 Risk of AAD on multivariate analysis*

To evaluate the potential confounding effect of variables on the risk of AAD, a multivariate analysis was performed that included known risk factors for AAD. Factors on the causal pathway were excluded from the analysis. The variables included were age, hospital length of stay, existing laxative use and duration of antibiotics. No factors were significant on multivariate analysis (Table 2.5).

**Table 2.5. Risk factors associated with AAD on multivariable analysis.**

| | **Regression Co-efficient** | **Standard error** | **Wald test statistic** | **Odds ratio** | **95% Confidence Interval** | **p-value** |
|---|---|---|---|---|---|---|
| **Age** | 0.014 | 0.011 | 1.65 | 1.01 | (0.99-1.04) | 0.20 |
| **Hospital length of stay** | 0.00 | 0.004 | 0.004 | 1.00 | (0.99-1.01) | 0.95 |
| **Existing laxative use** | 0.18 | 0.410 | 0.20 | 1.20 | (0.54-2.68) | 0.66 |
| **Duration of antibiotics** | 0.018 | 0.013 | 1.82 | 1.02 | (0.99-1.05) | 0.18 |

### *2.4.5 Comparison between centres*

The first ten centres were compared in terms of antibiotic use and rate of AAD and all rates were calculated as a ratio of the total number recruited per centre (Figure 2.3A). A positive correlation was suggested between the rate of AAD and number of antibiotics prescribed per patient per centre (r=0.4) but was not close to achieving statistical significance (p=0.28) (Figure 2.2B). The seven antibiotics most commonly prescribed were compared between centres (Figure 2.4). Co-amoxiclav (centres one to five and seven) and amoxicillin (centres six and eight to 10) were prescribed most often. The overall pattern of antibiotic use was similar between centre five (highest rate of AAD) and centre two (lowest rate of AAD), although centre five prescribed more doxycycline and less piperacillin-tazobactam. Penicillin was not prescribed to any patients recruited at centre seven.

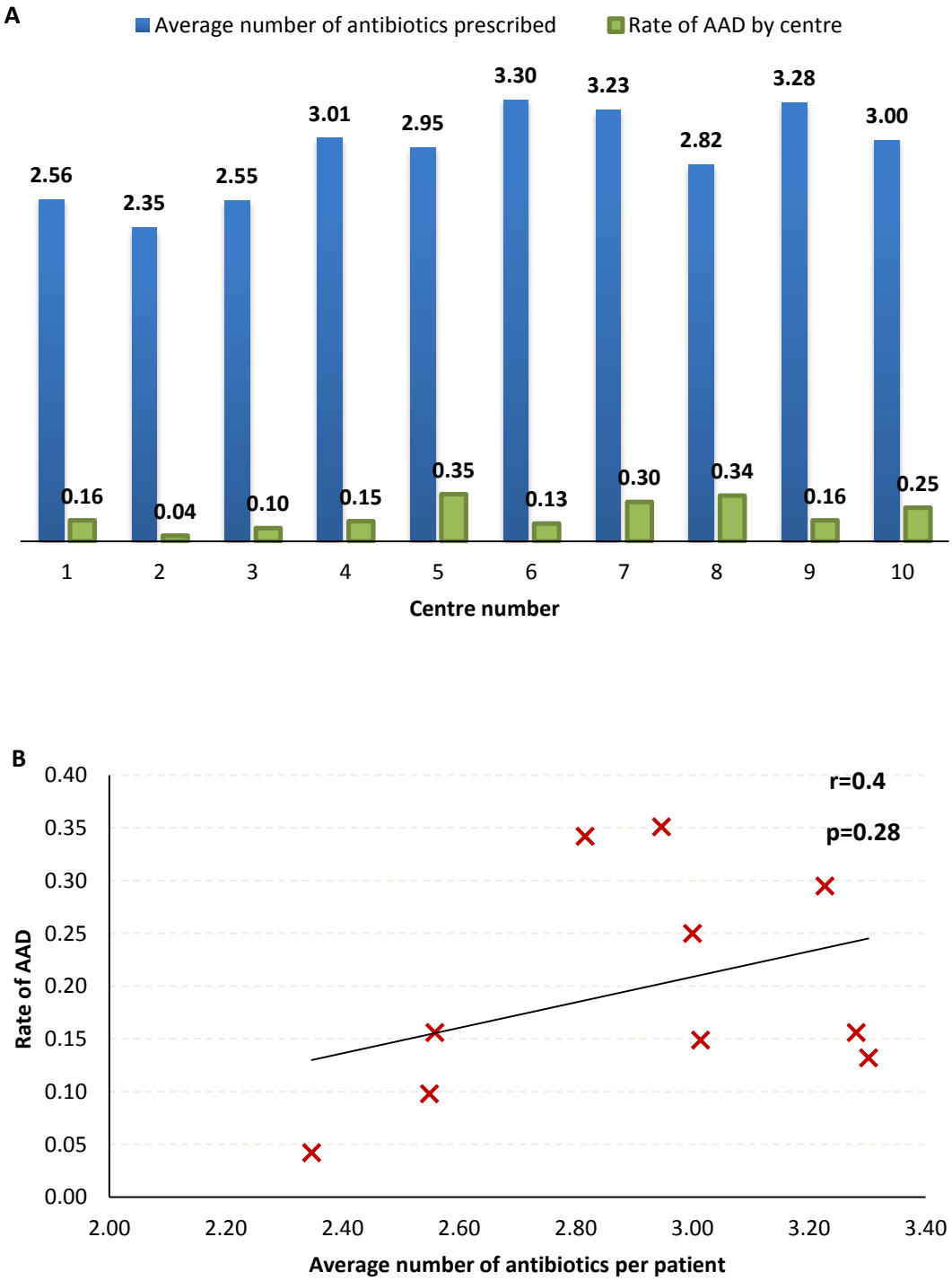



**Figure 2.3. Comparison of antibiotic use and rate of AAD by centre. A. The rate of AAD and total antibiotic use were normalised per centre and calculated based on the total numbers recruited in each centre. Rates are shown above each bar chart. B. Correlation between numbers of antibiotics prescribed and rate of AAD between centres. r=Pearson's correlation coefficient.**

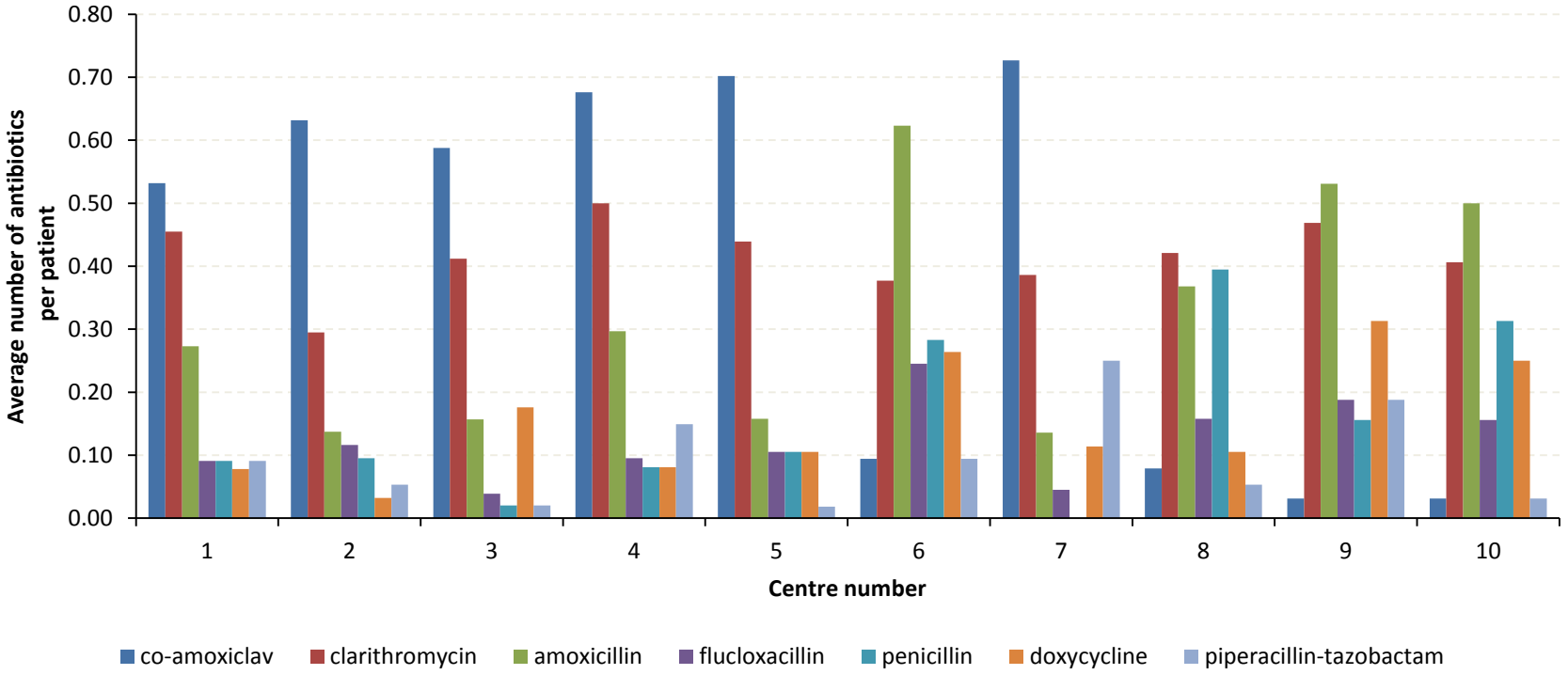



**Figure 2.4. Antibiotic prescribing patterns by centre. The seven most frequently prescribed antibiotics were compared between the first ten centres. Antibiotics are shown as a proportion of the total number recruited per centre.**

### *2.4.6 Detection of organisms in stool*

Stool samples were requested at baseline and at the onset of loose stool (type 5). A total of 324 stool samples were tested and consisted of 277 baseline stools and 23 loose stools. The remaining 24 samples were not recorded as being collected at baseline or at the onset of loose stool. All stool samples tested were negative for *Salmonella, Shigella, Campylobacter, E. coli* 0157, ova, cysts and parasites. Approximately one third of samples tested (113/324 [34.9%]) were positive for at least one of the remaining four organisms tested (*C. difficile, S. aureus, K. oxytoca* and *C. albicans*) (Figure 2.5).

#### *2.4.6.1 Baseline stools*

*C. albicans* was detected in 80/324 (24.7%) baseline stool samples. The remaining organisms were found in fewer samples: *C. difficile* 16/324 (4.9%), *S. aureus* 8/324 (2.5%) and *K. oxytoca* 7/324 (2.1%). Two different organisms were found in 10 stool samples and one sample contained three different organisms *(C. albicans, K. oxytoca and S. aureus).*

#### *2.4.6.2 Loose stools*

*C. albicans* was the commonest organism found in 13/14 (92.9%) loose stool samples. *C. difficile* and *K. oxytoca* were found in two stool samples and *S. aureus* was not detected in any loose stool.

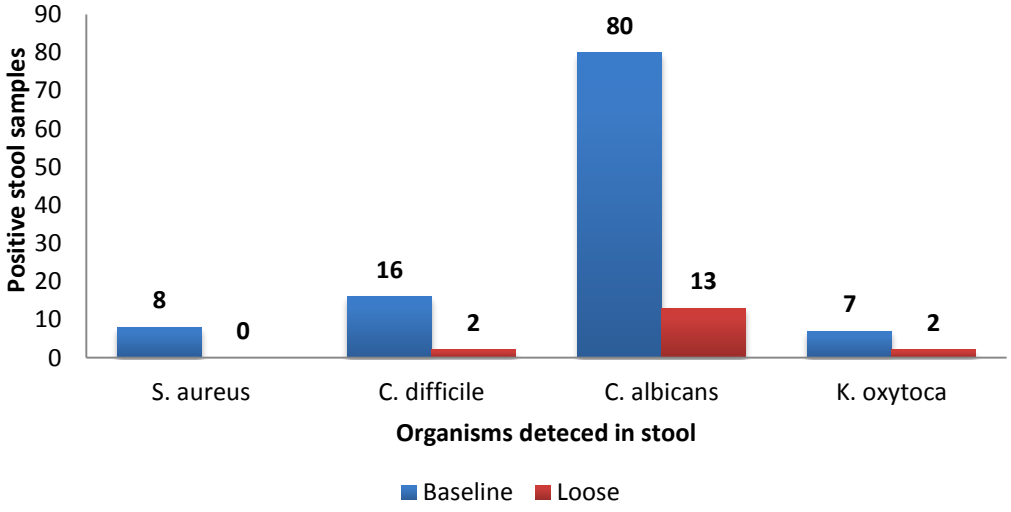



**Figure 2.5. Total number of positive stool samples. Stool samples were collected at baseline and at the onset of loose stool (type 5 on the Bristol Stool chart). The total number of stool samples that tested positive for each organism are shown above the bar charts.**

### *2.4.6.3 Organisms detected in patients with AAD*

There were 118 cases of AAD in the first 650 patients recruited and 30 stool samples were available for this group of patients (Figure 2.6). At baseline 22 stools were positive and *C. albicans* was found in 19/22 (86.4%) samples. *C. difficile*, *K. oxytoca* and *S. aureus* were each found in three baseline stool samples. Loose stool samples were available for eight AAD patients and *C. albicans* was found in 7/8 (87.5%) samples.

Paired stool samples (baseline and loose) were available for six patients. The same organism was detected in both samples for five out of the six patients (Table 2.6). In patient three *C. difficile* was isolated at baseline but not in the loose stool sample. *C. albicans* was detected in the loose stool sample.

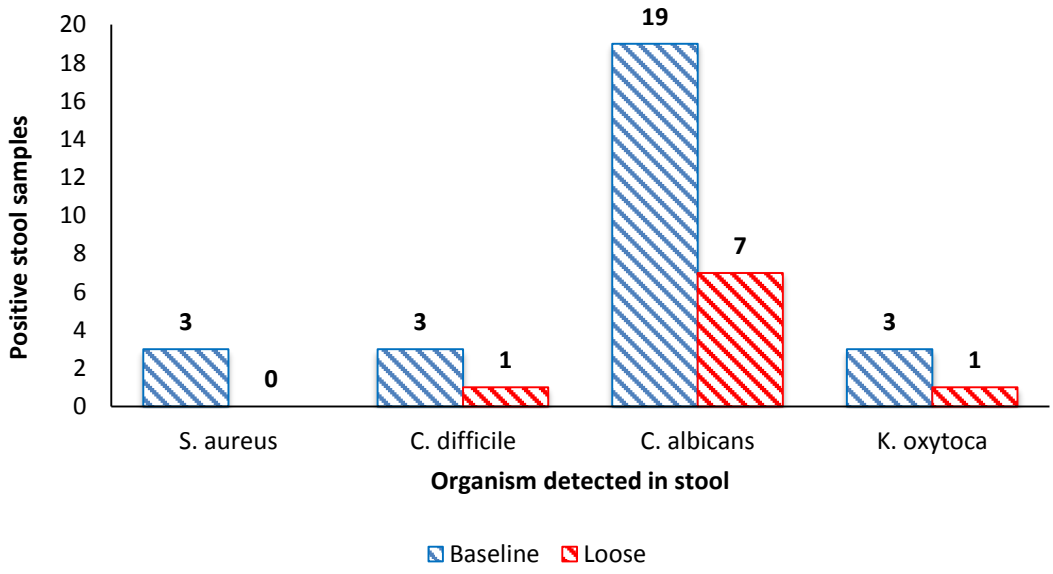



**Figure 2.6. Positive stool samples in AAD patients. Stool samples were collected at baseline and at the onset of loose stool (type 5 on the Bristol Stool chart). The total number of stool samples that tested positive for each organism are shown above the bar charts.**

**Table 2.6. Organisms detected in paired stool samples in AAD patients. Patient three was the only discordant pair of stool samples.**

| Patient number | Baseline stool | Loose stool |
|---|---|---|
| **1** | *C. albicans, C. difficile* | *C. albicans, C. difficile* |
| **2** | *C. albicans* | *C. albicans* |
| **3** | *C. difficile* | *C. albicans* |
| **4** | *C. albicans* | *C. albicans* |
| **5** | *C. albicans* | *C. albicans* |
| **6** | *C. albicans* | *C. albicans* |

### *2.4.7 Quality control testing of study product*

Samples from different batches were supplied from the main production plant in Paris and delivered to the microbiology laboratory at the Royal London Hospital, UK for testing. Samples were also collected at random from centres at the end of a two week randomisation block. There was no significant decline in the numbers of *L.casei* DN114001 and no bacteria other than *S. thermophilus, L. bulgaricus* and *L. casei* DN114001 were detected in any of the products tested.

#### *2.4.7.1 Taste test*

The taste test reported no difference in texture, consistency or flavour between the active and placebo product.

### *2.4.8 Safety*

All AEs and SAEs were recorded in the CRF. A total of 209 (32.2%) AEs and 110 (16.9%) SAEs were reported in the first 650 cases (Table 2.7). AEs reported in relation to the gastrointestinal tract accounted for 15.9% and included constipation, bloating and nausea. On univariate analysis, patients with three AEs were almost three times more likely to suffer from AAD (OR 2.84, 95% CI 0.66-12.12) although this did not reach statistical significance (p=0.16). In patients with two or more SAEs, there was a significant increase in the risk of AAD (OR 6.41, 95% CI 1.41-29.10, p=0.016). There were 22 deaths in total that corresponded to an overall mortality rate of 3.4%. Of the 22 deaths, seven were due to pneumonia, three due to cardiac causes, four caused by complications associated with malignancy, four caused by worsening sepsis and in the remainder of cases the cause was not known. A total of three deaths occurred in patients with AAD. No code breaks were requested and no SAEs or deaths were attributed to the study product. Patients recruited to the older age group accounted for a greater proportion of patients with AEs (141/650 [67.5%], p=0.048) and SAEs (81/650 [73.6%], p=0.006).

**Table 2.7. Adverse events, serious adverse events and number of deaths. Count and percentage (%) are shown.**

| | | Overall | AAD | Non-AAD | Odds ratio | 95% Confidence Interval | p-value |
|---|---|---|---|---|---|---|---|
| | **Total patients (%)** | **650 (100)** | **118 (18.2)** | **532 (81.8)** | | | |
| **Number of adverse events** | **None (%)** | 441 (67.8) | 77 (17.5) | 364 (82.5) | **Reference category** | | |
| | **One (%)** | 161 (24.8) | 27 (16.8) | 134 (83.2) | 0.95 | (0.59-1.54) | 0.84 |
| | **Two (%)** | 40 (6.2) | 11 (27.5) | 29 (72.5) | 1.79 | (0.86-3.74) | 0.12 |
| | **Three (%)** | 8 (1.2) | 3 (37.5) | 5 (62.5) | 2.84 | (0.66-12.12) | 0.16 |
| **Number of serious adverse events** | **None (%)** | 540 (83.1) | 93 (17.2) | 447 (82.8) | **Reference category** | | |
| | **One (%)** | 103 (15.8) | 21 (20.4) | 82 (79.6) | 1.23 | (0.73-2.09) | 0.44 |
| | **Two or more (%)** | 7 (1.1) | 4 (57.1) | 3 (42.9) | 6.41 | (1.41-29.1) | 0.016* |
| | **Deaths (%)** | 22 (3.4) | 3 (13.6) | 19 (86.4) | 0.70 | (0.21-2.42) | 0.58 |

### *2.4.9 Population screening logs*

Screening logs were used at each centre to assess patterns of recruitment and also provide a description of patient flow throughout the study. Data for five representative centres was available over a three month period (August 2012 to October 2012) (Table 2.8). During this period 32/1284 patients were recruited, which corresponded to 2.5% of total patients screened. A total of 1252 patients were excluded across the five centres, with 568/1252 (45.4%) excluded based on criteria listed in the protocol. The commonest reasons were existing gastrointestinal pathology (181/568 [31.9%]) and a history of diarrhoea in the preceding week (127/568 [22.4%]). Centre one excluded 38 patients with two exclusion criteria and four with at least three exclusion criteria. The remaining eligible patients (n=684) were excluded due to either logistical reasons (216/1252 [17.3%]) or other reasons (468/1252 [37.4%]) that included patients who were either too confused or too unwell as judged by the research nurse or principal investigator at each centre.

### *2.4.10 Study population*

Recruitment to the study proved difficult and was extended for a further 18 months. Therefore for the purpose of this thesis, a descriptive demographic analysis of blinded data was undertaken for the first 650 cases (Table 2.1). The overall median age was 73 years (interquartile range [IQR] 65-81) and equal numbers were recruited from each gender. Respiratory infections were the commonest infection on admission (57.4%) followed by urinary infections (15.7%) and patients were most frequently recruited from the acute medical ward. The intervention was taken for a median of 14 days (IQR 11-15.5) and patient compliance was 80.0% overall (defined as consumption of at least 50% of the study product).

#### *2.4.10.1 Withdrawal rate*

A total of 152/650 patients prematurely withdrew from the study, which corresponded to an overall withdrawal rate of 23.4%. Within this group compliance was poor with only 30.9% consuming at least 50% of the product. Patient choice accounted for 54.1% of cases with taste reported as the commonest reason for discontinuation of the study product. Clinical deterioration of patients was responsible for in 11.3% of cases of withdrawal and a similar number of patients were lost to follow-up. Logistical problems included: discharge or transfer to another hospital without the study product

on the ward (n=6) failure to administer product (n=4), failure to order a re-supply (n=2), refrigeration problems (n=3) and simultaneous consumption of the active product purchased from a shop (n=2).

### *2.4.11 Health economic analysis and quality of life scores*

The health economic analysis and interpretation of SF-12 scores will be performed at the end of the trial.

**Table 2.8. Population screening logs. The number of patients screened and reasons for exclusion are shown for five representative centres between August 2012 and October 2012. Abbreviations: gastrointestinal (GI), percutaneous endoscopic gastrostomy (PEG), nasogastric (NG).**

| | Centre number | | | | | |
|---|---|---|---|---|---|---|
| | **1** | **4** | **2** | **21** | **8** | |
| **Exclusion criteria listed in protocol** | | | | | | |
| GI Pathology or GI infection | 28 | 3 | 15 | 120 | 15 | 181 |
| Diarrhoea in preceding week | 59 | 6 | 3 | 40 | 19 | 127 |
| Unable to swallow (PEG/NG fed) | 28 | 0 | 5 | 24 | 3 | 60 |
| Cytotoxic therapy | 15 | 3 | 7 | 6 | 8 | 39 |
| Recent steroid use | 6 | 8 | 4 | 20 | 3 | 41 |
| Prosthetic heart valve or endocarditis | 3 | 2 | 2 | 18 | 1 | 26 |
| Surgery (past four weeks) | 5 | 5 | 4 | 9 | 0 | 23 |
| Immunosuppressed | 3 | 2 | 6 | 7 | 2 | 20 |
| Severe life-threatening illness | 1 | 0 | 7 | 1 | 6 | 15 |
| Pancreatic disease | 4 | 0 | 1 | 4 | 0 | 9 |
| Bowel surgery (past 12 weeks) | 2 | 0 | 0 | 3 | 0 | 5 |
| Foreign travel in past seven days | 0 | 1 | 3 | 3 | 0 | 7 |
| Recent steroid use | 5 | 0 | 0 | 0 | 4 | 9 |
| Included in another trial | 0 | 0 | 1 | 1 | 1 | 3 |
| Post-transplant | 1 | 0 | 0 | 2 | 0 | 3 |
| **Total excluded** | | | | | | **568** |
| **Other reasons for exclusion** | | | | | | |
| Patient choice | 20 | 2 | 7 | 13 | 12 | 54 |
| Hypersensitivity to milk products | 0 | 0 | 1 | 0 | 1 | 2 |
| Confused | 74 | 14 | 13 | 53 | 67 | 221 |
| Clinically unwell | 91 | 16 | 20 | 32 | 32 | 191 |
| **Total excluded** | | | | | | **468** |
| **Logistical reasons for exclusion** | | | | | | |
| Transfer to another hospital | 1 | 0 | 0 | 1 | 0 | 2 |
| Research nurse unavailable | 0 | 0 | 6 | 11 | 0 | 17 |
| Unable to randomise within 48 hours | 13 | 6 | 6 | 108 | 64 | 197 |
| **Total excluded** | | | | | | **216** |
| **Overall** | | | | | | |
| Number excluded | 359 | 68 | 111 | 476 | 238 | **1252** |
| Number randomised | 3 | 4 | 6 | 15 | 4 | **32** |

## 2.5 Discussion

Probiotics are live microorganisms that have the potential to counteract antibiotic disruption to the intestinal microbiota by competing with pathogens for nutrients and surface receptors (271)(272). Three large meta-analyses recently concluded that probiotics have a beneficial role in the prevention of AAD; however, they all highlighted that earlier studies have been limited by small sample size (254)(255)(257). The current Probiotic NU278 study was designed as a multicentre, double-blind, randomised, placebo-controlled trial that set-out to definitively address the role of the probiotic *L. casei* DN114001, which previously demonstrated a reduction in AAD in a smaller randomised controlled trial (235).

### *2.5.1 Changes to clinical trial design*

The study was designed to recruit patients at a rate of eight patients a fortnight per centre to ensure recruitment was completed over a two year period. However, recruitment to the study proved extremely challenging and resulted in an extension to the recruitment period by 18 months and a doubling of the number of active recruitment centres. The rate of recruitement was slow due to several logistical factors that included the set-up time of individual centres, time taken to obtain local research governance approval at sites and research nurse availability. In addition, the challenges to recruitment can partly be explained by the number of exclusion criteria and the nature of the study intervention. The exclusion criteria chosen in the current study were broader than those used previously by Hickson *et al.* and were comparable to criteria used in other probiotic studies (235). Within six months, the exclusion criteria were modified to include patients from Nursing and Residential Homes and patients with a Barthel Index <15, which allowed frailer patients to be recruited. However, these changes did not result in an increase in recruitment.

Patients with pre-existing gastrointestinal pathology or diarrhoea in the preceding week accounted for half of the total excluded patients (based on criteria listed in the protocol), but this was necessary as establishing causality of diarrhoea would be difficult in this group. Furthermore, the effect of the probiotic may have varied in this group, as the composition of the intestinal microbiota is known to differ in patients with inflammatory bowel disease (273). It is interesting that a large proportion of patients were not enrolled due to failure to randomise within 48 hours and this might

reflect changes to hospital practice that include a drive to discharge patients quickly following hospital admission. Although eligible for recruitment, patients perceived as 'too unwell' by the research team were not approached and this prevented the intervention from being tested in those patients that may have stood to gain the most benefit.

The nature of the study intervention was an important factor and several eligible older patients were not randomised as they reported a dislike of yoghurt. In contrast, other eligible patients chose to purchase the active product, which is already commercially available rather than risk receiving the placebo. There have been no studies exploring patient perception of probiotics or comparing efficacy of different delivery vehicles. However, previous studies have shown that older patients perceive the importance of individual medications based on three main areas; the drug's characteristics, their personal knowledge and attitudes and external opinions from the media, family and their physician (274). It is possible that more patients might have joined the current study if the product was delivered as a capsule. Patients might have identified more with a probiotic packaged in a similar way to their other medications and this would have differentiated the product from the commercially available form.

### *2.5.2 Change to sample size*

Calculation of sample size is based on a knowledge of endpoint variance and the effect size needed to adequately power the study (275). However, the variance in endpoint incidence can change as the epidemiology of a disease changes throughout the course of a long-term clinical trial. The epidemiology of CDI has changed dramatically since the study was designed with reported rates of CDI in England falling from 55,000 cases in 2007 to 13,352 in 2012 (15)(24). This has been accompanied by changes to antibiotic prescribing policies that could have conceivably impacted on the overall rate of AAD.

In order to maintain the validity and integrity of the trial, in December 2011 the independent TSC made the decision to change the sample size, to ensure that the clinical trial results would remain clinically valid. The increased withdrawal rate from 10% to 20% was also a factor for adjusting the sample size. The main reason for the increase in the withdrawal rate was patient choice with tase and a deterioration in clinical state most frequently reported. The primary outcome definition of AAD was

also changed in December 2011 as the original definition was too stringent and might have underestimated the incidence of AAD as only cases that remained symptomatic for a minimum of three days would be counted. As no guidelines or definitions currently exist for causes of AAD (excluding CDI), the revised trial definition was based on the European guidelines for CDI (165).

### *2.5.3 AAD*

Applying the definition to the first 650 cases resulted in an overall incidence of 18.2% AAD that is comparable to previously reported rates (107). The recurrence rate of AAD observed was low and might be related to the relatively short duration of follow-up of only three weeks after cessation of antibiotics. In the current study, it was surprising that antibiotic duration and drugs on admission were not associated with an increased risk of AAD on multivariate analysis, which is in contrast to other studies (276)(277). We have not addressed the timing of AAD and it is possible that in some cases antibiotics were discontinued or changed at the onset of AAD that may have affected the duration of antibiotic treatment. Although total number of antibiotics and overall duration were assessed, a more detailed analysis that accounts for cumulative changes in patterns of antibiotic exposure over time may be needed. This form of analysis was recently conducted by Stevens *et al*. who demonstrated a dose-dependent increase in the risk of CDI was significantly associated with an increase in the cumulative dose, number of antibiotics and days of exposure (278).

In the current study, clindamycin was the only antibiotic associated with AAD, although the numbers prescribed were small. During the 1970s and 1980s, this antibiotic was widely used to treat anaerobic infections, but was later shown to cause CDI in a hamster model of infection and was responsible for CDI outbreaks in four large hospitals (2)(279). More recently, healthy volunteers exposed to a short course of clindamycin demonstrated continued changes to the composition of their intestinal microbiota up-to two years after cessation of treatment. This could leave patients increasingly susceptible to colonisation and overgrowth of pathogens (280).

A non-significant positive correlation was suggested between the average number of antibiotics prescribed per patient and the rate of AAD in different centres (Figure 2.2). Antibiotic prescribing patterns showed variation between centres despite the introduction of national antibiotic prescribing policies; however, the overall pattern of

antibiotic use was similar in the centres with the lowest and highest reported rates of AAD. This may be explained by differences in other infection control measures that include the use of isolation wards, hand-washing and environmental cleaning, which were not recorded in this study. However, as almost 80% of AAD is due to a non-infective cause other possible explanations include differences in diet and under or over-reporting of loose stool by patients and research nurses in different centres (266).

During the current study, the commonest organism isolated in stool samples was *C. albicans.* This organism exists as part of the normal intestinal microbiota and levels have been shown to increase following antibiotic treatment, most likely due to a decline in colonisation resistance (281)(282).

The role of *C. albicans* overgrowth as a causative organism of AAD has been debated and the level of evidence is weak (107). Krause *et al.* demonstrated that although overall rates of *C. albicans* growth were increased in patients exposed to antibiotics, rates were comparable between patients who developed AAD and those that did not (283). However, in the present study, *C. albicans* was detected in five out of six paired stool samples and was the commonest organism in AAD patients. The organism may have contributed to AAD through increased production of secreted aspartyl proteinases that are capable of gastrointestinal mucin degradation (283). Alternatively, the increased detection of *C. albicans* may simply reflect the increased ability of the organism to colonise the gastrointestinal tract of elderly patients following antibiotic use. Case reports have suggested that *C. albicans* may contribute to prolonged refractory diarrhoea in elderly patients and cessation of symptoms has been seen in response to a course of anti-fungal treatment (284).

The evidence supporting *S. aureus* and *K. oxytoca* as aetiological causes of AAD is also limited. In this study, although three cases of *S. aureus* were detected at baseline in patients that developed AAD, none were found in loose stool. Furthermore, only leucotoxin and enterotoxin producing strains have been associated with AAD and these were not tested in the current study (285). *K. oxytoca* has been shown to cause a life-threatening haemorrhagic colitis and was found in the loose stool of one patient who developed AAD (286)(287). However, in the setting of mild-moderate AAD seen in the current study, the organism is unlikely to be significant. This is supported by a study of 371 patients on antibiotics that failed to detect a difference in the distribution

of *K. oxytoca* between patients that developed AAD and those that did not. This suggests that *K. oxytoca* may be a more important aetiological agent in patients who develop severe haemorrhagic colitis (288).

In the current study, the rate of asymptomatic carriage of *C. difficile* was 4.9% (detected in 16/324 baseline stools) which is slightly lower than the 7-26% previously reported in hospitalised patients (158)(159). In patients that developed AAD, *C. difficile* was detected in one set of paired samples (baseline and loose) that suggests a progression from asymptomatic carrier to active disease. This may have been influenced by failure to mount an effective immune response or due to continued disruption to the microbiota; therefore, it will be of interest to see whether this individual received the active or placebo product.

In one individual, although colonised with *C. difficile* at baseline, *C. albicans* was the only organism detected at the onset of loose stool. It is plausible that *C. albicans* was able to directly out-compete *C. difficile* for nutrients or binding space in this individual. Alternatively, *C. albicans* may have produced an inhibitory substance that indirectly inhibited *C. difficile,* which has been demonstrated in other bacteria (289).

### *2.5.4 Safety*

The overall safety profile in the current study was good with no AEs, SAEs or deaths related to the study product. A recent report documenting the safety profile of probiotics in preventing disease acknowledged that evidence from existing randomised controlled trials suggests probiotics used for the prevention of AAD have not been associated with any increased risks (290). However, the report also recommended that previous studies have not published the presence or absence of AEs and attention should be paid to different effects of individual strains. In the current study the product was well tolerated although a number of patients complained that they found the taste too sweet. A formal taste test was conducted and concluded no difference was detectable between the active and placebo product.

### *2.5.5 Health economic analysis*

A full health economics analysis will be conducted at the conclusion of the study to establish the cost-benefit ratio of the intervention. If improved outcome and reduced

cost per patient are achieved in the intervention arm, no further cost effectiveness analysis would be required, due to dominance of the intervention characterised by lower costs and better outcomes. The cost of introducing probiotics into routine clinical practice is expected to be low and any increased costs associated with the study product would most likely be off-set by reduced service use such as decreased length of stay.

### *2.5.6 Limitations*

A recent study by Hensgens *et al.* demonstrated that patients remained at risk from CDI for up-to three months after cessation of antibiotic therapy (291). The length of follow-up in the current study was only four weeks and may have been too short to detect all cases of AAD. Stool samples were not collected following patient discharge and therefore the incidence of infectious causes of AAD that includes CDI might have been underestimated in the current study. Collection of stool samples proved challenging even when patients were in-hospital and rectal swabs would have increased the numbers of samples available. Peri-rectal swabs are commonly used in studies performed in North America and have been shown to have comparable sensitivity and specificity to stool specimens for the diagnosis of CDI (292).

The current study did not attempt to investigate the mechanisms by which *L. casei* DN114001 might ameliorate the harmful effects of antibiotics on the host microbiota. Early studies relied heavily on culture-dependent techniques, which as discussed in the introduction, limited the evaluation of the microbiota *in vivo*. However, recent advances in high throughput sequencing technology have used metagenomics to provide a greater understanding of the role of the intestinal microbiota and outlined shifts in composition following antibiotic use (110). Therefore, future probiotic studies should examine serial stool samples using these techniques to evaluate possible temporal changes to the microbiota that may occur in response to probiotics.

## 2.6 Conclusion

The current study has evaluated the role of the probiotic *L. casei* DN114001 in preventing AAD. Recruitment to the study was challenging and the recruitment period was therefore extended by a further 18 months. The strengths of this study are the large sample size and wide geographical area. This probiotic has demonstrated a good safety profile and the observed mortality rate is comparable to other trials involving older patients. Analysis of the blinded data for the first 650 cases revealed patients that developed AAD were older with an increased length of stay. The potential ability of the probiotic to reduce overall length of stay through reduction in AAD is interesting from a health economics view-point and may have important financial implications. Once completed, this study stands to contribute significantly to the existing body of knowledge about the role of probiotics in preventing AAD. Future work should include carrying out a cumulative meta-analysis to assess the contribution of the current trial to the existing body of evidence.

# Chapter 3: The antibody response to TcdB in determining patient susceptibility

## 3.1 Statement of contribution

The indirect ELISA was developed from a protocol supplied by Dr. Amanda Taylor. Laboratory results for the Michigan cohort were collated by Dr. Krishna Rao, University of Michigan, USA. *C. difficile* PCR, culture and stool ELISA were performed by Dr. Mark Wilks at the Royal London Hospital, UK. The *C. difficile* toxins used in all experimental work were kindly supplied by Professor Klaus Aktories, University of Freiburg, Germany.

## 3.2 Introduction

Natural adaptive immunity to *C. difficile* occurs following *C. difficile* colonisation during infancy but may result from exposure to other clostridial species that possess cross-reacting antigens. Over 60% of the adult population possess antibodies to both serum and mucosal antibodies to both toxins in the absence of colonisation or active disease (127)(128). The humoral immune response may be important in patient susceptibility to CDI and a considerable body of evidence now exists to support its role in determining disease outcome.

Early work on *C. difficile* toxins focused on TcdA after animal models demonstrated TcdA, not TcdB, was responsible for intestinal damage and diarrhoea associated with CDI (63)(293). In a prospective study of 271 patients, median levels of IgG antibody against TcdA were significantly higher in asymptomatic patients compared to patients that developed active disease (129). Furthermore, lower antibody levels to TcdA have been associated with prolonged duration of diarrhoea and increased recurrence of CDI (131)(132).

The importance of TcdA in disease pathogenesis has recently been called into question by the demonstration that TcdB is necessary for virulence of *C. difficile* using isogenic mutants in a hamster model of infection (69)(70).This observation is supported by the circulation of *tcdA-/tcdB+* isolates in clinical practice, with *tcdB-* isolates yet to be found as causative agents of human disease (71). This is supported by earlier retrospective studies that correlated higher antibody titres to TcdB with protection from recurrence (134)(133). More recently, low levels of serum antibodies to an

epitope of the receptor binding domain of TcdB were significantly associated with recurrence (144). Evaluation of the antibody response to TcdB in the acute setting will improve our understanding of events that result in recurrence or prolonged complicated disease. Importantly, it would allow identification of patients most likely to benefit from novel immunotherapies such as vaccines and monoclonal antibodies.

In addition to the paradigm–shift in the relative importance of TcdB in disease pathogenesis, the nature of CDI has changed drastically since the previous studies were performed over a decade ago, with the emergence of hypervirulent strains such as ribotype 027 (294). *In vitro* studies have highlighted differences in toxicity and cell line specificity in the epidemic 027 strain when compared to historic strains (85). Therefore, strains may produce toxins that vary in antigenic potential that translates into a variation in the antibody response generated.

## 3.3 Aims

The purpose of this study was to re-evaluate the relationship between antibody responses to *C. difficile* toxins and development of symptomatic CDI among hospitalised patients. Currently no commercial assay exists for the detection of antibodies to *C.difficile* toxins in the blood. Therefore, the first aim was to develop a sensitive ELISA for detection of antibody responses to TcdB and TcdA in order to establish the relative importance of TcdB in determining patient outcome. The functionality of antibodies detected was assessed using a neutralisation assay.

## 3.4 Materials

All chemical reagents were supplied by Sigma (UK) unless otherwise stated.

### *3.4.1 Clostridial toxins*

Recombinant *C. difficile* TcdA and TcdB (strain VIP 10463) were expressed in *Bacillus megaterium* as C-terminal 6xHis-tagged proteins and purified by nickel affinity chromatography as published previously (295)(296). This work was carried out by Dr. Panagiotis Papatheodorou, University of Freiburg, Germany.

### *3.4.2 Tetanus toxin*

Tetanus Toxin was supplied by Sigma (UK).

### *3.4.3 Intravenous immunoglobulins*

Pooled IVIG was selected for standardisation of total Immunoglobulin (Ig) and IgG ELISAs (Vigam Liquid®). Pentaglobin was used for the IgM assay and colostrum for the IgA work.

**Table 3.1. Immunoglobulins used to standardise the ELISAs.**

| **Ig** | | **Composition** |
|---|---|---|
| **IgG** | Viagam Liquid® (Bioproducts Laboratory, UK) | 5% w/v solution. 2g Albumin, 2.4g Sucrose, 0.5g Glycine, 0.3g Sodium Acetate, 0.05g Sodium n-Octanoate. Total protein concentration 7g/L in 100mL |
| **IgM** | Pentaglobin (Biotest, UK) | Human plasma protein 50mg: IgM 6mg, IgA 6mg, IgG 38mg. Glucose monohydrate (27.5mg) and sodium chloride (78µmol) |
| **IgA** | Colostrum (Bioscience, UK) | 0.5mg pooled human colostrum, reconstituted with 150mM sodium chloride |

### *3.4.4 Buffers and solutions*

**Table 3.2. Buffers and Solutions.**

| Buffer and solutions | Composition |
|---|---|
| **Coomassie Blue Stain** | 50% v/v Ethanol (Merck), 7.5% v/v Glacial acetic acid, 0.06% w/v, Coomassie Brilliant Blue R250 |
| **Coomassie Destain** | 25% v/v Ethanol, 8% v/v Glacial acetic acid |
| **PBS** | 140mM NaCl, 2.7 mMKCl, 10 mM Na2HPO4, 1.8 Mm KH2PO4 |
| **SDS-PAGE Loading Buffer (2x)** | 125mM Tris-HClPh 6.8, 4.1% w/v SDS, 0.2% w/v Bromophenol blue, 20% v/v Glycerol, Dithiothreitol |
| **SDS Running Buffer** | 25mM Tris-HCl, 250mM Glycine, 0.1% w/v SDS |
| **Transfer Buffer** | 25mM Tris-HCl, 190mM Glycine, 0.05% w/v SDS, 20% Methanol |
| **2.5% Buffer** | PBS, 2.5% w/v Skimmed Milk powder |
| **5 % Buffer** | PBS, 5% w/v Skimmed Milk powder |
| **Developing Solution** | 50mM Citric acid, 100mM Na2HCO4 0.012% v/v H202, 0.5mg/m L o-phenylenediamine dihydrochloride |
| **PBS-T** | PBS, 0.05% v/v Tween 20 |
| **Blocking Buffer** | PBS, 0.05% v/v Tween 20 0.5% w/v 2% bovine serum albumin in PBS |
| **Stop Solution** | 10% Hydrochloric acid |

### *3.4.5 Cell culture reagents*

All cell culture reagents were supplied as part of a commercial kit (Diagnostic Hybrids, UK).

**Table 3.3. Cell culture reagents.**

| Reagent | Composition |
|---|---|
| **Toxin** | *C. difficile* toxin (TcdA+TcdB), 2% Fetal Bovine Serum, Gentamicin Sulphate (10µg/mL), Streptomycin Sulphate (50µg/m L) |
| **Antitoxin** | Purified antisera, 2% Fetal bovine serum, gentamicin sulphate (10µg/mL), Streptomycin sulphate (50µg/mL) |
| **Re-feed Medium** | EMEM, HEPES with FBS, Gentamicin, Penicillin/Streptomycin and Amphotericin B |
| **Dilutent** | PBS consisting of 8.5g/L NaCl, 1.14g/L Sodium Phosphate Dibasic, 0.015g/L Sodium Phosphate Monobasic, Phenol Red, Gentamicin Sulphate (10µg/mL), Amphotericin B (4µg/mL) |

### *3.4.6 Antibodies*

**Table 3.4. Antibodies used in ELISA and western blot.**

| Description | Isotype | Company |
|---|---|---|
| **Mouse monoclonal anti-*C. difficile* TcdB** | Mouse IgG1 | Serotec (UK) |
| **Goat anti-mouse IgG Peroxidase Conjugate** | Mouse IgG (whole molecule) | Sigma (UK) |
| **Goat antihuman IgG/A/M: HRP** | IgG | Serotec (UK) |
| **Goat antihuman IgG:HRP** | Human IgG-Fc | Bethyl Laboratories Inc. (UK) |
| **Goat antihuman IgM:HRP** | Human IgG-Fc | Bethyl Laboratories Inc. (UK) |
| **Goat antihuman IgA:HRP** | Human IgG-Fc | Bethyl Laboratories Inc. (UK) |

## 3.5 Methods

### *3.5.1 Patient recruitment*

#### *3.5.1.1 Brighton patients*

The Royal Sussex County Hospital, Brighton is an 800-bed acute general hospital on the south coast of England. Case patients were recruited from inpatients that had a confirmed positive *C. difficle* stool EIA (Premier TcdA and TcdB ELISA kit, Meridian Bioscience, USA) and had passed more than two liquid stools in the 24 hour period before assessment. Control subjects were diarrhoea-free inpatients in receipt of antibiotics and were confirmed negative for *C. difficile* colonisation by culture, PCR and stool ELISA. Written consent was required for participation in the study.

#### *3.5.1.2 Michigan Patients*

The University of Michigan Hospital is a 930-bed, tertiary care hospital in Ann Arbor, Michigan, USA. Cases and controls were identified from hospitalised adults who were tested for CDI at the discretion of their treating physician. Acute cases that tested positive for *C. difficile* toxins were confirmed by culture and PCR with no previous history of CDI. An equal number of age (plus or minus five years) and gender matched controls with *C. difficile* toxin negative diarrhoea were selected and confirmed as *C. difficile* negative by culture. Serum was collected from a cohort of patients presenting to hospital with a recurrence of CDI and from a cohort who subsequently developed recurrence. This work was carried out as part of the Enteric Research Integrative Network (ERIN) project. All serum samples were sent to Brighton and processed during the same period of time and under the same laboratory conditions as the Brighton samples.

### *3.5.2 Ethics approval*

Ethics approval was provided by the South East Ethics Committee (reference 09/H1102/63) and the University of Michigan Institutional Review Board (approval number HUM00033286).

### *3.5.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis*

Pre-cast 5% Tris-HCl gels (Biorad, USA) were inserted into a Biorad Mini Transfer Cell. Samples of TcdB were mixed with an equal volume of 2x sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and

incubated at 95°C for three minutes. A HiMark™ Pre-stained High Molecular Weight Protein Ladder (Invitrogen, UK) was included as a control. The samples (10µL) were loaded onto the precast gel and run at 20mV/gel in SDS-PAGE running buffer for 90 minutes.

### *3.5.4 Coomassie Blue staining*

Following SDS-PAGE, proteins were stained with Coomassie Blue stain for one hour. The gel was then rinsed in deionised water before using Coomassie destain. Excess dye was removed using tissue paper.

### *3.5.5 Western blot*

Proteins were separated by 5% SDS-PAGE and transferred to Amersham Hybond™-polyvinylidene difluoride (PVDF) transfer membrane (GE Healthcare, USA) using the Biorad™ Mini Trans Blot Wet transfer unit. The PVDF transfer membrane was activated by pre-soaking in 100% methanol for 15 seconds before soaking in transfer buffer for a further 20 minutes. The transfer membrane was placed on filter paper before placing the SDS-PAGE gel on top. A second piece of filter paper was placed on top and the HiMark™ was used as a protein transfer control. The transfer was performed at 100V/gel for one hour. The membrane was washed three times with PBS-Tween 20 (PBS-T) for five minutes before being blocked with 5% buffer for one hour at room temperature (RT) with constant shaking. The membrane was washed three times with PBS-T for five minutes before incubation with 1:200 mouse monoclonal anti-*C.difficile* TcdB antibody in 2.5% buffer and left at RT overnight on a rocking platform. The membrane was washed three times with PBS-T for five minutes before incubation with 1:10,000 goat anti-mouse IgG Peroxidase Conjugate antibody in 2.5% buffer for one hour at RT. Protein-antibody complexes were visualised by chemiluminescence using the Amersham ECL™ Western Blotting Analysis System (GE Healthcare, USA) and Konica Medical Film Processor SRX101A (Konica Minolta, USA).

### *3.5.6 Immunoblot*

A 3, 3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, USA) was applied to the transfer membrane after the western blot had been developed. On addition to the antibody-horse radish peroxidase (HRP) complex DAB forms a brown precipitate at the antigen location.

### *3.5.7 ELISA*

Maxisorb Immuno-assay ELISA plates were coated with 50µL of TcdA or TcdB (1µg/mL) and incubated at 4°C overnight. The plates were washed with PBS-T five times. Wells were blocked with 200µL blocking buffer for one hour at RT. The plates were washed again five times in PBS-T. Serial dilutions of serum (1:25 to 1:1,600) in blocking buffer (50µL) were added to each well in duplicate and incubated at RT for one hour. Washing was repeated as above. Detection of bound antibodies was achieved by incubation of wells with 50µL of goat-antihuman immunoglobulin HRP conjugate diluted 1:10,000 in blocking buffer at RT for one hour. After a final washing step, the protein: antibody complexes were detected by incubating plates with 50µL of developing solution in the dark for 20 minutes. The reaction was stopped by the addition of 50µL of stop solution to each well and the absorbance was read at 490 nm using mQuant Plate Reader (BioTek, USA). The negative controls included were PBS only, TcdB and no serum and TcdB and secondary antibody only. Pooled IVIG was chosen as a positive control and serial dilutions were used to establish a standard curve. The background signal for uncoated plates was subtracted for each sample. Two different methods were evaluated for expression of a positive antibody response.

#### *3.5.7.1 Optimisation of ELISA*

Serum from healthy adults was tested for total antibodies to TcdB at a series of two-fold dilutions (1:100 to 1:102,400) and repeated in triplicate over several days. Plasma and serum from the same individual was tested on different days. The effect of freeze-thawing on serum samples was also assessed.

##### *3.5.7.1.1 Data expression of ELISA Method 1*

Samples were tested at a dilution of 1:800 and compared to IVIG at the same dilution. An absorbance ratio of serum to IVIG was calculated and a positive sample was defined as a ratio ≥1.

##### *3.5.7.1.2 Data expression of ELISA Method 2*

Serial dilutions of patient serum (1:25 to 1:1,600) were compared to IVIG. A cut-off value, twice the background of the immunoglobulin standard curve, was chosen and

included on each plate. A positive test sample was a signal greater than 1:1,600 IVIG for TcdB and 1:3,200 IVIG for TcdA. Pentaglobin at a dilution of 1:800 was used to standardise the IgM ELISA and colostrum at a dilution of 1:1,600 for the IgA ELISA. For all analyses, samples negative at the detection limit of the assay (1:25) were assigned an arbitrary value of 1:12.5.

To test the specificity of differences in antibody responses to *C. difficile* toxins between cases and controls, antibody responses to Tetanus toxin (1µg/mL) were measured by ELISA performed in an identical manner.

### *3.5.8 Neutralisation assay*

#### *3.5.8.1 Optimisation*

To establish the concentration of TcdB for use in the neutralisation assay, serial dilutions (1µg/mL to 10pg/mL) of TcdB (125µL) were added to 125µL of specimen dilutent or 125µL antitoxin. Samples were left at RT for 30 minutes. Human Foreskin Fibroblast (HFF) cells in shell-vials were thawed from -80°C using a heat block at 37°C for four minutes. Maintenance medium (1mL) was removed from each vial and replaced with 800µL of cell culture re-feed medium. Samples (200µL) were added to each vial and incubated at 37°C. Neutralisation was defined as an absence of cell-rounding. An inverted light microscope (Axiovert 25 fluorescent microscope, Zeiss, UK) was used to look for evidence of cell rounding or neutralisation at 16 hours. Controls included were cells alone, toxin alone (TcdA + TcdB supplied by commercial assay), antitoxin alone and antitoxin and toxin. Serum (200µL) and HFF cells were incubated overnight to establish any direct effects on cells.

#### *3.5.8.2 IVIG and clinical samples*

Serial dilutions (1:20 to 1:1,600) of IVIG (125µL) were incubated with HFF cells and TcdB overnight using the method described above. A dilution of 1:20 IVIG was used as a positive control in all further neutralisation experiments instead of the antiserum supplied with the commercial assay kit. Samples (1:20) were incubated with HFF cells and TcdB or HFF cells alone. The negative controls were as described above.

### *3.5.9 Statistical analysis*

All data were analysed using GraphPad Prism™ (Graphpad Software, CA, USA) and SPSS version 20 (IBM®, UK). Continuous variables were compared by Mann

Whitney-U test and categorical variables using Fisher's exact test with a p-value of <0.05 used as a cut-off for statistical significance. All p-values between 0.05 and 0.09 were considered as statistical trends.

## 3.6 Results

### *3.6.1 Confirming the purity of TcdB*

SDS-PAGE was used to check the purity of the TcdB. After loading TcdB (0.9µg/mL) the gel was run at 20mv for 90 minutes before staining with Coomassie Blue.

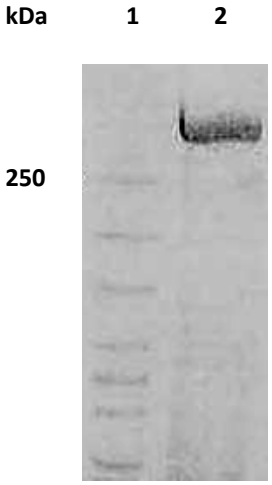



**Figure 3.1. SDS-PAGE and Coomassie Blue Staining. Samples of TcdB (10µL) were loaded onto a precast gel and visualised using Coomassie Blue. Lanes are as follows: (1) HiMark™ High Molecular Weight Protein Ladder (2) TcdB (0.9µg/mL). Image reproduced with the permission of Dr. Paniogiotis Papatheodorou.**

This method was used to establish the purity of TcdB. A band was detected at approximately 270kDa which corresponds to the molecular weight of TcdB.

### *3.6.2 Confirmation of TcdB by western blot*

A mouse monoclonal anti-*C. difficile* TcdB antibody was used to confirm the protein identified by SDS-PAGE was TcdB. A goat anti-mouse IgG Peroxidase Conjugate antibody was used for the detection of the primary antibody. After development of the western blot it was difficult to visualise protein due to non-specific binding. Application of a DAB substrate kit directly to the PVDF membrane allowed bands to be detected at approximately 268kDa, which corresponds to the molecular mass of TdcB (Figure 3.2).

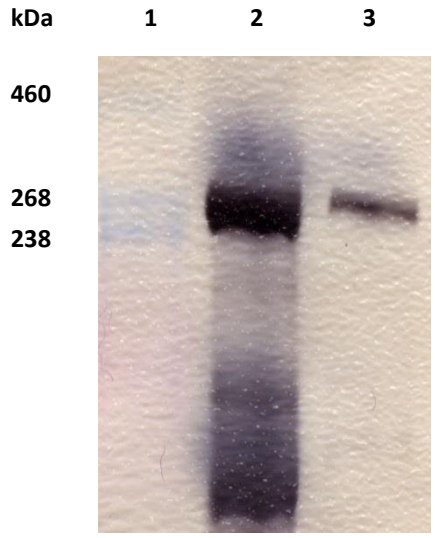



**Figure 3.2. Western blot detection of TcdB. Proteins were separated by 5 % SDS-PAGE and transferred to a PVDF transfer membrane before incubation with 1:200 mouse monoclonal anti-*C. difficile* TcdB antibody. Goat anti-mouse IgG Peroxidase Conjugate was used for detection of the primary antibody. DAB peroxidase kit was applied directly to the membrane following the western blot. Lanes are as follows: (1) HiMark™ High Molecular Weight Protein Ladder (2) 100µg/mL TcdB (3) 10µg/mL TcdB.**

### *3.6.3 Patient recruitment*

Serum samples were available for 20 cases of acute CDI and 18 control patients recruited in Brighton and 20 cases of acute CDI and 20 control patients recruited in Michigan (Table 3.5). Within both cohorts, cases and controls were of similar age and gender mix. Patients recruited to the study from Brighton were older than patients from Michigan, 79.5 years (interquartile range [IQR] 65.8-87.3) vs. 58.5 years (IQR 49-66.5) (p=0.002) and two-thirds of patients were male. Laboratory markers of inflammation and renal function were similar for cases and controls recruited in both Brighton and Michigan although serum albumin was lower in cases than controls in Brighton, 33g/L (IQR 28-37) vs. 39 g/L (IQR 37-41), p=0.004. Two cases recruited in Brighton died and five cases recruited in Michigan were admitted to the intensive care unit. One Michigan case subsequently required a colectomy and one died (p=0.18)

**Table 3.5. Demographic and laboratory data for acute cases of CDI and controls recruited in Brighton and Michigan. p-values are quoted for differences between cases and controls at each location. Medians and inter-quartile ranges (IQR) are shown. All laboratory markers were converted to the units used in Brighton. Normal ranges: white cell count (4-11), C-reactive protein (<5), urea (1.7-8.3), creatinine (62-106 ) and albumin (35-52).**

| | Brighton | | | Michigan | | |
|---|---|---|---|---|---|---|
| | **Cases** | **Controls** | **p-value** | **Cases** | **Controls** | **p-value** |
| **n** | 20 | 18 | - | 20 | 20 | - |
| **Age in years (IQR)** | 85 (65-88) | 77.5 (65.8-81.8) | p=0.39 | 58.5 (50-66) | 61 (51.5-66) | p=0.84 |
| **Male gender (%)** | 11 (55%) | 12 (67%) | p=0.39 | 12 (60%) | 12 (60%) | - |
| **White cell count $10^9$/L (IQR)** | 11 (7-12) | 13 (9-15.5) | p=0.25 | 5 (4-11) | 5 (2.5-11.5) | p=0.78 |
| **C- reactive protein mg/L (IQR)** | 48 (15.6-91.6) | 72.5 (23.3-144.3) | p=0.31 | 60 (50-100) | n/a | n/a |
| **Urea mmol/L (IQR)** | 6 (4-8) | 7 (6-8) | p=0.43 | 6.2 (2.9-12) | 8 (4.5-10.4) | p=0.27 |
| **Creatinine µmol/L (IQR)** | 78 (64-127) | 80 (70.5-104.5) | p=0.92 | 88.4 (61.9-154.7) | 66.3 (57.5-88.4) | p=0.08 |
| **Albumin g/L (IQR)** | 33 (28-37) | 39 (37-41) | **p=0.004*** | 29.5 (27-34.5) | 34 (30-36) | p=0.19 |
| **Complications** | 2 | 0 | p=0.17 | 5 | 3 | p=0.43 |

### *3.6.4 Development of an ELISA for the detection of antibodies to TcdB*

An ELISA was developed for the detection of the antibody response to TcdB in serum. The ELISA was initially performed using serum from healthy adults (Figure 3.3A). ELISA performance was unaffected by sample type (serum or plasma) or storage temperature (at 4°C overnight vs. immediately freeze-thawed) (Figure 3.3B and C). Two different methods were compared for antibody detection. The first method calculated an absorbance ratio of serum antibodies to IVIG at a dilution of 1:800, with a ratio of ≥1 classed as positive. Using this method only two clinical samples, case four and case nine, had detectable antibodies.

By contrast, no control samples had detectable antibodies (Table 3.6). The second method tested each sample over a wider range of dilutions (1:25 to 1:1,600) and compared absorbance values to a cut-off value of 1:1,600 for TcdB and 1:3,200 for TcdA that corresponded to a value twice the background of the IVIG standard curves (Figure 3.4). IVIG at the above dilutions was included as an internal control on each ELISA plate.

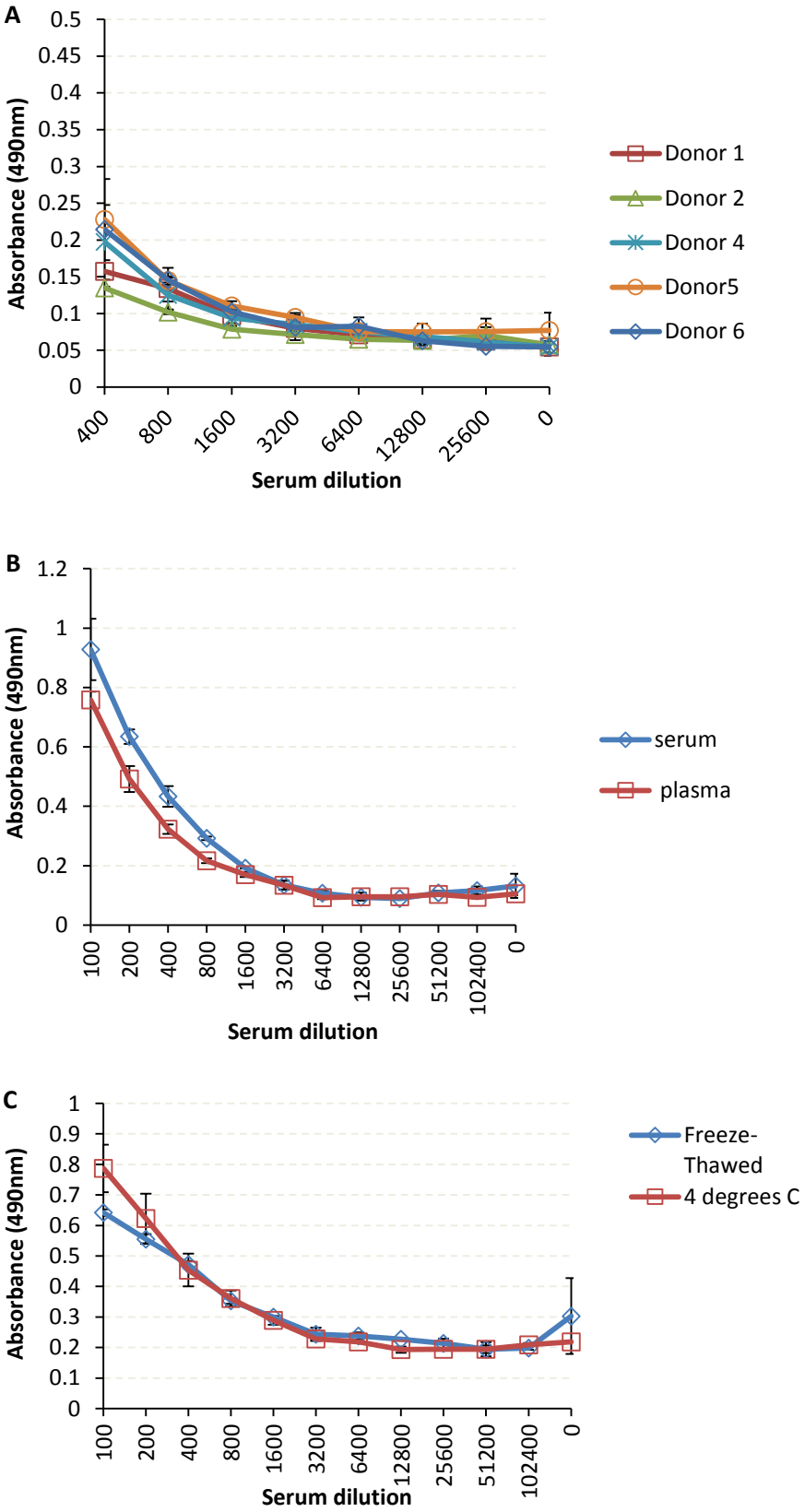



**Figure 3.3. Optimisation of ELISA. A. Serum from healthy adults B. Serum and plasma from the same individual C. Effect of freeze-thawing on antibody detection. Error bars are shown for standard deviation from the mean.**

**Table 3.6. Absorbance ratio of IVIG and serum at 1:800. Serum samples were tested at a dilution of 1:800 and compared to IVIG at the same dilution. A ratio ≥1 was classed as positive.**

| Cases | Absorbance | Ratio | Control | Absorbance | Ratio |
|---|---|---|---|---|---|
| **1** | 0.08 | 0.12 | 1 | 0.05 | 0.05 |
| **2** | 0.05 | 0.07 | 2 | 0.16 | 0.14 |
| **3** | 0.16 | 0.24 | 3 | 0.09 | 0.08 |
| **4** | 1.13 | **1.67** | 4 | 0.26 | 0.24 |
| **5** | 0.06 | 0.09 | 5 | 0.12 | 0.11 |
| **6** | 0.31 | 0.46 | 6 | 0.13 | 0.12 |
| **7** | 0.03 | 0.05 | 7 | 0.14 | 0.12 |
| **8** | 0.15 | 0.22 | 8 | 0.83 | 0.75 |
| **9** | 1.13 | **1.68** | 9 | 0.16 | 0.14 |
| **10** | 0.05 | 0.07 | 10 | 0.05 | 0.04 |
| **11** | 0.07 | 0.10 | 11 | 0.07 | 0.07 |
| **12** | 0.05 | 0.07 | 12 | 0.11 | 0.10 |
| **13** | 0.09 | 0.14 | 13 | 0.07 | 0.06 |
| **14** | 0.03 | 0.05 | 14 | 0.13 | 0.11 |
| **15** | 0.01 | 0.01 | 15 | 0.76 | 0.69 |
| **16** | 0.18 | 0.27 | 16 | 0.09 | 0.08 |
| **17** | 0.12 | 0.18 | 17 | 0.11 | 0.10 |
| **18** | 0.03 | 0.04 | 18 | 0.36 | 0.32 |
| **19** | 0.03 | 0.05 | | | |
| **20** | 0.07 | 0.10 | | | |

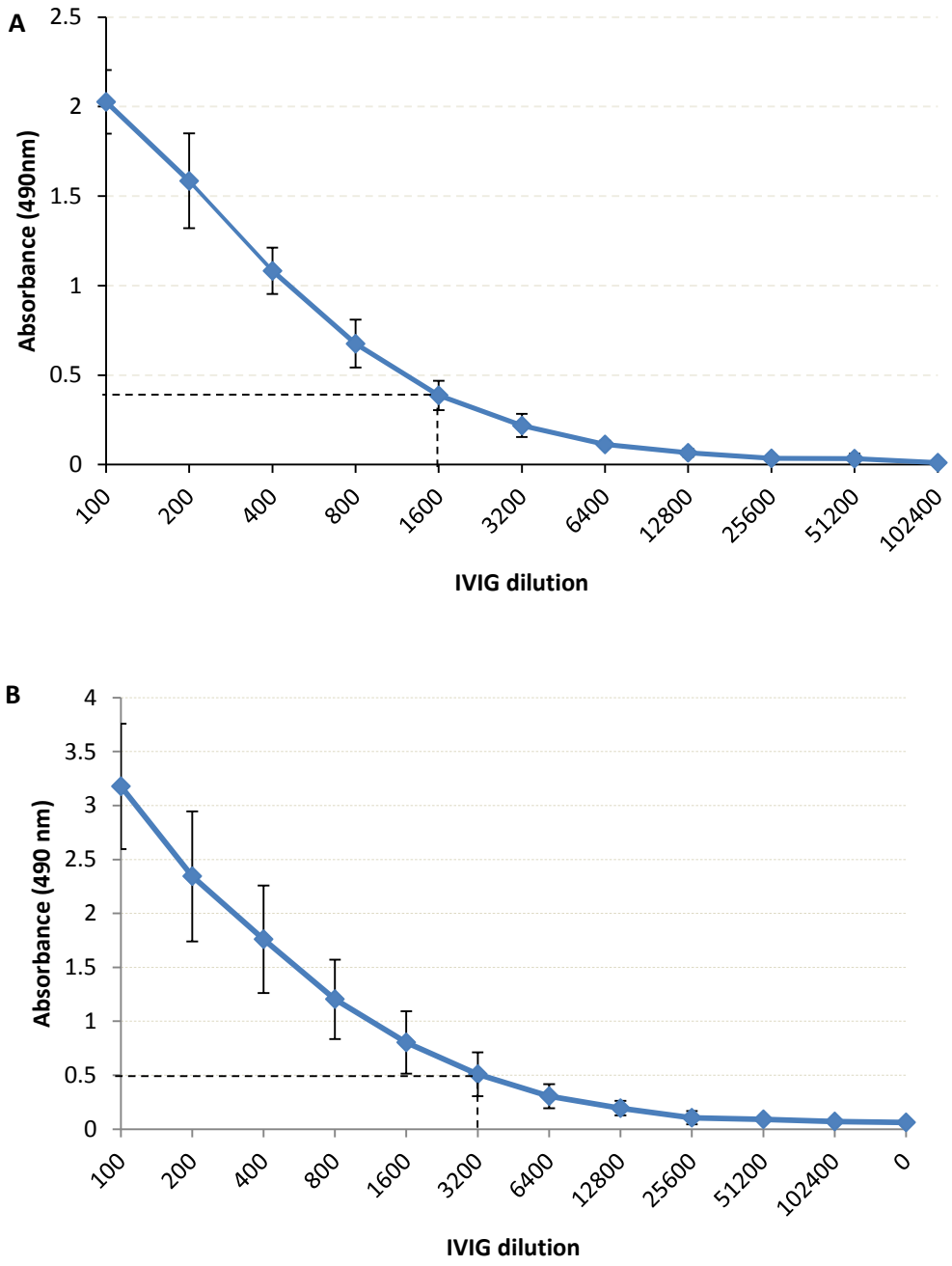



**Figure 3.4. Standard curve for IVIG. Serial dilutions of IVIG were tested in duplicate on three separate days and averaged to produce a standard curve. Error bars shown correspond to the standard deviation. A. TcdB. A dilution of 1:1,600 was chosen as a cut-off value (twice the background). B. TcdA. A dilution of 1:3,200 (twice the background) was chosen as a cut-off value.**

### *3.6.5 Serum antibody responses to TcdB*

In patients recruited in Brighton (Figure 3.5A and B), median total Ig titre against TcdA was similar among case and control patients (1:100, 95% CI 1:12.5-1:200 vs. control 1:50, 95% CI 1:12.5-1:200) (Table 3.7). In contrast, median antibody titres against TcdB were three-fold lower in cases than controls (1:18.75, 95% CI 1:12.5-1:100 vs. 1:200, 95% CI 1:50-1:200). Fewer cases than controls had an antibody titre above the detection limit of the assay; 10/20 cases (50%) vs. 15/18 controls (83%) (p=0.043).

Among patients recruited in Michigan (Figure 3.5C and D) the same trend occurred with only 12/20 cases (60%) vs. 18/20 (90%) controls having antibody titres against TcdB above the detection limit of the assay, although this was not statistically significant (p=0.065). Antibody levels to TcdB but not TcdA were lower in cases than controls (1:150, 95% CI 1:12.5-1:400 vs. 1:200, 95% CI 1:25-1:800) (Table 3.8). This difference was less marked than in the Brighton samples. Overall, Brighton cases had a lower titre of antibodies to TcdB than the Michigan cases (1:18.75, 95% CI 1:12.5-1:100 vs. 1:150, 95% CI 1:12.5-1:400).

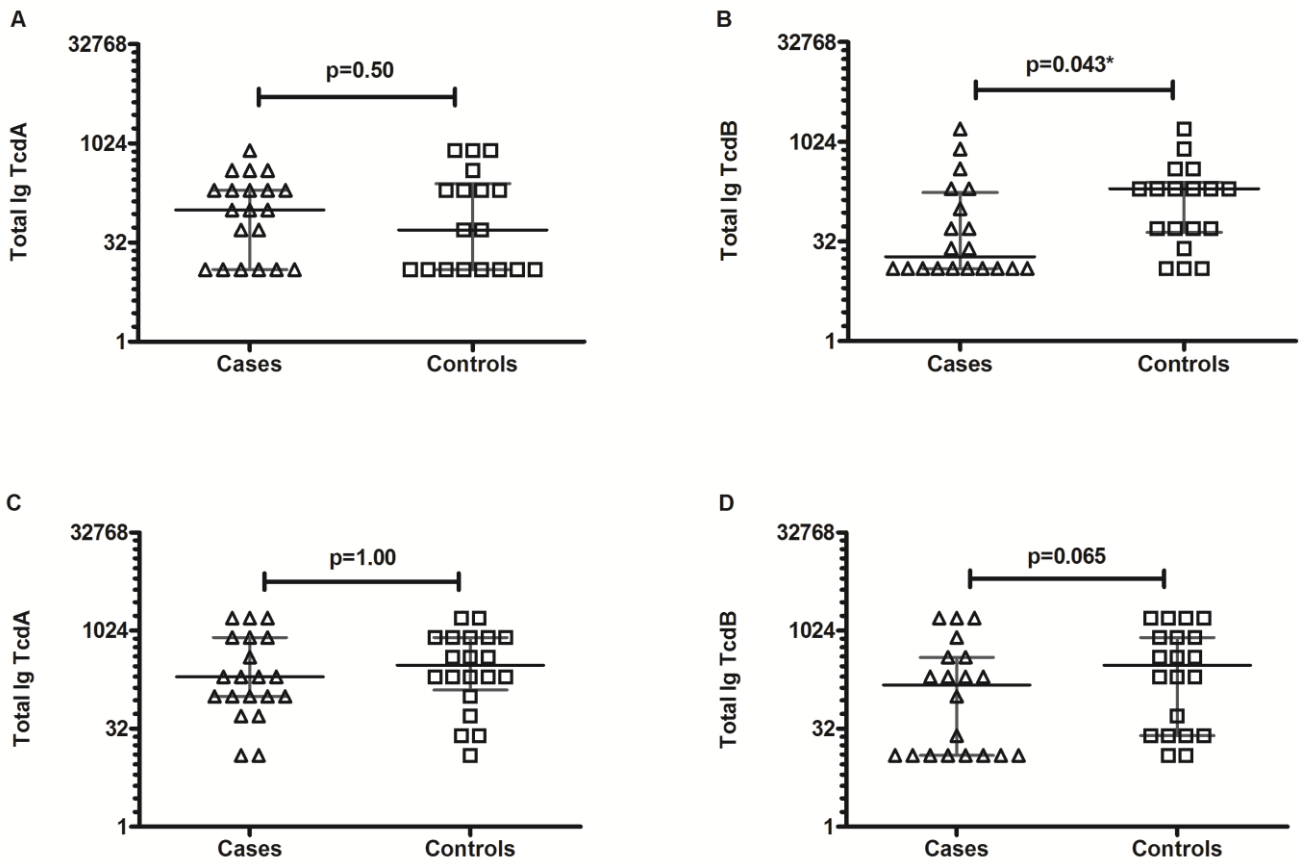



**Figure 3.5. Total Ig to TcdA and TcdB. Titres are expressed as dilutions using a logarithmic 2 scale along the y axis. All p-values were obtained using Fisher's exact test. Median titres are shown as black horizontal lines and error bars are shown in grey. A and B. Total antibody titres for cases and controls recruited in Brighton. C and D. Total antibody titres for cases and controls recruited in Michigan.**

**Table 3.7. Total Ig titres to TcdA and TcdB in Brighton cohort. Serum from 20 acute CDI cases and 18 age-matched controls was tested. The median antibody titre and interquartile range (IQR) for each group is shown at the bottom of the table. Antibodies below the level of detection of the assay were assigned a value of 1:12.5 and are shaded in grey.**

| | **Brighton** | | | |
|---|---|---|---|---|
| **Toxin** | **TcdA** | | **TcdB** | |
| | **Case** | **Control** | **Case** | **Control** |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:25 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:50 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:50 |
| | 1:50 | 1:12.5 | 1:12.5 | 1:50 |
| | 1:50 | 1:12.5 | 1:12.5 | 1:50 |
| | 1:100 | 1:50 | 1:12.5 | 1:200 |
| | 1:100 | 1:50 | 1:12.5 | 1:200 |
| | 1:100 | 1:200 | 1:12.5 | 1:200 |
| | 1:200 | 1:200 | 1:25 | 1:200 |
| | 1:200 | 1:200 | 1:25 | 1:200 |
| | 1:200 | 1:200 | 1:50 | 1:200 |
| | 1:200 | 1:400 | 1:50 | 1:400 |
| | 1:200 | 1:800 | 1:100 | 1:400 |
| | 1:400 | 1:800 | 1:200 | 1:800 |
| | 1:400 | 1:800 | 1:200 | 1:1,600 |
| | 1:400 | | 1:800 | |
| | 1:800 | | 1:1,600 | |
| **Median titre (IQR)** | **1:100 (1:12.5-1:200)** | **1:50 (1:12.5-1:200)** | **1:18.75 (1:12.5-1:150)** | **1:200 (1:50-1:200)** |

**Table 3.8. Total Ig titres to TcdA and TcdB in Michigan cohort. Serum from 20 acute CDI cases and 20 age-matched controls was tested. The median antibody titre and interquartile range (IQR) for each group is shown at the bottom of the table. Antibodies below the level of detection of the assay were assigned a value of 1:12.5 and are shaded in grey.**

| | **Michigan** | | | |
|---|---|---|---|---|
| **Toxin** | **TcdA** | | **TcdB** | |
| | **Case** | **Control** | **Case** | **Control** |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:25 | 1:12.5 | 1:12.5 |
| | 1:50 | 1:25 | 1:12.5 | 1:25 |
| | 1:50 | 1:50 | 1:12.5 | 1:25 |
| | 1:100 | 1:100 | 1:12.5 | 1:25 |
| | 1:100 | 1:200 | 1:12.5 | 1:25 |
| | 1:100 | 1:200 | 1:12.5 | 1:50 |
| | 1:100 | 1:200 | 1:12.5 | 1:50 |
| | 1:100 | 1:200 | 1:25 | 1:200 |
| | 1:200 | 1:200 | 1:100 | 1:200 |
| | 1:200 | 1:400 | 1:200 | 1:200 |
| | 1:200 | 1:400 | 1:200 | 1:400 |
| | 1:200 | 1:400 | 1:200 | 1:400 |
| | 1:400 | 1:800 | 1:200 | 1:400 |
| | 1:800 | 1:800 | 1:400 | 1:800 |
| | 1:800 | 1:800 | 1:400 | 1:800 |
| | 1:800 | 1:800 | 1:800 | 1:800 |
| | 1:1,600 | 1:800 | 1:1,600 | 1:1,600 |
| | 1:1,600 | 1:1,600 | 1:1,600 | 1:1,600 |
| | 1:1,600 | 1:1,600 | 1:1,600 | 1:1,600 |
| **Median titre (IQR)** | **1:200 (1:100-1:800)** | **1:300 (1:150-1:800)** | **1:150 (1:12.5-1:400)** | **1:200 (1:25-1:800)** |

### *3.6.6 Confirming the specificity of the antibody response to TcdB*

To confirm the specificity of the antibody response to *C.difficile* toxins, the ELISA was repeated using plates coated with Tetanus Toxin. Total Ig to Tetanus Toxin was similar in cases and controls (1:12.5 95% CI 1:12.5-1:25 vs. 1:12.5 95% CI 1:12.5-1:12.5) (Figure 3.6).

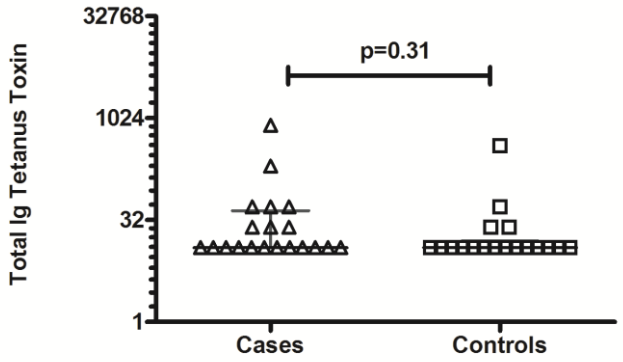



**Figure 3.6. Total Ig to Tetanus Toxin. Titres are expressed as dilutions using a logarithmic 2 scale along the y axis. Median titres are shown as black horizontal lines and error bars are shown in grey.**

### *3.6.7 Antibody-class specific response*

To identify which antibody class was predominantly responsible for the antibody response, samples from patients recruited in Brighton were tested for antibody-class specific responses to TcdB. Pentaglobin (1:800) was used to standardise the IgM assay, pooled human colostrum (1:1,600) for the IgA assay and IVIG (1:1,600) for the IgG assay (Figure 3.7). Significantly fewer cases had detectable IgA antibodies to TcdB compared to controls (7/20 cases [35%] vs. 13/18 controls [72%], p=0.028) (Figure 3.8). Antibody titres to IgG and IgM were similar in cases and controls and were only detected in a minority of samples (Table 3.9).

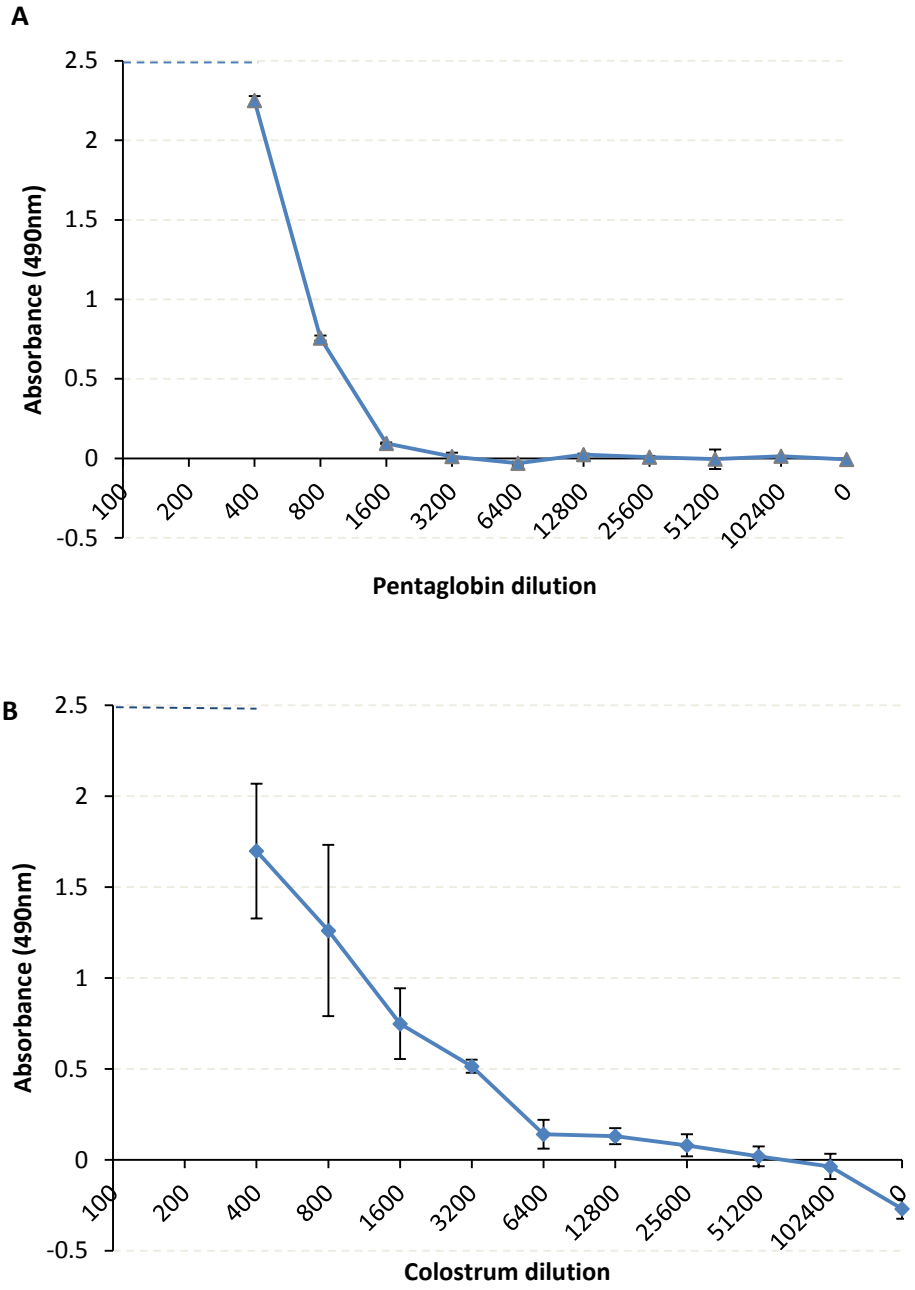



**Figure 3.7. Standard curve for pentaglobin and colostrum. Serial dilutions of pentaglobin and colostrum were tested in duplicate on three separate days and averaged to produce a standard curve. Error bars shown correspond to the standard deviation.A. Pentaglobin. A dilution of 1:800 (twice the background) was chosen as a cut-off value. B. Colostrum. A dilution of 1:1,600 (twice the background) was chosen as a cut-off value. The dashed line at dilutions of 1:100-1:200 corresponds to an absorbance too high to be read by the microplate reader.**

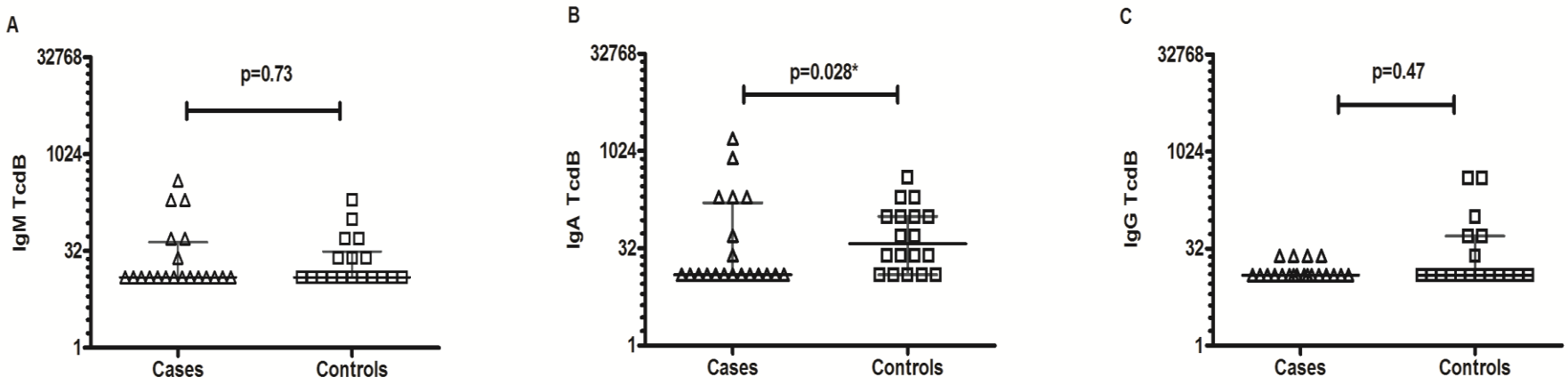



**Figure 3.8. Class-specific antibody response to TcdB. Serum samples from 20 acute CDI cases and 18 age-matched controls recruited in Brighton were tested. Titres are expressed as dilutions using a logarithmic 2 scale along the y axis. Median titres are shown as black horizontal lines and error bars are shown in grey. A. IgM response, B. IgA response C. IgG response. Pentaglobin (1:800) was used to standardise the IgM assay, colostrum (1:1,600) the IgA assay and IVIG (1:1,600) the IgG assay.**

**Table 3.9. Class-specific antibody titres to TcdB. IgM, IgA and IgG titres to TcdB were measured in the Brighton cohort. The median antibody titre and interquartile range (IQR) is shown at the bottom of the table. Antibodies below the level of detection of the assay were assigned a value of 1:12.5 and are shaded in grey.**

| | IgM | | IgA | | IgG | |
|---|---|---|---|---|---|---|
| | **Cases** | **Controls** | **Cases** | **Controls** | **Cases** | **Controls** |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:25 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:25 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:25 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:25 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:25 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:12.5 | 1:12.5 | 1:25 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:25 | 1:12.5 | 1:50 | 1:12.5 | 1:12.5 |
| | 1:12.5 | 1:25 | 1:12.5 | 1:50 | 1:12.5 | 1:25 |
| | 1:12.5 | 1:25 | 1:12.5 | 1:100 | 1:12.5 | 1:50 |
| | 1:25 | 1:50 | 1:25 | 1:100 | 1:12.5 | 1:50 |
| | 1:50 | 1:50 | 1:50 | 1:200 | 1:12.5 | 1:100 |
| | 1:50 | 1:100 | 1:200 | 1:200 | 1:25 | 1:400 |
| | 1:200 | 1:200 | 1:200 | 1:400 | 1:25 | 1:400 |
| | 1:200 | | 1:200 | | 1:25 | |
| | 1:400 | | 1:800 | | 1:25 | |
| **Median (IQR)** | **1:12.5 (1:12.5-1:37.5)** | **1:12.5 (1:12.5-1:25)** | **1:12.5 (1:12.5-1:125)** | **1:37.5 (1:12.5-1:100)** | **1:12.5 (1:12.5-1:12.5)** | **1:12.5 (1:12.5-1:50)** |

### *3.6.8 Antibody response to TcdB and recurrence of CDI*

To evaluate the role of the antibody response to TcdB in recurrence of CDI, two separate groups of patients and age-matched controls were recruited in Michigan. The first group included nine cases that were admitted to hospital with a recurrence of CDI. A third of this group were male and the median age was 58.5 years (IQR 50.8-64.3). The second group included 16 cases that subsequently developed a recurrence of CDI. This group were older with a median age of 66.5 years (IQR 58.3-72.8) and a quarter were male.

In patients presenting with a recurrence of CDI, total Ig response to TcdB was similar between cases and controls (1:100, 95% CI 1:12.5-1:800 vs 1:50, 95% CI 1:12.5-1:200). Median total Ig titres to TcdA were four-fold lower in cases than controls (1:100 95% CI 1:12.5-1:400 vs. 1:800 95% CI 1:400-1:1,600). The difference seen was not statistically significant (p=0.21) (Figure 3.9A and B). In patients that subsequently developed a recurrence of CDI, antibodies to both TcdA and TcdB were similar in cases and controls (Figure 3.9C and D).

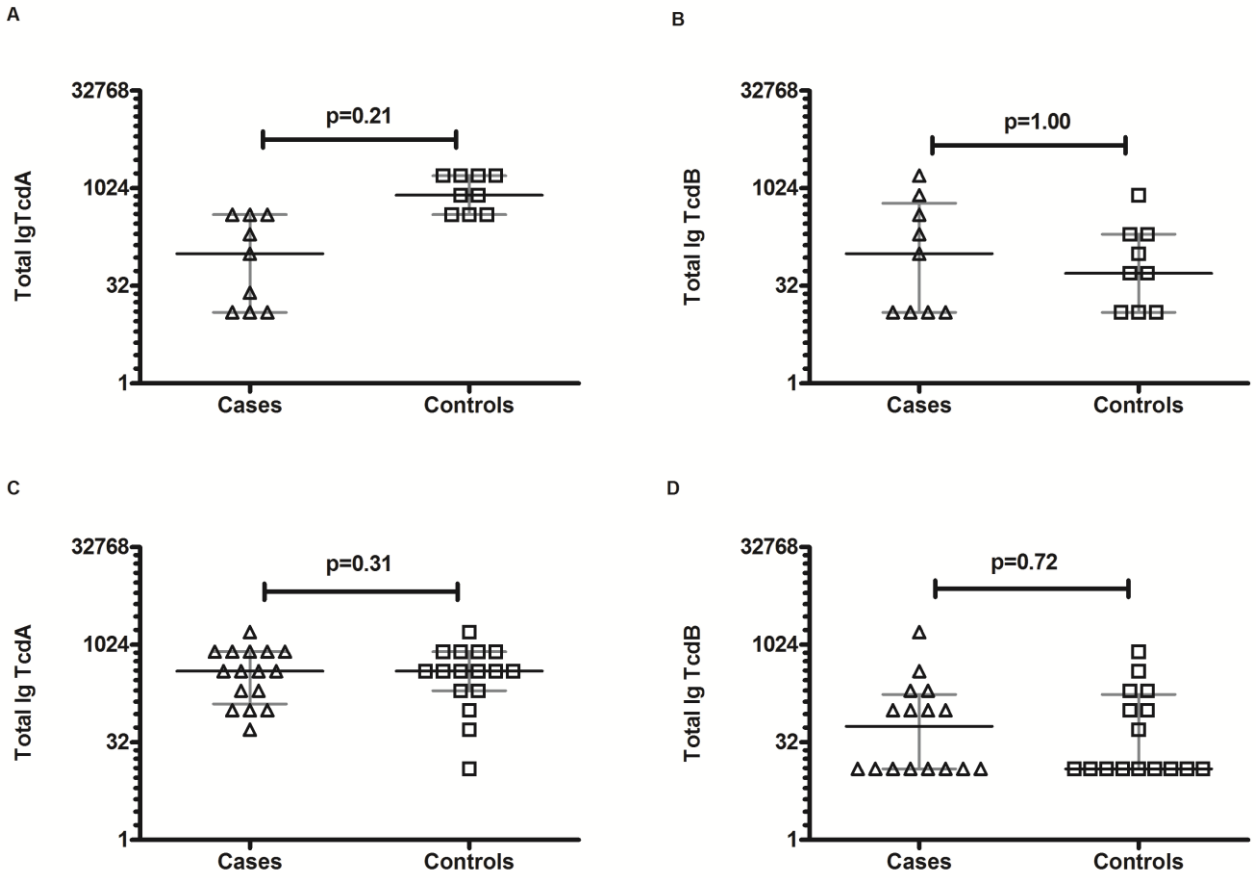


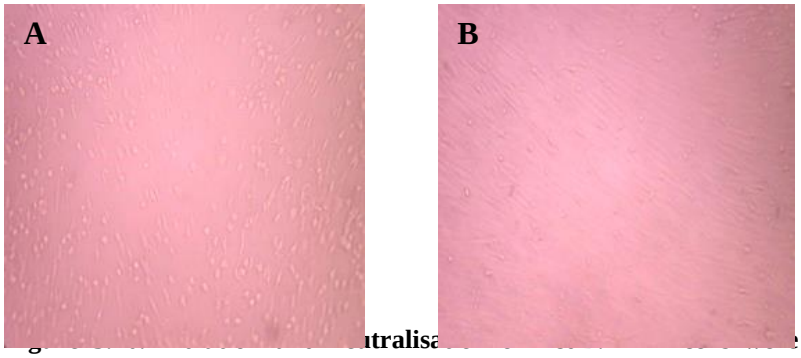

**Figure 3.9. Total Ig response to TcdA and TcdB in recurrence of CDI. Serum samples from two separate groups of patients recruited in Michigan were used to measure antibody responses to TcdA and TcdB in CDI recurrence. Titres are expressed as dilutions using a logarithmic 2 scale along the y axis. Median titres are shown as black horizontal lines and error bars are shown in grey. A and B. Patients that presented with a recurrence of CDI. C and D. Patients that subsequently developed a recurrence of CDI.**

### *3.6.9 Neutralisation assay*

A neutralisation assay was performed to evaluate the functionality of antibodies to TcdB detected by ELISA. TcdB causes disruption to the actin cytoskeleton resulting in cell rounding and this characteristic is a well-recognised sign of cytotoxicity. The ability of patient antibodies to neutralise this effect was used to determine their specificity.

#### *3.6.9.1 TcdB titration*

To establish the minimum concentration of recombinant TcdB that could be neutralised by the antiserum in the commercial kit, a series of titrations of TcdB were incubated with antiserum. HFF cells were examined at 16 hours using an inverted light microscope. The minimum concentration at which neutralisation occurred was 10pg/mL (Figure 3.10). This concentration was used in all remaining assays.




**tralisa incubated with different dilutions of TcdB alone and in combination with antiserum at 37°C. Cells were examined at 16 hours for evidence of neutralisation of cytotoxicity using an inverted microscope (magnification x32). A. HFF cells and TcdB (10pg/mL). B. HFF cells, TcdB (10pg/mL) and antiserum.**

#### *3.6.9.2 Detection of neutralising antibodies to TcdB in IVIG*

To confirm IVIG contained neutralising antibodies against TcdB, serial dilutions of IVIG (1:20 to 1:1,600) were incubated with HFF cells and TcdB (10pg/mL). Neutralisation only occurred at a dilution of 1:20 (Figure 3.11). Characteristic cell rounding caused by TcdB cytotoxicity took place at all other dilutions.

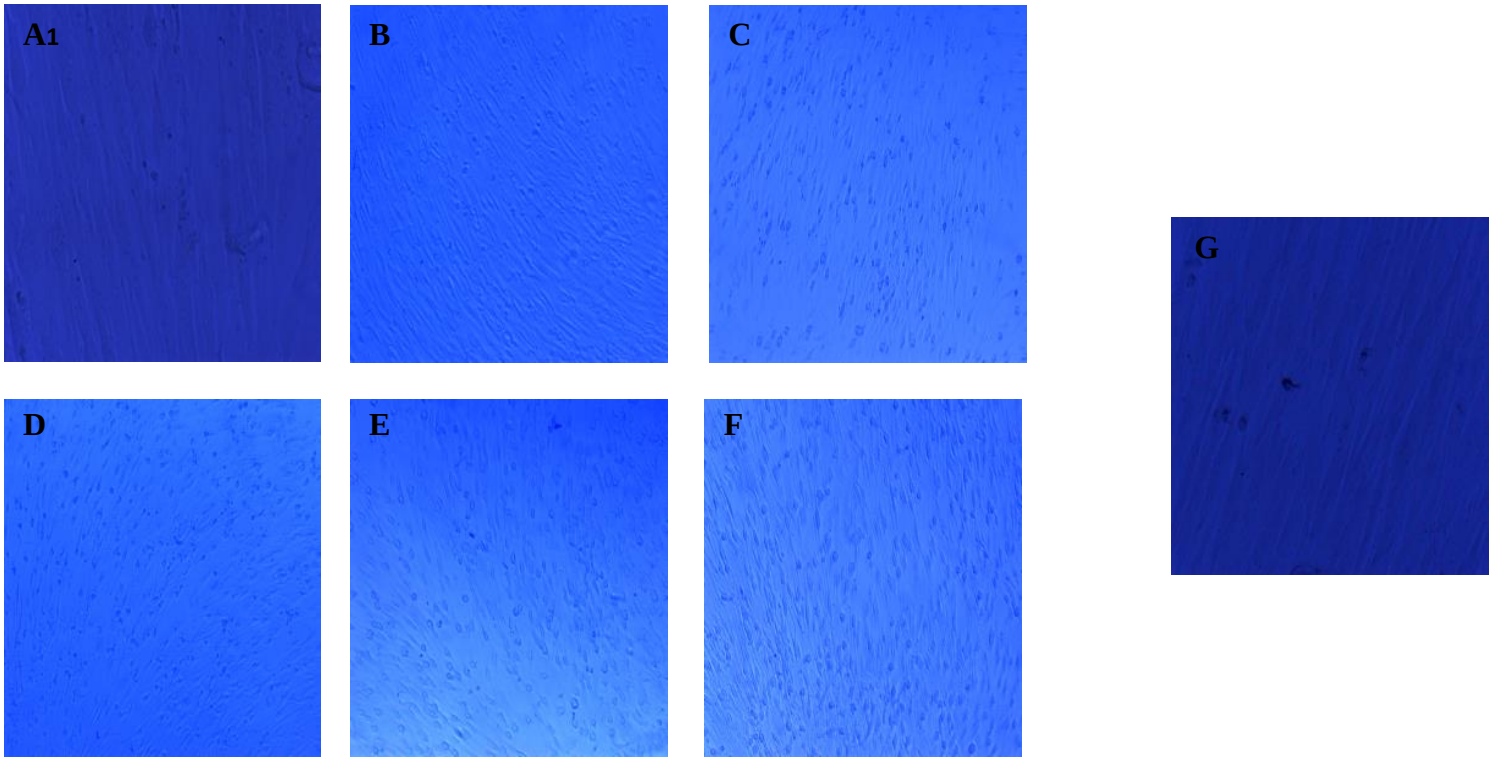



**Figure 3.11. IVIG neutralisation of TcdB. HFF cells were incubated at 37°C with TcdB (10pg/mL) and different dilutions of IVIG (1:20-1:800). A. 1:20 IVIG B. 1:50 IVIG C. 1:100 IVIG D. 1:200 IVIG E. 1:400 IVIG F. 1:800 IVIG G. Positive control (TcdB + commercial assay antitoxin).**

### *3.6.9.3 Detection of neutralising antibodies to TcdB in patient serum*

To establish if the antibodies to TcdB detected by ELISA were capable of neutralising TcdB, serum from the 20 acute CDI cases and 18 controls recruited in Brighton were tested. Samples at a dilution of 1:20 were incubated with TcdB (10pg/mL) and HFF cells at 37°C. IVIG (1:20) was included as a positive control. A positive result was indicated by the maintenance of a linear appearance of HFF cells, a borderline result was a mixture of linear and rounded cells and a negative result was shown by characteristic cell rounding that indicated TcdB cytotoxicity and loss of cellular structure. Only two cases, case three and case nine, neutralised TcdB cytotoxicity (Figure 3.12A). The remaining cases and controls were either borderline or negative and examples of each are shown below (Figure 3.12B and C).

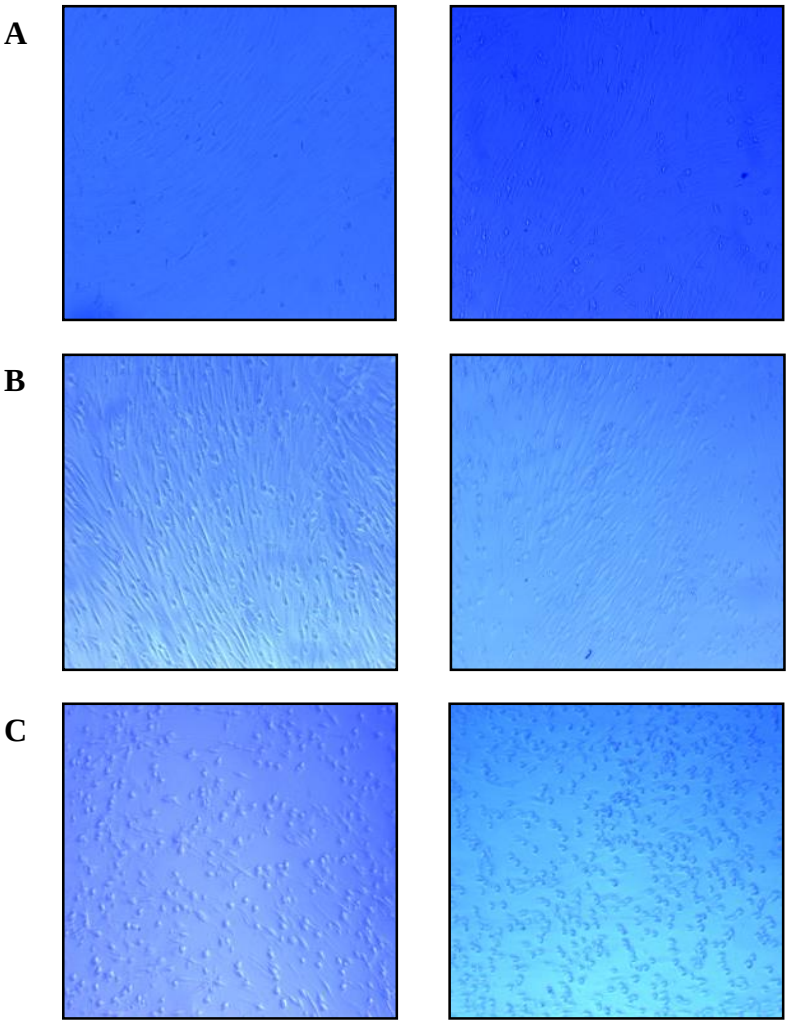



**Figure 3.12. Neutralisation of TcdB in patient serum. Serum from patients recruited in Brighton was diluted to 1:20 and incubated with HFF cells and TcdB (10pg/mL). IVIG (1:20) was included with each batch as a positive control. Negative controls were HFF cells and TcdB alone. A. Positive result (cases three and nine) B. Borderline result (case one and control 19) C. Negative result (case 20 and control 18).**

## 3.7 Discussion

The majority of healthy adults have detectable antibodies to *C. difficile* TcdA and TcdB in their serum that are thought to arise from colonisation in infancy or cross infection with other strains (127)(134) . The detection of antibodies to both toxins in the serum of healthy adults forms the rationale for the use of IVIG to treat refractory disease (141). The humoral immune response has been implicated in determining disease outcome in CDI and studies have demonstrated protection against recurrence is associated with antibody responses to TcdA, TcdB and several non-toxin antigens (Cwp66, Cwp84, FliC, FliD and the surface layer proteins) (132)(135)(144).

Previous work addressing the role of the humoral immune response in susceptibility to *C. difficile* has focused on responses to TcdA since in early animal models TcdA was required for infection, with TcdB unable to cause disease alone (63). However, few studies have assessed the contribution and exact role of the antibody response to TcdA and TcdB since the emergence of epidemic 027/NAP1/BI strains of *C. difficile*. Furthermore, recent studies using isogenic *C. difficile* mutants have demonstrated that TcdB is required for full virulence, supported by a lack of *tcdB*- strains in the clinical setting (69)(135).

During the past decade, stool assays have routinely been used as a diagnostic tool in CDI (297). However, currently no commercially available ELISA exists for the detection of serum antibodies to TcdA or TcdB. Previous studies have used similar ELISA protocols but have differed in their method of data expression (298).

The aim of this study was to reassess the role of serum antibody titres against TcdB in patient susceptibility to acute CDI. This was achieved by the development of a sensitive ELISA that allowed the detection of antibodies to TcdB and TcdA.

Two different methods of data expression were compared. The first involved comparison of absorbance readings at a single dilution (1:800). This resulted in a relative underestimation of lower antibody titres. The alternative method tested each serum sample across a range of dilutions and compared the absorbance to a single dilution of IVIG, identified using a standard curve. This method was previously used by Leav *et al.* to measure antibodies to TcdA and TcdB (144). In the current study, it allowed the detection of antibodies in samples that were determined negative using

the first method. However, the main disadvantage of this method is the increased resources required in terms of time and reagent costs (299).

In contrast to previous studies, this study demonstrated a difference in antibody titres to TcdB, but not TcdA, between acute CDI cases and controls. Fewer cases had detectable antibodies to TcdB than controls and the median antibody titre was also lower in cases. It is important that this trend was seen in two separate cohorts of patients that were recruited from different countries, with slightly different controls. The antibody response measured in this study reflects pre-formed antibody rather than a response to acute infection as serum was obtained within 72 hours of recruitment to the study, which corresponds to the approximate incubation period of CDI (158).

*C. difficile* cases recruited to the study had mild disease as evidenced by the fact that their average C-reactive protein levels, white cell counts and renal function test results were similar to controls. This may reflect a lack of severely ill patients recruited as written consent was required for participation in the study. Although lower titres of antibody to TcdB were found in cases at both locations, this difference was less marked in patients recruited in Michigan and could be explained by the fact that patients were on average 20 years younger than those recruited in Brighton. This is consistent with previous studies that have shown *C. difficile* patients in the USA are younger on average than in the UK (67.9 years vs. 78.0 years) (88)(300). To determine the specificity of the immune response, the ELISA was repeated in the Brighton cohort using plates coated with Tetanus Toxin produced by *Clostridium tetani.* The antibody titres were similar in cases and controls, which suggest the immune response measured was specific to TcdB and not representative of a generalised immune response in these patients.

In cases with lower antibody responses to TcdB, serum IgA levels were significantly lower than controls. IgA is the principle mediator of humoral immunity at the mucosal surface and exists in two forms, serum IgA and secretory IgA (sIgA) (301). Correlation between serum IgA and sIgA has been shown in animal models, although the strength of antibody response may vary dependent on the antigen (302). In the clinical setting, intestinal secretions obtained by colonic lavage were measured for TcdA-specific sIgA and found to display a similar pattern of antibody response to serum anti-toxin responses (303). Therefore, lower levels of serum IgA in the current study may reflect

a failed local mucosal immune response to TcdB-mediated intestinal inflammation or represent particularly high levels of TcdB locally that could neutralise IgA in the colon.

Cases recruited in Michigan that presented with a recurrence of CDI demonstrated lower antibodies to TcdA, but not TcdB, when compared with controls. These findings are similar to earlier studies that demonstrated lower IgG levels to TcdA were associated with an increased risk of recurrence (131)(132). Antibody titres to TcdA and TcdB were similar in patients that subsequently developed a recurrence of CDI. However, time to recurrence was variable amongst patients and only baseline serum samples were tested, with no convalescent or paired samples available. Previous infection with *C. difficile* does not appear to induce a protective serum antibody response and repeated exposure to the organism may lead to generation of an attenuated humoral response. Temporal changes in antibody responses to TcdB may also be relevant in determining patient susceptibility and future studies should include more frequent sampling of patients to try and delineate this response further.

Both TcdA and TcdB cause glucosylation of Rho proteins that results in disruption to the actin cytoskeleton with cell rounding, detachment and cell death (53)(304). Neutralisation cytotoxicity assays are routinely used to determine the functionality of antibodies that prevent cell rounding. Amongst the Brighton cohort, two thirds of patients (10 cases and 15 controls) had detectable antibodies to TcdB by ELISA. However, only two cases (case three and case nine) demonstrated neutralisation of TcdB. Both demonstrated high antibody titres (1:1,600) by ELISA; however, no correlation was observed in the remaining samples tested.

The disparity between antibody titre and functionality might be explained by differences in antibody binding to the recombinant TcdB used in the assay compared to TcdB *in vivo.* Furthermore, the TcdB concentration (10pg/mL) used may be different from that seen physiologically. It remains unclear if either toxin can enter the systemic circulation in humans. By contrast, both toxins have been detected in sera and body fluids of infected mice and piglets using an ultrasensitive assay, but to-date no such assay has been used in humans (126). The HFF-ready cell assay used has only been validated to confirm cytotoxicity in stool samples and has not been previously

used to test serum. Therefore, this particular cell line (HFF cells) may have variable sensitivity for use as a cytotoxicity assay with serum.

Finally, in an earlier study of CDI patients, only 1/18 acute sera tested were able to neutralise antibodies to TcdA compared to 5/14 convalescent sera and the presence of neutralising antibodies were found to be independent of the clinical course of disease (303). These findings support the current study's observations and suggest that serum antibodies in the acute phase may have other important roles, such as the immune clearance of toxins and that neutralisation of toxin may be more important at a later stage in the disease process.

Different *C. difficile* isolates are capable of synthesising toxins that display antigenic variation. The gene encoding TcdB is more variable than other genes within the PaLoc and marked sequence and functional differences have been demonstrated in epidemic 027/NAP1/BI TcdB compared to TcdB from historical non-027 strains (85). In this study, although isolates were not characterised, ribotype 027 strains were prevalent at both sites during the study period accounting for approximately one third of cases in Brighton and one sixth of cases in Michigan (90)(305). It is notable that 027 isolates from the USA and Europe are genetically highly similar and the same relationship between titre against TcdB and disease was shown in patients from the UK and the USA (306). An alternative explanation for the antibody responses to TcdB in the current study may be antigenic variation in the recombinant toxins used in our assay compared with previous studies. Both possibilities indicate that differences in antibody responses to *C. difficile* may arise from antigenic strain variation.

### *3.7.1 Limitations*

A limitation of the current study was the small number of patients recruited in both cohorts that might explain why the trend observed in the Michigan cohort did not reach statistical significance. Serum samples were only collected at baseline and more frequent sampling would have allowed delineation of the temporal antibody response to TcdB. The correlation between serum IgA and sIgA was previously demonstrated in the convalescent serum of CDI patients in an earlier study (303). However, stool samples were not collected in the present study and therefore, it was impossible to investigate if faecal IgA levels were correlated with serum IgA responses to TcdB in the setting of acute CDI.

### *3.7.2 Future work*

Previous studies have demonstrated the importance of the antibody reponse to TcdA in determining CDI outcome. The current study has highlighted that antibodies to TcdB are lower in patients compared to controls in the acute setting of CDI. This observation has important implications for both the development of novel immunotherapies and the application of treatment regimes. Future work could attempt to use peptide microarrays to compare differences in the qualitative antibody responses to TcdB between patients and controls to establish if differences exist in binding patterns. This information could be used to further refine vaccine development or be translated directly into clinical practice to predict which patients may respond to individual vaccines. Pre-formed antibody titres to TcdB measured by ELISA could also be used as a surrogate marker for vaccine efficacy by indicating which patients may require booster doses.

## 3.8 Conclusion

In summary, this study has demonstrated a novel finding that lower antibodies to TcdB and not TcdA are associated with patient susceptibility to acute CDI in two separate cohorts. This is in contrast to earlier studies that focused on TcdA and recurrence of CDI. Clinically relevant antigenic variation in TcdB might account for differences seen in this study as the majority of previous studies were performed before the emergence of 027/NAP1/BI strains. These findings can be used to identify which patients will benefit from novel immunotherapies. However, clinicians should be aware that antibody responses to TcdB could change as the epidemiology of CDI changes again and different strain types circulate.

# Chapter 4: Predicting recurrence of CDI

## 4.1 Introduction

### *4.1.1 Economic burden of recurrence*

Recurrence of CDI remains a problem affecting 10-30% of patients despite successful first-line treatment with metronidazole or vancomycin. Furthermore, following a first recurrence patients are at increased risk of additional episodes of CDI (172)(307). Recurrence of CDI is a significant economic burden due to frequent hospital readmissions combined with prolonged courses of treatment. McFarland *et al.* calculated the mean direct cost of a recurrent episode of CDI to be $4096.93 per patient, which did not account for the costs incurred through additional clinic visits or loss of patient earnings (172). A second study conducted in Canada estimated the cost of 10 readmissions per year from recurrence of CDI to be $99,887.58 based on annual antibiotic expenditure, mean cost per bed-days and mean length of stay per readmission (308).

An accurate estimation of the true economic burden of recurrence of CDI has been limited by a lack of consensus regarding the definition of recurrence, which differs between the European, American and Australasian clinical guidelines (19)(165)(309). This was highlighted in a recent systematic review of clinical and economic data from 14 countries in Europe that found a different definition of recurrence in each of the 39 studies reviewed (35). The time period between an initial episode of CDI and a recurrent episode varied from a minimum of 48 hours after cessation of initial CDI therapy to a period of 12 months. Lowest rates of recurrence were reported in Germany and Switzerland (3-4%) with the highest seen in Ireland (36%). However, the study in Ireland by Drudy *et al*. was conducted during an outbreak with a toxin variant ribotype 017 strain that may have led to an increased rate of testing and higher rates of detection of recurrence (310).

### *4.1.2 Pathogenesis of recurrence*

Symptomatic recurrence of CDI may occur by two mechanisms. Firstly, spores present in the colon during treatment may vegetate after treatment has finished leading to recrudescence of infection by the original strain. Secondly, patients who remain susceptible to infection after treatment may experience reinfection through acquisition

of a different strain. There is some evidence that early recurrences are more likely to be due to recrudescence of the original strain; however, it remains impossible to distinguish between the two mechanisms clinically (311)(312).

### *4.1.3 Reinfection vs. recrudescence of the original strain*

Several studies have used different typing techniques to demonstrate that reinfection accounts for up-to half of symptomatic recurrences of CDI. In an early study, Johnson *et al.* used REA to compare 50 isolates that were collected over a four year period from 11 patients (313). Five of the 11 patients (45.5%) experienced diarrhoea caused by a different infecting strain and these results were later repeated by another group who also used REA to show reinfection in 60% of recurrences (314). A subsequent study by Barbut *et al.* confirmed these findings in a larger cohort of 93 patients recruited from 20 centres across Canada. PCR ribotyping demonstrated reinfection with a different strain accounted for 48.4% of clinical recurrences and interestingly these patients had an increased length of stay in hospital compared to patients that experienced a recurrence due to the original strain (315).

More recently, paired isolates from patients recruited to the non-inferiority fidaxomicin randomised controlled trials were compared using REA (316). A total of 90 paired isolates were available for testing and in contrast to previous studies a higher proportion of recurrence was caused by the original *C. difficile* strain (83%). The reason for this observation is not easily explained. However, the commonest isolate identified was the epidemic 027/NAP1/BI strain and it is possible that what appeared to be recurrence caused by the original endogenous strain was in fact reinfection by a different isolate belonging to the same strain type. A similar observation was made by Wilcox *et al.* who found an endemic clone was responsible for 53% of isolates recovered from patients with recurrence of CDI and suggested that reacquisition of the same strain may have occurred (317).

This highlights the difficulty of distinguishing reinfection from recurrence caused by the same strain using current genotyping techniques that have limited discriminatory power and may underestimate the true rate of reinfection. However, it is hoped that the use of third generation whole genome sequencing platforms may help resolve this problem by providing a higher degree of genetic resolution (318).

### *4.1.4 Risk factors*

A number of risk factors for recurrence have been previously identified and include increased age, failure to mount effective antibody responses to TcdA and TcdB, underlying disease severity, leucocytosis and chronic renal failure (132)(176)(319). In a meta-analysis of risk factors for recurrence of CDI, 12 studies met the inclusion criteria and a total of 1,382 patients were included (177). Concomitant antibiotics after CDI diagnosis (OR 4.23, 95% CI 2.10-8.55, p<0.001), concomitant antacid medications (OR 2.15, 95% CI 1.13-4.08, p=0.019) and older age (OR 1.62, 95% CI 1.11-2.36, p=0.0012) were significantly associated with increased risk of recurrence of CDI. However, the most potent risk factor for developing CDI in hospital is contact with other patients with active disease. Therefore, it follows that if patients recovering from CDI remain susceptible to further infection through altered gut flora or lack of an adaptive immune response, then continued exposure to other patients who are shedding *C. difficile* may increase patient risk of symptomatic recurrence (320).

### *4.1.5 C. difficile pressure*

Continued exposure of patients to other symptomatic patients results in an increased risk of infection due to colonisation pressure, which is the risk of acquisition of a pathogen from surrounding infected or colonised patients. Colonisation pressure has been shown to be important in other health care associated infections (321)(322). For *C. difficile*, it can be difficult to assess the true colonisation pressure due to difficulties associated with asymptomatic carriers and shedders (323). Therefore, Dubberke *et al.* devised a modified pressure score that included only symptomatic patients who shared time on the same unit (320). Comparing *C. difficile* pressure to other risk factors that included age and gastric suppression, they demonstrated that *C. difficile* pressure remained the most important risk factor associated with CDI (OR 5.4, 95% CI 3.4-8.0).

### *4.1.6 Use of cohort wards*

Given the importance of *C. difficile* pressure, the rapid identification and isolation of symptomatic cases is central to efforts to reduce rates of CDI. Current guidance in both the UK and USA is that when single rooms are not available patients with CDI should be cohorted (19)(324). Since many hospitals lack sufficient side-rooms for isolation of all CDI cases, cohort nursing is recommended *'in either a designated ward*

*or in a designated bay on a ward'* (324). Therefore, it follows that cohorting of patients with *C. difficile* on the same unit might increase patient risk of recurrence if these patients remain exposed to *C. difficile* pressure once their symptoms have resolved.

### *4.1.7 Treatment*

Management of recurrence remains a therapeutic challenge and has previously been described in the main introduction (*1.10.2*)**.** In summary, a first recurrence is treated with the same antibiotic used for the initial episode of CDI and tapered or pulsed vancomycin is often used for refractory cases (19). Faecal microbiota transplants have also been used with success but standardised treatment protocols are lacking (209). Recently, the novel antibiotic fidaxomicin was licenced for the treatment of adult CDI and was associated with lower rates of recurrence, compared to vancomycin, in two randomised controlled trials (*1.10.1.1)* (194)(195). On further sub-group analysis, fewer patients in the fidaxomicin group experienced a second recurrence within 28 days (19.7% vs. 35.5%, 95% CI -30.4% to -0.3%, p=0.045) (325). However, as previously discussed, fidaxomicin use is currently on a named patient basis due to increased cost, with a single 10 day course costing £1350 (192). Therefore, early identification of those most likely to develop a recurrence of CDI is important, as they would stand to gain most benefit from fidaxomicin. Unfortunately, currently it remains impossible to predict which patients will recur.

### *4.1.8 Clinical prediction tools*

Clinical prediction tools have been used in community acquired pneumonia and in intensive care to categorise patients based on risk factors for disease (326)(327). Previous attempts at developing a clinical prediction tool for recurrence of CDI have been limited by small sample sizes and have not been validated in a second cohort of patients. The majority of scoring systems have attempted to predict CDI severity and only two scores have been published that have focused on recurrence of CDI (Table 4.1) (171)(176)(311).

Hu *et al.* developed a score that included age >65 years, severe or fulminant underlying illness (based on the Horn index) and concomitant antibiotic use. A second combined scoring system incorporated data on serum IgG titres against TcdA that were measured in an earlier study (132). The derivation cohort consisted of 44 patients and a validation cohort of 64 patients. Only 16 patients were used to develop the combined

score. The first predictive score correctly classified recurrence in 77.3% (95% CI 62.2-88.5) of patients in the derivation cohort and 71.9% (95% CI 59.2-82.4) of patients in the validation cohort. However, the combined score failed to perform well in the validation cohort and was only able to accurately predict recurrence in 69.2% of cases. This is likely to be due to the small number of serum samples available for testing IgG responses to TcdA in the validation cohort.

More recently, Eyre *et al.* proposed a clinical predictive score using data from electronic patient records, in contrast to previous scores that have used clinical data such as stool frequency and temperature (170)(311). The score assessed patient and health status, severity of initial disease, past health care exposure, antibiotic selection and timing of recurrence, with a maximum score of 15. At four months following a first episode of CDI, the absolute risk of recurrence increased by 5% for every one point increase in the scoring index. Although the scoring system performed well in a large well defined derivation cohort of 1,700 patients, it must still be validated in a separate cohort of patients before introduction into clinical practice.

**Table 4.1. Clinical prediction tools for recurrence of CDI.**

| Author | Study design | Variables in score | Interpretation |
|---|---|---|---|
| **Eyre, D *et al.* (2012)** | n=363<br>Retrospective study<br>Maximum score 15 | • Patient and health status (age, emergency admission)<br>• Severity of initial disease (stool frequency, admission with CDI, C-reactive protein)<br>• Past health exposure (type of past admission, total inpatient duration before admission)<br>• Antibiotic selection<br>• Susceptibility to diarrhoea several weeks after hospital exposure (primary CDI four to 12 weeks after hospital discharge) | The absolute risk of recurrence at four months increased by approximately 5% for every 1 point increase in score. |
| **Hu, M *et al.* (2009)** | n=44 (derivation cohort)<br>n=64 (validation cohort)<br>Retrospective study<br>Maximum score 4 | • Age >65years<br>• Horn index<br>• Concomitant antibiotics<br><br>Combined score: as above and IgG to TcdA <1.29 ELISA units | A score of:<br>≥2=high risk of recurrence<br><2=low risk of recurrence<br>The combined score failed to perform well in the validation cohort due to a small sample size. |

## 4.2 Aim

The aim of this study was to determine risk factors for symptomatic recurrence of CDI amongst patients admitted to the Royal Sussex County Hospital, Brighton and specifically to establish the impact of cohorting on the risk of recurrence.

## 4.3 Methods

A retrospective case note review was undertaken of all in-patients admitted to the Royal Sussex County Hospital, Brighton with a confirmed diagnosis of CDI on stool ELISA (Premier TcdA and TcdB ELISA, Meridian Bioscience, USA) between October 2008 and June 2011. Data were extracted until March 2012 to ensure a minimum follow-up time of eight months for each patient. Baseline information collected included medications on admission and the primary admission diagnosis, which was categorised from the admission clerking as respiratory tract infection, urinary tract infection, skin and soft tissue infection, gastrointestinal disease, renal disease and other causes. These included falls, endocrine abnormalities and acute confusion. Burden of comorbid disease was assessed using the Charlson Score and functional activity and frailty were assessed using the Barthel Index and Waterlow Score (328)(329)(330). All scores were calculated using routinely gathered clinical information where available. CDI severity was scored using the hospital's own criteria and was obtained from notes where possible.

### *4.3.1 Definitions*

#### *4.3.1.1 CDI recurrence*

Recurrence of CDI was defined as retreatment based on the clinical judgement of the physician in charge occurring more than 14 days after the day of diagnosis or a positive toxin EIA test on liquid stool more than 14 days after start of treatment. The primary end-point used was recurrence within 30 days of CDI diagnosis.

#### *4.3.1.2 CDI severity*

Severe disease was defined as a) the presence of ileus or toxic megacolon or b) the presence of more than two of: fever (>38$^{o}$C), ≥five stools passed per 24 hours, bloody diarrhoea, abdominal pain, distension, tenderness or ascites, cardiovascular compromise or dehydration requiring intravenous fluids, white blood cell count >15 or <5x10$^{9}$cells/L and albumin level <25g/L on the day of assessment. Patients were

treated for 14 days with metronidazole or if categorised as severe disease with vancomycin. In addition, baseline laboratory markers were recorded as the closest measurement to the positive stool sample (plus or minus two days).

#### *4.3.1.3 Cohort ward*

The cohort ward consisted of two four-bedded bays providing separate female and male areas, a double bay and one side room (Figure 4.1). All patients had their own commode, stethoscope and disposable bed curtains. Gloves and apron isolation precautions were in place for all patients.

### *4.3.2 Ethics approval*

The study was approved by the hospital research and development office as a service evaluation and therefore did not require a formal ethical review.

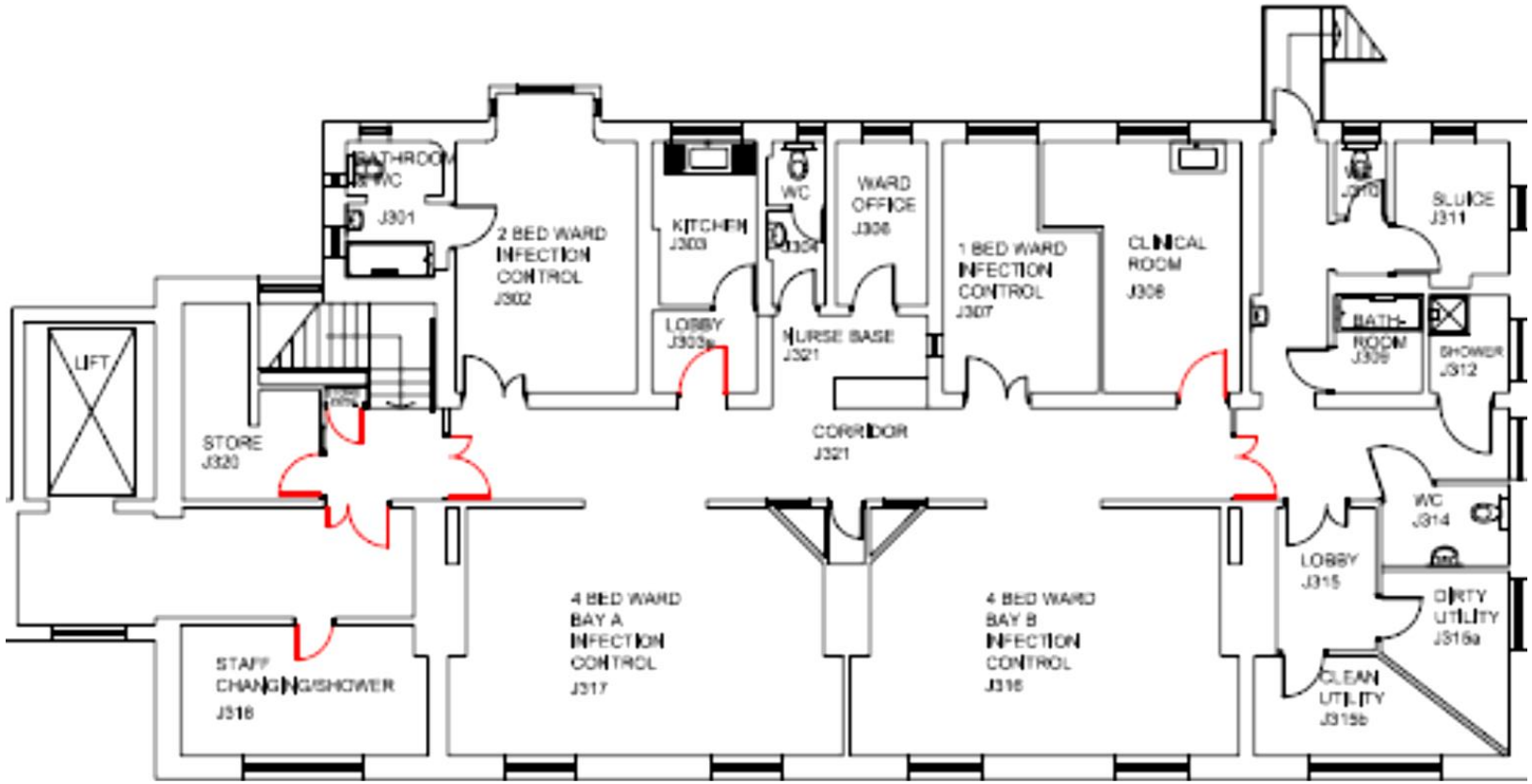



**Figure 4.1. Layout of cohort ward. The cohort ward is located on the second floor of the Royal Sussex County Hospital, Brighton and was introduced in 2008 as part of a bundle of infection control measures.**

### *4.3.3 Statistical analysis*

A p-value of <0.05 was used as a cut-off for statistical significance with values between 0.05 and 0.09 defined as a statistical trend. Mann Whitney U tests were used to compare non-parametric variables. A stepwise selection was used for the construction of the multivariate model and all variables with a p-value <0.1 were included. Receiver operating curves were initially used to dichotomise all continuous variables. However, as no difference in the sensitivity of the model was seen continuous variables were used in the logisitic regression. Kaplan-Meier survival curves were constructed to examine the relationship between variables and time to recurrence of CDI and differences were measured using the Log-rank test. All statistical analyses were performed using SPSS (version 20, IBM®, USA).

## 4.4 Results

### *4.4.1 Study population*

Between October 2008 and June 2011, 420 patients were diagnosed with CDI and 312 sets of case notes were available. Patients were followed up until March 2012 to allow a minimum follow-up period of eight months. Patients had a median age of 81 years (IQR 69-87) and 122 (45%) were male. There were a total of 188 deaths throughout the follow-up period giving a mortality rate of 60%. Death within 30 days was treated as a competing risk for recurrence of CDI within 30 days and therefore 64 cases were excluded. This left a total of 248 cases available for analysis. Four patients had multiple recurrences with one individual suffering four episodes during an eight month period.

### *4.4.2 CDI recurrence within 30 days*

Within 30 days of CDI diagnosis, 26 (10.5%) patients experienced a first recurrence of CDI. This group were older (83 years [IQR 76-91] vs. 81 years [IQR 69-87]) and frailer with higher Waterlow Scores (16 [IQR 9-20] vs. 13 [IQR 9-18.5] and lower Barthel Indices (10.5 [IQR 3-16] vs. 13 [IQR 6-18]) compared to those who did not experience a recurrence of CDI, although these differences were not statistically significant. The burden of underlying co-morbidity scored using the Charlson Score was similar between the groups (1 [IQR 0-2] and 2 [IQR 0-3]). Patients who experienced a recurrence within 30 days had an increased length of hospital stay of 40 days (IQR 28-70) compared with 32 days (18-53) in those that did not.

On univariate analysis (Table 4.2) patients admitted with a urinary tract infection were more likely to experience a recurrence within 30 days of initial CDI diagnosis (OR 5.16, 95% CI 2.10-12.64, p<0.001) as were patients managed on the *C. difficile* cohort ward (OR 3.77, 95% CI 1.37-10.35, p=0.010). Additional risk factors associated with recurrence but not statistically significant included antibiotics at the time of hospital admission (OR 2.06, 95% CI 0.64-6.63, p=0.23) severe CDI (OR 2.00, 95% CI 0.86-4.65, p=0.11) and concomitant antibiotics during CDI treatment (OR 2.07, 95% CI 0.91-4.72, p=0.083). In the 12 patients that received concomitant antibiotics and experienced a recurrence, one third received gentamicin and urinary tract infection was the commonest indication. Laboratory parameters taken at baseline were similar between patients that experienced a recurrence of CDI and those that did not (Table 4.3).

**Table 4.2. Risk factors for recurrence of CDI within 30 days. Count and percentage (%) or medians and interquartile range (IQR) are shown. Univariate analysis was used to calculate odds ratios, 95% CI and p-values. Age and Charlson score were analysed as continuous variables and odds ratio given are for every additional year and every additional point on the Charlson score. Other diagnoses on admission included falls, endocrine abnormalities and acute confusion. Patients were categorised as having severe CDI as described in the methods. a. Severity data were not recorded in three cases.**

| | | Overall | Recurrence | Odds ratio | 95% Confidence interval | p-value |
|---|---|---|---|---|---|---|
| | **Total number of patients (%)** | **248 (100)** | **26 (10.5)** | | | |
| | **Males (%)** | 112 (45.2) | 10 (8.9) | 0.74 | (0.32-1.69) | 0.47 |
| | **Age in years (IQR)** | 81 (70-87) | 83 (76-91) | 1.03 | (0.99-1.06) | 0.13 |
| **Medications on admission** | **Gastric suppression (%)** | 111 (44.8) | 11 (9.9) | 0.88 | (0.39-2.00) | 0.76 |
| | **Laxatives (%)** | 38 (15.3) | 5 (13.2) | 1.36 | (0.48-3.87) | 0.56 |
| | **Steroids (%)** | 16 (6.5) | 1 (6.2) | 0.55 | (0.070-4.36) | 0.57 |
| | **Antibiotics (%)** | 22 (8.9) | 4 (18.2) | 2.06 | (0.64-6.63) | 0.23 |
| **Diagnosis on admission** | **Chest infection (%)** | 41 (16.5) | 4 (8.9) | 0.91 | (0.30-2.79) | 0.87 |
| | **Urinary tract infection (%)** | 34 (13.7) | 10 (29.4) | 5.16 | (2.10-12.64) | **<0.001*** |
| | **Skin and Soft tissue infection (%)** | 8 (3.2) | 0 (0) | n/a | n/a | n/a |
| | **Gastrointestinal disease (%)** | 38 (15.3) | 2 (5.3) | 0.43 | (0.097-1.90) | 0.27 |
| | **Renal disease (%)** | 10 (4.0) | 2 (20) | 2.23 | (0.45-11.11) | 0.33 |
| | **Stroke (%)** | 12 (4.8) | 1 (8.3) | 0.77 | (0.95-6.20) | 0.80 |
| | **Surgical diagnosis (%)** | 49 (19.8) | 3 (6.1) | 0.50 | (0.14-1.74) | 0.27 |
| | **Other (%)** | 52 (21.0) | 5 (9.6) | 0.89 | (0.32-2.48) | 0.82 |
| **Comorbidity** | **Charlson Score (IQR)** | 2 (0-3) | 1 (0-2) | 0.73 | (0.55-0.97) | **0.029*** |

**Table 4.2. Continued.**

| | | Overall | Recurrence | Odds ratio | 95% Confidence interval | p-value |
|---|---|---|---|---|---|---|
| **Severity of CDI[a.]** | **Non-severe (%)** | 172 (69.4) | 14 (8.1) | **Reference category** | | |
| | **Severe CDI (%)** | 73 (29.4) | 11 (15.1) | 2.00 | (0.86-4.65) | 0.11 |
| | **Transferred to cohort ward (%)** | 138 (55.6) | 21 (15.2) | 3.77 | (1.37-10.35) | **0.010*** |
| **CDI treatment** | **Metronidazole (%)** | 89 (35.9) | 10 (11.2) | **Reference category** | | |
| | **Vancomycin (%)** | 159 (64.1) | 16 (10.1) | 0.88 | (0.38-2.04) | 0.77 |
| **Concomitant treatment** | **Enteral feeding (%)** | 27 (10.9) | 5 (18.5) | 2.17 | (0.74-6.31) | 0.16 |
| | **Gastric suppression (%)** | 58 (23.4) | 6 (10.3) | 0.98 | (0.37-2.57) | 0.97 |
| | **Antibiotics (%)** | 77 (31.0) | 12 (15.6) | 2.07 | (0.91-4.72) | 0.083 |

**Table 4.3. Baseline Laboratory parameters and recurrence of CDI within 30 days. Medians and interquartile range (IQR) are shown. Univariate analysis was used to calculate odds ratios, 95% CI and p-values. All blood tests were analysed as continuous variables and odds ratios are given for every unit increase. Normal ranges: white cell count (4-11), C-reactive protein (<5), urea (1.7-8.3), creatinine (44-80) and albumin (35-52).**

| | Overall | Recurrence | Odds ratio | 95% Confidence interval | p-value |
|---|---|---|---|---|---|
| **Total number of patients (%)** | **248(100)** | **26 (10.5)** | | | |
| **White blood cell count $10^9$/L (IQR)** | 10.2 (7.6-14.3) | 8.3 (6.8-12.1) | 0.99 | (0.92-1.05) | 0.66 |
| **C-reactive protein mg/L (IQR)** | 67.0 (35-111.8) | 80.0 (26.0-89.9) | 1.00 | (1.00-1.01) | 0.64 |
| **Urea mmol/L (IQR)** | 7.4 (4.8-10.9) | 7.0 (5.7-8.4) | 1.00 | (0.93-1.07) | 0.88 |
| **Creatinine µmol/L (IQR)** | 79.5 (62.0-122.3) | 77.0 (63.5-103.5) | 1.00 | n/a | 0.40 |
| **Albumin g/L (IQR)** | 32.0 (27.0-35.0) | 32.0 (29.0-36.0) | 1.00 | (0.93-1.07) | 0.92 |

### *4.4.3 Cohort ward*

Of the 248 CDI cases, 138 (55.6%) were transferred to the *C. difficile* cohort ward and the remainder were managed on the ward where their CDI diagnosis was made (Table 4.4). Patients transferred to the cohort ward were older than those that remained on other wards (84.5 years [IQR 78-90] vs. 83years [IQR 75-87], p=0.005) and more likely to have underlying congestive cardiac failure and dementia. Patients with renal pathology on admission were more likely to remain on the renal specialist unit (OR 0.082, 95% CI 0.010-0.66, p=0.018) and no patients with severe chronic renal failure were transferred to the cohort ward.

Patients admitted to the cohort ward were almost twice as likely to have severe CDI as defined by the hospital criteria (OR 1.95, 95% CI 1.10-3.46, p=0.022) and were more likely to receive vancomycin first line (OR 1.59, 95% CI 0.94-2.68, p=0.083). Fewer patients on the cohort ward (n=25) received concomitant gastric suppression compared to those on other wards (n=33) (OR 0.52, 95% CI 0.29-0.94, p=0.029). Patients that experienced a recurrence had an increased length of stay on the cohort ward of 26 days (IQR 19-40) compared to only 15 days (IQR 7.5-28.5) in patients that did not experience a recurrence of CDI (OR 1.02, 95% CI 1.00-1.04, p=0.033). Once transferred, the majority of patients remained on the cohort ward until discharge from hospital (104/138 [75.4%]) with only a minority (27/138 [19.6%]) transferred back onto general hospital wards. Seven patients (5.1%) died on the cohort ward.

A Kaplan Meier curve was used to assess the effect of transfer to the cohort ward on time to recurrence. Data was censored at the end of the follow-up period (Figure 4.2). It was impossible to estimate median times to recurrence because less than 50% of patients experienced recurrence. The 10th percentile points are estimated as 28 days for patients transferred to Grant ward and 145 days for those not transferred. That is, 90% of patients are expected not to experience a recurrence for at least 28 days if they are transferred to the cohort ward, but for at least 145 days if they are not transferred (p=0.004).

**Table 4.4. Risk factors associated with management on the cohort ward. Count and percentage (%) or medians and interquartile range (IQR) are shown. Univariate analysis was used to calculate odds ratios, 95% CI and p-values. Abbreviations: CVA (cerebrovascular accident), COPD (Chronic obstructive pulmonary disease), CCF (congestive cardiac failure). Other diagnoses on admission included falls, endocrine abnormalities and acute confusion. Patients were categorised as having severe CDI as described in the methods. a. severity data were not recorded in three cases.**

| | | Transferred to cohort ward | Not transferred to cohort ward | Odds ratio | 95% Confidence interval | p-value |
|---|---|---|---|---|---|---|
| | **Total number of patients (%)** | **138 (55.6)** | **110 (44.4)** | | | |
| | **Males (%)** | 55 (49.1) | 57 (50.9) | 0.62 | (0.37-1.02) | 0.061 |
| | **Age (years, IQR)** | 84.5 (78-90) | 83 (75-87) | 1.03 | (1.01-1.04) | **0.005*** |
| **Medications on admission** | **Gastric suppression** | 63 (56.8) | 48 (43.2) | 1.05 | (0.63-1.74) | 0.85 |
| | **Laxatives (%)** | 22 (57.9) | 16 (42.1) | 1.11 | (0.55-2.24) | 0.76 |
| | **Steroids (%)** | 8 (50) | 8 (50) | 0.79 | (0.29-2.16) | 0.64 |
| | **Antibiotics (%)** | 12 (54.4) | 10 (45.5) | 0.95 | (0.40-2.29) | 0.91 |
| **Diagnosis on admission** | **Chest infection (%)** | 25 (61) | 16 (39) | 1.30 | (0.66-2.58) | 0.45 |
| | **Urinary tract infection (%)** | 27 (79.4) | 7 (20.6) | 3.58 | (1.49-8.57) | **0.004*** |
| | **Skin and Soft tissue infection (%)** | 3 (37.5) | 5 (62.5) | 0.47 | (0.11-2.00) | 0.30 |
| | **Gastrointestinal disease (%)** | 22 (57.9) | 16 (42.1) | 1.11 | (0.55-2.24) | 0.76 |
| | **Renal disease (%)** | 1 (10) | 9 (90) | 0.082 | (0.01-0.66) | **0.018*** |
| | **Stroke (%)** | 5 (41.7) | 7 (58.3) | 0.55 | (0.17-1.79) | 0.32 |
| | **Surgical diagnosis (%)** | 26 (53.1) | 23 (46.9) | 0.88 | (0.47-1.64) | 0.69 |
| | **Other (%)** | 30 (57.7) | 22 (42.3) | 1.11 | (0.60-2.06) | 0.74 |

**Table 4.4. Continued.**

| | | Transferred to cohort ward | Not transferred to cohort ward | Odds ratio | 95% Confidence interval | p-value |
|---|---|---|---|---|---|---|
| **Comorbidities** | **CVA (%)** | 26 (53.1) | 23 (46.9) | 0.89 | (0.47-1.66) | 0.71 |
| | **COPD (%)** | 28 (62.2) | 17 (37.8) | 1.39 | (0.72-2.70) | 0.33 |
| | **CCF (%)** | 11 (84.6) | 2 (15.4) | 4.68 | (1.01-21.56) | **0.048*** |
| | **Dementia (%)** | 21 (84) | 4 (16) | 4.76 | (1.58-14.30) | **0.006*** |
| | **Leukaemia (%)** | 1 (33.3) | 2 (66.7) | 0.39 | (0.035-4.41) | 0.45 |
| | **Malignant lymphoma (%)** | 10 (71.4) | 4 (28.6) | 2.07 | (0.63-6.79) | 0.23 |
| | **Myocardial Infarction (%)** | 8 (66.7) | 4 (33.3) | 0.38 | (0.11-0.13) | 0.12 |
| | **Ulcer disease (%)** | 9 (52.9) | 8 (47.1) | 0.69 | (0.26-1.85) | 0.46 |
| | **Diabetes (%)** | 13 (38.2) | 21 (61.8) | 1.34 | (0.64-2.81) | 0.44 |
| | **Moderate renal failure (%)** | 10 (50) | 10 (50) | 0.78 | (0.31-1.95) | 0.60 |
| | **Severe renal failure (%)** | 0 (0) | 17 (100) | n/a | n/a | **<0.0001*** |
| | **Cancer (%)** | 15 (48.4) | 16 (51.6) | 0.72 | (0.34-1.52) | 0.39 |
| | **Charlson Score (IQR)** | 1 (0-2) | 2 (0-3) | 0.97 | (0.86-1.09) | 0.57 |
| **Functional status & frailty** | **Barthel Index (IQR)** | 13 (7-17) | 11 (6-19) | 1.02 | (0.96-1.08) | 0.54 |
| | **Waterlow Score (IQR)** | 13 (9-19) | 14 (10-20) | 0.98 | (0.94-1.02) | 0.36 |
| **Severity of CDI[a.]** | **Non-severe (%)** | 88 (51.2) | 84 (48.8) | **Reference category** | | |
| | **Severe CDI (%)** | 49 (67.1) | 24 (32.9) | 1.95 | (1.10-3.46) | **0.022*** |
| **CDI treatment** | **Metronidazole (%)** | 43 (48.3) | 46 (51.7) | **Reference category** | | |
| | **Vancomycin (%)** | 95 (59.7) | 64 (40.3) | 1.59 | (0.94-2.68) | 0.083 |
| **Concomitant treatment** | **Enteral feeding (%)** | 11 (40.7) | 16 (59.3) | 0.51 | (0.23-1.15) | 0.10 |
| | **Gastric suppression (%)** | 25 (43.1) | 33 (56.9) | 0.52 | (0.29-0.94) | **0.029*** |
| | **Antibiotics (%)** | 39 (50.6) | 38 (49.4) | 0.75 | (0.44-1.28) | 0.29 |

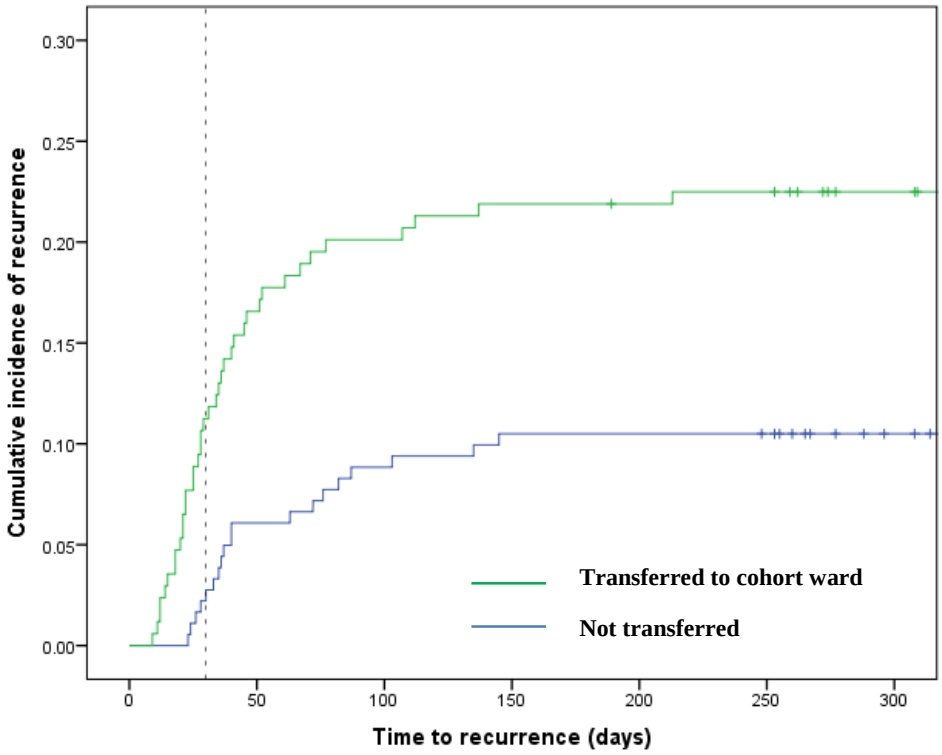



**Figure 4.2. Transfer to cohort ward and time to recurrence. The Kaplan Meier plot shows the cumulative incidence of recurrence of CDI stratified by transfer to the cohort ward. Recurrence was defined as a positive repeat stool EIA test, after an interval of 14 days or re-treatment based on the clinical judgement of the physician in charge. Data was censored at completion of follow-up. Crosses represent censored data. The dashed line indicates recurrence at 30 days.**

### *4.4.4 Recurrence of CDI within 30 days on multivariate analysis*

A multivariate model was constructed that included any variables with a p-value of <0.1. Using this criteria, urinary tract infection on admission, transfer to the cohort ward, concomitant antibiotics and Charlson Score were included (Table 4.5). Transfer to the cohort ward (OR 3.33, 95% CI 1.15-9.65, p=0.027), urinary tract infection on admission (OR 4.0, 95% CI 1.56-10.35, p=0.004), concomitant antibiotics (OR 2.52, 95% CI 1.03-6.13, p=0.043) and a lower Charlson Score (OR 0.71, 95% CI 0.51-0.99, p=0.040) remained significantly associated with recurrence of CDI within 30 days.

**Table 4.5. Recurrence of CDI within 30 days on multivariate analysis. Factors with a significance <0.1 on univariate analysis were included in the model. The Charlson Score was included as a continuous variable and odds are given for every point increase in theCharlson Score.**

| | **Odds ratio** | **95% Confidence Interval** | **p-value** |
|---|---|---|---|
| **Transferred to cohort ward** | 3.33 | (1.15-9.65) | **0.027*** |
| **Urinary tract infection** | 4.01 | (1.56-10.35) | **0.004*** |
| **Concomitant antibiotics** | 2.52 | (1.03-6.13) | **0.043*** |
| **Charlson score** | 0.71 | (0.51-0.99) | **0.040*** |

## 4.5 Discussion

Symptomatic recurrence of CDI affects up-to one third of patients despite successful treatment of the initial infection and remains a therapeutic challenge due to a lack of evidence supporting any one specific treatment strategy (331). Early identification of those patients at greatest risk is of paramount importance in guiding early changes to treatment. The two main mechanisms are recurrence caused by persistence of an endogenous strain or reinfection following acquisition of a new strain (332). Risk factors consistently shown to be associated with recurrence include use of concomitant antibiotics, concomitant gastric suppressants and older age (177). However, previous studies have identified other factors that include failure to mount an effective immune response to TcdA and TcdB, severity of underlying illness and chronic renal failure (176)(319). Given the heterogeneous nature of risk factors identified, the aim of this study was to examine risk factors for symptomatic CDI recurrence in patients admitted locally to the Royal Sussex County Hospital, Brighton over a 32 month period.

The observed recurrence rate within 30 days was only 10.5%, which is much lower than the 10-30% rate frequently quoted in the literature. However, the study included patients admitted to hospital at a time of rapidly falling CDI rates in the UK that followed the introduction of increased infection control measures. If reinfection contributes significantly to recurrence then recurrence rates would be expected to fall as rates of primary CDI infection decline. The incidence of recurrence in the current study is similar to that detected in the largest recurrence

study in the UK, which reported rates of 7% and 16% at one and two months respectively (311).

The Charlson Score is a measure of underlying comorbidity that was originally validated for use in oncology patients (328). It was surprising that an increase in Charlson Score, suggesting more comorbidity, was associated with a reduced risk of recurrence. However, this might be explained by the fact that the nature of comorbidity is more important than the overall number of comorbidities and only certain conditions, such as chronic renal failure, have been associated with severe CDI (319)(333).

The Horn Index is a generic four-level index of disease severity that uses a physician's clinical judgement to classify a patient's overall condition into different levels of disease severity (334)(335). Previous studies have used a modified Horn Index to demonstrate that underlying severe disease on admission is associated with an increased risk of recurrence of CDI (176)(132). In the current study, it was impossible to use this index given the retrospective nature of data collection from case notes.

### *4.5.1 Urinary tract infection on admission*

The effect of presenting complaint on recurrence was assessed and showed urinary tract infection on admission was significantly associated with recurrence. Given that certain antibiotics have been associated with delayed recovery to the host microbiota, this may reflect the nature and duration of the antibiotics used in these patients. Another plausible explanation is that older patients are often diagnosed with recurrent episodes of urinary tract infections that are managed with repeated courses of antibiotics in quick succession. This may result in incomplete recovery of the intestinal microbiota and leave patients increasingly susceptible to CDI. Furthermore, urinary tract infections in the older population are known to frequently cause delirium that necessitates admission to hospital. This further increases their burden of healthcare contact and exposure to possible environmental and horizontal transmission of CDI (336).

Patients experiencing recurrence were older and frailer, suggested by higher Waterlow Scores and lower Barthel Indices. Kyne *et al*. demonstrated that patients

with lower Barthel Indices (median score of eight) at the onset of CDI were more likely to develop severe disease (defined as a prolonged course or associated with development of toxic megacolon) and Tanner *et al*. demonstrated the ability of the Waterlow Score to predict development of CDI (329)(337). The association with recurrence for both scores did not reach statistical significance but this may be a consequence of missing data, as scores were only available if they had been recorded in the case notes.

No association was seen between baseline laboratory parameters and CDI recurrence, which is in contrast to other studies that have shown leucocytosis, raised C-reactive protein and renal dysfunction are associated with CDI recurrence (311)(319). The problems associated with using laboratory markers as predictors of severity and recurrence in CDI were recently demonstrated by Bauer *et al.* who found a high degree of variability in levels measured at the time of diagnosis that affected whether cases were classified as severe or non-severe (319).

### *4.5.2 Concomitant antibiotics*

The current study found an association between concomitant antibiotics prescribed for infections other than CDI and increased risk of recurrence (OR 2.52, 95% CI 1.03-6.13, p=0.043) that confirms the findings of a number of previous studies (132)(172)(338). Although clinical guidelines advocate stopping concomitant antibiotics at the earliest opportunity, this is often impossible in CDI patients who frequently develop additional hospital acquired infections due to increased lengths of stay in hospital.

Fidaxomicin has been associated with lower rates of recurrence compared to vancomycin (194)(195). In a follow-up study by Mullane *et al*., the effect of concomitant antibiotics on global cure and recurrence in patients recruited to the main fidaxomicin studies was investigated (339). Overall, recurrence rates were higher in patients receiving concomitant antibiotics compared to those that did not irrespective of antimicrobial treatment. However, recurrence was lower in patients treated with fidaxomicin compared to those receiving vancomycin (16.9% v. 29.2%), which may reflect the ability of fidaxomicin to cause minimal disruption to the microbiota (196).

### *4.5.3 Cohort ward*

Previous studies have demonstrated that exposure to symptomatic CDI patients can leave other patients at increased risk of developing CDI due to increased colonisation pressure (323). Guidelines in both the UK and USA support the isolation of patients with suspected CDI at the earliest opportunity. However, if no isolation rooms are available patients should be nursed on a specialist cohort ward (13)(19). In 2008, a cohort ward was opened at the Royal Sussex County Hospital, Brighton in addition to the introduction of a restrictive antibiotic prescribing policy. Following these interventions, rates of CDI fell from 1.30 cases per 1000 bed days to 0.69 cases per 1000 bed days that suggested a positive role for cohorting, which has been observed in other hospitals (340)(341).

In the current study, patients managed on the cohort ward were prescribed fewer concomitant gastric suppressants, which have been associated with an increased risk of CDI (342). However, patients admitted to the cohort ward were at an increased risk of recurrence within 30 days compared to those that remained on other wards. Once on the cohort, patients that experienced a recurrence of CDI had an increased length of stay on the cohort ward compared to those that did not recur and the vast majority of patients remained on the cohort until they were discharged from hospital.

Patients are often considered non-infectious once diarrhoea has resolved. However, Sethi *et al*. reported 56% of patients had asymptomatic stool shedding of *C. difficile* in the weeks following treatment and found skin and environmental contamination rates of 58% and 50% respectively (343)(344). Asymptomatic shedders are likely to contribute to transmission of *C. difficile* in hospitals and subsequently contribute to rates of symptomatic disease (162). Reinfection is a well-established cause of recurrence of diarrhoea in patients following treatment of *C. difficile*. Patients that remain in hospital following an episode of CDI remain increasingly susceptible to reinfection due to continued exposure to environmental spores and recurrent courses of antibiotics that continue to disrupt the host microbiota. The proportion of recurrences caused by reinfection as opposed to recurrence due to an endogenous strain increases with time following the primary episode and it has been suggested that recurrences beyond eight weeks be considered reinfection (345). However,

Figueroa *et al*. recently reported re-infection rates of 13.3% in the two weeks after completion of treatment; therefore, it is plausible that patients admitted to a *C. difficile* cohort ward remain exposed to an increased risk of reinfection compared to patients who are not cohorted (316).

### *4.5.4 Limitations*

The major limitations of this study are its retrospective nature, relatively small sample size and being conducted in one centre. Previous studies have identified increasing age and initial disease severity as important risk factors for recurrence. The association between these factors and recurrence did not achieve statistical significance in this study and is likely to be because it was underpowered to confirm these effects. Due to the retrospective nature of the study it was difficult to establish the previous burden of hospital admission or antibiotic exposure, which have previously been associated with recurrence (311).

As typing of isolates was not performed, it was impossible to establish the mechanism of recurrence. The majority of cases of CDI admitted to the Royal Sussex County Hospital during the study period were caused by ribotypes 027 and 106 , which is similar to the pattern seen across the UK during this period (24)(90). However, a highly discriminatory typing technique is needed to distinguish between relapse and reinfection. Whole genome sequencing allows the true genetic relatedness of individual strains to be identified. Third generation rapid bench-top sequencers can generate rapid and precise sequencing and have already been used to demonstrate true transmission events for *C. difficile* and *S. aureus*, which will improve hospital infection control in the future (318).

### *4.5.5 Development of a clinical prediction tool*

After the identification of potential risk factors for recurrence it was hoped that a clinical predictive tool could be developed. However, this was not possible due to the small sample size and the low number of outcome events. It has been recommended that the minimum number of events per variable should be between 10 and 20 as fewer than this can result in the selection of variables by chance that may not actually be of any real significance (346)(347).

## 4.6 Conclusion

This is the first study to establish an association between cohorting of patients and increased risk of recurrence of CDI. Currently, a paucity of data exists in relation to the optimal time that patients should be discharged from a cohort ward and this study provides an important reminder to clinicians to reassess the management of CDI. Although this observation has been made in a relatively small cohort of patients it is biologically plausible and difficult to account for by selection bias. This provides a dilemma for the management of *C. difficile* patients in hospitals that often lack sufficient side rooms and emphasises the importance of single-room isolation for patients with CDI. An association between urinary tract infection on admission and recurrence was observed that has not previously been reported. Concomitant antibiotics are a well-recognised risk factor for recurrence and this study confirms this association. Although the recurrence rate of 10.5% in the current study was much lower than previously observed, this likely reflects changes in CDI epidemiology and infection control measures that have been introduced since earlier studies.

It was not possible to develop a clinical predictive tool for recurrence of CDI due to the small sample size and low event rate. This may be overcome through collaborative work and increasing the overall sample size; however, it may be that development of a simple clinical scoring system is not possible and future work should focus on possible biomarkers for predicting CDI response.

# Chapter 5: Concluding remarks

Over the past decade *C. difficile* has emerged to become the commonest cause of nosocomial diarrhoea in the developed world (4). Efforts to reduce CDI have focused on improving infection control measures and optimising diagnostic testing, which have met with some success, as demonstrated by the continued decline in UK rates over the past three years (15). However, the epidemiology of CDI is changing and has been characterised by the recent emergence of different strains associated with severe disease. Furthermore, there has been an increase in the number of community cases and a suggestion that animal and environmental reservoirs of disease may exist (26)(31). Earlier work using stochastic modelling has suggested a role for patient susceptibility in CDI (7)(348)(349).

Therefore, this thesis has focused on three different areas of patient susceptibility that may offer an effective alternative strategy for reducing transmission of CDI. The first two studies examined the role of the host microbiota and the humoral response to TcdB in acute CDI and the final study identified novel risk factors for the recurrence of CDI.

## 5.1 Role of probiotics in preventing AAD

The Probiotic NU278 study was designed to definitively address whether or not the probiotic *L. casei* DN114001 was able to prevent AAD and CDI, based on the observations of an earlier study by Hickson *et al.* (235). Recruitment proved extremely challenging and resulted in an extension to the recruitment period that meant the final results were unavailable at the time of writing this thesis. Recruitment difficulties were due to a combination of factors that included the older age of the target population, the nature of the intervention (yoghurt vs. capsule) and the exclusion criteria used. However, the exclusion criteria chosen were similar to other probiotic studies in this area and similar difficulties in recruitment have been reported in other trials (personal communication Dr. C. Selinger, Clinical Research Fellow for VSL3# probiotic study) (350). The incidence of 18.2% AAD in the current study was comparable to rates reported in the literature and a non-significant positive correlation was suggested between the rate of AAD and the average number of antibiotics prescribed per patient in different centres.

The main strength of the current study is the large sample size, which is over twice the size of previous trials in this area. The trial was conducted over a large geographical area in both small District General Hospitals and large Teaching Hospitals that allows the findings to be applied to different patient groups.

At the conclusion of the study, the health economic analysis will be important to establish if *L. casei* DN114001 affects morbidity in terms length of stay and patient quality of life. Irrespective of the outcome, a cumulative meta-analysis should be conducted as the trial is likely to have a significant impact on the overall relative risk and conclusions reported in previous probiotic meta-analyses.

## 5.2 The antibody response to TcdB in determining patient susceptibility

A significant body of evidence now exists to support the role of the humoral response in determining CDI outcome. The central aim of this study was to establish the role of antibodies to TcdB in determining patient susceptibility in acute CDI. Currently no commercial validated assay exists for the detection of antibody responses to TcdA or TcdB. This study developed and optimised a sensitive ELISA that was used to measure antibody responses to both TcdA and TcdB in a case-control study. Lower total antibodies to TcdB, not TcdA, were found in patients with acute CDI. This response appeared to be predominantly IgA mediated and clinically relevant antigenic variation in TcdB might account for differences seen. The findings presented here are novel and in contrast to earlier studies that focused on TcdA and on CDI recurrence. Importantly, the same trend was observed in two separate cohorts (Brighton, UK and Michigan, USA) and was specific for *C. difficile* TcdB.

These findings demonstrate the importance of the antibody response to TcdB in acute CDI and could be used to identify which patients are likely to benefit from novel immunotherapies. For example, patients with lower antibody levels may require increased doses of monoclonal antibodies or booster vaccinations, should either treatment become available for CDI.

## 5.3 Predicting a recurrence of CDI

Previously identified risk factors for recurrence of CDI include concomitant antibiotics that cause continued perturbation to the host microbiota, patient failure to mount an effective antibody response to TcdA and TcdB and older age (132)(144) (177). A retrospective case note review of known patients with CDI was conducted to identify risk factors for CDI. The study supported previous observations that concomitant antibiotics were associated with an increased risk of recurrence. In addition, the study demonstrated a novel association between urinary tract infection on admission and increased risk of recurrence that has not been previously reported. However, the most striking finding in this study was that patients nursed on a cohort ward were at an increased risk of recurrence of CDI, which can be explained by their continued exposure to *C. difficile* colonisation pressure.

Cohort wards are recommended in all CDI guidelines as a way of reducing transmission of CDI (13)(19). The current study has important implications for infection control management. Where possible, patients should be discharged back to individual rooms within the hospital at the earliest opportunity, although this is often not possible due to a limited number of side-rooms. Alternatively, consideration should be given to the design of cohort wards, such as how different bays are utilised, in order to limit the potential reinfection of patients due to horizontal transmission of CDI.

## 5.4 Final remarks

This thesis has investigated three patient susceptibility factors that could act as targets for reducing the rate of CDI. Prevention of antibiotic disruption to the host microbiota using the probiotic *L. casei* DN114001 has been investigated in the largest probiotic study to date and will significantly contribute to the existing body of evidence. The importance of TcdB in CDI pathogenesis has been demonstrated by the novel observation that lower antibody responses to TcdB not TcdA are found in patients with acute CDI, which will be important when considering which patients will benefit from novel immunotherapies. Finally, this thesis is the first study to demonstrate an association between cohorting of patients and recurrence of CDI and reminds clinicians that patients on cohort wards should be discharged in a timely manner to limit possible reinfection.

Taken together, this body of work contributes to a rapidly emerging understanding that patient susceptibility is a crucial factor in determining risk of infection, risk of severe disease and risk of recurrence following treatment in CDI. Therefore, future strategies that focus on targeting patient susceptibility may prove to be more effective in controlling a disease that remains a significant cause of mortality and morbidity.

# Chapter 6: Glossary

Adverse event

This refers to any unwanted effect in a subject during a clinical study.

Bacteriocins

Antimicrobial peptides synthesised by bacteria that can inhibit the growth of other bacterial strains.

B Cell

A lymphocyte of the immune system with principle functions in the humoral immune response including acting as an APC and production of antibodies.

Cell cytotoxicity assay

Reference test used to confirm the diagnosis of *C. difficile* that involves the application of stool filtrate onto a monolayer of cells. A positive test is represented by characteristic cell rounding due to the cytotoxicity of TcdB.

Chimera vaccine

A vaccine that combines the binding domains of TcdA and TcdB.

Colonisation pressure

The risk of acquisition of a pathogen from surrounding infected or colonised patients.

Colonisation resistance

The mechanism by which the normal intestinal microbiota prevents colonisation and overgrowth of pathogens through competition for nutrients, space and binding sites.

Cytokine

Signalling molecule secreted by a cell for regulation and intercellular communication.

Enterotypes

Intestinal microbial communities that differ in their composition and functional structure.

Enzyme immunoassay (EIA)

This assay is used to detect the presence of TcdA and TcdB.

FOXP3

Transcription factor forkhead box P3 is involved in the regulation and development of regulatory T cells.

Glutamate dehydrogenase (GDH)

This antigen is produced in large amounts by all *C.difficile* strains (toxin and non-toxin-producing). The GDH test identifies the presence of the organism and not its pathogenic potential.

IgA

Immunoglobulin A class of antibody that plays an important role in mucosal immunity. Two forms exist, serum IgA and secretory IgA.

IgG

Immunoglobulin G class of antibody involved predominantly in the immune response to pathogens. This is the most abundant antibody isotype.

IgM

Immunoglobulin M class of antibody that is produced during the acute phase of the immune response.

Immunosenescence

This term is used to define the age-related changes that occur in the immune system.

Interleukins

Group of cytokines that are involved in the modulation of the inflammatory immune response.

Metagenomics

The use of high-throughput molecular sequencing to study the genetics of microorganisms recovered directly from human (or environmental) samples.

Microbiota

The collective name for the total number of organisms that colonise the human body.

Multilocus strain typing (MLST)

Typing technique that characterises strains based on nucleotide sequences of housekeeping gene fragments.

Multilocus variable number tandem repeat analysis (MLVA)

PCR is used to detect variation in the number of tandem repeated DNA sequences that exist at different loci within a bacterial genome. This produces a unique MLVA profile that can easily be compared between laboratories via central databases. The technique is useful to investigate outbreaks of CDI.

Mucins

A family of proteins that are responsible for gel formation that includes mucus.

Nucleic Acid Amplification Technique (NAAT)

This technique detects the presence of TcdA and TcdB genes. PCR is a type of NAAT.

PaLoc

Open reading frame that contains genes involved in the expression of TcdA and TcdB.

Placebo

An inactive agent designed to balance the perceived effect of an intervention.

Polymerase chain reaction (PCR)

A molecular technique that amplifies DNA by using specific primers and the enzyme DNA polymerase to produce millions of copies of a DNA sequence.

Pulse field gel electrophoresis (PFGE)

Restriction enzymes are used to splice a bacterial genome into large fragments of DNA that are then transferred to a polyacrylamide gel. An alternating current separates fragments based on their molecular size in to multiple bands that represent the entire chromosome.

Restriction endonuclease analysis (REA)

This technique is similar to PFGE but involves more frequent cutting of the genome that results in the generation of hundreds of fragments that can be difficult to interpret. This is further confounded by the presence of extra-chromosomal DNA.

Serious adverse event

This refers to any event during a clinical trial that is considered to be life-threatening, results in disability or permanent injury, hospital readmission, prolonged stay in hospital or death.

S-Layer

A paracrystalline outer surface-array that surrounds some species of bacteria.

S-Layer Cassette

This contains the genes that encode *C. difficile* surface layer proteins, the adhesin Cwp66 and a secretory enzyme.

Stratified randomisation

The method of grouping individuals at the start of a clinical trial based on clinical features that may alter outcome risk.

T Cell

A lymphocyte of the immune system with principle functions in adaptive immunity including cytokine production, cytotoxic activity and regulatory function.

Toll like receptor

Immune receptors that are able to detect conserved molecular patterns on pathogens and activate the innate immune response.

Toxinotype

The name given to groups of *C. difficile* strains with identical changes in their PaLoc. Toxinotypes form the basis of one typing method in CDI and currently 31 different toxinotypes exist.

T Regulatory Cell

Subset of T cell with regulatory and suppressive function falling into the categories of naturally-occurring and inducible regulatory T cells.

# Chapter 7: Appendices

## 7.1 Publications

Papers

- Islam J, Cohen J, Rajkumar C, Llewelyn MJ. Probiotics for the prevention and treatment of *Clostridium difficile* in elderly patients. Age Ageing. 2012 Nov; 41(6):706-11.

- Islam J, Taylor AL, Rao K, Huffnagle G, Young VB, Rajkumar C, Cohen J, Papatheodorou P, Aronoff DM, and Llewelyn MJ. Role of the Humoral Immune Response to *Clostridium difficile* Toxins A and B in Susceptibility to *Clostridium difficile Infection*: A Case-control Study. Submitted to Anaerobe.

- Islam J, Cheek E, Navani V, Rajkumar C, Cohen J and Llewelyn MJ. Influence of cohorting patients with *Clostridium difficile* infection on risk of symptomatic recurrence. Accepted May 2013 J. Hosp. Infect.

Oral presentations

- Antibiotic associated diarrhea: Are probiotics the answer? Anaerobe Society of the Americas, San Francisco, California, USA (June 2012).

- The role of probiotics in preventing antibiotic associated diarrhea. British Geriatric Society Annual Conference, Brighton, UK (November 2010)

Poster presentations

- Predicting recurrence following a first episode of *Clostridium difficle* infection. Infectious Disease Society of America ID:Week, San Diego, California, USA (November 2012)

- Development of an ELISA for the detection of antibodies to Toxin B in Clostridium difficile infection. Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Illinois, USA (September 2011). Awarded an ICAAC Fellowship.

## 7.2 Probiotic NU278 Exclusion Criteria

- Patients on PEG or NG feed
- Diarrhoea on admission or within the preceding week
- Severe life-threatening illness
- Subjects with allergy or hypersensitivity to any component of the study product (e.g. allergy or hypersensitivity to milk proteins)
- Subjects who have had any surgery in the last four weeks
- Subjects enrolled in another clinical study in the last four weeks
- Subjects presenting with a severe evolving or active pathology or infection of the gastrointestinal tract such as: inflammatory bowel disease, diverticular disease, biliary tract disease or liver cirrhosis
- Any clinical condition affecting the pancreas including acute and chronic pancreatitis
- Patients who have had a surgical operation on their bowels in the preceding three months
- A medical condition such that the life expectancy of the patient is predicted at less than three months by the admitting consultant and validated by a member of the trial team
- Immuno-suppressed patients (e.g. HIV) and patients on cytotoxic drugs
- Steroid use of prednisolone greater than 10mg a day (or equivalent of dexamethasone) continuously for greater than two weeks prior to entering the trial
- Post-transplant patients
- Patients with prosthetic heart valves or a history of endocarditis
- Patients who have consumed probiotic drinks containing live organisms or over the counter probiotic preparations daily for the past seven days
- Foreign travel in the last seven days

## 7.3 Barthel Index

| **MOBILITY** | | **BOWELS** | |
|---|---|---|---|
| Immobile | 0 | Incontinent | 0 |
| Wheelchair independent | 1 | Occasional accident (Once week) | 1 |
| Walks with 1 person | 2 | Continent | 2 |
| Independent +/- aid | 3 | | |
| **CHAIR / BED TRANSFER** | | **BLADDER** | |
| Unable / needs hoist | 0 | Incontinent / catheterised | 0 |
| Major help 1-2 | 1 | Occasional accident (once day) | 1 |
| Minor help | 2 | Continent / able to use device if necessary | 2 |
| Independent | 3 | | |
| **FEEDING** | | **TOILET USE** | |
| Dependent: Needs to be fed | 0 | Dependent, unable to manage without major help | 0 |
| Needs help e.g.cutting up food | 1 | Needs some help | 1 |
| Independent | 2 | Independent | 2 |
| **GROOMING** | | **DRESSING** | |
| Dependent needs some help | 0 | Dependent | 0 |
| Independent | 1 | Needs help e.g.with buttons, zips | 1 |
| | | Independent | 2 |
| **BATHING** | | **STAIRS** | |
| Dependent | 0 | Unable | 0 |
| Independent | 1 | Needs Help | 1 |
| | | Independent | 2 |

## 7.4 Short form 12 (SF-12)

# Your Health and Well-Being

**This survey asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities.**
***Thank you for completing this survey!***

**For each of the following questions, please tick the one box that best describes your answer.**

**1. In general, would you say your health is:**

| Excellent | Very good | Good | Fair | Poor |
|---|---|---|---|---|
| ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |

**2. The following questions are about activities you might do during a typical day. Does <u>your health now limit you</u> in these activities? If so, how much?**

| | Yes, limited a lot | Yes, limited a little | No, not limited at all |
|---|---|---|---|
| a Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf | ☐ 1 | ☐ 2 | ☐ 3 |
| b Climbing several flights of stairs | ☐ 1 | ☐ 2 | ☐ 3 |

**3. During the <u>past 4 weeks</u>, how much of the time have you had any of the following problems with your work or other regular daily activities <u>as a result of your physical health</u>?**

| | All of the time | Most of the time | Some of the time | A little of the time | None of the time |
|---|---|---|---|---|---|
| a <u>Accomplished less</u> than you would like | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |
| b Were limited in the <u>kind</u> of work or other activities | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |

**4. During the <u>past 4 weeks</u>, how much of the time have you had any of the following problems with your work or other regular daily activities <u>as a result of any emotional problems</u> (such as feeling depressed or anxious)?**

| | All of the time | Most of the time | Some of the time | A little of the time | None of the time |
|---|---|---|---|---|---|
| a <u>Accomplished less</u> than you would like | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |
| b Did work or other activities <u>less carefully than usual</u> | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |

**5. During the <u>past 4 weeks</u>, how much did pain interfere with your normal work (including both work outside the home and housework)?**

| Not at all | A little bit | Moderately | Quite a bit | Extremely |
|---|---|---|---|---|
| ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |

6. **These questions are about how you feel and how things have been with you <u>during the past 4 weeks</u>. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the <u>past 4 weeks</u>…**

| | All of the time | Most of the time | Some of the time | A little of the time | None of the time |
|---|---|---|---|---|---|
| a Have you felt calm and peaceful? | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |
| b Did you have a lot of energy? | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |
| c Have you felt downhearted and low? | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |

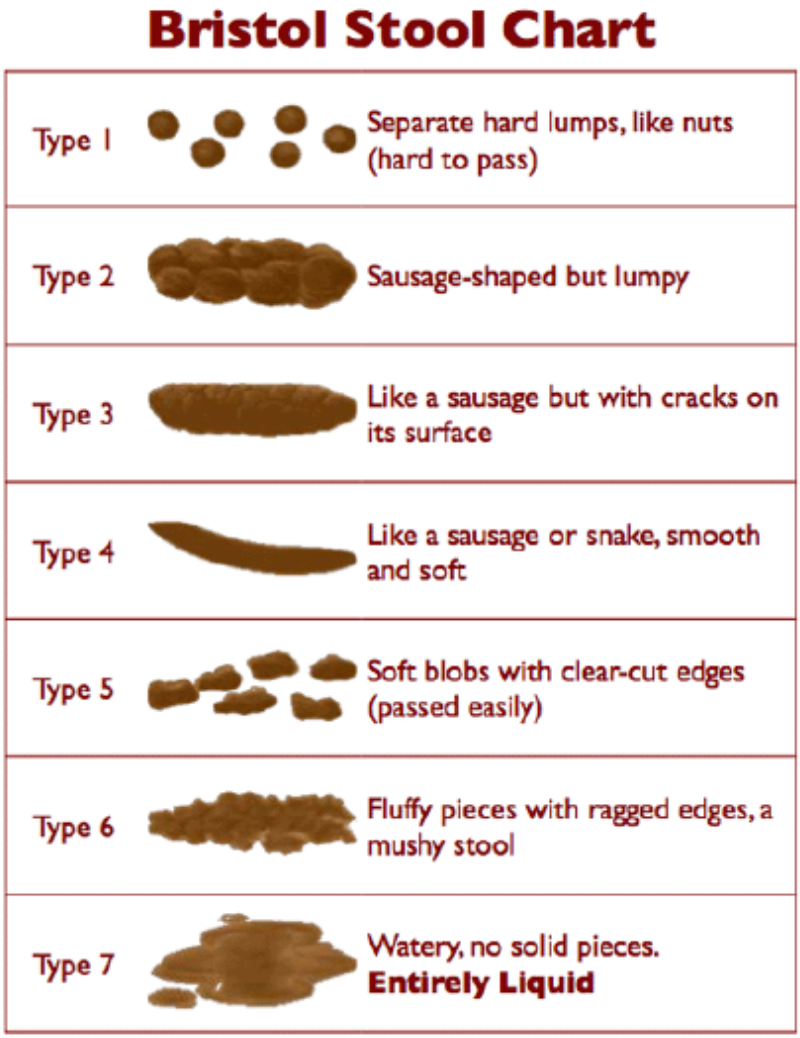
7. **During the <u>past 4 weeks</u>, how much of the time has your <u>physical health or emotional problems</u> interfered with your social activities (like visiting with friends, relatives, etc.)?**

| All of the time | Most of the time | Some of the time | A little of the time | None of the time |
|---|---|---|---|---|
| ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |

***Thank you for completing these questions!***

## 7.5 Bristol stool chart

The Bristol Stool Chart or Bristol Stool Scale is a medical aid designed to classify faeces into seven groups. It was developed by K. W. Heaton and S. J. Lewis at the University of Bristol and was first published in the Scandinavian Journal of Gastroenterology in 1997.1 The form of the stool depends on the time it spends in the colon.



## 7.6 Microbiology SOP

### SOP for receipt and processing of faecal specimens for Probiotic NU278 Study

### Introduction

Most of the procedures used for culture, identification and sensitivity testing of organisms, disposal of waste etc are performed in accordance with Barts and the London, NHS Trust. Microbiology SOPs. These SOPs are available in Room 304 and on the Q drive.

This SOP contains information which is specific to this study.

### Health & Safety

All work involving the handling of specimens is performed in a Class 1 safety cabinet. Protective coats and gloves are worn when handling specimens and faecal organisms.

### Methods

1. Receipt of specimens

Faecal samples are collected by recruiting centres and sent to the central Microbiology Laboratory at the Royal London Hospital by City Sprint Couriers.

Upon receipt, the date and time of receipt is recorded in the diary in Rm 304 and stored in the -80 freezer

2. Sample processing:

2.1 Samples are removed from the -80˚C freezer and thawed at room temperature by leaving in the Class 1 cabinet for approximately 15 minutes.

2.2 The weight of the sample is determined by weighing an empty plastic universal bottle using the Mettler PM 600 balance, 'taring' the balance and then weighing the bijou containing the sample to determine the weight of the contents.

2.3 This is then diluted 1:10 (w/v) in a cryopreservative broth (Brain heart infusion broth (Oxoid) containing 10% glycerol (w/v) (Sigma Aldrich) + 6 sterile glass balls (3.5 to 4.5mm diameter, VWR), mixed by vortexing for 10 seconds and processed as outlined below.

2.4 Example of weight calculation e.g weight of faecal sample is 0.35g and then the volume of liquid to be added is 3.5 ml minus 0.35ml =3.25 ml, giving a 1:10 dilution.

2.5 They are then vortexed briefly and further tenfold serial dilutions made in Brain Heart Infusion broth (Oxoid) by adding 100ul of sample to 900ul of BHI in a sterile Eppendorf microtube (1.5ml) from $10^{-1}$ to $10^{-6}$

2.6 100 ul of the -1, -3 and -6 dilutions are inoculated onto the surface of the following media:

- Saborauds Agar for the detection of *Candida albicans*, incubate for 2 days at 32˚C
- 5% NaCl agar ( Oxoid) for the detection of staphylococci, incubate overnight at 37˚C
- MacConkey agar (Oxoid) for detection of *Klebsiella*, incubated at 37°C aerobically for 24-48 hours in aerobic conditions.

**3. Detection of *C. difficile***

An aliquot of the 1/10 dilution of the faeces is mixed with an equal volume of alcohol and left for 30 minutes, then subcultured onto CCFA (E &O) and incubated for 48hours anaerobically at 37˚C.

**4. Examination of culture plates**

4.1 After incubation for the times and under the conditions described above, plates are removed from the incubator and different colony types enumerated.

4.2 Different colony types may be Gram stained or identified directly by MALDI-TOF using the laboratories SOP for MALDI-TOF.

4.3 All organisms will be put through the MALDI-TOF for identification; this has been extensively validated for these organisms.

4.4 Any *C difficile* obtained are inoculated into RCM (Southern Group Laboratories), incubated for 48h at 37˚C and tested for toxin production by testing the supernatant using the Alere QuickCheck C diff toxin LFD card.

5. Recording of microbiology results

5.1 Fill in the worksheet and file

**6. Storage of plates**

After processing faecal plates will be kept in appropriately labelled containers at >4°C for one week. At the end of the week plates will be put into autoclave bags and autoclaved prior to incineration.

**7. Storage of isolates from specimens**

Isolates of *C. albicans, K.oxytoca, S. aureus* and *C difficile* will be emulsified into microbank storage vials (Prolabs) broth and stored in Freezer 3 at -80°C.

**8. Balance calibration.**

The balance is serviced annually under a service contract. Every time the balance is used it should be checked. This may conveniently be done by weighing a sterile universal container or bijou bottle. The weight of an RBI bijou bottle is 4.6g +/- 0.5g. A Labplex Universal container should weigh 10.5g +/- 0.5g.

**9. Gilson pipettes**

Pipettes are serviced and calibrated annually under a service contract.

**10. New Brunswick U410 -80 freezer**

This is connected to an alarm system. Outside normal laboratory working hours, the alarm will signal the on call BMS who will take appropriate action.

**MW Feb 2012**

## 7.7 Quality control for testing viable counts of NU278 study product

**Method**

a. Label six 1.5ml sterile microtubes, from -1 to -6.

b. Add 900µl of sterile pre-reduced BHI (Oxoid) to each tube.

c. Perform serial dilutions by adding 100ul from the bijou bottle containing the yoghurt contents to tube -1, mix by pipetting and vortex (10 secs). Change pipette tips between dilutions. Carry on the dilutions to -6.

d. Plate out the –4 to -6 dilutions by spreading 100µl volumes onto the surface of the following media:

M17 : for Streptococcus thermophilus

MRS-A : for Lactobacillus bulgaricus

MRS-N : for Lactobacillus casei DN114001

Plate out the -1 and -2 dilutions on MacConkey and Blood agar to detect other contaminating bacteria. Standard methods were used for the preparation of the media.

e. Spread the liquid using a sterile plastic spreader and incubate MRS agar for 48hours in an anaerobic chamber at 37˚C. M17 plates should be incubated at 44˚C for four days. Blood agar plates and MacConkey agar are incubated for 48hours anaerobically.

f. Remove the plates from the chamber and count the number of colonies.

g. Colonies are identified by MALDI-TOF using the Barts and the London NHS Trust SOP.

h. Multiply the number of colonies from a plate that has between 20 and 200 colonies by the dilution factor and x10 to allow for the volume of 100 µl sample plated out and record the total number of viable bacteria in the original sample.

i. The detection of any bacteria other than those listed and any deviations in counts of from the range listed (6.0 $\log^{10}$cfu/ml of *S. thermophilus* and *L. bulgaricus* and 8.0 $\log^{10}$cfu/ml of *L. casei* DN114001) should be reported at once to the Trial Centre at the Royal Sussex County Hospital, Brighton.

**MW Feb 2012**

# Chapter 8: References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