Evaluation of the host response to *Clostridium difficile* infection

Thesis submitted in accordance with the requirements of

the University of Liverpool for the degree of

Doctor in Philosophy

by

Andrew Swale

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Declaration

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

Andrew Swale

This research was carried out in the Department of Molecular and Clinical Pharmacology, in the Institute of Translational Medicine, at the University of Liverpool.

Contents

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Finally, I would like to dedicate this thesis to my Mum, my Dad and my sister Emma: I hope I've made you proud.

Abstract

Despite several interventional measures, *Clostridium difficile* infection (CDI) continues to be a major problem for healthcare services worldwide. Clinical classification of patients at initial disease presentation is very challenging which makes it complex to accurately predict who will respond favourably to the treatment or have adverse outcomes such as recurrence. This thesis is based upon work undertaken on a prospective CDI cohort, which was the preferred study design, as it allowed for careful assessment of both clinical and biological factors.

In order to identify clinical risk predictors for poor CDI outcomes, such as mortality and recurrence, clinical and laboratory variables were analysed, and predictive models derived. Although some similarities were identified in the risk factors in our cohort when compared with previous published studies, overall, the potential for external replication was poor, indicating that many of the models had internal validity, but little external validity. We also attempted to assess clinical prediction rules, and applied to our dataset. Again, it was not possible to replicate the findings of the prediction rules. Most studies, including ours, are small with less than 500 evaluated patients, which may be the major factor in limiting their generalisability. Future studies need to focus on much larger cohorts.

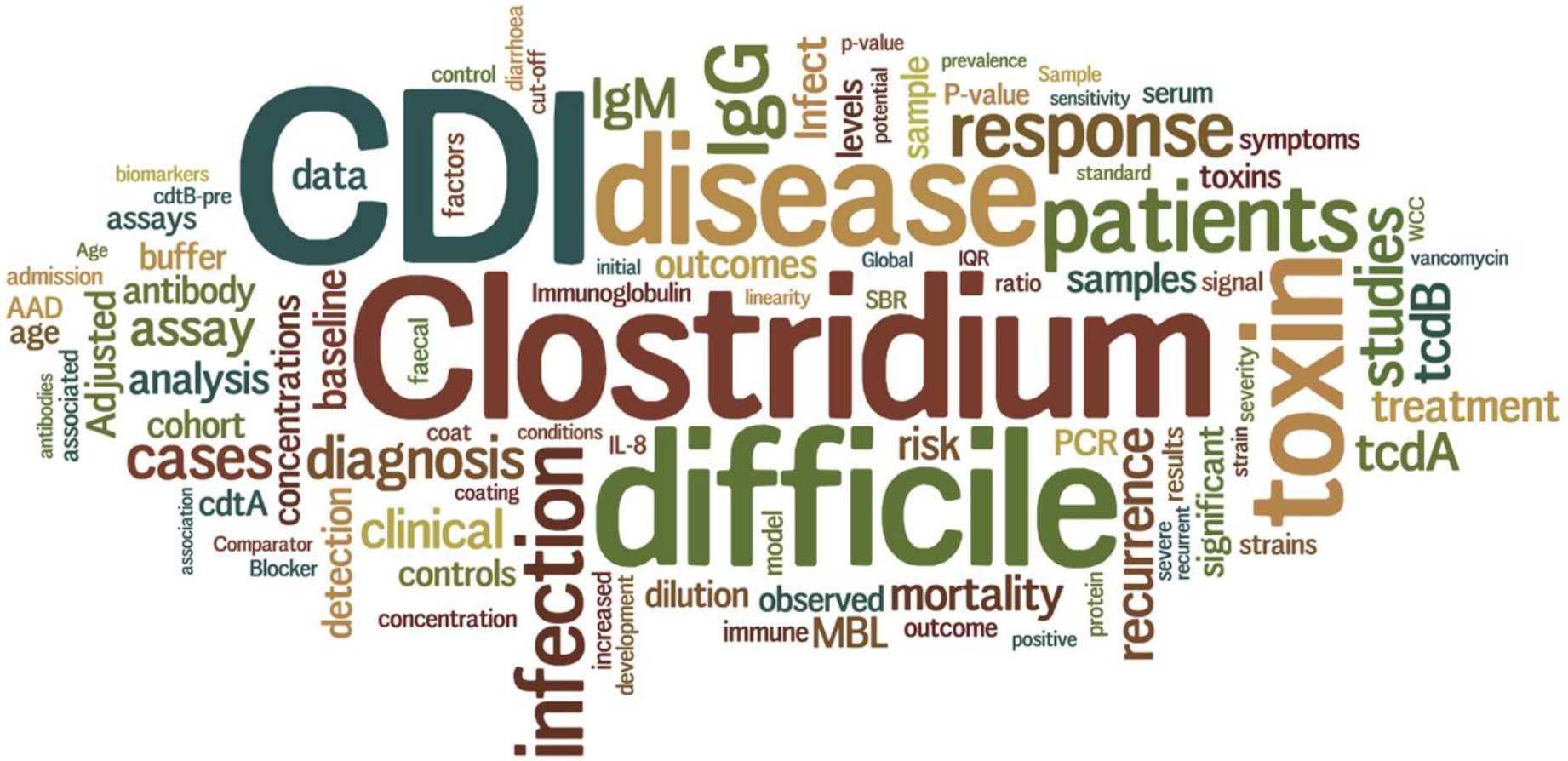
The genetic polymorphism rs4073/-251T>A in the pro-inflammatory IL-8 gene has previously been reported to predispose to CDI. We were unable to replicate these findings using both a discovery cohort (286 CDI cases versus 135 AAD controls; p=0.84) and a replication cohort (100 CDI cases versus 170 healthy controls; p=0.87), and no association was found upon meta-analysis with the original study data (OR, 1.72; 95% CI, 0.63-4.71). We also failed to replicate previous findings of a significant association between faecal IL-8 concentration and IL-8 rs4073 genotype in a sub-set of our CDI patients (p=0.28). These findings suggest that this polymorphism is unlikely to constitute a major risk factor for CDI.

Faecal calprotectin and faecal lactoferrin have been used as biomarkers in inflammatory bowel disease. We analysed these biomarkers in CDI cases compared with a group of diarrhoea control inpatients. There was a significant difference between cases and controls ($p<0.0001$; ROC>0.85), but there was no association with CDI clinical outcomes, including severity, recurrence, and length of stay, suggesting a limited applicability of both faecal biomarkers for disease stratification.

An effective CDI vaccine would constitute an important breakthrough for tackling the disease, but progress in this area has been hampered in part due to the lack of reliable methods for quantitating toxin-specific immune-mediated responses. We have developed novel and enhanced assays to measure immune response to the major *C. difficile* toxin epitopes (tcdA, tcdB, cdtA and cdtB). Whilst lower anti-tcdA and anti-tcdB IgG titres correlated with severe disease at baseline ($p<0.01$ and $p=0.04$), lower anti-tcdB IgM titres were associated with recurrence ($p=0.04$) and decreased levels of anti-cdtB (the binding precursor of binary toxin) was linked with prolonged disease ($p=0.01$). Nonetheless, our overall findings did not confirm previous associations with disease recurrence, mortality or prolonged disease, which is probably related to the fact that we did not have access to longitudinal samples.

The role of mannose binding lectin (MBL), a lectin protein whose deficiency has been linked with several acute infections, was investigated in CDI due to its immunomodulatory properties and association with inflammation and innate immunity. We demonstrated that MBL concentration, but not genotype, was a significant predictor of 90-day CDI recurrence at both <50 ng/ml (OR=3.18, $P<0.001$) and <100 ng/ml (OR=2.61, $P<0.001$). MBL seems to acts as an immunomodulator of CDI disease course, but not as a predisposing factor.

In conclusion, the work in this thesis has focused on clinical and biological factors associated with differing clinical outcomes in patients with CDI. Further work is needed to define host factors that modulate disease severity, and how they interact with the bacterium, in order to better understand the pathogenesis of disease, allow for stratification of treatment and improve clinical outcomes.


CDI
Clostridium
difficile
disease
infection
toxin
response
patients
data
factors
IgM
IgG
Infect
levels
sample
p-value
prevalence
Sample
P-value
sensitivity
serum
symptoms
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Publications and communications

Published papers

Swale A[#], Miyajima F[#], Kolamunnage-Dona R, Roberts P, Little M, Beeching NJ, Beadsworth MB, Liloglou T, Pirmohamed M. (2014). SERUM MANNOSE-BINDING LECTIN CONCENTRATION, BUT NOT GENOTYPE, IS ASSOCIATED WITH CLOSTRIDIUM DIFFICILE INFECTION RECURRENCE: A PROSPECTIVE COHORT STUDY. Clin Infect Dis, 59(10):1429-36

Swale A[#], Miyajima F[#], Roberts P, Hall A, Little M, Beadsworth MB, Beeching NJ, Kolamunnage-Dona R, Parry CM, Pirmohamed M. (2014). CALPROTECTIN AND LACTOFERRIN FAECAL LEVELS IN PATIENTS WITH CLOSTRIDIUM DIFFICILE INFECTION (CDI): A PROSPECTIVE COHORT STUDY. PLoS One, 9(8): e106118

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Miyajima F, Roberts P, **Swale A**, Price V, Jones M, Horan M, Beeching N, Brazier J, Parry C, Pendleton N, Pirmohamed M. (2011). CHARACTERISATION AND CARRIAGE RATIO OF CLOSTRIDIUM DIFFICILE STRAINS ISOLATED FROM A COMMUNITY-DWELLING ELDERLY POPULATION IN THE UNITED KINGDOM. PLoS One, 6(8):e22804

Manuscripts in preparation

Miyajima F[#], **Swale A**[#], Roberts P, Little M, Beadsworth MB, Beeching NJ, Kolamunnage-Dona R, Parry CM, Pirmohamed M. (2014). PREDICTING POOR DISEASE OUTCOMES IN A PROSPECTIVE COHORT OF CDI PATIENTS.

Poster presentations

Swale A[#], Miyajima F[#], Roberts P, Price V, Little M, Kolamunnage-Dona R, Parry CM, Pirmohamed M.: ASSOCIATION OF FAECAL LACTOFERRIN AND CALPROTECTIN IN A PROSPECTIVE CLOSTRIDIUM DIFFICILE (CDI) COHORT. ClostPath 2013, 22-26 October 2014, Palm Cove, Australia

Miyajima F, **Swale A**, Little M, Kolamunnage-Dona R, Schwan C, Shone C, Papatheodorou P, Pirmohamed M.: TOWARDS A HOST BIOMARKER PROFILING STRATEGY FOR CLOSTRIDIUM DIFFICILE INFECTION (CDI). ClostPath 2013, 22-26 October 2014, Palm Cove, Australia

Swale A, Miyajima F, Schreiber F, Little M, Dougan G, Parry CM, Lawley T, Pirmohamed M.: STRATEGY FOR THE IDENTIFICATION OF HOST FACTORS ASSOCIATED WITH CLOSTRIDIUM DIFFICILE INFECTION. 5TH NIHR annual trainee meeting, 19-20 September 2011, Leeds, UK

Swale A, Miyajima F, Roberts P, Farragher T, Little M, Beeching NJ, Parry CM, Pirmohamed M.: CLOSTRIDIUM DIFFICILE INFECTION (CDI) – INVESTIGATION OF RISK FACTORS ASSOCIATED WITH DISEASE ACTIVITY, DURATION AND SEVERITY IN A LARGE PROSPECTIVE COHORT. 1st NIHR Biomedical Research Centre Experimental Medicine Summer School, 17-18 June 2010, Berkhamsted, UK [**Awarded 'Highly commended poster'**]

Abbreviations

AAD	Antibiotic-associated diarrhea
ACG	American College of Gastroenterology
AUC	Area under the curve
Blocker 1	PBST + 1% BSA + 1% Milk powder
BMI	Body mass index
bp	Base pairs
BSA	Bovine serum albumin
CA-CDI	Community-acquired *Clostridium difficile* infection
Casein Blocker	1x Tris Buffered Saline with 1% Casein
CCI	Charlson Comorbidity Index
CCNA	Cell cytotoxicity neutralisation assay
CD	Compact disc
C. diff	*Clostridium difficile*
CDI	*Clostridium difficile* infection
CDRN	*Clostridium difficile* Ribotyping Network
CDT	*Clostridium difficile* transferase, or 'binary toxin'
cdtA	Binary toxin A
cdtA+B-act	*Clostridium difficile* binary toxin A plus active *Clostridium difficile* binary toxin B
cdtB	Binary toxin B
cdtB-act	Active *Clostridium difficile* binary toxin B
cdtB-pre	*Clostridium difficile* binary toxin B component - precursor
CdtLoc	Binary toxin locus
COPD	Chronic obstructive pulmonary disease
CPR	Clinical prediction rule
CRP	C-reactive protein
CT	Computed tomography
ECL	Electrochemiluminescence
eGFR	Estimated glomerular filtration rate
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
FBS	Foetal bovine serum
Fc	Fragment, crystallisable
FcR	Fc Receptor
FDA	Food and Drug Administration
FMT	Faecal microbiota transplantation
FP	Fluorescent polarisation
GDH	Glutamate dehydrogenase
GI	Gastrointestinal

GoF	Goodness-of-fit
GWAS	Genome-wide association study
HA-CDI	Hospital-acquired *Clostridium difficile* infection
Hb	Haemoglobin
HDU	High dependency unit
HIV	Human immunodeficiency virus
HRL	Hybridoma Reagent Laboratory
HRP	Horseradish peroxidase
HWE	Hardy-Weinburg equilibrium
IBD	Inflammatory bowel disease
ICU	Intensive care unit
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-23	Interleukin-23
IL-8	Interleukin-8
IVD	*In vitro* diagnostic
IVIG	Intravenous immunoglobulin
LCT	Large clostridial toxin
LD	Linkage disequilibrium
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LOO	Leave-one-out
LSR	Lipolysis-stimulated lipoprotein receptor
MAbs	Monoclonal antibodies
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MBL	Mannose-binding lectin
MIC	Minimum inhibitory concentration
MK2	MAPK-activated protein kinase-2
MLST	Multi-locus sequence typing
MMD	Minimum detectable dose
mMLVA	Modified multiple-locus variable number tandem repeat analysis
MSD	Meso Scale Discovery
NAAT	Nucleic acid amplification test
NICE	UK National Institute for Health and Care Excellence
NIH	National Institutes of Health
NPV	Negative predictive value
PaLoc	Pathogenicity locus
PBS	Phosphate buffer solution
PBST	Phosphate buffer solution + 0.05% Tween20
PBST-coat	Phosphate buffer solution + 0.1% Tween20
PCT	Procalcitonin

PE	Phycoerythrin
PFGE	Pulse field gel electrophoresis
PMC	Pseudomembranous colitis
PPI	Proton pump inhibitor
PPV	Positive predictive value
QC	Quality control
RCT	Randomised controlled trial
REA	Restriction endonuclease analysis
RIA	Radio immunoassay
RLBUHT	Royal Liverpool & Broadgreen University Hospitals Trust
ROC	Receiver operating characteristic
SBR	Signal-to-background ratio
SDS	Sodium dodecyl sulfate
S-layer	Surface layer
SLP	Surface layer protein
SNP	Single nucleotide polymorphism
TC	Toxigenic culture
tcdA	*Clostridium difficile* toxin A
tcdB	*Clostridium difficile* toxin B
TMB	3,3′,5,5′-Tetramethylbenzidine
TPA	Tripropylamine
UK	United Kingdom
US	United States
VRE	Vanomycin-resistant enterococci
WCC	White cell count
WGS	Whole genome sequencing
WTH	Wirral University Teaching Hospital

Chapter 1

General Introduction

Contents

1.1 Overview

The anaerobic, Gram-positive, spore-forming bacillus *Clostridium difficile* (*C. diff*) was first identified in 1935 as a component of the faecal microflora of healthy newborns. So named due to the difficulty involved in its isolation and culturing, it was not until the 1970s that a link was established between the microorganism, antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (PMC) (Bartlett *et al*., 1978; Bartlett *et al*., 1977; Larson *et al*., 1977).

Three decades later and *Clostridium difficile* infection (CDI) is now regarded as the major cause of PMC and accounts for 15-39% of all cases of AAD (Dubberke and Wertheimer, 2009; McFarland, 2009; Viswanathan *et al*., 2010). The dramatic increase in incidence and severity observed across both healthcare and community settings has largely been attributed to the emergence of hypervirulent strains of *C. diff*, and is associated with increased hospitalisation times, costs, morbidity, and mortality among patients (Dubberke *et al*., 2008; Kuijper *et al*., 2006).

1.2 Clinical disease

Asymptomatic colonisation by *C. diff* is not frequent amongst healthy adults, varying between 1.6-4% (Miyajima *et al*., 2011; Rea *et al*., 2012). However, disruption of gut flora by drugs or pathological processes may lead to a dysbiotic status, which in turn facilitates the establishment of *C. diff*. This significantly predisposes patients to progress to CDI, and it is widely recognised that the main at-risk group are the hospitalised elderly receiving antibiotic therapy (Bassetti *et al*., 2012).

Symptomatic patients exhibit a broad range of clinical manifestations, from mild, watery diarrhoea to life-threatening fulminant PMC that can lead to severe complications, including toxic megacolon, septic shock and death (Rupnik *et al*., 2009). Recently updated guidelines from Public Health England (Public Health England, 2013) categorised CDI individuals using the following definitions: -

- **Mild CDI:** Typically associated with <3 stools of type 5–7 on the Bristol Stool Chart (Lewis and Heaton, 1997) per day; not associated with a raised white cell count (WCC)
- **Moderate CDI:** Typically associated with 3–5 stools per day and a raised WCC that is <15 $x10^9$/L
- **Severe CDI:** Associated with a WCC >15 $x10^9$/L, or an acutely rising serum creatinine (i.e. >50% increase above baseline), or a temperature of >38.5°C, or evidence of severe colitis (abdominal or radiological signs). The number of stools may be a less reliable indicator of severity.
- **Life-threatening CDI:** Includes hypotension, partial or complete ileus or toxic megacolon, or computed tomography (CT) evidence of severe disease

One of the most challenging aspects of CDI concerns the recurrence of disease after apparent completion of successful initial therapy (Barbut *et al*., 2000; Johnson, 2009). Reported recurrence rates have been extremely variable ranging between 5-47% depending on the clinical definition and evaluation period employed (Aslam *et al*., 2005; Cocanour, 2011; Cohen *et al*., 2010; van Nispen tot Pannerden *et al*., 2011). It is generally defined as the presence of another CDI episode within a given period, typically 4-12 weeks following the onset of the previous episode (Bauer *et al*., 2011; D'Agostino *et al*., 2014; Kyne *et al*., 2001). It can be linked with either a relapse with the initial infecting strain or a re-infection with a new strain (Barbut *et al*., 2000; Johnson *et al*., 1989; O'Neill *et al*., 1991; Wilcox and Spencer, 1992), and both have the potential to affect clinical care and management.

1.3 Pathogenesis

Infection usually arises in susceptible individuals through the ingestion of environmental spores, shed by both infected and asymptomatic individuals (Lawley *et al*., 2009; McFarland *et al*., 1989; Shaughnessy *et al*., 2011). Hence the logical strategy is to target spore-mediated transmission, but the spores are highly resistant to desiccation, chemicals and extreme temperatures. Further to this, they can potentially persist for months or even years, as opposed to the

pathogen in its vegetative state, which perishes rapidly once subject to aerobic conditions. After resisting the acidity of the stomach, the spores pass through to the small intestine where favourable conditions including activators present in the bile and gastric juice lead to their germination into the vegetative form. Disruption of the normal intestinal microbiota or 'gut flora', typically by exposure to antimicrobial agents (see Figure 1.1), allows the vegetative cells to further invade the mucus layer, thus adhering to the surface of epithelial cells and establishing themselves in the gut (Karjalainen *et al.*, 1994).

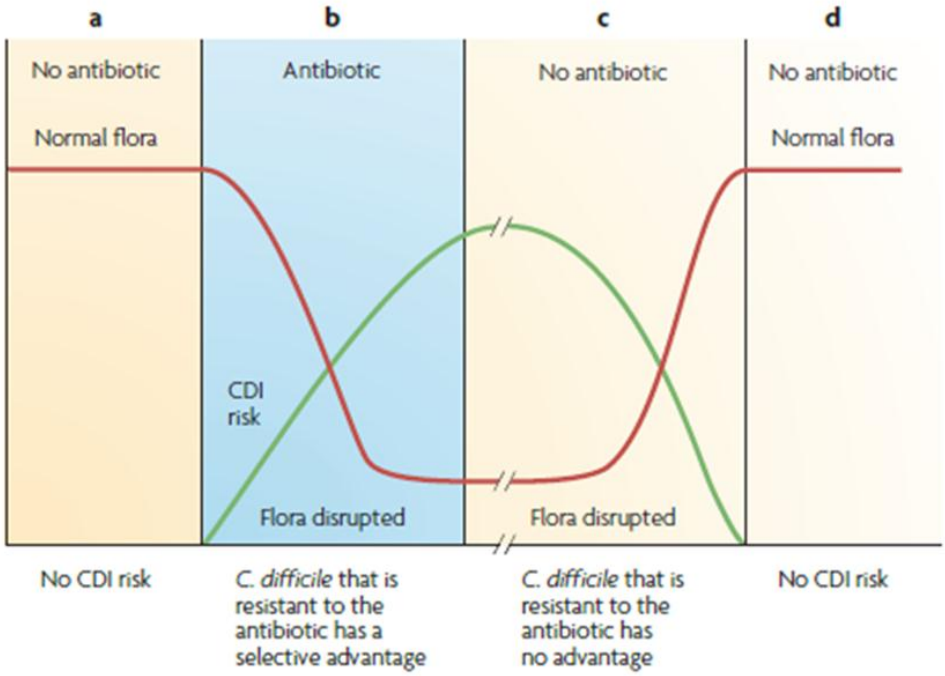


Figure 1.1 – The effect of antibiotics on the normal gut flora and the risk of *Clostridium difficile* infection (CDI) (taken from (Rupnik *et al.*, 2009)

*Patients are resistant to CDI if the diversity of the gut flora is not disrupted by antibiotics (**a**). Once antibiotic therapy starts, infection by C. diff strains resistant to the antibiotic is greatly maximised (**b**). When the antibiotic therapy ceases, the levels of the antibiotic in the gut diminish rapidly, but the microflora remains in a dysbiotic state for a variable period of time (indicated by the break in the graph), depending on the antibiotic given (**c**). During this time, patients can be infected with either resistant or susceptible C. diff strains. Finally, after the microflora recovers, colonisation resistance to C. diff is restored (**d**).*

Vegetative cells subsequently produce and secrete a number of virulence factors, increasing as they enter the stationary phase of their growth and ultimately promoting intestinal damage and disease. It is generally accepted that CDI pathogenesis is multifactorial; it is dependent upon alterations in the gut flora, virulence factors produced by the infecting strain and host immune response and susceptibility factors (Barbut *et al.*, 2000; McFarland *et al.*, 1989). Figure 1.2 summarises the *C. diff* infection cycle.

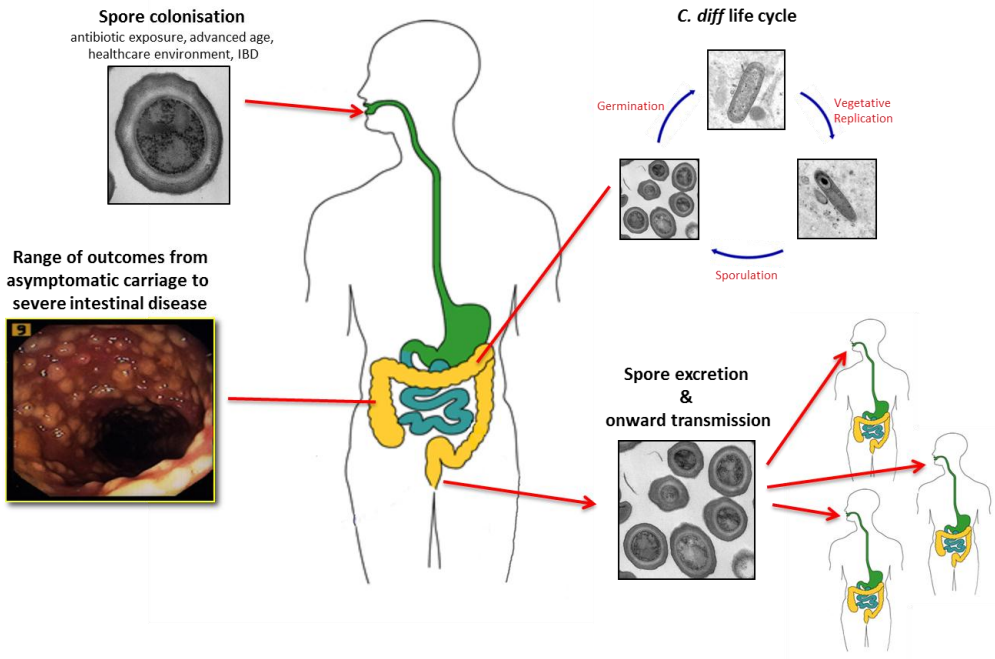


Figure 1.2 – The infection cycle of *Clostridium difficile*

Both biochemical and molecular studies have shown that the major clinical signs and symptoms of CDI can be explained largely by the actions of two high molecular weight exotoxins, the enterotoxic toxin A (tcdA) and the cytotoxic toxin B (tcdB) (Jank *et al.*, 2007; Rupnik *et al.*, 2009; Thelestam and Chaves-Olarte, 2000) (Figure 1.3a). Genetic inactivation of the *tcdA* and *tcdB* genes in a hamster model of infection has shown that they are essential for the occurrence of the disease (Kuehne *et al.*, 2010). As well as tcdA and tcdB, specific strains of

C. diff also produce a third toxin: a *C. difficile* transferase, termed 'binary toxin' (CDT) [Figure 1.3b]. It is thought that this may act synergistically with tcdA and tcdB further increasing their glucosylating potency and therefore resulting in an increase in disease severity (Barbut *et al*., 2005). More recent research has identified that GTPase-independent toxin virulence mechanisms may also be important in CDI pathogenesis (Chumbler *et al*., 2012).

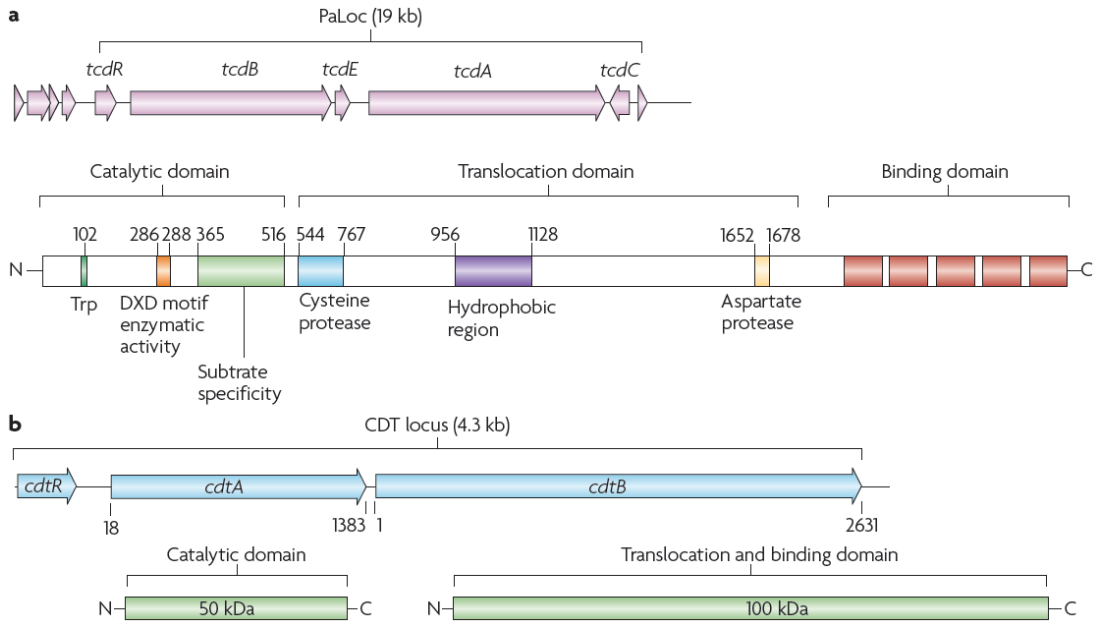


Figure 1.3 - Toxins produced by *Clostridium difficile* (taken from (Rupnik *et al*., 2009))

a: *Two large toxins, toxin A and toxin B (tcdA and tcdB), are encoded on the pathogenicity locus (PaLoc), which comprises five genes. In non-toxigenic strains, this region is replaced by a short 115 bp sequence. Both toxins are single-chain proteins, and several functional domains and motifs have been identitifed. tcdB is shown in detail below the PaLoc*

b: *A third toxin, the binary toxin or CDT, is encoded on a separate region of the chromosome (CdtLoc) and comprises three genes. The binary toxin is composed of two unlinked proteins, cdtB and cdtA. cdtB has a binding function and cdtA is the enzymatic/catalytic component.*

1.3.1. Glucosylating toxins A & B

Both tcdA and tcdB are large molecular-weight protein toxins of approximately 308 and 270 kDa, respectively (Voth and Ballard, 2005). Differing in length, with tcdA about 350 amino acids longer than tcdB, both belong to a group of large clostridial toxins (LCTs) related to *Clostridium sordellii*, *Clostridium novyi* and *Clostridium perfringens* (Amimoto *et al*., 2007; von Eichel-Streiber *et al*., 1996). LCTs are single-chain proteins consisting of three main functional domains: an amino-terminal binding domain with characteristic tandem repeats, a carboxy-terminal catalytic domain and a putative translocation domain (von Eichel-Streiber *et al*., 1996), with structural studies having further elucidated the role of several other functional motifs (Jank *et al*., 2007) (Figure 1.3a).

The binding domain interacts with putative cell-surface carbohydrate receptors allowing the toxins to bind to host cells. Seven potential carbohydrate binding sites have been proposed for tcdA (Greco *et al*., 2006; Ho *et al*., 2005). In animals the tcdA receptor carries the trisaccharide Gal1(α1–3)Gal(β1–4) GlcNac (Krivan *et al*., 1986) but this is probably not present in humans where the glycoprotein gp96 is thought to act as a co-receptor (Na *et al*., 2008). No receptor has yet been identified for tcdB, which has been shown to bind more effectively to the basolateral side of the host cell as opposed to the apical site favoured by tcdA (Thelestam and Chaves-Olarte, 2000).

After cell binding and subsequent endocytosis, a decrease in endosomal pH induces a conformational change in the toxin enabling the amino-terminus to insert into the endosomal membrane via the formation of a pore (Jank and Aktories, 2008). The host cell is able to initiate autocatalytic cleavage of the amino-terminal region proximal to the cysteine protease site which in turn releases the carboxy–terminal catalytic domain into the cytoplasm leaving the remainder of the toxin polypeptide attached to the membrane (Reineke *et al*., 2007). The catalytic domain is responsible for glycosylating small GTPases of the Rho and Ras families in host cells, resulting in their irreversible inactivation. This disrupts cell signaling pathways, cytoskeleton integrity and tight junctions (Reinert *et al*., 2005), resulting in decreased trans-epithelial resistance, fluid

accumulation and destruction of the intestinal epithelium (Riegler *et al*., 1995; Rupnik *et al*., 2009; Thelestam and Chaves-Olarte, 2000). In clinical terms, the complete mucosal damage is associated with severe colitis, pseudomembrane formation and the typical diarrhoea that characterises CDI (Fiorentini *et al*., 1998; Kreimeyer *et al*., 2011; Poxton *et al*., 2001; Voth and Ballard, 2005). The toxins also elicit the release of inflammatory cytokines from the intestinal epithelial cells, mast cells and macrophages, promoting an influx of inflammatory cells and fluid secretion that results in recruitment and activation of neutrophils and propagates a vicious cycle of colonic inflammation (Pothoulakis, 2000; Sebaihia *et al*., 2006; Sun *et al*., 2010).

The genes *(tcdA* and *tcdB)* encoding the respective toxins are contained within the pathogenicity locus (PaLoc), a critical region of the genome for toxin production and regulation (Lyerly *et al*., 1988; Rupnik *et al*., 2009). The PaLoc also contains the gene for a putative holin (*tcdE*) (Tan *et al*., 2001) thought to facilitate release of tcdA and tcdB, and two regulatory genes (*tcdC* & *tcdR*), which encode a negative regulator (Matamouros *et al*., 2007) and an alternative sigma factor involved in positive transcriptional regulation (Mani and Dupuy, 2001), respectively. Present at the same chromosomal integration site in all toxigenic *C. diff* strain types that have been analysed to date, the PaLoc is non-existent in non-toxigenic strains ($tcdA^-/tcdB^-$), being replaced by 115 base pair (bp) of non-coding sequence (Rupnik *et al*., 2009). Changes in the DNA sequence of the Paloc are observed across different *C. diff* strains and can be defined as different toxinotypes (Rupnik, 2008), with polymorphisms appearing more frequently in *tcdA* than in *tcdB*, especially deletions (Rupnik *et al*., 1998; van den Berg *et al*., 2004). Punctual and tandem repeat deletions in *tcdC* could lead to a lack of negative regulation and thus increased production of both tcdA and tcdB. Factors outside of the PaLoc, such as CodY (Dineen *et al*., 2007), may also be involved with the regulation of toxin synthesis.

The relative importance of tcdA and tcdB to disease pathogenesis remains unclear, with both toxins extensively studied ever since *C. diff* was confirmed as a major etiological agent of PMC and AAD.

Early research suggested a key role for tcdA over tcdB. Initial experiments employing intragastric challenge of hamsters suggested that tcdA alone displayed enterotoxic effects that resulted in haemorrhagic fluid secretion, inflammation and necrosis of intestinal tissue, compared to tcdB alone, which had no effect unless applied with a sub-active concentration of tcdA (Lyerly *et al*., 1985). It was then postulated that clinically significant disease occurs only for toxigenic strains of *C. diff* that produce both tcdA and tcdB (Bartlett, 1992). The lethal effects of tcdB were shown to depend on the initial damage to the surface of the intestinal cells caused by tcdA (Depitre *et al*., 1993; Lyerly *et al*., 1988), as opposed to the fact that tcdA did not appear to be dependent upon the action of any other virulence factors (Bartlett, 1994).

However, more recent evidence further supports an essential role for tcdB over tcdA. Indeed, since the 1990's experiments on human colonic tissue indicated that tcdB was significantly more potent than tcdA in causing mucosal necrosis and decreasing barrier function (Riegler *et al*., 1995). Furthermore, it has now been repeatedly reported that tcdA-/tcdB+ strains can consistently cause PMC and the full range of the CDI spectrum (Johnson *et al*., 2001; Kato *et al*., 1998; Limaye *et al*., 2000), with several outbreaks documented (Alfa *et al*., 2000; Drudy *et al*., 2007; Kuijper *et al*., 2001; Sato *et al*., 2004). These strains are distinguished by deletions in tcdA resulting in non-production of biologically active toxin (Sambol *et al*., 2000). Further investigation of the role of these toxins in disease pathogenesis has been facilitated by the more recent development of two systems for the genetic manipulation of *C. diff* (Heap *et al*., 2007; Lyras *et al*., 2009a), with a comparison of mutants lacking one of the toxins, which revealed that tcdA−/tcdB+ mutants retain the ability to kill hamsters, whereas tcdA+/tcdB− mutants were not virulent (Lyras *et al*., 2009b).

All naturally occurring pathogenic strains produce tcdB (but not necessarily tcdA) thus suggesting that tcdB has a more active role than was previously believed. This is further supported by evidence from clinical epidemiology studies (Loo *et al*., 2011), as well as porcine and humanised animal models (Savidge *et al*., 2003; Steele *et al*., 2013). The current impression is that both

toxins may cause severe *C. diff* colitis (Kuehne *et al*., 2010), with the relative importance of each toxin in disease pathogenesis still under intense debate.

1.3.2 Binary toxin and non-toxin antigens

In addition to tcdA and tcdB, further virulence factors have been implicated in CDI pathogenesis, including CDT binary protein and surface layer proteins (SLPs), both of which are involved in adherence to host epithelial cells and modulation of inflammatory and antibody responses (Madan and Jr, 2012; Vedantam *et al*., 2012), with evidence suggesting that *C. diff* strains that adhere better to human intestinal cell lines are more virulent in hamsters (Dingle *et al*., 2011b).

Present in 6-12% of *C. diff* clinical isolates including hypervirulent PCR ribotype 027 strains (Sundriyal *et al*., 2010), CDT belongs to the family of ADP-ribosylating toxins and is composed of two separate toxin proteins, binary toxin A (cdtA) and binary toxin B (cdtB) (Figure 1.3b). cdtB is the larger binding domain (~50kDa in its mature form), activated by serine proteases and docking to host cells via a lipolysis-stimulated lipoprotein membrane receptor (Papatheodorou *et al*., 2011), which allows translocation of the catalytic ADP-ribosyltransferase, cdtA, into the cytosol, ultimately inducing depolymerisation of the actin cytoskeleton. Genes coding for CDT are located within the binary toxin locus (CdtLoc) and are distinct to the PaLoc (Popoff *et al*., 1988), and multiplex PCR-based diagnostic tests are now able to individually detect the presence of both loci.

Until recently, the extent to which CDT was contributing to *C. diff* pathogenicity was largely unclear. Although CDT is cytotoxic in tissue cultures and enterotoxic in a rabbit ileal loop assay, *C. diff* strains that are tcdA-/tcdB-/CDT+ are able to colonise in hamsters, but fail to produce disease (Bacci *et al*., 2011; Geric *et al*., 2006; Perelle *et al*., 1997; Viscidi *et al*., 1981). However, a recent study by Schwan *et al*. found that CDT induces formation of microtubule-based protrusions that wrap and embed *Clostridia*, thereby acting in synergy through increased pathogen adherence (Schwan *et al*., 2009). Furthermore,

neutralisation of CDT in the gut of mice caused a significant decrease in colonisation of the caecal content, indicating that the increased adherence facilitates colonisation (Schwan *et al*., 2009). There are multiple epidemiological studies linking CDT with increased patient mortality (Bacci *et al*., 2011; Barbut *et al*., 2007; Goldenberg and French, 2011; Walker *et al*., 2013), but further studies are needed to confirm its significance as an important virulence factor.

SLPs are a crucial group of proteins for the adherence of *C. diff* to the intestinal epithelium and subsequent gut colonisation (Calabi *et al*., 2002). *C. diff* is unusual in expressing two of these, which are of varying size in a number of strains and arise from post-translational cleavage of a single precursor, SlpA (Eidhin *et al*., 2006). These comprise the surface layer (S-layer) of *C. diff* vegetative cells, an exterior protein coat lying above the peptidoglycan layer of the Gram-positive cell wall common in many bacteria (Calabi *et al*., 2001). As well as mediating adhesion to enteric cells (Calabi *et al*., 2002), the S-layer has also been shown to infer protection from phagocytic attack or avoidance of the immune system (Sára and Sleytr, 2000). Considerable variation exists between SlpA across different strains, including the hypervirulent 027 strains (Eidhin *et al*., 2006; Fagan *et al*., 2009). Preliminary results indicate that the altered SlpA in hypervirulent strains is associated with increased adherence to human intestinal epithelial cells (Rupnik *et al*., 2009), and SlpA is now being considered as a potential vaccine candidate. Other possible virulence factors include bacteriophages, cell wall proteins and other non-toxin antigens, which are currently under scrutiny (Emerson *et al*., 2009; Govind *et al*., 2009; Lawley *et al*., 2009; Stabler *et al*., 2009).

1.3.3 Sporulation

Before the *C. diff* toxins can exert their effects, ingestion and germination of spores in the intestinal tract is required (Kelly and LaMont, 1998). *C. diff* produces highly resistant, infectious spores that promote transmission within the healthcare setting, as well as across greater distance through subject carriers (Clements *et al*., 2010). Thus sporulation and germination are major regulators of virulence and propagation of the disease.

The spore is the dormant form of *C. diff* and as described above, in their metabolically inactive state they can potentially persist for months or even years, which is similar to other Gram-positive spore formers. Due to their anaerobic nature, vegetative cells are unable to survive outside of the host and therefore spores are the most important form of transmission and perpetuation of the organism. Both *in vivo* and *in vitro* studies demonstrate that germination can be induced in response to bile derivatives; a clear indicator that they are adapted for life in the gastrointestinal (GI) tract (Sorg and Sonenshein, 2008, 2009). Epidemic strains of *C. diff,* such as PCR-ribotype 027/NAP1/BI, show increased sporulation levels *in vitro,* which may be associated with their increased propagation (Fawley *et al*., 2007; Merrigan *et al*., 2010). Furthermore, differences in sporulation capacity between strains have been associated with increased pathogenicity (Merrigan *et al*., 2010). A study focusing on a mouse model described a highly contagious "super-shedder state" of spores, mediated by antibiotics and characterised by a dramatically low microbiota diversity index, overgrowth of *C. diff* and excretion of an extremely high load of spores (Lawley *et al*., 2009). Furthermore, they found that spore-mediated transmission to antibiotic-treated immune-compromised mice resulted in severe, often fatal, intestinal disease, in contrast to a more moderate level of mucosal intestinal inflammation observed in immune-competent mice.

More in-depth investigations of factors affecting both sporulation and germination have the potential to provide further insight into pathogenistic mechanisms and unveil novel treatment options.

1.4 Epidemiology

CDI remains the most common cause of hospital-acquired diarrhoea and in the past decade it has already surpassed methicillin-resistant *Staphlococcus Aureus* as the most common hospital-acquired healthcare facility-associated infection in the United States (US) (Miller *et al*., 2011). In the US, the number of CDI discharge diagnoses in hospitalised patients rose from 139,000 in 2000 to 336,000 in 2009 (Centers for Disease Control and Prevention, 2012), with

similar trends having been observed across Canada (Pépin *et al*., 2004) and Europe (Freeman *et al*., 2010; Kuijper *et al*., 2008; Kuijper *et al*., 2006).

1.4.1 Emergence of hypervirulent strains: 2002-2007

In England, the prevalence of CDI gradually increased in the 1990s, with a pronounced increase in rates observed between 2001 and 2007 (Figure 1.4). In 2007, over 55,000 cases of CDI were reported, with more than half of acute NHS trusts reporting 2 or more cases per 1,000 admissions in patients over 65 years (Health Protection Agency, 2009a).

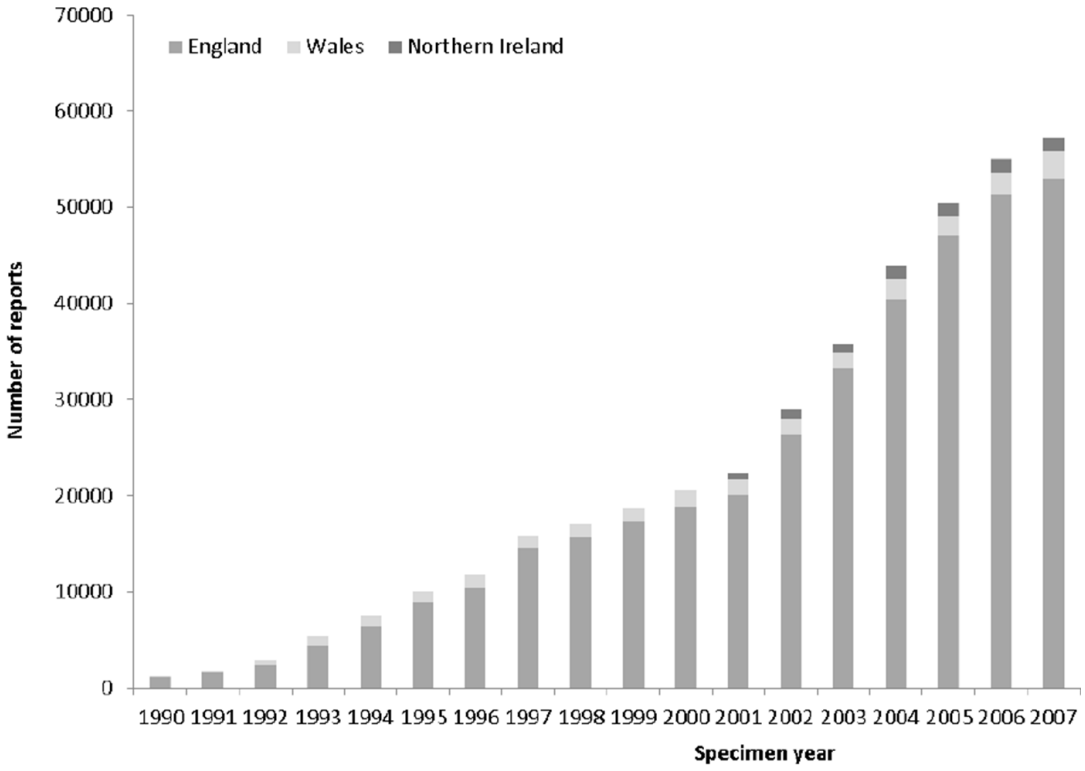


Figure 1.4: Voluntary laboratory reports of *C. difficile* positive faecal specimens: England, Wales and Northern Ireland* 1990-2007 (adapted from Public Health England, 2014) [*Northern Ireland reports included from 2001]

This is consistent with the increases in CDI prevalence and severity observed in the last decade across many European countries (Barbut *et al*., 2011; Freeman *et al*., 2010; Kuijper *et al*., 2006). Amongst the most important developments in the field was the emergence of an epidemic, hypervirulent strain of *C. diff* which accounted for numerous outbreaks worldwide and triggered the introduction of profound changes in healthcare practice, such as the adoption of new testing algorithms, thorough risk assessment for the management of patients, deep cleaning measures, and reviews into antibiotic policy (Kuijper *et al*., 2006; Warny *et al*., 2005).

First emerging in 2002/2003 (Clements *et al*., 2010), this epidemic strain, commonly referred to as 027/NAP1/B1 (dependent on the employed typing method; see section 1.6.7), was first recognised as the cause of the notable CDI outbreaks documented in Canada in 2003, and thereafter in the United Kingdom (UK), the Netherlands, Belgium and France (Kuijper *et al*., 2007; Pépin *et al*., 2004). Through whole genome sequencing and phylogenetic analysis, a recent paper was able to define the global population structure of PCR ribotype 027, and track its subsequent spread through the global healthcare system (He *et al*., 2013). Voluntary surveillance and strain typing has coincided with the increases in CDI prevalence observed from 2003 onwards, which may have resulted in the increases in severe disease, treatment failure, disease recurrence and mortality (Kuijper *et al*., 2006; Ricciardi *et al*., 2007; Warny *et al*., 2005). Strain characteristics postulated to contribute to the observed hypervirulence include:

- Hyperproduction of both tcdA and tcdB
- Ability to also produce CDT
- Complex genetic profile
- Antibiotic resistance
- Increased sporulation rates

Compared to other strain types, this epidemic PCR-ribotype 027 strain has been shown to produce up to 16- and 23-fold more tcdA and tcdB, respectively (Warny *et al*., 2005), which may be the result of both accelerated kinetics and sustained production (Freeman *et al*., 2007). Normally, toxin synthesis in

common strains increases as bacteria enter the stationary phase, but with hypervirulent strains this is likely to occur in both the exponential and stationary growth phases (Freeman *et al*., 2007). As well as hyperproduction of both tcdA and tcdB, the synthesis and release of CDT (see section 1.3.2) may be a contributing factor for the hypervirulence of these strains.

Interestingly, the PCR ribotype 027 strain and another so-called hypervirulent strain, here designated as PCR-ribotype 078, appear to be genetically divergent from other strains (Dingle *et al*., 2011a), and comparative molecular studies across multiple strains have identified that their observed hypervirulence may be a result of mutations in the *tcdC* gene, the negative regulator located in the PaLoc locus (Carter *et al*., 2011). However, historical isolates containing similar *tcdC* mutations were not responsible for outbreaks at that time (McDonald *et al*., 2005b). It is therefore thought that the associated epidemic behaviour may be related to expression of multiple genetic elements, with five unique genetic regions identified in epidemic 027 strains (transcriptional regulators, a dual-component regulatory system and a novel phage island) that are absent in non-epidemic 027 strains (Stabler *et al*., 2009).

Unlike historical 027 strains, hypervirulent 027 demonstrates high levels of antibiotic-resistance, most notably to fluoroquinolones and erythromycin (Drudy *et al*., 2006; Spigaglia *et al*., 2008). There are also concerns regarding this strain's reduced susceptibility to metronidazole since a poor response rate to metronidazole treatment was observed in the publicised 027-associated Canadian outbreaks of 2004 (Pépin *et al*., 2004). A recent study of 398 European *C. diff* isolates demonstrated that in general, 027 isolates had a two-fold higher minimum inhibitory concentration (MIC) of metronidazole, capable of inhibiting 90% of bacterial isolates, than non-027 isolates (Debast *et al*., 2013). However, another study failed to identify any significant differences in clinical outcome across metronidazole-treated CDI patients infected with reduced versus fully susceptible strains (Purdell *et al*., 2011). In addition, the 027 strain has been associated with an increased *in vitro* sporulation rate in both the presence/absence of non-chloride cleaning agents (Akerlund *et al*., 2008), with the subsequent production of more spores resulting in increased

environmental contamination, improved survival and the potential for further spread and sharing.

1.4.2 Decreasing incidence: 2007-2014

Due to the dramatic increases in incidence and severity of CDI, surveillance studies to monitor this and the associated spread of hypervirulent strains have been established since 2007 at both regional and national levels in Europe and North America (Wilcox *et al*., 2012). In England, a *Clostridium difficile* Ribotyping Network (CDRN) was created, stringent trajectory targets were established and all NHS hospitals were required to report all cases of CDI (mandatory notification status).

Since 2006, the National Reference Laboratory of the Netherlands has observed a decrease in 027-associated CDI in hospitals, but an increase across other healthcare facilities such as nursing homes (Hensgens *et al*., 2009). Other European countries, such as Belgium, have remained relatively static (Viseur *et al*., 2011). However, the most substantial reduction in the overall incidence of CDI has occurred in England and Wales: voluntary reports peaked in 2007, but by 2010 had decreased by ~60% (Figure 1.5) (Public Health England, 2014). The most recent reports from 2013 demonstrated a 9.8% decrease from 2012 (13,547 versus 15,011; Figure 1.5) (Public Health England, 2014). This reduction is coincident with control of the epidemic 027 strain and substantial decrease in 027-associated CDI cases: the 027 strain was responsible for 55% of all samples submitted to the CDRN in 2007-2008, subsequently decreasing to 36% and 21% of samples submitted in 2008-2009 and 2009-2010, respectively (Wilcox *et al*., 2012). These findings coincided with a decrease in the number of CDI-attributed deaths in England, falling by 70% between 2007 and 2010 (7,916 versus 2,335 death certificates) (Office of National Statistics, 2011).

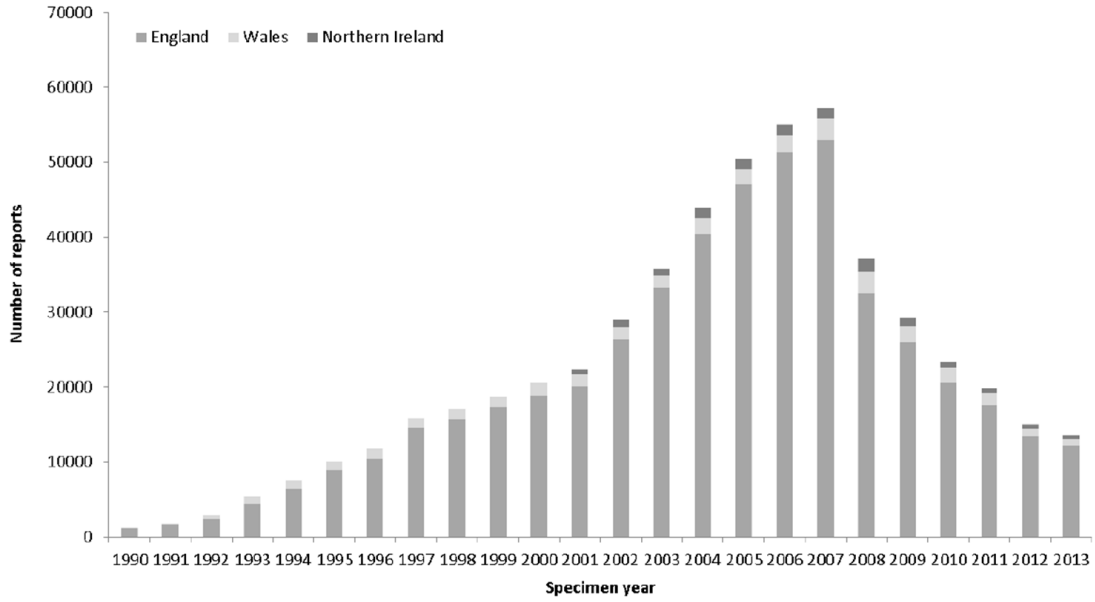


Figure 1.5: Voluntary laboratory reports of *C. difficile* positive faecal specimens: England, Wales and Northern Ireland 1990-2013* (taken from (Public Health England, 2014)**

**Date from 2013 are provisional (date was extracted on 15th January 2014); **Northern Ireland reports included from 2001;*

Whilst the decrease in CDI-related complications is likely due to the observed reduction in cases accounted by hypervirulent, epidemic strains, the overall substantial decrease in CDI incidence is likely due to increased clinical vigilance (Wilcox *et al*., 2012): the introduction of enhanced infection control measures such as hand washing and more effective isolation of infected patients most likely limited the spread of spores within the hospital environment (Price *et al*., 2010). Furthermore, strict antibiotic stewardship reduced the use of treatment linked with drugs such as fluoroquinolones, cephalosporins and clindamycin (Talpaert *et al*., 2011).

Focusing on infection control measures will have been aided by the provision of timely information on the PCR ribotypes responsible for both individual cases, and clusters (Wilcox *et al*., 2012). Interestingly, it is also thought that the

observed emergence of less virulent PCR ribotypes, such as 012, 017, 019, 036, 078 and 153 (Dawson *et al*., 2011; Knetsch *et al*., 2011), may also be involved in the reduction of severe cases across Europe (Goorhuis *et al*., 2008b). An increase in the prevalence of PCR ribotype 078, the predominant strain of *C. diff* in livestock such as calves and pigs, has been observed in both hospital and community settings (Goorhuis *et al*., 2008a) and a 2008 Pan-European surveillance study found that PCR ribotype 078 was the third most prevalent ribotype, accounting for 8% of all regional cases (Bauer *et al*., 2011). PCR ribotypes 014/020 (16%) and 001 (10%) were the two most frequently observed, with PCR ribotype 027 now accounting for just 5% (Figure 1.6).

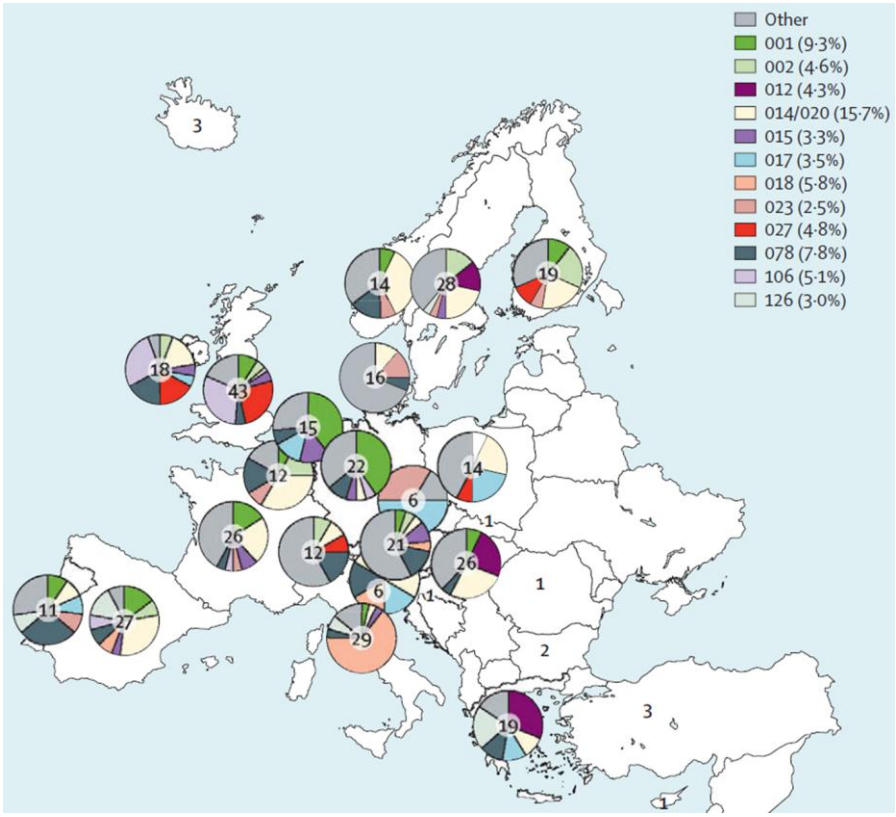


Figure 1.6: Geographical distribution of *Clostridium difficile* PCR ribotypes in European countries with more than five typable isolates, November, 2008 (taken from (Bauer *et al*., 2011)

Pie charts show proportion of most common PCR ribotypes per country. The number in the centre of pie charts is the number of typed isolates in the country.

1.4.3 Community-acquisition

Although first described in the late 1980s/early 1990s, relatively few studies describe the epidemiology of CA-CDI. Despite being reported at a considerably lower rate than hospital-acquired CDI (HA-CDI), the incidence of CA-CDI has steadily increased with time: a recently published US population-based study showed a 5.3-fold increase in CA-CDI from 1991 to 2005, with CA-CDI accounting for a large proportion (41%) of the overall cases across this time period (Khanna *et al*., 2012b). Possible sources of CDI within the community include animals (e.g. pets), food (meat & vegetables) and the environment (soil & water) (al Saif and Brazier, 1996) but although similar PCR ribotypes were found, the basis for direct transmission has yet to be elucidated.

Compared with HA-CDI patients, CA-CDI patients are younger (50 years versus 72 years), more likely to be female (76% versus 60%) and suffer from less comorbidities (Jones *et al*., 2013; Khanna *et al*., 2012b). This is consistent with findings that CA-CDI affects groups previously considered 'low-risk' such as children or pregnant females (Centers for Disease Control and Prevention, 2005), with paediatric studies showing a 12.5-fold increase in the incidence of CA-CDI over the last twenty years (Khanna *et al*., 2013a; Kim *et al*., 2008). Patients with CA-CDI are also less likely to have been exposed to antibiotics (78% versus 94%) (Jones *et al*., 2013), corresponding with US reports that 35% of their 7.7 cases per 100,000 persons per year received no antibiotics within at least 42 days of *C. diff* detection (Bassetti *et al*., 2012). With no CA-CDI outbreaks having been reported to date, it is thought that host factors resulting in increased susceptibility may be of greater importance than overall level of exposure to the organism (Jones *et al*., 2013).

1.5 Risk factors

With dramatic increases in the incidence of CDI observed globally, marked efforts have been made to identify risk factors for both susceptibility to *C. diff* acquisition and disease severity including complications, disease recurrence and mortality. Discussed below are some of the traditionally reported risk factors, including age at the time of diagnosis, medication exposure

(antibiotics/proton pump inhibitors (PPIs)) and hospitalisation history. Additional risk factors include, but are not limited to, underlying comorbidities such as malignancies, immunosuppression and inflammatory bowel disease (IBD), length of hospital stay, contact with active carriers, hypoalbuminaemia and enteral tube feeding (Khanna and Pardi, 2014).

1.5.1 Age

Increasing age is a consistently noted risk factor, likely due to the inability of older patients in mounting a sufficient immune response upon first exposure to the *C. diff* toxins, which unsurprisingly also results in a higher rate of disease recurrence in these patients (Dial *et al*., 2004; Kyne *et al*., 2001).

Reports demonstrate between a 10- and 20-fold increased risk for patients aged 60-90 years compared to the younger population (Bartlett and Gerding, 2008; Brown *et al*., 1990; Kelly and LaMont, 1998), with 90% of all CDI-related deaths occurring in persons aged 65 years and above (Brown *et al*., 1990). Furthermore, a two- to three-fold increase in mortality has been observed in elderly CDI patients infected with the PCR-ribotype 027 strains (Miller *et al*., 2010b).

1.5.2 Antibiotics

As described in Section 1.3 and illustrated in Figure 1.1, disruption of the normal gut flora allows the vegetative cells to penetrate the mucus layer, adhere to the surface of epithelial cells and fully establish themselves in the gut (Karjalainen *et al*., 1994). Prior use of antibiotics has been well documented as a major cause of dysbiosis as it can particularly facilitate colonisation by strains resistant to the administered agent. Prior antibiotic exposure is therefore seen as a predominant risk factor for CDI, with CDI accounting for 15-20% of all cases of AAD (Bartlett and Gerding, 2008; Cohen *et al*., 2010). The majority of antibiotics have been reported to cause CDI, though some obviously carry higher risk than others. Ampicillin/amoxicillin, clindamycin and cephalosporins (in particular third-generation) have been consistently implicated, and more recently fluoroquinolones (Gaynes *et al*., 2004; Johnson *et al*., 1999; Loo *et al*.,

2005; Muto *et al*., 2005; Pépin *et al*., 2005; Riley, 1996; Thomas *et al*., 2002). Epidemic strains of *C. diff* have differing antibiotic resistance profiles to their historical counterparts. Though once susceptible, emerging epidemic strains are able to evolve and acquire resistance, as with PCR-ribotype 027 and the various classes of fluoroquinolones, including gatifloxacin and moxifloxacin (Johnson *et al*., 1999; McDonald *et al*., 2005a).

It has been estimated that the use of broad spectrum antibiotics increases CDI risk by 8-fold to 10-fold from the time of the initial exposure up to one month post-administration, and up to 3-fold for the following two months (Hensgens *et al*., 2012), though this is dependent on the antibiotic type administered (Merrigan *et al*., 2003a; Merrigan *et al*., 2003b). Increased risk has also been demonstrated for use of multiple antibiotics, as well as increased length of treatment (>10 days) (Brown *et al*., 1990; Gerding *et al*., 1986). Continued use of non-CDI antibiotics both during and post-CDI treatment is linked with poor disease prognosis: one study demonstrated a significant increase in disease recurrence within CDI patients receiving concomitant non-CDI antibiotics post-therapy (Drekonja *et al*., 2011). Furthermore, two phase III trials comparing fidaxomicin to vancomycin demonstrated that patients receiving concomitant non-CDI antibiotics during therapy had a decreased cure rate and increased time to resolution of diarrhoea (Mullane *et al*., 2011). Patients receiving concomitant non-CDI antibiotics post-therapy in these two trials also showed a (non-significant) increase in disease recurrence (Mullane *et al*., 2011).

Despite the high frequency of cases linked with their exposure, CDI can also occur without pre-exposure to antimicrobials: a 2005 study demonstrated that 24% of CDI patients had no prior exposure, with a further 9% receiving antibiotics for only 3 days or less (Centers for Disease Control and Prevention, 2005). Interestingly, of these patients with no exposure, 75% were either hospitalised or had had close contact with an individual suffering from a diarrhoea-related illness.

1.5.3 Gastric acid suppression

As described in Section 1.3, the initial step in the CDI infection cycle is the ingestion of *C. diff* spores, which must subsist into their activated vegetative form before adherence/colonisation and subsequent infection can take place. After resisting the acidity of the stomach, the spores pass through to the small intestine where favourable conditions, including bile acids/salts facilitate this germination. It has therefore been hypothesised that a reduction in stomach acidity, through the use of suppressive medication including H2-receptor antagonists and PPIs, may enhance survival of the *C. diff* spores in the GI tract thus increasing their ability to convert to the vegetative form.

Although a number of recent studies have demonstrated that a significant relationship between PPI use and CDI has been limited to univariate analysis and is not significant within multivariable models (Khanna *et al*., 2012a; Leekha *et al*., 2013; Leonard *et al*., 2012), there is evidence that acid-suppressive therapy may constitute a risk factor for CDI (Dial *et al*., 2005; Dial *et al*., 2006; Howell *et al*., 2010; Loo *et al*., 2011; Stevens *et al*., 2011), with some meta-analyses having identified a significant relationship (Janarthanan *et al*., 2012; Kwok *et al*., 2012; Leonard *et al*., 2007). Howell *et al* observed a dose response effect, whereby the risk of CDI increased as with the degree of acid suppression, defined as (i) none, (ii) H2 receptor antagonists, (iii) once daily PPI and (iv) more frequent PPI (Howell *et al*., 2010). PPIs have also been identified as potential risk factors for CDI-related outcomes including complications, recurrence and mortality (Kim *et al*., 2010; Kim *et al*., 2012; Morrison *et al*., 2011), but associations remain controversial and inconclusive.

Despite a lack of conclusive evidence, in 2012 the US Food and Drug Administration (FDA) issued a warning regarding the prescription of PPIs in relation to CDI, and updated guidance on the management of CDI in the UK also reinforced the concept: "*Given that acid suppression drugs, especially PPIs, may be over-prescribed and frequently not reviewed to determine if long-standing prescriptions are still justifiable, consideration should be given to*

stopping/reviewing the need for PPIs in patients with or at high risk of CDI" (Public Health England, 2013).

1.5.4 Hospitalisation & healthcare acquisition

The combination of a spore-contaminated environment, sub-optimal hand hygiene of healthcare employees and a highly susceptible patient population, especially the elderly, make recent hospitalisation a further significant risk factor for acquisition of CDI (Bassetti *et al*., 2012). Recent hospitalisation also seems to modulate the risk in asymptomatic patients: Minnesota researchers identified toxigenic *C. diff* in 9.7% of 320 asymptomatic individuals, with multivariate analysis revealing recent hospitalisation (within 3 months), chronic dialysis and use of corticosteroids as the three main risk factors for colonisation by *C. diff* (Leekha *et al*., 2013).

Although the majority of CDI infections are hospital-acquired, in 2010 the Centers for Disease Control and Prevention demonstrated that 94% of overall CDI cases were healthcare-associated, with the onset in 75% occurring outside of a hospital setting (Centers for Disease Control and Prevention, 2012). Another study demonstrated that as many as 25% of all CDI cases develop in nursing home patients (Centers for Disease Control and Prevention, 2012). Notably, the majority of cases occurring in a non-hospital healthcare setting involve recently hospitalised individuals (Guerrero *et al*., 2011; Kim *et al*., 2011). Therefore, caution must be taken with residents of long-term care facilities involving concentrated elderly populations, polypharmacy and high antibiotic use, and frequent hospital visits and nosocomial exposure (Iv *et al*., 2014).

Available data on risk factors has largely been derived from studies on healthcare-associated CDI. However, with the increasing incidence of CA-CDI (Gerding *et al*., 1986) and a lack of understanding of preponderant risk factors within this population, it is believed that cases of CA-CDI may have different underlying causes. Recent studies have demonstrated that up to 94% of CA-CDI patients had had recent outpatient or emergency room visits, which suggests

that short-term healthcare without hospitalisation may also constitute a risk factor for CDI in this population (Centers for Disease Control and Prevention, 2012; Chitnis *et al*., 2013; Khanna *et al*., 2013a; Lessa, 2013).

1.6 Diagnosis

Diagnosis of CDI is usually based on the combination of clinical history, the presence of diarrhoea and positive laboratory confirmation. Although CDI is a classic example of health-care-associated diarrhoea, reliably distinguishing *C. diff* from other causes is only possible through the use of laboratory tests. Many alternative laboratory screening methods are currently being employed, summarised in Table 1.1, such as direct culture and the glutamate dehydrogenase (GDH) antigen assay, which both target the organism itself; and the cell cytotoxicity neutralisation assay (CCNA) and enzyme-linked immunosorbent assay (ELISA), which instead detect the presence of the *C. diff* toxins in the specimens. A third group of tests detect the presence of the toxin genes via molecular methods, such as nucleic acid amplification tests (NAATs) (Kufelnicka and Kirn, 2011). Test performance varies widely and there is now increasing recognition of the advantages and disadvantages of each approach (Wilcox, 2011; Wilcox *et al*., 2010). CDI diagnosis remains a difficult issue for hospital diagnostic laboratories due to the lack of a single accepted gold standard (Curry, 2010). Indeed, a recent study evaluating multiple methods concluded that clinical outcomes differed according to the testing method employed, with multi-stage algorithms recommended over stand-alone approaches (Planche *et al*., 2013).

In addition to high sensitivity, another crucial requirement for an ideal screening test is a rapid turnaround time (Fenner *et al*., 2008; Department of Health, 2012; Sharp *et al*., 2010; Swindells *et al*., 2010). Although some may argue that immediate results are not essential for CDI unless a patient has PMC or toxic megacolon (Wilkins and Lyerly, 2003), a rapid turnaround for CDI could reduce unnecessary antibiotic treatment that arises through the initiation of pre-emptive antibiotic therapy based on clinical evidence alone. Furthermore, a delay in results could potentiate transmission and hamper patient management.

Table 1.1 – Diagnosing *Clostridium difficile* infection (adapted from (Rupnik *et al.*, 2009)

Question to be answered	Detection method	Advantages	Disadvantages
Is the organism present?	Culture	- Sensitive, but presence does not equate with infection as many *C. diff* strains are non-toxigenic - Useful for epidemiological investigation and surveillance	- Slow turnaround times (days) - Suboptimal sensitivity in inexperienced hands - Requires anaerobic culturing capability
	Antigen (GDH) detection	- High negative predictive value* - Rapid detection (hours)	- Not specific for *C. diff* and therefore requires supplementary testing
Is *C. diff* toxin present?	Cytotoxin assay	- Sensitive - High specificity for infection	- Slow turnaround times (minimum 1–2 days) - Requires access to and/or experience of cell culture methods
	Enzyme immunoassays	- Familiar methodology that can be used widely - Rapid (hours)	- Variable sensitivity and specificity resulting in low positive predictive values, especially in populations with low prevalence of CDI - Requires laboratory facilities
	Membrane assays	- Does not necessarily require laboratory facilities - Rapid (minutes to hours)	- Variable sensitivity and specificity resulting in low positive predictive values, especially in populations with low prevalence of CDI
Does the organism have the capacity to produce toxin?	Cytotoxigenic culture	- High sensitivity	- Uncertain specificity for infection - Slow turnaround times (days)
	PCR detection of *tcdB* gene	- High sensitivity - Rapid (hours)	- Uncertain specificity for infection - Requires laboratory and molecular expertise - High cost

*C. diff: Clostridium difficile; CDI: Clostridium difficile infection; GDH: Glutamate dehydrogenase; PCR: Polymerase chain reaction; tcdB: Toxin B; *There are recent contradictory data regarding assay sensitivity;*

1.6.1 Cell cytotoxicity neutralisation assay & toxigenic culture

CCNA was the accepted gold standard for many years. This comprises a two step approach where a cytotoxicity assay is firstly used to assess the ability of suspect faecal filtrates in causing apoptotic cell rounding, followed by an anti-toxin neutralisation step that confirms whether or not this effect can be reversed through inoculation with a controlled amount of toxin-specific antibodies. The method has high specificity, can detect toxin (primarily tcdB) in the stool as low as 10 picograms (Iv *et al*., 2014) and a variety of cell lines can be used, including human foreskin cell monolayers and in-house cell lines such as Chinese hamster ovary K-1 cells (Bassetti *et al*., 2012). However, CCNA is the least-controlled test and combines high expense with a slow turnaround time (minimum of 2 days). Furthermore, there is a lack of standardisation across laboratories, with non-specific reactions commonplace in some, and the need for technical expertise and cell culture facilities means that application of this test is generally unavailable outside of a dedicated research facility or reference laboratory (Bassetti *et al*., 2012; Iv *et al*., 2014).

More recently, toxigenic culture (TC) has been considered the method of choice due to the belief that it is more sensitive (Curry, 2010), though less specific, than CCNA when used in experienced laboratories. Stool is cultured for *C. diff* on specific, selective media such as Cycloserine–Cefoxitin–Fructose agar that allows recovery in the presence of enteric microbiota (George *et al*., 1979), and the organism's ability to produce toxin is subsequently tested. Compared to CCNA, TC appears easier to apply on a routine basis and with the availability of internal control reference strains and the quality standards exerted by several brands on the production of effective selective media, TC also appears to be more reliable and reproducible. The 2010 guidelines provided by the Society for Healthcare Epidemiology of America/Infectious Diseases Society of America noted that "the sensitivity and specificity of stool culture followed by identification of a toxigenic isolate as performed by an experienced laboratory provides the standard against which other clinical tests should be compared" (Cohen *et al*., 2010). However, TC also comes with its limitations. The dual step approach of stool culture plus toxigenic culture means that turnaround time can

be anything up to a week, as opposed to the 24 and 48-hour values for positive and negative cytotoxicity assays, respectively. Most importantly, TC is only able to identify the potential of a strain to produce toxin but it does not measure actual toxin levels in the stool, which may lead to false conclusions. This is especially true when considering rates of asymptomatic colonisation, which may vary between 7% in hospitalised patients on admission (Kyne *et al*., 2000b) and 20% amongst elderly nursing home patients (Simor *et al*., 2002).

Despite their high specificity and sensitivity, the turnaround times and technical demands posed by both CCNA and TC tests make them increasingly impractical for the routine diagnosis of CDI and management of patients.

1.6.2 Enzyme immunoassay-detection of toxins A & B

Toxin enzyme immunoassays (EIAs) are fast, convenient and inexpensive: unlike TC and CCNA they do not require specialist equipment/staff and they have a turnaround time of less than 24 hours. Advantages such as these resulted in their widespread uptake and they are now used by up to 90% of clinical diagnostic laboratories (Bartlett and Gerding, 2008). However, in terms of sensitivity and specificity, the assay performance of commercially-available *C. diff* toxin EIAs is considered sub-optimal when compared to gold-standard methods TC and CCNA. Although generally showing high specificity (~95%), sensitivity is low to moderate (60-90%) with some studies reporting sensitivity as low as 38% (Ticehurst *et al*., 2006): whereas CCNA has a reportedly lower limit of detection of around 10 picograms of toxin, the EIA usually requires 100-1,000 picograms (Bartlett and Gerding, 2008).

Positive predictive values (PPVs) and negative predictive values (NPVs) of all commercial EIAs may also vary depending upon the prevalence of the condition being detected. For instance, if positivity rates observed for *C. diff* in a given population decrease to 5-10%, assay PPV could be as low as 50% (American Society for Microbiology, 2010), thus potentially deeming them unacceptable for diagnostic purposes (Planche *et al*., 2008). Insufficient NPV results in false diagnosis of CDI, which can result in isolation of patients who are uninfected, leading to the misclassification of uninfected patients with those suffering from

active disease, unnecessary use of antibiotics and a delay in finding the true causes of the diarrhoea (Iv *et al*., 2014).

Such lack of sensitivity has resulted in the current consensus that *C. diff* toxin EIAs should no longer be recommended as a standalone test (American Society for Microbiology, 2010). Confirmation of initial positive diagnoses, arising from one or more rapid sensitive screening methods and resulting in a two-step diagnostic algorithm, is strongly recommended and already in routine use across several laboratories.

1.6.3 Detection of Glutamate Dehydrogenase antigen

The lack of sensitivity and low NPV outlined above for toxin detection by EIA has led to a search for more accurate methods. GDH is a common, cell wall-associated antigen expressed by all *C. diff* strains and as it is produced in significantly higher quantities than the toxins it results in a significantly more sensitive assay. Indeed, early studies reported sensitivity rates as high as 100% for *C. diff* detection (Peterson and Robicsek, 2009). Favoured due to their diagnostically-compatible turnaround time of 15-45 minutes, rapid GDH screening tests are increasingly popular, especially in tandem with the toxin EIA, to increase the sensitivity of current diagnostic algorithms.

However, as with TC, GDH tests do not directly detect the presence of the toxin protein, nor can they distinguish between toxigenic (toxin-producing) and non-toxigenic strains (Carman *et al*., 2012; Willis and Kraft, 1992). With approximately 20% of GDH positive-patients carrying a non-toxigenic strain (McFarland *et al*., 1989; Wilkins and Lyerly, 2003), the greatest utility of this assay appears to be as a primary screening step in a diagnostic algorithm. In such a scenario, GDH-negative specimens can be used to rule out negative cases, while GDH-positive specimens are then subject to a second test confirming/denying toxin production, thus giving increasing confidence for the diagnosis of the disease.

1.6.4 Nucleic acid amplification tests

With the advent of diagnostic PCR-based commercial kits, PCR assays have now been applied for the detection of *C. diff*. Different regions have initially been targeted (Alonso *et al*., 1999; Arzese *et al*., 1995; Peterson *et al*., 2007) but since all pathogenic strains to date are able to synthesise tcdB, screening of the *tcdB* gene region (which is responsible for encoding the toxin) has become the mainstream standard. Although the cost can be significantly greater than for toxin EIA, the *tcdB* PCR-based assay is fast (turnaround time of 2 hours) and a meta-analysis comparing data from the four FDA-approved PCR assays against gold-standard TC returned a pooled sensitivity and specificity of 92 and 94%, respectively (O'Horo *et al*., 2012). Of these 4 approved assays, the Xpert *C. diff* assay (Cepheid, CA, USA) has been widely evaluated for routine diagnostics with its utility proven by various authors in comparative studies to both CCNA/TCA and EIA-based tests (Babady *et al*., 2010; Huang *et al*., 2009; Novak-Weekley *et al*., 2010; Tenover *et al*., 2010).

Similarly to TCs, NAATs are able to identify toxigenic strains but do not measure the presence of the actual toxin in the stool and as a result concerns have been raised regarding over diagnosis due to the potential inclusion of asymptomatic carriers and issues related to the mandatory notification of CDI and inter-hospital performance comparisons (Iv *et al*., 2014). Interestingly, the prevalence of positive samples has in fact increased since the introduction of PCR as a detection method, with some studies noting an increase from 6.5 to 15% (Fong *et al*., 2011). Secondly, findings that 56% of patients will be PCR-positive for months or years after completing therapy (Sethi *et al*., 2010) means that PCR cannot be used for suspected recurrence. Thirdly, the high expense involved with the necessary equipment limits its presence to a small handful of laboratories, though as these systems become increasingly validated and adopted, the likelihood is that their price will begin to drop. Finally, a recent meta-analysis of CDI diagnostic results obtained by PCR between 1995–2010 found that the observed specificities and sensitivities were highly dependent upon the CDI prevalence rate, with decreasing prevalence resulting in decreased assay performance (Deshpande *et al*., 2011). Despite these

limitations, PCR may be diagnostically advantageous in epidemic conditions and multiples studies have demonstrated elevated sensitivity, specificity and test-retest reliability, hinting that PCR is fast becoming an alternative gold standard to stool culture (Khanna *et al*., 2012c; Sloan *et al*., 2008).

A variant method of the above involves detection of the toxin gene using loop-mediated isothermal amplification (O'Horo *et al*., 2012). Targeting a conserved, 204 bp region of the *tcdA* gene within the PaLoc, an FDA-licensed, commercial kit based on this method now exists (Illumigene, Meridian Bioscience, Europe). The Illumigene test is simple with a rapid turnaround time (one hour) and performance figures against the gold standards CCNA and TC have been promising (92% sensitivity, 98% specificity, 99% NPV and 84% PPV, respectfully; (Bamber *et al*., 2012)). However, since it is NAAT based, the main issue concerns its inability to specifically detect the *C.diff* toxins, which are the key causative agents of the disease.

1.6.5 Non-laboratory testing: radiologic and endoscopic diagnosis

Although the majority of CDI diagnosis takes place within a laboratory setting, the diagnosis of severe forms of the disease can sometimes be complemented by both radiology and endoscopy. Though not normally required for diagnosis of CDI, findings from abdominal CT scans such as the presence of pleural effusion and thickening of the colonic wall have both been associated with the development of severe disease. CT findings also correlate with other factors associated with severe disease including immunosuppression, leukocytosis and hypoalbuminemia (Valiquette *et al*., 2009). Lower GI endoscopy is able to highlight the presence of pseudomembranes or inflammation, as well as to collect tissue/stool samples for diagnostic purposes. It is recommended by The American College of Gastroenterology (ACG) guidelines when rapid diagnosis is necessary, for the exclusion of other coexisting etiologies including cytomegalovirus colitis, ischemic colitis or IBD, and when clinical suspicion is high but stool tests are inconclusive (Burkart *et al*., 2011; Fekety, 1997).

1.6.6 Algorithmic approaches

With the incidence of CDI cases remaining relatively high in several developed countries, accurate and reliable laboratory diagnosis of CDI continues to be a priority. The limitations outlined above regarding the use of standalone tests based on toxin EIAs, GDH and PCR have led to the investigation of multiple algorithmic strategies for a confident CDI diagnosis. Such approaches are not novel and have become established for the diagnosis of other diseases, such as human immunodeficiency virus and syphilis.

A recent study evaluated several diagnostic algorithms and concluded that diagnosis of CDI is improved by the use of two-stage algorithms (Davies *et al*., 2012). Two of the more commonly recommended laboratory parameters are reliant on the initial detection of faecal GDH in stools, and NAAT, such as PCR, for the confirmation and detection of toxigenic *C. diff* strains (Crobach *et al*., 2009; Surawicz *et al*., 2013). In England, updated guidance on the diagnosis and reporting of CDI (Department of Health, 2012) has been derived from a recent observational diagnostic NHS study of 12,441 specimens, resulting in the subsequent adoption of their recommended two-step algorithm (Figure 1.7) by affiliated NHS laboratories commencing in April 2012 (Department of Health, 2012; Wilcox, 2012). Wilcox *et al*. evaluated the effectiveness of routine screening assays for GDH, the toxin gene and toxin itself, and found that an EIA for GDH detection or NAATs for toxin gene detection (including tcdB PCR) followed by confirmation of stool toxin (either by a relatively sensitive toxin EIA or CCNA) was the most effective testing algorithm in accurately distinguishing patients with and without CDI, resulting in enhanced specificity and PPV (90%) (Wilcox, 2012). This algorithm also identifies 'potential *C. diff* excretors', i.e. individuals with diarrhoeal samples that contain *C. diff* but without demonstrable toxin levels, who may be a relevant source of transmission of *C. diff* to susceptible patients.

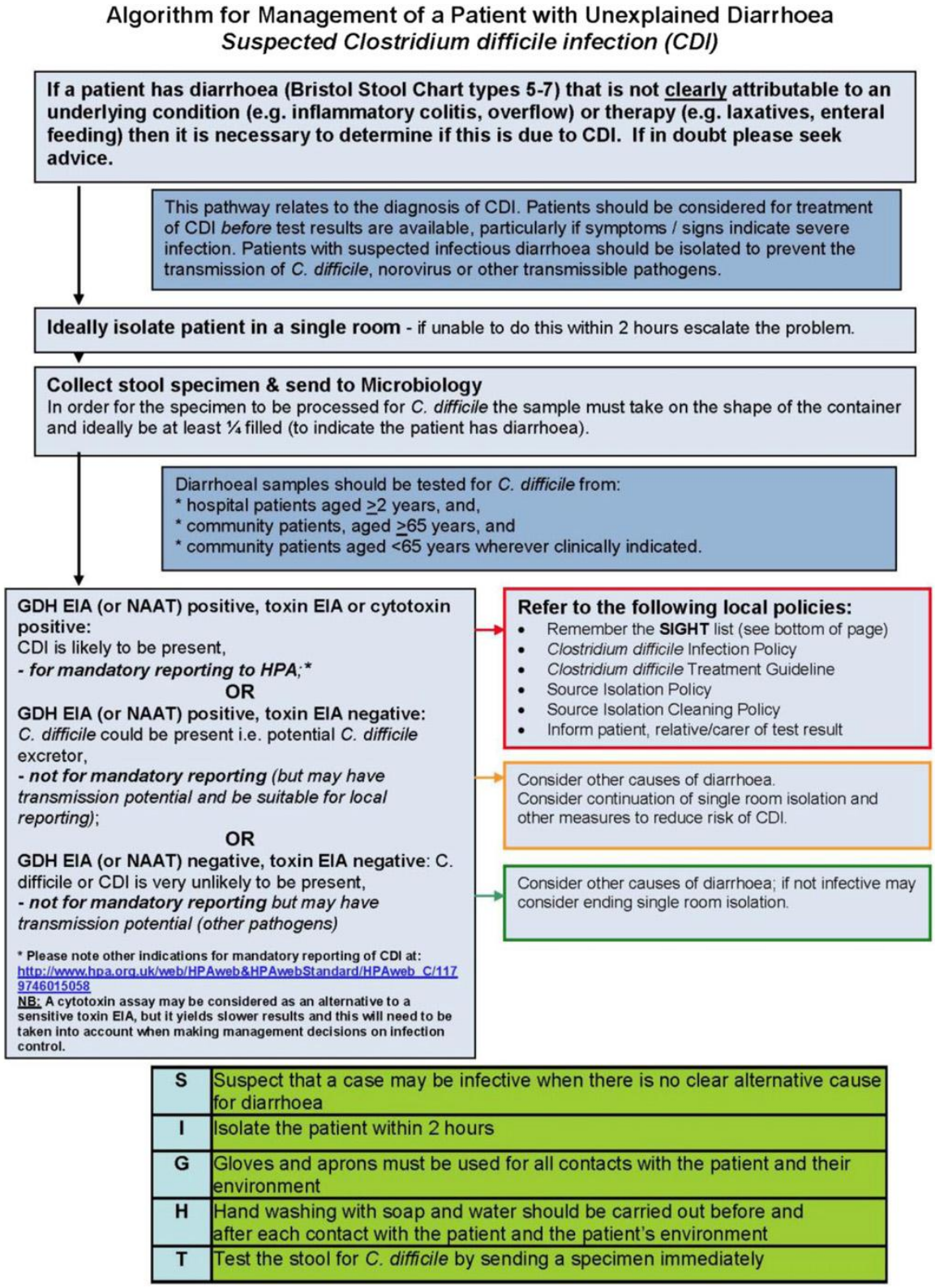


Figure 1.7 - UK algorithm for CDI diagnosis taken from (Department of Health, 2012)

1.6.7 Complimentary Tests

In practical terms, the simple detection of *C. diff* toxins combined with clinical evidence is all that is required for diagnostic purposes. However, characterisation of the strain through molecular typing, though generally not affecting therapeutic decision, can be useful for epidemiological surveys, outbreak monitoring and infection control (Janezic *et al*., 2012; Koene *et al*., 2012; Mulvey *et al*., 2010).

Several molecular typing methods have been introduced to study the epidemiology of *C. diff* (Table 1.2). Agarose gel-based PCR ribotyping is currently the most common method used across Europe to type *C. diff*, rising to prominence in 2003/4 with the emergence of the hypervirulent strain, PCR ribotype 027, whereas North America favour pulse field gel electrophoresis (PFGE). Usage of different typing methods results in different nomenclatures, which can complicate inter-laboratory data exchange. The epidemic strain responsible for the increase in CDI rates observed across North American hospitals, characterised as PCR ribotype 027 by PCR ribotyping, can also be characterised as B1 by restriction endonuclease analysis (REA) and Type 1 by PFGE (specifically North American pulse-field Type 1, or NAP1) (McDonald *et al*., 2005a). More recent developments include modified multiple-locus variable number tandem repeat analysis (mMLVA), capillary PCR-ribotyping and whole genome sequencing (WGS).

WGS is increasingly being used for the study of *C. diff* transmission. Unlike multi-locus sequence typing (MLST) or PCR ribotyping, which are hampered by the large numbers of patients who share a genotype and hospital-based contact, WGS is able to show that substantial genetic diversity exists even within isolates of the same genotype (Didelot *et al*., 2012). Whilst most episodes of *CDI a*re believed to result from recent acquisition within a health care setting, recent research using WGS has found that nosocomial transmission between symptomatic CDI cases contributes far less to current rates of infection than has been widely assumed (Didelot *et al*., 2012; Eyre *et al*., 2013). In particular, Eyre *et al*. found that 45% of all cases included in their study were genetically distinct

from all previous cases, clarifying the importance of future research into other transmission routes from genetically diverse sources other than symptomatic patients (Eyre *et al*., 2013). WGS can also be used with regards to disease recurrence, whereby it is able to distinguish between recurrences occurring due reinfection with a new strain or due to relapse with the same strain. This was recently used to demonstrate that was able to demonstrate that fidoxamicin is superior to vancomycin for preventing both reinfection and relapsing infection (Eyre *et al*., 2014).

Reducing costs and high discriminatory power means it is likely that sequencing-based methods will be increasingly applied for C*. diff* epidemiological studies (Eyre *et al*., 2012a; Walker *et al*., 2012). Promising results from the initial WGS studies, described above, suggest that these approaches will become increasingly established in the next decade.

Table 1.2 – Summary of typing methods employed for characterisation of *Clostridium difficile* strains

Technique	Target	Summary
PCR ribotyping	16S-23S ribosomal RNA spacer region	Specific primers used for PCR-mediated amplification of the DNA encoding the target regions. Generates a few DNA bands as visualised by gel electrophoresis; the DNA band patterns are referred to as ribotypes
PFGE	SmaI restriction sites	Enzyme cuts bacterial genome at target sites giving large DNA fragments. These are then slowly separated in a polyacrylamide gel, submitted to an electrical field in which the voltage repeatedly switches. The fragments migrate varying distances according to size and are visualised by DNA staining to reveal differences in banding patterns
MLVA	DNA repeat units	Involves counting the numbers of repeat alleles in the genome for a series of predefined, conserved loci that are amplified by PCR. Requires expensive equipment but is highly discriminatory, and produces a consistent numerical result (code) for each strain that should be comparable between different laboratories
REA	HindIII restriction sites	Relies on more frequent cutting of the bacterial genome than PFGE, resulting in large numbers of DNA fragments. These fragments are separated by electrophoresis in an agarose gel. This method is usually highly discriminatory, but produces complex DNA banding patterns that can be difficult to interpret and reproduce
Toxinotyping	B1 & A3 fragments of PaLoc	RFLP-PCR based method where strains are assigned to 27 variant toxinotypes (I-XXVII), according to the lengths and restriction patterns of the two target fragments from the PaLoc when compared to the VPI 10463 reference strain
MLST	Housekeeping loci	Similar in principle to MLVA
AFLP	PstI & MseI restriction sites	Restriction enzymes cut genomic DNA, which is followed by ligation of adaptors to the ends of the restriction fragments. A subset of the restriction fragments are then amplified using primers that are complementary to the adaptor and part of the restriction site fragments, with the DNA visualised following gel electrophoresis

AFLP: Amplified fragment length polymorphism; DNA: Deoxyribonucleic acid; MLST: Multi-locus sequence typing; MLVA: Multiple locus variable number tandem repeat analysis; PaLoc: Pathogenicity locus; PCR: Polymerase chain reaction; PFGE: Pulse field gel electrophoresis; REA: Restriction endonuclease analysis; RFLP: Restriction fragment length polymorphism; RNA: Ribonucleic acid;

1.7 Management

Since this is an air borne transmissible disease, in order to prevent widespread transmission within hospitals, appropriate infection control measures should be implemented even before diagnosis of CDI has been confirmed. Updated measures for CDI prevention and control have been previously outlined in European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines (Vonberg *et al*., 2008), and termed a 'bundle approach'. These generally include: -

(a) Contact precautions such as the use of gowns and gloves, which has been shown to decrease CDI from 7.7 to 1.5 cases per 1,000 discharges (Johnson *et al*., 1990)

(b) Isolation and cohort nursing, thought to minimise horizontal transmission with patients in single rooms having a lower rate of transmission than those in double rooms (7% versus 17%) (McFarland *et al*., 1989)

(c) Hand washing with soap and water, as alcohol rubs are ineffective against *C. diff* (Wullt *et al*., 2003), by all persons in contact with the patient including family and visitors

(d) Environmental decontamination using chlorine-containing compounds or vaporised hydrogen peroxide (Goulioуris *et al*., 2011), with such standardised cleaning protocols having reduced infection rates on wards with high incidence of HA-CDI (Orenstein *et al*., 2011).

With previous studies having suggested that the use of concomitant antibiotics is associated with a decreased cure rate as well as an increased risk of recurrent CDI (Garey *et al*., 2008; Mullane *et al*., 2011), it has been postulated that upstream antibiotic selection for non-clostridial infections may affect the risk for CDI infection (Malkan and Scholand, 2012). Therefore, it is imperative that such antibiotics should be discontinued whenever possible or, if absolutely necessary, conservative approaches should be in place, such as prescription for the shortest duration possible and/or the selection of targeted narrow spectrum antibiotics. This is known as antibiotic stewardship, supported by

evidence that restriction of high-risk antibiotics has been shown to reduce CDI risk (Aldeyab *et al*., 2012). There also remains the possibility for CDI-targeted antibiotic therapy to be started in elderly and severely ill patients who have pending stool test results but a high clinical suspicion of CDI due to risk factors and ongoing symptoms (Surawicz *et al*., 2013). Additional treatment measures may also include supplementary replacement of fluid and electrolytes, avoidance of anti-motility medications and a review of PPI use in high-risk patients (Bauer *et al*., 2009; Cohen *et al*., 2010; Janarthanan *et al*., 2012; Martinez *et al*., 2012; Surawicz *et al*., 2013).

1.8 Treatment

Metronidazole and vancomycin have been the mainstay treatment options for CDI for the last 30 years, which is rare for a common infectious disease in a developed country (Pepin, 2006) and is partially due to the lack of development of significant resistance (Iv *et al*., 2014). Indeed, from a number of recent studies there is no evidence for increased resistance to either metronidazole or vancomycin (Aspevall *et al*., 2006; Bourgault *et al*., 2006; Hecht *et al*., 2007). However, there are significant unmet medical and therapeutic needs including disease recurrence, meaning that a consensual drug of choice for treating CDI remains controversial. As such, the evolution of epidemic so-called hypervirulent strains have recently sparked doubts due to their increased resistance to a number of key antibiotics, such as quinolones and macrolides (Bourgault *et al*., 2006) This is consistent with results from complete genome sequencing of *C. diff* that revealed the presence of dynamic elements with the potential of developing antibiotic resistance (Sebaihia *et al*., 2006).

More recently, a wide array of antibiotics and alternative therapies have been studied and as the epidemiology of CDI evolves, so should its treatment. Novel therapies or pre-existing strategies for CDI treatment have two main goals: eradicating the organism to ameliorate the infection, (despite continuation of concomitant therapy), and reducing the incidence of disease recurrence. This section summarises the current treatment options being pursued for the treatment and prevention of CDI.

1.8.1 Antibiotic therapy

In addition to the two mainstays across all guidelines (vancomycin and metronidazole), a new FDA-approved drug has been brought to the market that is the only one shown to beat oral vancomycin in clinical trials: fidaxomicin. A number of alternative therapies used in unusual circumstances have occasionally been tested for CDI, though with limited supporting evidence, such as nitazoxanide, rifaximin, teicoplanin and tigecycline (Bartlett, 2009; Johnson and Wilcox, 2012).

1.8.1.1 Vancomycin and Metronidazole

Although not FDA-approved for treating CDI, metronidazole is an inexpensive and generally effective treatment. Although under normal circumstances it is almost completely absorbed in the upper gastrointestinal tract, therapeutic levels are generally achieved in the faeces during diarrhoeal illnesses due to enhanced secretion across a more permeable gut mucosa (Bolton and Culshaw, 1986). However, these levels can be modest and have been found to decrease to undetectable levels as mucosal inflammation improves and diarrhoea resolves (Bolton and Culshaw, 1986). Notably, metronidazole treatment has been shown to be less effective for CDI linked to specific strain variants, such as PCR-ribotype 027 (Freeman *et al*., 2007). By contrast, due to negligible absorption by the intestinal tract, colonic levels of vancomycin are approximately 1,000-fold higher than the MIC of vancomycin for *C. diff* (Baird, 1989), meaning that suppression of *C. diff* to an undetectable level and resolution of diarrhoea occur more rapidly (Al-Nassir *et al*., 2008; Wilcox and Howe, 1995). A reliable but more costly treatment, vancomycin was the first, and until very recently, the only, FDA-approved drug for the management of CDI. Although concerns have previously been raised over links between vancomycin use and promotion of colonisation and transmission of vanomycin-resistant enterococci (VRE), results from a recent retrospective analysis indicate such concerns may be misplaced (Miller *et al*., 2010a).

Despite having been the mainstays for treatment of CDI since 1978, until 2007 only two prospective randomised trials compared vancomycin and

metronidazole, which incidentally demonstrated cure rates above 90% and no relevant differences between the two treatment options (Teasley *et al*., 1983; Wenisch *et al*., 1996). However, these studies were not blinded or placebo-controlled and the treatments were not stratified by disease severity. Several studies have highlighted vancomycin's superiority over metronidazole (Al-Nassir *et al*., 2008; Lahue and Davidson, 2007; Wilcox and Howe, 1995; Zar *et al*., 2007), and results from a more recent clinical study further support this (Johnson *et al*., 2014). Metronidazole was inferior to vancomycin for achieving clinical success, with subgroup analyses demonstrating a similar trend in both moderate and severe patients, in CDI caused by PCR-ribotype 027, in patients over 65 years and for the treatment of first CDI recurrence (Johnson *et al*., 2014). Furthermore, in a retrospective case record analysis, symptomatic response time was shown to be significantly shorter for patients treated with vancomycin as opposed to metronidazole (Wilcox and Howe, 1995).

Progress has certainly been made, and current guidelines now reflect research arising from randomised, clinical trials that looked at stratification of treatments based on key outcomes, such as disease severity and recurrence. In mild-to-moderate CDI, oral metronidazole (e.g. 250–500 mg 3–4 times a day for 10–14 days) is considered equivalent to vancomycin (Cohen *et al*., 2010; Surawicz *et al*., 2013; Zar *et al*., 2007), and is recommended for patients with first infection or first recurrence of mild/moderate CDI in the absence of contraindications (Cohen *et al*., 2010). Second or later recurrence of CDI should be treated with vancomycin, using a tapered and/or pulse regimen, as per recommendation by ESCMID. Oral vancomycin is also recommended for the treatment of severe CDI (125 mg 4 times a day for 10 days) due to its superior cure rates in these patients (97% versus 76%) (Cohen *et al*., 2010; Surawicz *et al*., 2013). In seriously ill patients with severe-complicated CDI, a higher dose (250–500 mg) in combination with intravenous metronidazole is recommended (Cohen *et al*., 2010).

1.8.1.2 Fidaxomicin

Whilst generally effective in controlling *C.diff* levels, vancomycin and metronidazole are broad-spectrum antibiotics that significantly prolong colonic dysbiosis, a side effect that may predispose patients to disease recurrence. The macrocyclic antibiotic fidaxomicin, recently approved in Europe and North America for the treatment of CDI, has a narrow spectrum of activity against Gram-positive aerobic and anaerobic bacteria, including *C. diff* (Gerber and Ackermann, 2008), and is seemingly less disruptive to the commensal microbiota. Furthermore, it has a bactericidal mechanism of action (Venugopal and Johnson, 2012), a safety profile comparable to that of vancomycin (Weiss *et al*., 2012), and undetectable serum levels, whilst often achieving high faecal concentrations that average greater than 10,000 times the MIC for *C. diff* (Sears *et al*., 2012). Fidaxomicin delivered close to full protection in a hamster model of CDI (Johnson, 2007) and there are signs for its role in reducing toxin re-expression and CDI recurrence (Louie *et al*., 2012).

The majority of the attention surrounding fidaxomicin stems from the findings of two prospective, multi-centric, double-blind, randomised phase III trials, which demonstrated its non-inferiority to vancomycin for clinical cure rate, but superiority in reduction of recurrence and sustained clinical response (cure without recurrence during the 30 day follow-up) upon meta-analysis (Crook *et al*., 2012). Interestingly, when the meta-analysis was limited to the epidemic PCR-ribotype 027 strain, a 22% non-significant reduction in persistent/recurrent diarrhoea was observed (Crook *et al*., 2012). Although this lack of association may in fact be due to lack of power, the finding demonstrates that treatment of PCR-ribotype 027-associated cases remains more challenging. Most recently, fidaxomicin was shown to be successful in resolving CDI in an *in vitro* gut model, with observations of supra-MIC levels and prevention of spore recovery, therefore underscoring the *in vivo* observations that fidaxomicin is associated with a reduced risk of disease recurrence (Chilton *et al*., 2014).

There are calls for prescription of fidaxomicin in the treatment of severe CDI patients at high risk of recurrence (Hu *et al*., 2009; Wilcox *et al*., 2012).

However, the current major stumbling block for its routine use concerns its extremely high costs. Its current average wholesale price is 135 US dollars (Lancaster and Matthews, 2012), compared to 0.72 for a 500 mg dose of metronidazole and 31.81 US dollars for a 125mg vancomycin capsule (Lancaster and Matthews, 2012). Therefore, the estimated costs for a 10-day course of fidaxomicin therapy (2 times daily) would be $2700, compared to the $22 and $1270 associated with metronidazole (3 times daily) and vancomycin 125 mg (4 times daily), respectively (Lancaster and Matthews, 2012). The pharmaceutical company responsible for its retail is now developing several strategies to assist in cost reduction (Iv *et al*., 2014), with further studies needed on its cost-effectiveness in CDI.

1.8.1.3 Others

A variety of other antibiotics have been explored for the treatment of CDI. These are summarised in Table 1.3.

1.8.2 Non-antibiotic therapy

1.8.2.1 Surgical management

Surgical intervention is an aggressive alternative therapy and tends to be restricted to patients suffering from severe-complicated CDI, with colectomy having long been the procedure of choice. Indications include failure to respond to maximal medical management, caecal dilatation larger than 10 cm and presence of bowel perforation. A review of 165 CDI cases requiring admission to the intensive care unit (ICU) during the Quebec epidemic season between 2003-2005 observed a significant decrease in mortality in individuals who had undergone a colectomy compared to those treated medically (Lamontagne *et al*., 2007). However, that study was retrospective in nature and there are conflicting data regarding the preferred surgical procedure (Dallal *et al*., 2002; Koss *et al*., 2006; Neal *et al*., 2011). Most recently, a novel colon-sparing surgical procedure has been proposed as an interesting alternative to total colectomy (Neal *et al*., 2011). While further studies are needed to evaluate the beneficial

impact of this approach, colectomy remains the procedure of choice (Surawicz *et al*., 2013).

Even after surgical intervention, mortality rates remain quite high (averaging over 50% in some series) (Dallal *et al*., 2002). With early surgery having been shown to be superior to delayed surgery in improving patients' outcomes, early surgical consultation should be pursued for all severe-complicated CDI patients.

Table 1.3 – Overview of alternative antibiotics used for treatment of *Clostridium difficile* infection

Antibiotic	Details	Pros	Cons	Considerations
Nitazoxanide	Anti-parasitic drug	Highly active *in vitro* against *C. diff* Shown to prevent colitis in the hamster model Shown to be as effective as vancomycin & metronidazole for treatment of CDI	Lack of safety and efficacy data Has not been directly compared against other drugs	May currently only be considered as an alternative in patients unresponsive to standard therapy who may not be suitable candidates for FMT
Rifaximin	Broad spectrum antimicrobial	Excellent *in vitro* activity against *C. diff* Not thought to significantly alter gut microbiota Studies demonstrate similarity to vancomycin for resolution of diarrhea and rates of recurrence	Inferiority to vancomycin for achievement of clinical success Potential for resistance	Not currently recommended as a monotherapy for CDI May be used at the end of primary treatment with vancomycin in an attempt to decrease recurrences ('rifaximin chaser')
Teicoplanin	Semi-synthetic glycopeptide antibiotic	Spectrum of activity similar to that of vancomycin Equivalent, or in some cases superior, to vancomycin Licensed indication for treatment of CDI since 2013	Lack of availability in the US High associated costs	The cons associated with teicoplanin limit its use
Tigecycline	Broad-spectrum glycylcycline antibiotic	Achieves fecal concentrations well above MIC for *C. diff* Doesn't induce *C. diff* toxin production *in vitro* Several reports demonstrated success in severe CDI	Not licensed for this indication No clinical trials conducted to date Recent report refuted its efficacy	Despite initial promise, caution is urged for indiscriminate off-label use. Data from prospective clinical trials is needed.

C. diff: Clostridium difficile; CDI: Clostridium difficile infection; FMT: Faecal microbiota therapy; MIC: Minimum inhibitory concentration; US: United States;

1.8.2.2 Reestablishment of colonic microflora

As discussed previously, the human colonic microflora is one of the most important natural barriers against colonisation and infection by *C. diff.* Alterations in the balance of intestinal flora, such as those triggered by broad spectrum antimicrobials, have a critical role in disease pathogenesis (Na and Kelly, 2011). The host's inability to restore gut microfloral balance is a commonly observed feature of disease recurrence (Lawley and Walker, 2013; Newton *et al*., 2013). Therefore, it is worth considering measures that promote restoration of gut microbiota diversity for future management of CDI patients. Such therapies have gained ground in recent years, and two approaches have been the subject of intense debate: the use of probiotics, and faecal microbiota transplantation (FMT).

Probiotics

Probiotics are living microorganisms that confer a health benefit to the host (World Health Organisation, 2001), with increasing evidence for their use in a variety of gastrointestinal conditions (Floch *et al*., 2011). A number of probiotic strains have been tested across CDI studies, most commonly *Lactobacilli* (especially *Lactobacillus rhamnosus GG*) and the yeast *Saccharomyces boulardii* (Parkes *et al*., 2009; Tasteyre *et al*., 2002). Activity against *C.diff* and other opportunistic pathogens, as well as modulation of host response, has been demonstrated experimentally using both *in vitro* studies and animal models (Castagliuolo *et al*., 1999; Chen *et al*., 2006; Qamar *et al*., 2001; Trejo *et al*., 2006), and several recent meta-analyses have endorsed potential benefits for the use of probiotics (Martin *et al*., 2013). However, these meta-analyes are limited due to a lack of standardisation across studies and the administration method employed.

Overall, there is a lack of large randomised controlled trials (RCTs) that include CDI treatment as a primary outcome, and results obtained from the existing RCTs are variable. Thus current evidence for the use of probiotics in treating CDI is limited and they cannot currently be recommended (Cohen *et al*., 2010; Public Health England, 2013). Further research involving large and well-

controlled studies is clearly needed to fully determine the efficacy of this approach. One such study completed recruitment in 2012 but the results have yet to be published (Allen *et al*., 2012).

Faecal Microbiota Transplantation

In contrast, FMT appears highly effective for the treatment of CDI. First used over 50 years ago to successfully treat PMC that was later found to be due to CDI (Eiseman *et al*., 1958), this process is based on the concept that microorganisms in the stool of a healthy donor can re-establish microfloral diversity and suppress *C. diff*. In such an approach, the donor faeces is initially screened for transmissible infectious pathogens and then transferred into a CDI patient's lower proximal, lower distal or upper GI tract via one of many employed methods including enema, nasogastric tube and colonoscopy.

FMT has been proposed as an alternative treatment for patients suffering from both severe unresponsive and recurrent disease (Pacheco and Johnson, 2013; Russell *et al*., 2010), and existing data are very compelling. One study was able to show that the post-transplant bacterial composition of the recurrent CDI-recipient's stool becomes remarkably similar to that of the healthy donor (Khoruts *et al*., 2010). Recent meta-analyses of previous case series have reported clinical 'cure' rates of over 90% for refractory CDI (Guo *et al*., 2012; Kassam *et al*., 2013; Sofi *et al*., 2013). Notably, the first randomised controlled trial recently demonstrated that FMT following antibiotic treatment with an oral glycopeptide is highly effective in treating patients with multiple recurrent CDI (van Nood *et al*., 2013).

Despite these promising results demonstrating FMT's benefit to a large number of patients, the procedure is still regarded as a last resort treatment. However, recently published ECSMID guidelines strongly support FMT in combination with oral antibiotic therapy for multiple recurrent CDI cases, especially for those who have been unresponsive to repeated antibiotic therapy (Debast *et al*., 2014). The recent introduction of a US FDA mandate requiring approval of FMT as a drug (Food and Drug Administration, 2013) may further restrict its use though it is hoped that similarly efficacious synthetic bacterial mixtures can be

developed for treating CDI in the future with investigative studies already underway.

1.7.8.3 Binding agents

Non-absorbable, anion exchange resins that remove cytotoxic activity through binding of *C. diff* toxins have also been used to treat CDI. Advantages of such a method include no disturbance of the normal intestinal flora (potentially decreasing the risk of recurrent disease), no underlying resistance issues, the availability of a non-antibiotic therapy for an antibiotic-induced disease, and relatively lower costs (Taylor and Bartlett, 1980). In the early stages cholestyramine and colestipol (Kreutzer and Milligan, 1978) were investigated but their observed activity was modest and thus other candidates were pursued, such as Tolevamer. Despite promising results in phase II (Louie *et al*., 2006), Tolevamer's performance was overshadowed in phase III studies by comparatively superior antimicrobial standard therapy with either metronidazole or vancomycin (Johnson *et al*., 2014). Designing non-antibody molecules that are able to sustainably bind with high affinity and avidity to *C. diff* toxins whilst at the same time avoiding other substrates circulating in the gut is extremely challenging and it is thought that monoclonal antibodies (MAbs) (see section 1.7.8.4) constitute a more realistic approach for toxin immobilisation or neutralisation (Lowy *et al*., 2010).

1.7.8.4 Immunotherapy

The prominent role of humoral immunity in neutralisation and clearance of *C.diff* toxins is widely accepted. As such, mounting a robust *C. diff* anti-toxin response has been shown to confer protection against the development of CDI following nosocomial colonisation with the organism (Kyne *et al*., 2000a, 2001), with mildly affected patients displaying high levels of anti-toxin immunoglobulin G (IgG) (Kyne *et al*., 2001; Viscidi *et al*., 1983; Warny *et al*., 1994) and recurrent patients having comparatively lower anti-toxin Ig titres (Katchar *et al*., 2007; Kyne *et al*., 2001). Based upon this, early researchers hypothesised that anti-toxin neutralising antibodies could potentially become a primary line for treating CDI given their specificity for the toxins. Termed

immunotherapy, methods currently being used include the introduction of intravenous immunoglobulins or monoclonal antibodies (known as passive immunisation), and vaccination (known as active immunisation).

Passive immunisation

Intravenous immunoglobulin (IVIG) involves the administration of a blood product containing the pooled, polyvalent IgG antibodies isolated from multiple blood donors. This therapy has been used sporadically for the treatment of CDI since 1991 (Stanley *et al*., 2013), and its use for treating severe refractory and recurrent CDI has been met with mixed feelings by the scientific community due to variable rates of success. Although 15 small, mostly retrospective and non-randomised reports have documented successful treatment of protracted, recurrent or severe CDI using IVIG, there are no randomised control trials endorsing its benefits (Abougergi *et al*., 2010). Furthermore, meta-analysis of these studies concluded that there is a lack of evidence to prescribe its use, though it is acknowledged that this is in part due to lack of consensus on indications, dosage, and/or data from trials tailored in design (Abougergi *et al*., 2010). When combined with the fact that the cost of an individual IVIG approaches $10,000 per treatment course, clear cut proof of efficacy is important. A prospective study was undertaken evaluating the utility of IVIG in combatting severe CDI (Juang *et al*., 2007), but these results were inconclusive and could not demonstrate an obvious role for IVIG in ameliorating symptoms. In 2013, the ACG assessed its use for recurrent CDI, with subsequently published guidelines concluding that it does not have a significant role as a sole therapy agent in part due to a potential lack of specificity (Surawicz *et al*., 2013).

Results from administration of mAbs directed against tcdA and tcdB seem slightly more promising. Having previously reduced hamster mortality following intra-peritoneal injection (Babcock *et al*., 2006a), the most successful study to date involves concurrent administration of two full MAbs (one against tcdA, the other against tcdB) in 200 symptomatic CDI patients concomitantly receiving either metronidazole or vancomycin (Lowy *et al*., 2010). This randomised, double-blind, placebo-controlled study found that overall

recurrence rate was remarkably lower in the MAb group compared to those receiving placebo (7 vs. 25%; p<0.001) (Lowy *et al*., 2010). Notably, it has also been shown that MAbs have a long serum half-life, typically 14-21 days for human IgG1 (Wang *et al*., 2008), which is a significant time frame given the typical duration of a primary episode and potential disease recurrence periods. Collectively, these results suggest that MAbs may represent a useful treatment option for CDI, with single infusions or sub-cutaneous injections offering sustained protection against primary and recurrent infections, respectively. Although this approach is not yet commercially available, it is now being pursued by large pharmaceutical companies with a phase III study underway to further establish its efficacy and safety (Khanna and Pardi, 2014).

Active immunisation

The current chief approach considered for the prevention of CDI is through the development of a vaccine, with substantial progress having been made in recent years. In 2005, Sougioultzis *et al.* reported successful prevention of further recurrence in 3 separate patients suffering from recurrent CDI who had been vaccinated with intramuscular administration of tcdA and tcdB (Sougioultzis *et al*., 2005). In 2010, Leav *et al*. showed that their toxoid vaccine was capable of inducing serum anti-tcdA and anti-tcdB IgG antibody levels that were typically associated with protection against recurrent CDI (Leav *et al*., 2010). More recently, development of a chimeric anti-toxin vaccine using an endotoxin-free expression system derived from *Bacillus megaterium* has begun, with initial data showing its capability for neutralising anti-toxin production and preventing spore-induced recurrence (Wang *et al*., 2012).

Another pharmaceutical company, Sanofi Pasteur, has recently completed six Phase I trials across 200 individuals using bivalent formalin-inactivated vaccines against tcdA and tcdB, with the development of detectable specific antibodies (termed seroconversion) in 75% of participants by day 70 (Foglia *et al*., 2012). With their initial studies having verified safety and immunogenicity, a phase II trial of the vaccine assessing prevention of primary CDI is currently ongoing with 650 at risk US adults (Sanofi Pasteur). Other major vaccine

manufacturers are currently at a pre-clinical stage, utilising a similar parenteral approach. The major challenge for vaccine studies is related to their prospects for delivering immunity against all major nosocomial strains of *C. diff* as well as fully preventing CDI in a clinical setting for both primary and recurrent cases.

1.8.3 Summary

A recent Cochrane review of 15 studies concluded that a formal recommendation for a consensual antibiotic therapy of CDI could not be made (Nelson *et al*., 2011). Current comparison across studies is challenging due to a lack of standardisation. In particular, the use of varying definitions of disease severity result in the definition of phenotypically distinct populations, which in turn result in mixed conclusions, and this is especially true for small treatment groups (Wilcox, 2014). Therefore, the use of a standardised severity definition across all drug trials would certainly be beneficial but the lack of independently validated prediction tools that robustly define therapeutic clinical outcomes in CDI makes the task extremely difficult.

Despite the observed limitations, there is an urgent need for clear cut and updated guidelines for the treatment of CDI. Guidelines are now stratifying treatment recommendations based on clinically defined sub-groups, including non-severe disease, severe disease, first recurrence and multiple recurrences. Recently updated ESCMID guidelines (Debast *et al*., 2014) are summarised in Figure 1.8.

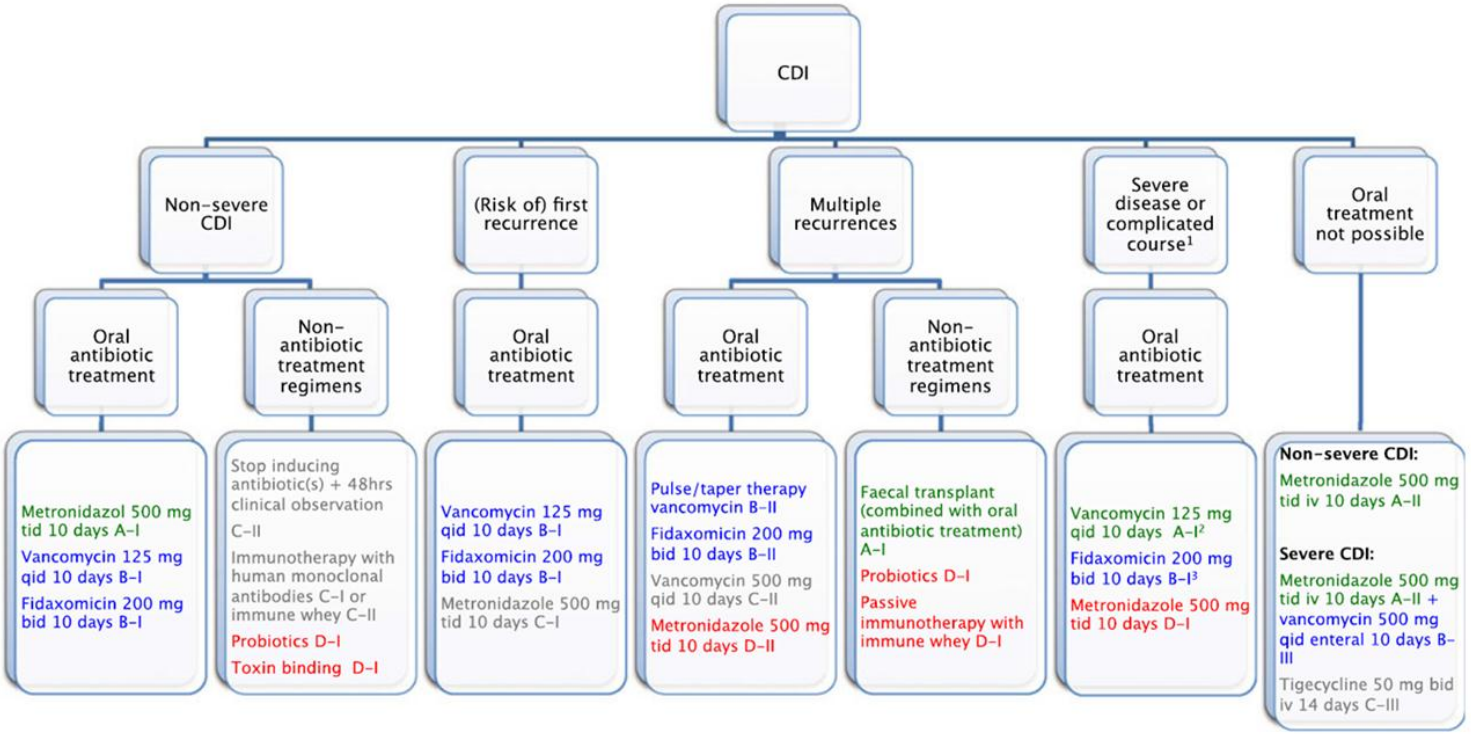


Figure 1.8 – Schematic overview of therapeutic regimens for *Clostridium difficile* infection (taken from (Debast *et al.*, 2014)

[1] Severe CDI or complicated course: surgical therapy not included in this overview; [2] It can be considered to increase the oral dosage of vancomycin to 500 mg four times daily for 10 days (B-III); [3] There is no evidence that supports the use of fidaxomicin in life-threatening CDI (D-III); Strength of recommendation (SOR) A = green (strongly suggests a recommendation for use); SoR B = blue (moderately supports a recommendation for use); SoR C = grey (marginally supports a recommendation for use); SoR D = red (recommendation against use).

1.9 Prognosis

As outlined in section 1.8, guidelines for the stratification of treatment have become reliant upon disease outcomes. This is mainly due to the dramatic increases observed in disease severity, recurrence and mortality in the last decade. Although the majority of patients tend to initially respond well to either vancomycin or metronidazole, a significant amount of patients suffer from recurrence, with reported rates varying between 5 and 50% (Aslam *et al*., 2005). Recurrent CDI remains a substantial challenge as it inevitably increases transmission periods and an initial recurrence constitutes a predisposing state that is often followed by further similar episodes. Pooled attributable mortality, within 90 days of diagnosis, has risen from 3.64% prior to the year 2000 to 8.03% post-2000 (Karas *et al*., 2010). It has been shown that hospitalised patients with CDI are 2.74 times more likely to die during their hospitalisation stay than all other non-CDI patients (Wenisch *et al*., 2012), and absolute mortality within 30 days is increased by 10% in the presence of CDI (Loo *et al*., 2005; Oake *et al*., 2010).

At initial disease presentation most patients display similar symptoms and therefore predicting who may progress to experience unfavourable outcomes is difficult, though much needed. Identifying clinical parameters or host-related factors associated with adverse outcomes would improve the management of CDI in the early stages, and would enable the promotion of novel interventions for the prevention of recurrence (Johnson, 2009; Louie *et al*., 2011) or alternatively more aggressive treatment for patients at most risk of clinical complications (Bauer *et al*., 2009; Cohen *et al*., 2010). Progress within diagnosis of CDI (described in section 1.6) has resulted in molecular testing for *tcdA* and *tcdB* becoming more common in the US, whilst toxin detection remains the test of choice in Europe (Burnham and Carroll, 2013; Crobach *et al*., 2009). Use of these different tests has also generated an increasing need for treatment stratification based on defined clinical criteria/host biomarkers (Boone *et al*., 2014).

A myriad of variables have been associated with increased CDI severity, recurrence and mortality, including increasing age, concomitant antibiotics and/or PPIs use, underlying comorbidities, fever, abdominal pain, increased WCC, elevated serum creatinine, decreased serum albumin, admission to ICU, PCR-ribotype and previous episodes of CDI (Belmares *et al*., 2007; Bishara *et al*., 2008; Fujitani *et al*., 2011; Garey *et al*., 2008; Henrich *et al*., 2009; Hu *et al*., 2009; Keddis *et al*., 2012; Khanna *et al*., 2013b; Miller *et al*., 2010b; Pant *et al*., 2010; Pépin *et al*., 2004; Rodríguez-Pardo *et al*., 2013; Walker *et al*., 2013; Zar *et al*., 2007). A great deal of attention has been placed on information available at the time of diagnosis such as clinical/admission data and blood biomarkers routinely measured during a patient's hospitalisation in order to facilitate development of simple scoring systems, or clinical prediction rules (CPRs) that could be used at the patient's bedside for both prognostic care and treatment guidance, as well as evaluating response to therapy. Prevalent across varying areas of clinical medicine, multiple CPRs are in routine use across gastroenterology specialties (Forrest *et al*., 2005; Malinchoc *et al*., 2000; Rockall *et al*., 1996). Despite the existence of several CPRs for the prediction of CDI outcomes, none have gained widespread clinical acceptance, with a recent systematic review of 13 identified CPRs utilised for unfavourable CDI outcomes concluding that current implementation of existing CPRs is limited by several methodological issues, heterogeneity of phenotype definition and a lack of statistical power (Abou Chakra *et al*., 2012).

Other biomarkers not routinely assessed in a clinical setting have been experimentally investigated, although only a small number of associations have been identified to date. Host anti-toxin immunoglobulin response has been shown to play a key role in influencing the duration of disease (Warny *et al*., 1994), and determining the risk of recurrence (Kyne *et al*., 2001; Warny *et al*., 1994) and mortality (Solomon *et al*., 2013). As well as the induction of an anti-toxin response, some attention has been given to the release of pro-inflammatory cytokines *in vitro* elicited by toxin exposure, thus constituting plausible candidates (Hippenstiel *et al*., 2000; Ishida *et al*., 2004). The single nucleotide polymorphism (SNP) rs4073/-251T>A within the gene encoding

pro-inflammatory cytokine interleukin-8 (IL-8) remains the only genetic association with CDI reported to date, with the AA genotype being shown to increase the odds of developing CDI, as well as experiencing recurrent disease, by at least 3-fold (Garey *et al*., 2010; Jiang *et al*., 2006; Jiang *et al*., 2007). The increase in secretion of pro-inflammatory cytokines results in augmented intestinal inflammation (Kelly and Kyne, 2011; Savidge *et al*., 2003), and consequently two faecal biomarkers widely used in IBD as indicators of intestinal inflammation, lactoferrin and calprotectin (D'Incà *et al*., 2008; García-Sánchez *et al*., 2010; Jones *et al*., 2008; Langhorst *et al*., 2008; Schoepfer *et al*., 2009; Schoepfer *et al*., 2010; Schoepfer *et al*., 2008; Sipponen *et al*., 2008a; Sipponen *et al*., 2008b; Sipponen *et al*., 2008c; van Langenberg *et al*., 2010), have been pursued for the study of CDI, with positive associations in a number of studies (Archbald-Pannone *et al*., 2010; Boone *et al*., 2013; El Feghaly *et al*., 2013; LaSala *et al*., 2013; Shastri *et al*., 2008; Vaishnavi *et al*., 2000; Whitehead *et al*., 2014). Most recently, associations with poor CDI outcomes have been identified with interleukin-23 (IL-23) (Buonomo *et al*., 2013) and procalcitonin (PCT) (Rao *et al*., 2013).

1.10 Aims of the thesis

Despite the considerable progress made in relation to CDI diagnosis and treatment, the increasing burden of disease-related outcomes including recurrence and mortality means that a lack of robust prognostic markers remains a priority area for significant advances. Although numerous clinical investigations have been conducted for CDI, there remains no single parameter, or combination of parameters, validated for the stratification of patient therapy, with the majority of studies suffering from suboptimal study design, inconsistent outcome definitions, small sample size, and a lack of standardisation (Abou Chakra *et al*., 2012).

A similar situation can be seen with the associations identified for non-routinely measured biomarkers. Genetic associations with IL-8 have not been independently replicated, and studies assessing faecal biomarkers such as lactoferrin and calprotectin suffer from a wide array of limitations. Since the

majority of the significant associations regarding host anti-toxin response were identified, the CDI research landscape has undergone a significant reshuffle: the emergence of epidemic, CDT-producing strains has resulted in a need for re-evaluation of existing concepts. Furthermore, the question remains as to whether more specific CDI biomarkers are yet to be discovered or whether the current methodologies simply lack the necessary credentials to achieve validation. This is of particular importance within vaccine development, whereby quantitation of antibody response is hampered by reliance on limited in-house ELISA methods and the absence of more innovative supporting approaches.

Using a prospective cohort of carefully phenotyped individuals, this thesis aims to: -

1. Determine independent risk predictors of CDI disease outcomes such as severe-complicated disease, recurrence and mortality, and to assess the consistency of existing clinical prediction rules for each individual outcome.

2. Evaluate the IL-8 genetic variant implicated in predisposing to CDI and recurrent disease, relate it to faecal IL-8 levels and undertake a meta-analysis using available literature.

3. Simultaneously evaluate both faecal lactoferrin and calprotectin in order to investigate whether these faecal biomarkers would add clinical value in the stratification of complicated CDI patients

4. Develop a novel, sensitive assay for the quantification of host immune response to tcdA, tcdB, and CDT, the latter being a novel approach previously not reported in the literature.

5. Evaluate the potential role of mannose-binding lectin (MBL), a key activator of the complement system and modulator of inflammation, as a

novel diagnostic/prognostic candidate for CDI and related outcome measures.

6. Investigate the role of immunoglobulin G- and immunoglobulin M-driven responses to both tcdA and tcdB, as well as the previously uninvestigated CDT, as predictors of poor CDI outcomes.

Chapter 2

PhD/Cohort study overview

Contents

2.1 Background

My PhD formed part of an ongoing NIHR-funded research study that was set-up in 2008 as part of Liverpool's Biomedical Research Centre status at the Royal Liverpool University Hospital: "*Clostridium difficile*-associated toxin disease: development of a tool to predict individual susceptibility based on environmental and genetic factors".

I was originally employed as a research technician to process the samples from this and other departmental studies in April 2008. In September 2009, my PhD began on a part-time basis alongside this role, funded through the Liverpool BRC. However, in late 2011 it was announced that Liverpool had not been renewed and as a BRC centre and from 1st April 2012 I would have no further funding. I was therefore encouraged to apply for an NIHR Biomedical Research Fellowship in order to obtain the necessary funding to continue my PhD. I was successful in my application (ref: BRF-2011-028) and received 18 months funding on a full-time basis, commencing 1st April 2012.

2.2 Study overview

2.2.1 Study design

At study inception, we aimed to prospectively recruit 300 CDI cases and 300 AAD controls, based upon the following power analysis taken from the initial study application:

"We are unable to provide realistic power calculations in advance. However, we can calculate power for some simplistic analyses. In particular, for the power calculations below, we consider carrier / non-carrier analysis for a single SNP and a binary outcome (e.g. benefit/no benefit) or time to event outcome. Thus, the values given below are expected to substantially understate the true power of the studies, since combinations of SNPs will be analysed simultaneously via haplotype and regression-based analyses, and quantitative response variables will be available. A key variable is the frequency of variant carriers among patient. For rarer variants to be clinically important, their effect size (odds ratio (OR) or

hazard ratio (HR)) must be large. We therefore specify two benchmarks for the power analyses: we seek to have good power for (a) OR/HR=3 and a rare variant (p=5%); (b) OR/HR=2 for a common variant (p=20%). Note that these effect sizes are for a single causal variant, we expect to realise much larger overall effect sizes via combinations of causal variants. The assumed type 1 error is 5%."

2.2.2 Patient recruitment

An overview of the recruitment process is provided in Figure 2.1. Ethical approval was obtained from the Liverpool Research Ethics Committee (reference number 08/H1005/32) and each patient provided written informed consent prior to recruitment. Blood and faecal specimens were collected from patients at study entry.

2.2.2.1 Case and control definitions

CDI cases:

- Positive laboratory diagnosis of CDI (TOX A/B II, Techlab, Blacksburg)
- Aged ≥18 years
- Healthcare-associated diarrhoea
    - ≥3 liquid stools in 24 hours preceding assessment
    - Onset after being in hospital for >48 hours
- Recent exposure (within 30 days) to antimicrobials and/or PPIs

AAD controls:

- Negative laboratory diagnosis of CDI (Toxin EIA A/B)
- Aged ≥18 years
- Healthcare-associated diarrhoea
    - ≥3 liquid stools in 24 hours preceding assessment
    - Onset after being in hospital for >48 hours
- Recent exposure (within 30 days) to antimicrobials and/or PPIs
- Strict exclusion criteria (see Figure 2.3)

Samples were then subject to microbiological culture. Any AAD control samples testing positive for culture were excluded from the study.

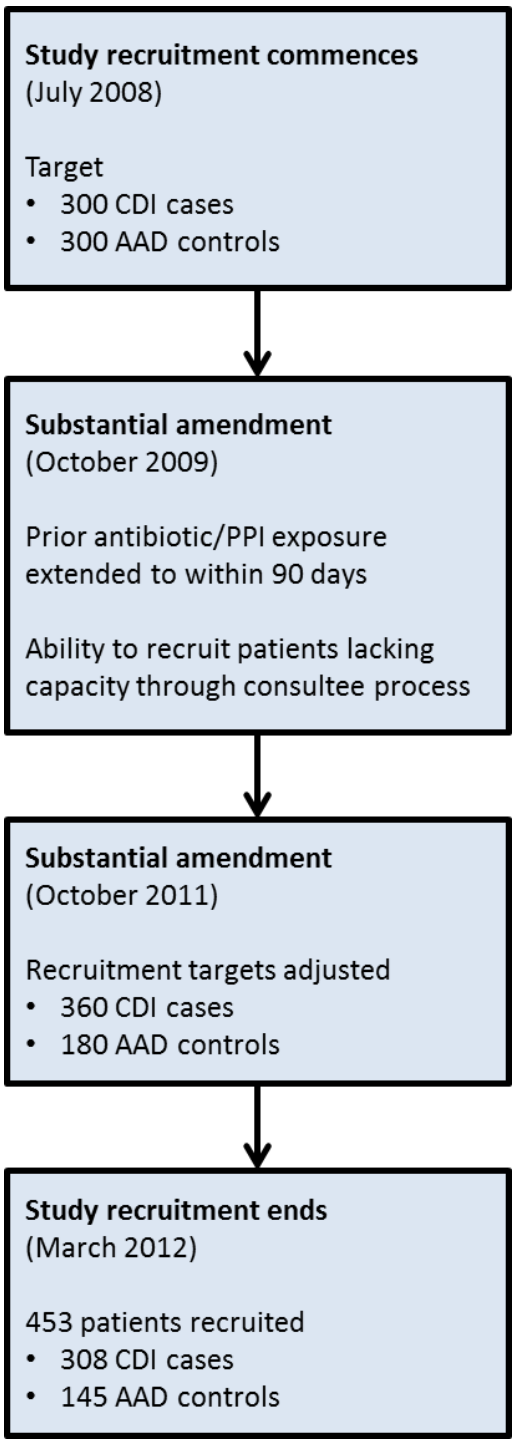


Figure 2.1 – Overview of recruitment process for CDI cohort study

2.2.2.2 Amendments

The adjustment of prior antimicrobial/PPI exposure to within 90 days outlined in the first substantial amendment (see flowchart) was necessary as throughout the study we noticed a significant number of new CDI cases in our Trust, where patients had no antibiotics in the previous 30 days while having a documented history of antibiotic usage in the 90 day period before the index date. The 3-month guideline was also corroborated by the infection control team in our Trust and backed by reports from the recent literature (Dial *et al*., 2006; Dial *et al.*, 2008).

This substantial amendment also allowed us to recruit patients who lacked capacity through the involvement of a consultee. Until that date, approximately 40% of patients approached had been ineligible to participate as they lacked capacity to consent. The reasons for lack of capacity varied and included acute confusion due to deranged liver/kidney function, dehydration, and long term cognitive impairment. It is extremely important to recruit from these vulnerable groups as they are more likely to suffer from serious symptoms and comprise an important group within the spectrum of the disease phenotype. By excluding those patients who cannot consent, we are in danger of getting selection bias which will lead to limited generaliseability. Using the consultee process we managed to recruit 13 patients to our study.

The adjustment of recruitment targets outlined in the second substantial amendment (Figure 2.1) was based on a revision of the existing cohort at that time, as well as on power calculations based on our current recruitment rates, timelines and overall numbers recruited to that date:

- Inability to identify and recruit adequate numbers of AAD controls meeting our inclusion criteria meant that, given our timelines, we felt that we would have been unlikely to reach the target of 300. We therefore chose to recruit additional cases (360 as opposed to the original target of 300) as a compensatory measure in order to nullify any loss of power

- Power calculations estimated that the revised recruitment would have 90% power to detect an effect size of OR≥2.89 assuming an event frequency around 5% (f~5%); and OR≥1.96 for a relatively common event (f~20%). We believed that the revised figures (360 CDI cases and 180 AAD controls) would be sufficiently powered for our investigation. Figure 2.2 shows the effect size able to detect with increasing power for both a relatively rare event (f=5%) and a common event (f=20%), with the increase in the number of cases compensating for the reduced number of controls.

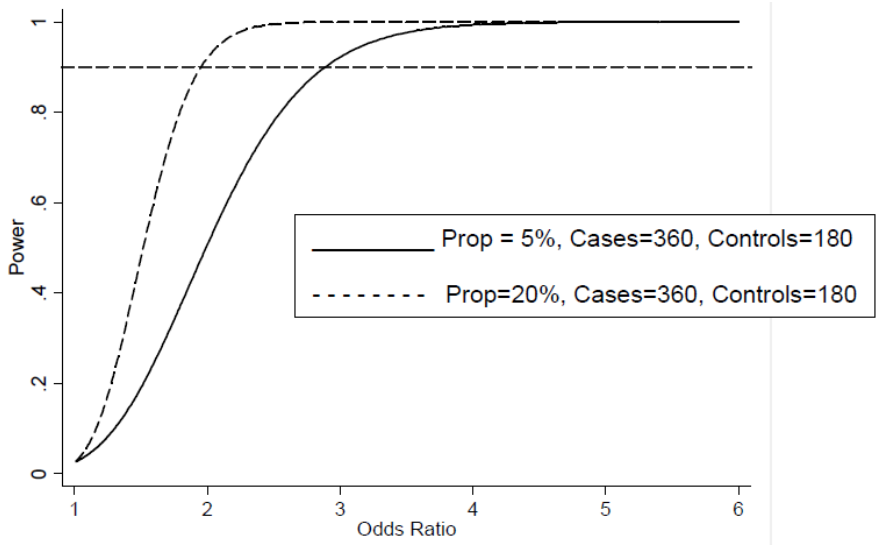


Figure 2.2 – Effect size able to detect with increasing power for both a relatively rare and a common event

2.2.3 Final cohort

As outlined in Figure 2.1, a total of 453 patients (308 CDI cases and 145 AAD controls) were prospectively recruited between July 2008 and March 2012, across two large hospital sites in Merseyside: the Royal Liverpool and Broadgreen University Hospitals Trust (RLBUHT) and Wirral University Teaching Hospital (WTH). The screening process involved in recruitment of this final cohort is summarised in Figure 2.3.

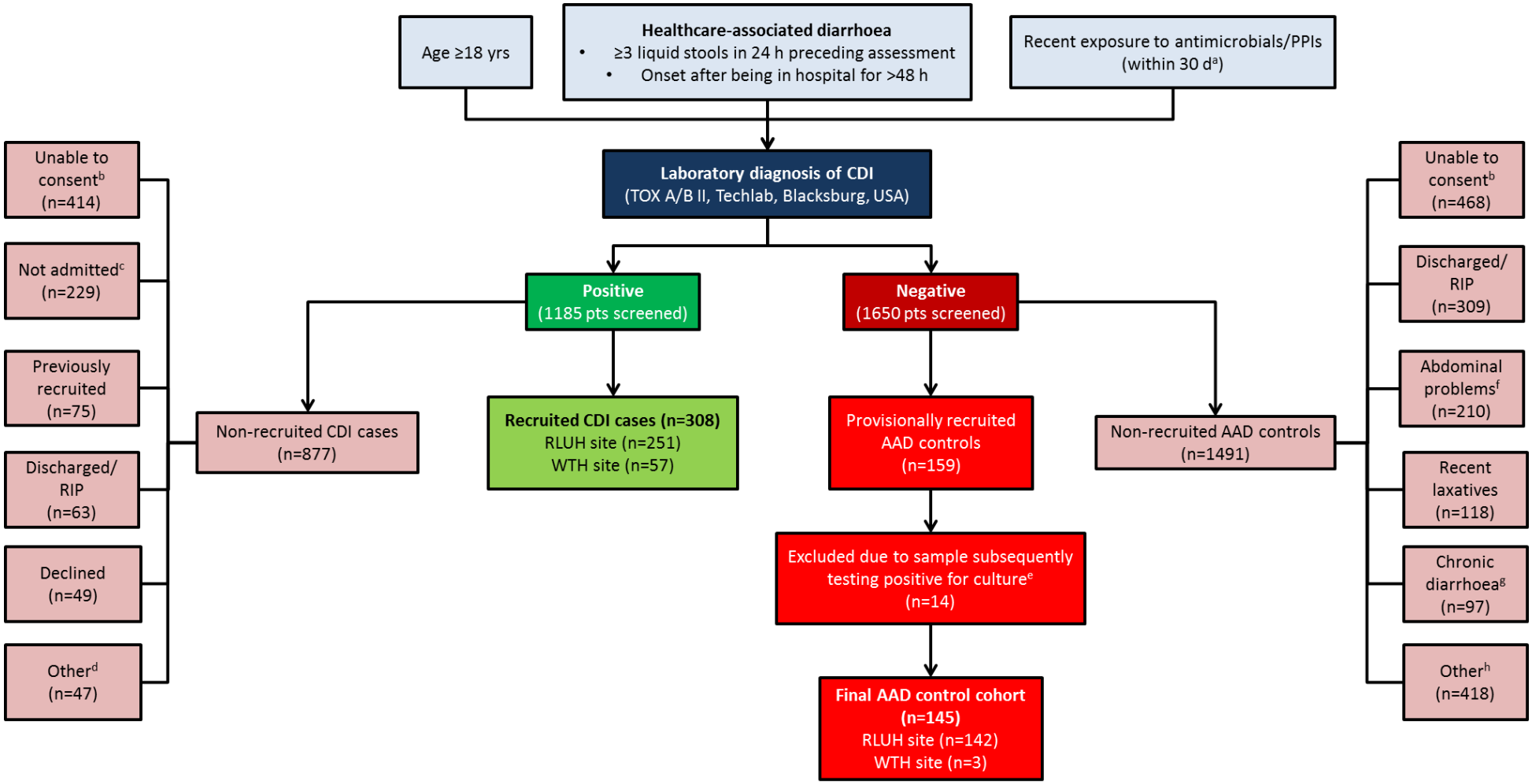


Figure 2.3 – Overview of recruitment screening (July 2008 – March 2012)

a: Adjusted to within 90 days (see flowchart); **b**: Includes cognitive impairment, confusion, dementia, lacking capacity, unresponsiveness, poorly, inability to communicate; **c**: Includes community patients and those admitted to sites to which we did not have recruitment access (e.g. Cardiothoracic centre); **d**: Includes annual leave/other work commitments of recruitment staff, decreased/low Hb; **e**: Patients provisionally recruited as AAD controls were also excluded if they developed CDI-positive diarrhoea during the study period; **f**: Also includes bowel, gastric and pancreatic problems, as well as colostomy, recent abdominal surgery, overflow diarrhoea, stoma and IBD; **g**: Diarrhoea that preceded antibiotics; **h**: Includes patients previously recruited to the study, those who have previously tested CDI positive, those on recent chemotherapy and other drug-related interactions, those who declined, those with nastrogastric and enteral feeding, those not admitted (e.g. outpatients or day cases), those with no evidence of diarrhoea (i.e. single stool), those with decreased/low Hb and those with known infections (e.g. Salmonella, Influenza, Rotovirus, Norovirus)

2.2.4 Data collection

Relevant information on demographics, admission and clinical history was collected for each patient and recorded using a standardised research pro-forma. Clinical progress was monitored for a period of 30 days. If the patient was discharged from hospital prior to final follow-up, we attempted in every case to obtain data from the hospital, general practitioner or the patient (the latter by a telephone call).

2.2.5 Microbiological methods

Faecal samples were tested for *C. diff* toxin using a TOX A/B II ELISA kit according to the manufacturer's instructions (Techlab, Blacksburg, USA). Toxin positive samples were screened for faecal leukocytes (an indicator of intestinal inflammation) by microscopy of a wet preparation of faecal sample. Specimens were cultured for *C. diff* using Brazier's cefoxitin-cycloserine egg yolk agar (Lab M Ltd, Bury, UK) and incubated for 48 hours at 37°C in an anaerobic chamber. Isolates were identified by characteristic smell, colonial morphology and fluorescence under long wave UV light. Identification was confirmed using a latex agglutination test for *C. diff* somatic antigen (Oxoid, Basingstoke, UK). Isolates were stored on PROTECT beads (Technical Services Consultants Ltd, Heywood, UK) at -70°C.

The bacteria were recovered from storage at a later date for PCR-ribotyping. Isolates were sub-cultured onto fastidious anaerobe agar (Bioconnections, Wetherby, UK) and incubated for 48 h at 37°C in an anaerobic chamber. PCR ribotyping was performed using standard methods (Health Protection Agency, 2009b) and compared to the ten commonest ribotypes circulating in the UK (Health Protection Agency, 2009b).

2.3 Cohort use across experimental chapters

A breakdown of the total sample numbers used across each of the experimental chapters is provided in Figure 2.4. Experimental work was conducted as recruitment was ongoing, which is why specific chapters (in particular Chapters 4 & 5) employ a smaller cohort. Figure 2.4 also contains information pertaining to the year during which the corresponding experimental work was carried out.

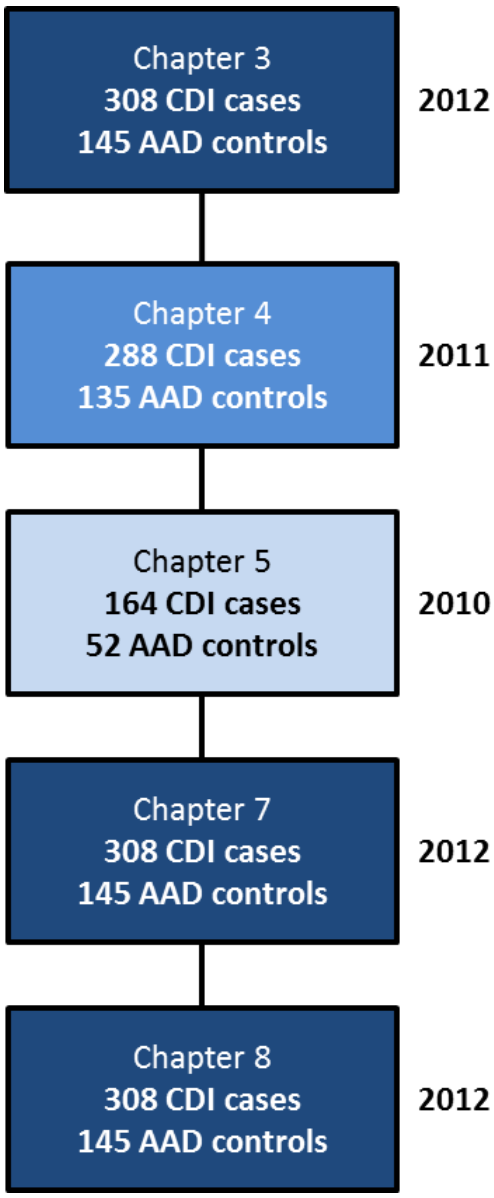


Figure 2.4 – Overview of sample sizes employed across experimental chapters

2.4 Responsibility breakdown

Study design was developed by the Principal Investigator (Professor Munir Pirmohamed), alongside the Study Lead (Dr Fabio Miyajima), Study Administrator (Ms Anita Hanson) a Microbiology Consultant (Dr Christopher Parry) and two leading Infectious Disease consultants (Dr Nicholas Beeching and Dr Mike Beadsworth).

Patient recruitment was carried out by a senior research nurse (Mrs Margaret Little), with blood samples processed by myself whilst faecal samples were processed (including microbiological culture and ribotyping) by two members of the RLUH Microbiology team (Mr Paul Roberts and Miss Valerie Price). Appropriate data was collected by myself, Mrs Margaret Little, Dr Fabio Miyajima and Mr Paul Roberts.

Experimental work contributing to this thesis was conducted by myself, with minor assistance from Dr Fabio Miyajima. The statistical analysis for the data generated through this work was conducted by myself, with advice provided by the statistician on my supervisory board (Dr Ruwanthi Kolamunnage-Dona), Dr Fabio Miyajima and a further member of my supervisory board (Dr Ana Alfirevic). However, the meta-analysis in Chapter 4 was conducted by Dr Ruwanthi Kolamunnage-Dona.

This thesis was written by myself, and reviewed by all members of my supervisory board (Professor Munir Pirmohamed, Dr Fabio Miyajima, Dr Ana Alfirevic and Dr Ruwanthi Kolamunnage-Dona).

Chapter 3

Predicting poor disease outcomes in a prospective cohort of CDI patients

Contents

3.1 Introduction

As discussed in Chapter 1 (section 1.9), complications arising from CDI, such as recurrence and mortality, are common albeit very difficult to predict. This constitutes a stumbling block for the stratification of patients and provision of more personalised clinical care. Given the characteristic symptoms of the disease, a multitude of studies have attempted to ascertain key risk factors for selected outcome measures, such as recurrence, severe-complicated disease and mortality, which are summarised in Tables 3.1, 3.2 and 3.3, respectively. It can be noted that the most commonly associated variables across the multiple CDI outcomes are advanced age, elevated white cell blood count and serum levels of creatinine and hypoalbuminaemia. However, the studies are mainly retrospective in nature, and suffer from both a lack of standardised disease outcome measures and a high degree of heterogeneity for the variables selected. Furthermore, very few studies have statistically assessed the predictive capability of their own models. Most studies have simply reported associations with potential risk predictors, but an association does not necessarily imply clinical usefulness.

Conversely several authors have proposed the development of a scoring system, or CPR, that could be used at the bedside for the prediction of unfavourable patient outcomes. Table 3.4 depicts a number of CPRs proposed for recurrence, severe-complicated CDI and mortality. The majority of these have not been validated, either internally or externally, and many are deemed overcomplicated due to the adoption of too many parameters. As a result, a single and reliable CPR is yet to gain widespread clinical acceptance. Using a prospective cohort of carefully phenotyped CDI patients, this study sought to:

- Determine independent risk predictors of previously studied CDI disease outcomes (severe-complicated disease, recurrence and mortality), as well as prolonged duration of disease symptoms.
- Assess the existing CPRs for each previously investigated disease outcome using our own patient cohort, for which we have the adequate information.

Table 3.1 – Review of previous studies of *Clostridium difficile* infection investigating independent risk factors for disease recurrence

Study	Years	Country	Study design	N	Average age	Definition, days	No. of relapses (%)	Significant multivariate variables
Do *et al*. (1998)	1993-4	Canada	R	59	77	45 (FD)	13[a]	History of increased creatinine Increasing WCC (at diagnosis) Community-acquisition
Kyne *et al*. (2001)	1998	US	P	44	69	60 (FD1)	22 (50)	Increasing age Severe/Extremely severe disease Concomitant antibiotics IgG and IgM against tcdA
Pepin *et al*. (2005)	1991–2004	Canada	R	845[b]	-	60 (FD)	243 (29)	Year of diagnosis Increasing age Prolonged hospitalisation
Cadena *et al*. (2010)	2003-5	US	R	129	67	90 (FD)	38 (29)	Recent fluoroquinolone use
Jung *et al*. (2010)	1998-2008	South Korea	R	117[b]	64	90 (FT)	13 (13)	Recent surgery prior to CDI
Kim *et al*. (2010)	2006-7	Korea	R	125	68	90 (FC)	27 (22)	Increasing age Decreased serum albumin (at diagnosis) Concurrent PPI
Bauer *et al*. (2011)	2008	34 European	R	484	-	90 (FD)	86 (18)	Recent use of ceftazidime Recent CDI episodes
Choi *et al*. (2011)	2008-2010	Korea	R	84	63	60 (FT1)	11 (13)	Stool VRE colonisation

Table 3.1 (continued) - Review of previous studies of *Clostridium difficile* infection investigating independent risk factors for disease recurrence

Study	Years	Country	Study design	N	Average age	Definition, days	No. of relapses (%)	Significant multivariate variables
Eyre *et al*. (2012)	2006-10	UK	P	1678	77	≥14 (FD)	363 (22)	Emergency admission Previous gastro ward admission(s) Diagnosis at admission Increasing age Previous total hours in hospital Recent inpatient before diagnosis
Petrella *et al*. (2012)[c]	2006-9	Canada/Europe	R	794	N/A	28 (FT)	150 (19)	Strain type (REA group) Recent CDI episode Concomitant antibiotics Treatment choice
Freedberg *et al*. (2013)	2009-12	US	R	894	64	90 (FD)	167 (19)	Black race Increasing age Increasing Charlson Comorbidity Index
Lavergne *et al*. (2013)	2009-2010	Canada	P	121	77	60 (FT)	40 (33)	Increasing age Female gender Positive anti-toxin serology Lymphopenia at treatment completion
Rodriguez-Pardo *et al* (2013)	2009	Spain	P	348	72	56 (FD)	63 (18)	Increasing age PPI use post-diagnosis Increasing WCC (at diagnosis)

***CDI**: Clostridium difficile infection; **FC**: From cure; **FD**: From diagnosis; **FD1**: From discharge; **FT**: From treatment completion; **FT1**: From treatment initiation; **Gastro**: Gastroenterology; **IgG**: Immunoglobulin G; **IgM**: Immunoglobulin M; **N**: Number; **N/A**: Not Available; **P**: Prospective; **PPI**: Proton pump inhibitor; **R**: Retrospective; **REA**: Restriction endonuclease analysis; **UK**: United Kingdom; **US**: United States; **tcdA**: Clostridium difficile toxin A; **VRE**: Vancomycin-resistant enterococci; **WCC**: White cell count;*

[a] This was a case-control study involving 13 recurrent cases and 46 randomly-selected non-recurrent cases; [b] Study was restricted to patients receiving metronidazole as CDI therapy; [c] Patients included in this study were enrolled in 2 phase III clinical trials, comparing the efficacy and safety of fidaxomicin versus vancomycin;

Studies conducted exclusively in populations with specific pathologies or those undergoing specific procedures/surgery, such as organ transplants, CT-scans, endoscopies, colectomy etc, were excluded. Studies were also excluded that used only univariate comparisons between groups, or hypothesis-driven studies focusing upon the risk associated with one or two specific variables.

Table 3.2 - Review of previous studies of *Clostridium difficile* infection investigating risk factors for severe-complicated disease

Study	Years	Country	Study design	N	Average age	Definition	Nº (%) severe disease	Significant multivariate variables
Andrews *et al.* (2003)	1995–9	Canada	R	153	63	Inpatient mortality (attributable) ICU admission Colectomy Hospital stay >14 days	4 (28.8)	Increasing age Increasing comorbidity illness Recent CDI episode
Pepin *et al.* (2004)	1991-2003	Canada	R	1675	-	30-day mortality (attributable, FD) Toxic megacolon Perforation Colectomy Septic shock	183 (10.9)	Increasing age Hospital acquisition Recent surgery Recent tube feeding Immunosuppression Increasing peak WCC Increasing peak creatinine Vancomycin as initial treatment
Hardt *et al.* (2008)	2003-6	Germany	R	124	76	Heart rate bpm/systolic BP mmHg >1.5 (at diagnosis)	27 (22.0)	Charlson Comorbidity Index Increasing CRP (at diagnosis)
Cloud *et al.* (2009)	2004-6	US	R	272	67	Inpatient mortality (attributable) ICU admission Toxic megacolon Colectomy	60 (22.1)	Increasing WCC at diagnosis Increasing peak creatinine
Gravel *et al.* (2009)	2004-5	Canada	P	1430	70	30-day mortality (attributable, FO) ICU admission Colectomy	82 (5.7)	Increasing age Admission from other hospital/LTCF Liver disease Vancomycin as initial treatment Change in CDI treatment
Gujja & Friedenberg (2009)	2003-8	US	R	200	66	Mortality (attributable, FI) Colectomy	32 (16)	Increasing WCC (at treatment initiation) Creatinine >50% increase over baseline

Table 3.2 (continued) - Review of previous studies of *Clostridium difficile* infection investigating risk factors for severe-complicated disease

Study	Years	Country	Study design	N	Average age	Definition	Nº (%) severe disease	Significant multivariate variables
Henrich *et al.* (2009)	2006-7	US	R	336	64	30-day mortality (attributable, FO) ICU admission Colectomy Perforation	41 (12.2)	Increasing age Small bowel obstruction/ileus Abnormal abdominal CT scan
Bauer *et al.* (2011)	2008	34 European	R	442	71	90-day mortality (attributable, FD) ICU admission Colectomy	44 (10)	Increasing age PCR ribotype (018/056)
Fujitani *et al.* (2011)	2006	US	P	184	70	30-day mortality (attributable, FD) ICU admission Toxic megacolon Perforation Refractory colitis	19 (10)	Abdominal distention Increasing temperature Increasing WCC (at diagnosis) Decreasing albumin (at diagnosis)
Manek *et al.* (2011)	2007-8	Canada	R	305	71	Mortality (all cause, BT) ICU admission Severe hypokalemia Toxic megacolon Perforation Lower GI bleeding	97 (27)	Previous CDI episodes Confusion Increasing systolic BP Increasing WCC (at diagnosis) Vancomycin as initial treatment Concomitant antibiotics
Morrison *et al.* (2011)	2004-8	US	R	485	53	Mortality (attributable) ICU admission Surgery Toxic megacolon	47 (10)	Increasing age Recent acid suppression use
Walk *et al.* (2012)	2000-6	US	R	310	57	30-day mortality (attributable, FD) ICU Admission Surgery	34 (11)	Altered WCC (at diagnosis) Altered albumin level (at diagnosis)

Table 3.2 (continued) - Review of previous studies of *Clostridium difficile* infection investigating risk factors for severe-complicated disease

Study	Years	Country	Study design	N	Average age	Definition	Nº (%) severe disease	Significant multivariate variables
Wenisch *et al.* (2012)	2009-10	Austria	R	133	74	30-day mortality (attributable, FO) ICU admission Surgical intervention	24 (18.1)	Severe diarrhoea Chronic pulmonary& renal disease Diabetes mellitus
Khanafer *et al.* (2013)	2007-11	France	R	40	63	30-day mortality (attributable, FD) ICU admission Colectomy Toxic megacolon Colitis/Perforation Septic shock	15 (37.5)	Male gender Increasing CRP Recent fluoroquinolone exposure
Rodriguez-Pardo *et al.* (2013)	2009	Spain	P	348	72	30-day mortality (FD) Colectomy	53 (15)	Concomitant antibiotics Increasing Charlson Score Increasing age
Shivashankar *et al.* (2013)	2007-10	US	R	1446	63	30-day mortality (FD) ICU admission Colectomy	487 (33.7)	Increasing peak WCC (within 7 days of diagnosis) Increasing peak creatinine (≥1.5 fold baseline) Increasing age Concomitant narcotics & acid suppression
Hensgens *et al.* (2014)	2006-9	Netherlands	P	395	65	30-day mortality (attributable, FD) ICU admission Colectomy	47 (12)	Increasing age Admission due to diarrhoea Diagnosis at ICU Recent abdominal surgery Hypotension

***BP**: Blood pressure; **BT**: Before treatment completion; **CDI**: Clostridium difficile infection; **CRP**: C-reactive protein; **CT**: Computerised tomography; **FD**: From diagnosis; **FI**: From initiation of therapy; **FO**: From onset of diarrhoea; **GI**: Gastrointestinal; **ICU**: Intensive care unit; **LTCF**: Long term care facility; **N**: Number; **P**: Prospective; **PCR**: Polymerase chain reaction; **R**: Retrospective; **UK**: United Kingdom; **US**: United States; **WCC**: White cell count;*

Studies conducted exclusively in populations with specific pathologies or those undergoing specific procedures/surgery, such as organ transplants, CT-scans, endoscopies, colectomy etc, were excluded. Studies were also excluded that used only univariate comparisons between groups, or hypothesis-driven studies focusing upon the risk associated with one or two specific variables.

Table 3.3 - Review of previous studies of *Clostridium difficile* infection investigating risk factors for mortality

Study	Years	Country	Study Design	N	Average age	Definition	No. of deaths (%)	Significant multivariate variables
Kenneally *et al*. (2007)	2004-5	US	R	278[a]	64	30-day (all cause, FD)	102 (36.7)	Septic shock ICU transfer Increasing APACHE II score
Lamontagne *et al*. (2007)	2003-5	Canada	R	165[a]	75	30-day (all cause, FI)	87 (53)	Leukocytosis using peak value Increasing peak lactate Increasing age Immunosuppression Septic shock Colectomy
Marra *et al*. (2007)	2002-5	US	R	58[a]	56	Inpatient (attributable)	16 (27.6)	Increasing age Increasing SOFA score at onset
Bishara *et al*. (2008)	1999-2000	Israel	P	52	74	28-day (all cause, FH)	8 (15.4)	Elevated serum urea Lack of occult blood in stool
Labbe *et al*. (2008)	2000-4	Canada	R	230	-	30-day (all cause, FD)	55 (23.9)	PCR ribotype 027 Increasing age Increased Charlson Index Increased recent hospitalisation
Cloud *et al*. (2009)	2004-6	US	P	272	67	Inpatient (all-cause)	33 (12.1)	Increased WCC (at diagnosis) Male gender
Cober *et al*. (2009)	2006	US	R	70[b]	84	90-day (all cause, FD)	12 (17.1)	Coronary artery disease

Table 3.3 (continued) - Review of previous studies of *Clostridium difficile* infection investigating risk factors for mortality

Study	Years	Country	Study Design	N	Average age	Definition	No. of deaths (%)	Significant multivariate variables
Sailhamer *et al*. (2009)	1996-2007	US	R	199[c]	68	Inpatient (attributable)	69 (34.7)	Increased age Altered peak WCC Increased peak neutrophil bands Vasopressors Intubation Vancomycin
Zilberberg *et al*. (2009)	2004-5	US	R	148[d]	76	30-day (all cause, FD)	67 (45.3)	Increased age No history of CRD Lack of leucocytosis Septic shock
Bhangu *et al*. (2010)	2006-7	UK	R	158	82	30-day inpatient (all cause, FD)	60 (38)	Increased CRP (at diagnosis) Decreased albumin (at diagnosis) Increased urea (at diagnosis) Increased WCC (at diagnosis) General surgery: Medicine
Cadena *et al*. (2010)	2003-5	US	R	129	67	90-day (all cause, FD)	38 (29)	Severe disease
Dudukgian *et al*. (2010)	1999-2006	US	R	398	59	Inpatient (attributable)	41 (10.3)	APACHE II score ASA class Pre-existing organ dysfunction Concomitant steroid use
Pant *et al*. (2010)	-	US	R	184	-	30-day inpatient (all cause, FD)	23 (13.6)	Recent renal failure
Wilson *et al*. (2010)	2007-8	UK	P	128	83	30-day (all cause, FD)	46 (35.9)	Ischemic heart disease Hypoalbuminemia (at diagnosis)

Table 3.3 (continued) - Review of previous studies of *Clostridium difficile* infection investigating risk factors for mortality

Study	Years	Country	Study Design	N	Average age	Definition	No. of deaths (%)	Significant multivariate variables
Morrison *et al*. (2011)	2004-8	US	R	485	53	Attributable	23 (4.7)	Increased age Prior acid suppression use
Welfare *et al*. (2011)	2002-9	UK	R	2761	82	30-day (all cause, FD)	-	Increased age Cancer Cognitive impairment Comorbidities
Khan *et al*. (2012)	2006-9	Qatar	R	123	51	30-day (all cause, FD)	38 (30.9)	Occurrence among Qataris Prolonged hospitalisation Positive stool occult blood test Increased WCC Septic shock
Venugopal *et al*. (2012)	2005-6	US	P	118	68	30-day (all cause, FD)	29 (24.6)	Recent ICU stay
Bloomfield *et al*. (2013)	2010	UK	P	131	74	30-day inpatient (all cause, FD)	13 (9.9)	Increased WCC (at diagnosis) Decreased albumin (at diagnosis)
Inns *et al*. (2013)	2009-11	UK	R	1426	77	30-day (all cause, FD)	366 (25.7)	PCR ribotype (015 & 027) Increased age Hospital-acquired
Kim *et al*. (2013)	2005-10	South Korea	R	536	64	30-day (all cause, FT)	48 (9)	Malignant comorbidity Decreased albumin Increased WCC ICU admission Treatment response failure

Table 3.3 (continued) - Review of previous studies of *Clostridium difficile* infection investigating risk factors for mortality

Study	Years	Country	Study Design	N	Average age	Definition	No. of deaths (%)	Significant multivariate variables
Solomon *et al.* (2013)	2008-9	UK	P	86	75	30-day (all cause, FD)	14 (16.3)	Increased peak WCC Increased peak Creatinine Low peak day 12 IgG anti-tcdA titre
Boone *et al.* (2014)	2010-11	US	P	210	60	100-day (all cause, FD)	50 (24)	Increased age ICU treatment Increased Charlson Index

***APACHE**: Acute Physiology and Chronic Health Evaluation; **ASA**: American Society of Anesthesiologists; **CRD**: Chronic renal disease; **CRP**: C-reactive protein; **FD**: From diagnosis; **FH**: From hospitalisation; **FI**: From ICU admission; **FT**: From treatment completion; **ICU**: Intensive care unit; **IgG**: Immunoglobulin G; **N**: Number; **P**: Prospective; **PCR**: Polymerase chain reaction; **R**: Retrospective; **SOFA**: Sequential Organ Failure Assessment; **tcdA**: Clostridium difficile toxin A; **UK**: United Kingdom; **US**: United States; **WCC**: White cell count;*

[a] Only included patients in intensive care; [b] Restricted to patients aged ≥80 yrs; [c] Restricted to patients with fulminant disease defined by the need for colectomy and admission to the intensive care unit as a result of their infection; [d] A re-analysis of Kenneally et al (2009) but restricted to patients ≥65 yrs;

Studies conducted exclusively in populations with specific pathologies or those undergoing specific procedures/surgery, such as organ transplants, CT-scans, endoscopies, colectomy etc, were excluded. Studies were also excluded that used only univariate comparisons between groups, or hypothesis-driven studies focusing upon the risk associated with one or two specific variables.

Table 3.4 – Existing clinical prediction rules for poor disease outcome in *Clostridium difficile infection*

Study (Country)	Variables	Points	Interpretation
Recurrence			
Hu *et al* 2009 (US)[a]	Age >65 yrs	1	Score ≥2: High risk
	Horn Index: Severe or Fulminant disease	1	
	Additional antibiotics post-CDI therapy	1	
D'Agostino *et al* 2014 (US)[b]	Age ≥75 yrs	1	Predicts recurrence risk when prescribing specific treatment
	Serum creatinine at baseline ≥106 umol/L	2	
	Number of unformed bowel movements ≥10	1	
	Prior episode of CDI	1	
Severe-complicated disease			
Rubin *et al* 1995 (USA)[c]	Age >90 yrs	1	Score >4: Severe disease
	Albumin <30 g/L	1	
	WCC >25 or <1.5 x10^9/L	1	
	≥5% increase in Haematocrit	1	
	Clindamycin use	1	
	Immunosuppressive medication use	1	
	Antiperistaltic/narcotic use	1	
	Baseline/development of depressed mental status	1	
	Renal insufficiency	1	
	COPD	1	
	Abdominal pain	1	
	Abdominal distension	1	
	Abdominal tenderness	1	
Drew *et al* 2009 (IRE)[d, e]	WCC ratio day 1: 2 days previous (0.5-1.5/>1.5-2 or <0.5/>2-4/>4)	0/1/2/3	Score ≥4: Risk of severe complications
	Urea day 1 (<10/10-20/>20 mmol/L)	0/1/2	
	WCC day 1 (4-10/>10-20 or <4/>20-30/>30 x10^9/L)	0/1/2/3	
	Albumin day 1 (>30/24-30/<24 g/L)	0/1/2	
Lungulescu *et al* 2011 (US)[c]	History of malignancy	1	Score ≥2: Risk of severe consequences
	WCC >20 x10^9/L (at admission)	1	
	Albumin <30 g/l (at admission)	1	
	Creatinine (at admission) >1.5 fold baseline	1	
Hensgens *et al* 2014 (NET)	Age (≤49 yrs/50-84 yrs/≥85 yrs)	0/1/3	Score ≥4: High risk
	Department of diagnosis (other/Surgery/ICU)	0/0/3	
	Recent abdominal surgery (90 days)	-3	
	Hypotension (at diagnosis)	2	
	Diarrhoea is reason for admission	2	
Van Der Wilden *et al* 2014 (US)	Age >70 yrs	2	Score ≥6: High risk
	WCC ≥20 or <2 x10^9/L (at diagnosis)	1	
	Cardiorespiratory failure	7	
	Diffuse abdominal tenderness on PE	6	

Table 3.4 (continued) – Existing clinical prediction rules for poor disease outcome in *Clostridium difficile infection*

Study (Country)	Variables	Points	Interpretation
Mortality			
Zilberberg *et al* 2009 (US)	Absence of history of respiratory disease	3	Score ≥6: 60% risk Score ≥8: 80% risk
	Age ≥75 yrs	3	
	Septic shock	1	
	APACHE II score 20+	1	
Bhangu *et al* 2010 (UK)	Age ≥80 yrs	1	Score 0-1: 22% risk Score 2-3: 55% risk Score 4-5: 89% risk
	Severe disease (sepsis/peritonitis/≥10 stools in 24 h)	1	
	WCC ≥20 x10^9/L or CRP ≥150 mg/L (72 h diagnosis)	1	
	Urea ≥15 mmol/L (72 h diagnosis)	1	
	Albumin ≤20 g/L (72 h diagnosis)	1	
Welfare *et al* 2011 (UK)	Age 60-79 yrs	3	Score ≤3: <22% risk Score = 8: 66% risk
	Age ≥80 yrs	4	
	Presence of renal disease	2	
	Presence of cancer	2	
Butt *et al* 2013 (UK)	Albumin ≤24.5 g/L (48 h diagnosis)	1	Increasing score: Increasing risk
	CRP >228 mg/L (48 h diagnosis)	1	
	WCC >12 x10^9/L (48 h diagnosis)[f]	1	

***APACHE**: Acute Physiology & Chronic Health Evaluation; **CDI**: Clostridium difficile infection; **COPD**: Chronic obstructive pulmonary disease; **CRP**: C-reactive protein; **h**: hours; **ICU**: Intensive care unit; **IRE**: Ireland; **NET**: Netherlands; **PE**: Physical examination; **UK**: United Kingdom; **US**: United States; **WCC**: White cell count; **yrs**: Years;*

[a] This study produced two prediction rules - here we highlight their 'Clinical prediction rule', as opposed to their 'Combined prediction rule' that included an extra variable: 'Anti-tcdA IgG <1.29 ELISA units'; [b] This study utilised data from two clinical trials and their outcome was not to assess prediction of recurrence but to in fact produce a risk of recurrence for two separate treatment choices (fidaxomicin and vancomycin) dependent upon defined clinical variables; [c] Retrospectively derived; [d] Letter to the Editor; [e] Scoring system is to be used for assessment at days 1 & 3. We have presented day 1 only; [f] Initially included respiratory rate but excluded as not present for validation;

3.2 Methods

3.2.1 Identification of independent risk factors associated with poor disease outcomes

3.2.1.1 Study design

Recruitment of patients was conducted using the criteria defined in Chapter 2. 308 CDI cases and 145 AAD controls were recruited from July 2008 to March 2012.

3.2.1.2 Data collection

Data for approximately 50 variables were analysed across the four primary outcomes, encompassing information on demographics, medication, clinical characteristics and underlying comorbidities, laboratory results, microbiology, current admission and CDI disease outcomes.

Charlson Comorbidity Index (CCI) was derived on an individual basis without age adjustment, as per its original development (Charlson *et al*., 1987) as well as current research references (Boone *et al*., 2014; Caplin *et al*., 2011; Daskivich *et al*., 2014). Blood markers measurements were taken ±2 days of the positive *C. diff* test date. Analysis of concomitant immunosuppressive therapy included both anti-neoplastic therapy and glucocorticoids alongside standard immunosuppressive drugs. For data gathering prior to the patients' current infection, a cut-off of 90 days was employed. An episode was considered nosocomial in acquisition if the diarrhoea arose ≥3 days from the day of hospital admission.

3.2.1.3 Definition of outcomes

Severe-complicated CDI was considered when patients met one of four eligibility criteria: -

1. Death directly due to or contributed to by CDI, according to death certificates, within 30 days of CDI diagnosis (attributable mortality)

2. Admission to ICU or high dependency unit (HDU) prior to cure
3. CT evidence of severe disease prior to cure, including colitis, perforation, ileus and toxic megacolon
4. Need for colectomy prior to cure

Recurrent CDI was defined as the development of subsequent CDI episodes up to a period of 90 days following diagnosis of the initial episode, with both relapses and re-infections included. In addition to monitoring attributable mortality, all-cause mortality within 30 days of CDI diagnosis was recorded as a primary outcome. Duration of symptoms, defined as the number of days from CDI diagnosis until diarrhoea resolution, was recorded and then dichotomised into episodes lasting more or less than 10 days.

3.2.1.4 Statistical analysis

The characteristics of the cohort were described by medians and interquartile ranges for continuous variables and proportions for categorical variables. Logistic regression models were used to identify the factors that predicted the development of primary outcome measures; this included a range of potential predictors (demographic, clinical and disease-specific), all initially examined by univariate models. Predictors significant at the 20% level (to account for correlations between predictors) were included in the initial multivariate models. A reduced multivariate analysis (again only retaining variables at 20% level) was conducted to minimise the number of variables and proportion of missing data in order to determine independent predictors (at the 5% significance level). Predictors were excluded from the multivariable models if there was a strong colinearity (>0.8) between them or on the grounds of biological plausibility.

To assess the performance of these models, pseudo R^2 values were produced for each multivariate model, Cragg & Uhler's R^2 (logistic). Hosmer-Lemeshow Goodness of Fit (GoF) test and the area under the ROC curve were used to assess the fit of the multivariate logistic regression model. Given that the exclusion of subjects with missing data can lead to biased estimates, as well as reducing

statistical efficiency particularly in multivariate models, we have imputed missing data using switching regression, an iterative multivariate regression technique that retains an element of random variation in the estimates. These imputed data were used in the final multivariate models to assess if the overall results were affected by data missingness.

Next, a resampling approach was used to randomly split the dataset in two halves and test the calibration of our models by performing a fitness tests on both subgroup samples (training and testing sets). We have also carried out a K-fold leave-one-out cross-validation of the multivariate models to estimate the expected level of fit of the model to an independent sample of patients, i.e. independent to the cohort used to derive the model. The cross-validation methods cited above were chosen as they were more appropriate for smaller datasets and provided estimated goodness-of-fit statistics (i.e. Hosmer-Lemeshow Statistic and AUC). All analyses were undertaken using Stata, version 9.2.

3.2.2 Assessment of existing clinical prediction rules for poor disease outcomes

A systematic review was performed using an electronic search of all studies published since January 1978 (the year that C. difficile was identified as the etiological agent of pseudomembranous colitis (Bartlett *et* al., 1979; Chang *et* al., 1978) for the three primary outcome measures (recurrence, mortality and severe-complicated CDI). Pubmed was the electronic database used and the keywords employed were, "Clostridium difficile AND Recurrence/Mortality/Severe/Complicated AND predict/rule/risk index/risk score/risk model/risk scale". The search was limited to studies published in English and conducted in humans, aged ≥18 years. In addition, the reference lists of identified CPRs were searched manually (crossreferencing). The final electronic search was performed on 31st May 2014.

The following data was extracted into a standardised matrix: year of publication, location, definition of the outcome(s) of interest, sample size,

average age of cohort, frequency of the outcome(s) of interest, the variables included in the clinical prediction rule and the corresponding points/cut-offs used to produce the risk score.

Only CPRs that incorporated variables available to us from our cohort could be assessed despite the extensive collection of data in place for this study. Risk scores for this cohort were calculated using the chosen CPRs and statistical analysis was conducted to identify the area under the curve (AUC), sensitivity, specificity, PPV and NPV. Where available, these were then compared against those of the original study.

3.3 Results

3.3.1 Patient characteristics

Within our cohort, the median duration of diarrhoeal symptoms was 7 days with 109/274 (40%) experiencing prolonged disease (≥10 days), whilst 83/220 (38%) suffered from disease recurrence within 90 days of diagnosis, and 43/256 (17%) developed severe-complicated disease. All-cause 30-day mortality was 9% (26/305), with an attributable mortality rate of 2% (7/305). Table 3.5 shows a general description of the cohort.

Table 3.5 – Characteristics of all CDI patients (n=308)

	N (%) or Median (IQR)
Demographics	
Age at baseline	74.7 (61.3-81.1)
Gender: Female	177/308 (57)
Smoking: Pack years	10.3 (0.0-35.0)
Body Mass Index	23.6 (20.3-27.7)
Medication information	
Number of co-medications at baseline	3.0 (0.0-4.0)
Taking fluoroquinolones prior to CDI	107/308 (35)
Taking PPIs prior to CDI	208/306 (68)
Concomitant antibiotics	152/308 (49)
Concomitant immunosuppressants	52/307 (17)
Clinical characteristics & underlying comorbidities	
Number of stools at baseline	5.0 (3.0-6.0)
Fever (≥36.8°C)	18/293 (6)
Charlson Comorbidity Index score*	1.0 (0.0-2.0)
Diabetes	58/307 (19)
Hypotension	36/296 (12)
Current malignancy	8/308 (3)
Respiratory comorbidities at baseline	167/307 (54)
GI comorbidities at baseline	179/307 (58)
Recent abdominal surgery (90 days)	34/298 (11)
Laboratory results at baseline	
Hb (mmol/L)	10.8 (9.6-11.9)
WCC (10^9/L)	11.8 (8.3-17.7)
Neutrophils (10^9/L)	9.0 (6.0-14.8)
Platelets (10^9/L)	294.0 (211.0-392.0)
C-reactive protein (mg/L)	71.0 (31.0-140.0)
Creatinine (mmol/L)	81. 0 (59.0–133.0)
eGFR (ml/min/1.73m^2)	67.8 (41.5-100.1)
Albumin (g/L)	30.0 (25.0-34.0)
Sodium (mmol/L)	135.0 (132.0-138.0)
Potassium (mmol/L)	3.8 (3.4-4.2)
Urea (mmol/L)	6.3 (4.1-10.7)

Table 3.5 (continued) – Characteristics of all CDI patients (n=306)

	N (%) or Median (IQR)
Microbiological information	
Presence of faecal leukocytes	162/313 (52)
Toxin OD	2.5 (0.7-3.0)
Presence of PCR ribotype 027	89/283 (31)
Current admission information	
Length of hospitalisation prior to diagnosis, days	8.0 (1.0-20.0)
Admitted via an emergency ward	167/256 (65)
Admitted with diarrhoea	113/308 (37)
Suffered from previous CDI	46/284 (16)
Recent CDI infection (90 days)	28/292 (10)
Recent ICU/HDU admission (90 days)	46/250 (18)
Nosocomial admission	204/306 (67)
Duration of symptoms prior to diagnosis, days	2.0 (0.0-5.0)
CDI outcome information	
ICU admission due to infection	8/246 (3)
Severe-complicated CDI	43/256 (17)
90-day recurrence	83/220 (38)
30-day all-cause mortality	26/305 (9)
Prolonged symptoms (≥10 days)	109/274 (40)
Duration of symptoms	7.0 (4.0-12.0)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **eGFR**: Estimated glomerular filtration rate; **GI**: Gastrointestinal; **Hb**: Haemoglobin; **HDU**: High dependency unit; **ICU**: Intensive care unit; **IQR**: Interquartile range; **N**: Number; **OD**: Optical density; **OR**: Odds ratio; **PPI**: Proton pump inhibitor; **WCC**: White cell count;*

**Charlson Comorbidity Index is calculated without age adjustment (see Methods)*

3.3.2 Univariate risk factors for poor disease outcomes

Table 3.6 illustrates univariate associations for clinical variables in patients suffering poor disease outcomes. A full breakdown for each individual disease outcome can be found in Appendices 1-4.

Recurrent patients, in comparison to their non-recurrent counterparts, were significantly older (77.5 versus 69.6 years; p<0.01), more likely to have taken fluoroquinolones within 90 days of diagnosis (48 versus 31%; p=0.01) and less likely to have had abdominal surgery within the previous 90 days (4 versus 17%; p=0.01). They also had increased baseline WCC (13.1 versus 10.6 10^9/L; p=0.05), neutrophils (10.1 versus 8.0 x 10^9/L; p=0.03), creatinine (95.5 versus 71.5 mmol/L; p=0.04) and urea (7.6 versus 5.3 mmol/L; p<0.01), and decreased eGFR (58.5 versus 76.2 ml/min/1.73m^2; p=0.02).

Patients with severe-complicated disease, in comparison to their non-severe counterparts, were less likely to have taken PPIs within 90 days of diagnosis (50 versus 71%; p=0.01), were taking less medications at diagnosis (2.0 versus 3.0; p<0.01) and were more likely to be suffering from hypotension (24 versus 10%; p=0.02) or a GI comorbidity (77 versus 58%; p=0.02). They also had increased baseline neutrophils (12.7 versus 8.7 10^9/L; p=0.01), C-reactive protein (CRP) (108.5 versus 66.0 mg/L; p=0.01) and urea (8.2 versus 6.0 mmol/L; p=0.02), and decreased haemoglobin (Hb) (9.9 versus 10.9 mmol/L; p=0.03) and albumin (27.0 versus 31.0 g/L; p<0.01).

Patients who died within 30 days, compared to those who survived, were older (79.4 versus 74.4 years; p=0.02), had a decreased body mass index (BMI) (20.6 versus 23.9; p=0.01), a higher median CCI score (2.0 versus 1.0; p=0.01) and were more likely to have a respiratory comorbidity (77 versus 53%; p=0.02).

Patients suffering from ≥10 days of symptoms, in comparison to those having shorter episodes, had increased baseline neutrophils (10.3 versus 8.6 x 10^9/L; p=0.05), CRP (90.5 versus 61.0 mg/L; p=0.01), decreased Hb (10.3 versus 10.9 mmol/L; p=0.03), and an increased duration of diarrhoea prior to testing positive for *C. diff* (2.0 versus 1.0 days; p=0.01).

Table 3.6 – Univariate analysis of clinical variables in CDI patients across poor disease outcomes

	OR (95% CIs)			
	Recurrence	**Severe-complicated**	**Mortality**	**Prolonged disease**
Demographics				
Age at baseline: per decade	1.44 (1.19-1.76)	0.91 (0.76-1.10)	1.49 (1.07-2.07)	1.16 (0.99-1.35)
Gender: Female	0.84 (0.49-1.46)	1.75 (0.87-3.55)	0.86 (0.38-1.92)	0.95 (0.58-1.55)
Smoking pack years: per year increase	1.00 (0.99-1.01)	1.00 (0.98-1.01)	1.01 (1.00-1.02)	1.00 (0.99-1.01)
Body Mass Index	0.99 (0.95-1.03)	0.99 (0.94-1.04)	0.88 (0.81-0.96)	0.99 (0.95-1.03)
Medication information				
Number of co-medications at baseline	1.04 (0.89-1.20)	0.76 (0.63-0.91)	0.90 (0.74-1.11)	0.95 (0.84-1.07)
Taking fluoroquinolones prior to CDI	2.10 (1.20-3.70)	0.74 (0.36-1.49)	1.42 (0.63-3.21)	1.15 (0.69-1.91)
Taking PPIs prior to CDI	1.17 (0.66-2.10)	0.41 (0.21-0.81)	1.07 (0.45-2.54)	0.82 (0.49-1.37)
Concomitant antibiotics	1.18 (0.68-2.03)	1.04 (0.54-2.00)	1.45 (0.65-3.28)	0.82 (0.50-1.33)
Concomitant immunosuppressants	1.53 (0.75-3.12)	1.57 (0.73-3.40)	0.38 (0.09-1.66)	1.25 (0.66-2.38)
Clinical characteristics & underlying comorbidities				
Number of stools at baseline	0.99 (0.90-1.08)	1.05 (0.95-1.17)	0.95 (0.81-1.11)	1.05 (0.97-1.14)
Fever (≥36.8°C)	0.94 (0.30-2.92)	2.41 (0.79-7.36)	0.64 (0.08-5.00)	2.66 (0.94-7.54)
Charlson Comorbidity Index score	0.98 (0.81-1.19)	0.93 (0.75-1.15)	1.31 (1.07-1.61)	0.90 (0.77-1.06)
Diabetes	1.14 (0.57-2.30)	1.12 (0.48-2.61)	1.01 (0.36-2.80)	0.94 (0.51-1.76)
Hypotension	0.96 (0.40-2.28)	2.76 (1.15-6.62)	1.41 (0.46-4.37)	1.33 (0.63-2.84)
Current malignancy	1.10 (0.18-6.74)	3.12 (0.72-13.58)	3.79 (0.73-19.82)	2.06 (0.45-9.38)
Respiratory comorbidities at baseline	1.61 (0.92-2.82)	1.75 (0.87-3.49)	2.97 (1.16-7.62)	1.13 (0.70-1.84)
GI comorbidities at baseline	0.74 (0.42-1.28)	2.43 (1.14-5.20)	0.91 (0.40-2.07)	1.54 (0.93-2.53)
Recent abdominal surgery (90 days)	0.19 (0.06-0.67)	1.55 (0.62-3.87)	0.31 (0.04-2.37)	0.83 (0.38-1.80)

Table 3.6 (continued) – Univariate analysis of clinical variables in CDI patients across poor disease outcomes

	OR (95% CIs)			
	Recurrence	**Severe-complicated**	**Mortality**	**Prolonged disease**
Laboratory results at baseline				
Hb: per unit (mmol/L) decrease	0.97 (0.83-1.13)	1.26 (1.02-1.55)	0.96 (0.76-1.22)	1.18 (1.02-1.36)
WCC: per 10^9/L increase	1.02 (1.00-1.05)	1.01 (0.99-1.03)	0.99 (0.94-1.03)	1.00 (0.99-1.02)
Neutrophils: per 10^9/L increase	1.03 (1.00-1.06)	1.04 (1.01-1.07)	0.99 (0.95-1.04)	1.03 (1.00-1.05)
Platelets: per 10^9/L increase	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (0.99-1.00)	1.00 (1.00-1.00)
C-reactive protein: per ten unit (mg/L) increase	1.02 (0.99-1.05)	1.05 (1.01-1.08)	0.99 (0.94-1.04)	1.04 (1.01-1.07)
Creatinine: per unit (mmol/L) increase	1.00 (1.00-1.01)	1.00 (1.00-1.00)	1.00 (0.99-1.00)	1.00 (1.00-1.00)
eGFR: per ten unit (ml/min/1.73m^2) decrease	1.08 (1.01-1.16)	0.96 (0.90-1.03)	0.94 (0.88-1.02)	1.02 (0.97-1.07)
Albumin: per unit (g/L) decrease	1.03 (0.98-1.08)	1.11 (1.05-1.19)	1.01 (0.94-1.08)	1.04 (1.00-1.08)
Sodium: per unit (mmol/L) increase	0.99 (0.93-1.05)	1.01 (0.93-1.09)	1.01 (0.92-1.12)	0.98 (0.92-1.03)
Potassium: per unit (mmol/L) increase	0.90 (0.57-1.43)	1.50 (0.87-2.59)	0.92 (0.46-1.85)	0.95 (0.63-1.42)
Urea: per unit (mmol/L) increase	1.08 (1.03-1.14)	1.06 (1.01-1.11)	1.02 (0.97-1.08)	1.04 (1.00-1.08)
Microbiological information				
Presence of faecal leukocytes	1.30 (0.75-2.27)	0.59 (0.30-1.15)	0.70 (0.31-1.61)	1.22 (0.75-1.99)
Toxin OD	0.86 (0.68-1.09)	1.16 (0.90-1.51)	1.07 (0.80-1.44)	0.95 (0.79-1.14)
Presence of PCR ribotype 027	1.52 (0.84-2.73)	0.57 (0.25-1.29)	1.08 (0.44-2.63)	1.45 (0.84-2.51)
Current admission information				
Length of hospitalisation prior to diagnosis, days	1.01 (1.00-1.02)	1.00 (0.99-1.02)	0.99 (0.97-1.01)	1.01 (1.00-1.02)
Admitted via an emergency ward	1.57 (0.87-2.82)	1.09 (0.50-2.38)	2.41 (0.78-7.39)	1.03 (0.59-1.81)
Admitted with diarrhoea	1.22 (0.70-2.15)	1.04 (0.53-2.04)	0.74 (0.31-1.75)	1.01 (0.62-1.67)
Suffered from previous CDI	1.42 (0.66-3.04)	1.64 (0.71-3.78)	0.97 (0.32-2.97)	1.47 (0.75-2.89)
Recent CDI infection (90 days)	2.23 (0.88-5.65)	1.74 (0.65-4.65)	0.38 (0.05-2.95)	1.76 (0.74-4.15)

Table 3.6 (continued) – Univariate analysis of clinical variables in CDI patients across poor disease outcomes

	OR (95% CIs)			
	Recurrence	**Severe-complicated**	**Mortality**	**Prolonged disease**
Current admission information (continued)				
Recent ICU/HDU admission (90 days)	1.01 (0.50-2.01)	1.57 (0.66-3.77)	0.22 (0.03-1.66)	1.04 (0.51-2.13)
Nosocomial admission	0.92 (0.52-1.62)	1.17 (0.58-2.34)	1.77 (0.69-4.55)	1.02 (0.62-1.70)
Duration of symptoms prior to diagnosis, days	1.03 (1.00-1.06)	1.00 (0.97-1.03)	1.00 (0.96-1.04)	1.04 (1.01-1.07)

Red: *p<0.05;* ***Orange:*** *0.05≤p<0.10;* ***Yellow:*** *0.10≤p<0.20;* ***Grey:*** *p≥0.2;*

CDI*: Clostridium difficile infection;* ***CI****: Confidence interval;* ***eGFR****: Estimated glomerular filtration rate;* ***GI:*** *Gastrointestinal;* ***Hb****: Haemoglobin;* ***HDU****: High dependency unit;* ***ICU****: Intensive care unit;* ***IQR****: Interquartile range;* ***N****: Number;* ***OD****: Optical density;* ***OR****: Odds ratio;* ***PPI****: Proton pump inhibitor;* ***WCC****: White cell count;*

3.3.3 Multivariate risk factors

3.3.3.1 90-day recurrence

Multivariate findings are summarised in Table 3.7. Consistent with previous literature, we identified independent associations with increased age (OR, 1.51; 95% CI, 1.21-1.90) (Eyre *et al*., 2012b; Freedberg *et al*., 2013; Kim *et al*., 2010; Kyne *et al*., 2001; Lavergne *et al*., 2013; Pepin *et al*., 2005; Rodríguez-Pardo *et al*., 2013), recent fluoroquinolone exposure (OR, 2.11; 95% CI, 1.09-4.09) (Cadena *et al*., 2010) and an increased duration of hospitalisation prior to diagnosis (OR, 1.02; 95% CI, 1.00-1.03) (Eyre *et al*., 2012b). We also identified an inverse association with patients having recently had abdominal surgery (OR, 0.26; 95% CI, 0.07-0.99). Whilst this has not specifically been associated with disease recurrence, several studies report an association with complicated disease (Bhangu *et al*., 2010; Gravel *et al*., 2009; Hensgens *et al*., 2014; Pépin *et al*., 2004). It is speculated that these patients are often younger and fitter than their counterparts, which was indeed the case in our cohort where mean age (65.4 versus 70.5 years; p=0.09) and median CCI (0.0 versus 1.0; p=0.01) significantly differed between patients with and without previous surgery, respectively.

Multiple studies have also identified associations with an increased WCC (Do *et al*., 1998; Rodríguez-Pardo *et al*., 2013), recent CDI infection (Bauer *et al*., 2011; Petrella *et al*., 2012), the presence of comorbidities (Freedberg *et al*., 2013; Kyne *et al*., 2001) and concomitant antibiotics (Kyne *et al*., 2001; Petrella *et al*., 2012) (Table 3.1). An increase of both WCC and neutrophils was associated on univariate analysis, but only neutrophil count was included in the initial multivariate model given the better performance and high degree of collinearity between them; however this failed to reach significance in the final reduced model (p=0.19; Table 3.7). Recent CDI infection and the presence of an underlying respiratory comorbidity were both associated in our univariate analysis (Table 3.6; Appendix 1) and therefore included in the initial multivariate model. These again did not independently predict recurrence in the final reduced model (p=0.35 and p=0.37, respectively; Table 3.7). No difference

was found with concomitant antibiotics upon univariate analysis (p=0.56; Table 3.6; Appendix 1).

Table 3.7 - Multivariate analysis of possible risk factors for 90-day disease recurrence

Variable	P-value	OR (95% CIs)
Initial model (n=178)		
Age at baseline: per decade	0.01	1.39 (1.08-1.79)
Taken fluoroquinolones prior to CDI	0.05	2.11 (1.01-4.42)
Respiratory comorbidities at baseline	0.37	1.40 (0.67-2.95)
Neutrophils: per 10^9/L increase	0.83	1.00 (0.95-1.04)
eGFR: per ten unit (ml/min/1.73m^2) decrease	0.71	0.98 (0.87-1.10)
Urea: per unit (mmol/L) increase	0.37	1.04 (0.96-1.12)
Recent abdominal surgery (90 days)	0.20	0.41 (0.10-1.63)
Presence of PCR ribotype 027	0.81	1.09 (0.53-2.25)
Length of hospitalisation prior to diagnosis, days	0.11	1.01 (1.00-1.03)
Admitted via an emergency ward	0.42	1.38 (0.64-3.00)
Recent CDI infection (90 days)	0.35	1.74 (0.55-5.50)
Duration of symptoms prior to diagnosis, days	0.07	1.04 (1.00-1.08)
Reduced model (n=200)		
Age at baseline: per decade	<0.01	1.51 (1.21-1.90)
Taken fluoroquinolones prior to CDI	0.03	2.11 (1.09-4.09)
Recent abdominal surgery (90 days)	0.05	0.26 (0.07-0.99)
Neutrophils: per 109/L increase	0.19	1.02 (0.99-1.05)
Length of hospitalisation prior to diagnosis, days	0.02	1.02 (1.00-1.03)
Duration of symptoms prior to diagnosis, days	0.12	1.03 (0.99-1.07)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **eGFR**: Estimated glomerular filtration rate; **OR**: Odds ratio; **PCR**: Polymerase chain reaction;*

3.3.3.2 Severe-complicated disease

Multivariate findings are summarised in Table 3.8. Consistent with previous literature, an independent association was found with hypoalbuminaemia (OR, 1.14; 95% CI, 1.04-1.25) (Fujitani *et al*., 2011; Walk *et al*., 2012). Other independent predictors identified were the presence of an underlying GI comorbidity (OR, 3.00; 95% CI, 1.00-8.99) and current malignancy (OR, 10.11; 95% CI, 1.28-78.92), which is in line with reports linking underlying comorbidities with CDI clinical complications (Gravel *et al*., 2009; Wenisch *et al*., 2012). Recent PPI exposure (OR, 0.27; 95% CI, 0.10-0.75) was found to have a protective effect, which is in contrast with claims suggesting that it is a risk factor (Morrison *et al*., 2011) but is consistent with previous literature suggesting a role for PPIs in shielding the gut mucosa (Tsuji *et al*., 2002).

Multiple studies have also identified associations with increased age (Andrews *et al*., 2003; Bauer *et al*., 2011; Gravel *et al*., 2009; Henrich *et al*., 2009; Hensgens *et al*., 2014; Morrison *et al*., 2011; Pépin *et al*., 2004; Rodríguez-Pardo *et al*., 2013; Shivashankar *et al*., 2013) and elevated WCC at diagnosis (Cloud *et al*., 2009; Fujitani *et al*., 2011; Gujja and Friedenberg, 2009; Manek *et al*., 2011) (Table 3.2). Similarly to disease recurrence, an increase of both WCC and neutrophils was associated on univariate analysis, but only neutrophil count was included in the initial multivariate model given the better performance and high degree of collinearity between them; however this failed to reach significance in the initial multivariate model (p=0.65; Table 3.8). No significant difference was found with increased age upon univariate analysis (p=0.33; Table 3.6; Appendix 2).

Table 3.8 - Multivariate analysis of possible risk factors for severe-complicated disease

Variable	P-value	OR (95% CIs)
Initial model (n=155)		
GI comorbidities at baseline	0.02	5.86 (1.40-24.49)
Hb: per unit (mmol/L) decrease	0.14	1.31 (0.91-1.88)
Neutrophils: per 109/L increase	0.65	0.98 (0.91-1.06)
Albumin: per unit (g/L) decrease	0.04	1.13 (1.01-1.26)
C-reactive protein: per ten unit (mg/L) increase	0.18	1.05 (0.98-1.14)
Urea: per unit (mmol/L) increase	0.02	1.13 (1.02-1.26)
Potassium: per unit (mmol/L) increase	0.20	2.02 (0.69-5.95)
Number of co-medications at baseline	0.05	0.71 (0.50-1.00)
Taking PPIs prior to CDI	0.03	0.20 (0.05-0.86)
Respiratory comorbidities at baseline	0.50	1.57 (0.42-5.82)
Gender: Female	0.02	5.56 (1.28-24.18)
Presence of faecal leukocytes	0.15	0.40 (0.11-1.39)
Presence of PCR ribotype 027	0.73	0.79 (0.20-3.05)
Fever (≥38.6°C)	0.07	6.30 (0.84-47.41)
Current malignancy	0.11	11.10 (0.61-203.23)
Hypotension	0.65	1.45 (0.29-7.28)
Reduced model (n=183)		
GI comorbidities at baseline	0.05	3.00 (1.00-8.99)
Hb: per unit (mmol/L) decrease	0.07	1.34 (0.98-1.84)
Albumin: per unit (g/L) decrease	0.01	1.14 (1.04-1.25)
Urea: per unit (mmol/L) increase	0.11	1.06 (0.99-1.14)
Potassium: per unit (mmol/L) increase	0.14	1.84 (0.83-4.10)
Taking PPIs prior to CDI	0.01	0.27 (0.10-0.75)
Gender: Female	0.06	2.84 (0.97-8.26)
Fever (≥38.6°C)	0.07	4.16 (0.90-19.18)
Current malignancy	0.03	10.11 (1.28-79.82)
Hypotension	0.15	2.61 (0.71-9.59)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **GI**: Gastrointestinal; **Hb**: Haemoglobin; **OR**: Odds ratio; **PCR**: Polymerase chain reaction; **PPI**: Proton pump inhibitor;*

3.3.3.3 30-day mortality

Multivariate findings are summarised in Table 3.9. Not surprisingly, we identified independent associations with increased age (OR, 1.46; 95% CI, 1.02-2.08), which corroborates with several other reports (Boone *et al*., 2014; Inns *et*

al., 2013; Labbé *et al*., 2008; Lamontagne *et al*., 2007; Marra *et al*., 2007; Morrison *et al*., 2011; Sailhamer *et al*., 2009; Welfare *et al*., 2011; Zilberberg *et al*., 2009), as well as with increased CCI score (OR, 1.31; 95% CI, 1.03-1.67) (Boone *et al*., 2014; Labbé *et al*., 2008). We also identified an independent association with low BMI (OR, 0.87; 95% CI, 0.78-0.96).

Multiple studies have also identified associations with PCR-ribotype 027 (Inns *et al*., 2013; Labbé *et al*., 2008), elevated WCC at diagnosis (Bloomfield *et al*., 2013; Cloud *et al*., 2009; Solomon *et al*., 2013), hypoalbuminaemia at diagnosis (Bloomfield *et al*., 2013; Kim *et al*., 2013; Wilson *et al*., 2010) and the presence of septic shock (Kenneally *et al*., 2007; Khan *et al*., 2012; Lamontagne *et al*., 2007; Sailhamer *et al*., 2009; Zilberberg *et al*., 2009) (Table 3.3). However, we did not confirm association with the former three upon univariate analysis (p=0.87, p=0.53 and p=0.79, respectively; Table 3.6; Appendix 3), whilst for septic shock a comparison was not possible to be conducted as this information was not available from our cohort.

Table 3.9 - Multivariate analysis of possible risk factors for 30-day mortality

Variable	P-value	OR (95% CIs)
Initial model (n=141)		
Age at baseline: per decade	0.85	1.05 (0.66-1.66)
Body Mass Index	0.03	0.87 (0.77-0.98)
Charlson Comorbidity Index score	0.04	1.41 (1.02-1.94)
Smoking pack years: per year increase	0.10	1.02 (1.00-1.04)
Platelets: per 10^9/L increase	0.24	0.97 (0.93-1.02)
Respiratory comorbidities at baseline	0.43	1.81 (0.41-7.99)
Admitted via an emergency ward	0.30	2.26 (0.49-10.55)
Reduced model (n=286)		
Age at baseline: per decade	0.04	1.46 (1.02-2.08)
Body Mass Index	0.01	0.87 (0.78-0.96)
Platelets: per 10^9/L increase	0.08	0.97 (0.93-1.00)
Charlson Comorbidity Index score	0.03	1.31 (1.03-1.67)

***CI**: Confidence interval; **OR**: Odds ratio;*

3.3.3.4 Prolonged disease

Multivariate findings are summarised in Table 3.10. To the best of our knowledge, no previous studies have focus upon the identification of independent risk predictors for this outcome. As expected, length of diarrhoeal symptoms prior to CDI diagnosis was directly associated with prolonged disease and was considered a significant independent predictor (OR, 1.04; 95% CI, 1.01-1.08). Likewise, neutrophilia (OR, 1.03; 95% CI, 1.00-1.06) and decreased Hb (OR, 1.21; 95% CI, 1.02-1.43) also produced independent associations.

Table 3.10 - Multivariate analysis of possible risk factors for prolonged disease

Variable	P-value	OR (95% CIs)
Initial model (n=170)		
Age at baseline: per decade	0.28	1.14 (0.90-1.45)
GI comorbidities at baseline	0.08	1.91 (0.92-3.93)
Duration of symptoms prior to diagnosis, days	0.03	1.04 (1.00-1.08)
Neutrophils: per 109/L increase	0.02	1.06 (1.01-1.11)
Albumin: per unit (g/L) decrease	0.86	1.01 (0.94-1.07)
Length of hospitalisation prior to diagnosis, days	0.42	1.01 (0.99-1.02)
Hb: per unit (mmol/L) decrease	0.12	1.18 (0.96-1.46)
Urea: per unit (mmol/L) increase	0.21	1.04 (0.98-1.09)
Presence of PCR ribotype 027	0.99	1.01 (0.47-2.15)
C-reactive protein: per ten unit (mg/L) increase	0.31	1.02 (0.98-1.07)
Fever (≥38.6°C)	0.18	2.43 (0.66-9.01)
Recent CDI infection (90 days)	0.73	1.23 (0.37-4.05)
Number of stools at baseline	0.69	1.02 (0.91-1.15)
Reduced model (n=237)		
Age at baseline: per decade	0.19	1.14 (0.94-1.37)
Duration of symptoms prior to diagnosis, days	0.01	1.04 (1.01-1.08)
Presence of PCR-ribotype 027	0.19	1.51 (0.82-2.77)
Neutrophils: per 10^9/L increase	0.04	1.03 (1.00-1.06)
Urea: per unit (mmol/L) increase	0.07	1.04 (1.00-1.09)
Length of hospitalisation prior to diagnosis, days	0.07	1.01 (1.00-1.03)
Hb: per unit (mmol/L) decrease	0.03	1.21 (1.02-1.43)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **GI**: Gastrointestinal; **Hb**: Haemoglobin; **OR**: Odds ratio; **PCR**: Polymerase chain reaction;*

3.3.3.5 Statistical assessment and validation of multivariate models

A statistical assessment of the performance of the derived multivariate models was conducted, which showed that patient drop-outs due to missing data was 9, 29, 6 and 14% of the total cohort for 90-day recurrence, severe-complicated disease, 30-day mortality and prolonged disease, respectively (Table 3.11). Multiple imputation of missing values, whilst narrowing CIs and minimising the impact of missing data, demonstrated that the non-imputed and imputed datasets were comparable with the vast majority of independent predictors identified remaining unchanged (see Appendices 9-12).

ROC AUC values were obtained for each of the primary outcome measures and while model performance for 90-day recurrence, 30-day mortality and prolonged disease was considered acceptable (0.75, 0.78 and 0.70, respectively), the severe-complicated model provided the most fitting results (0.86). Furthermore, the Hosmer-Lemeshow test showed that the models generated for all four outcomes could not be rejected after splitting the data into ten subgroups (p=0.62, p=0.30, p=0.90 and p=0.52, respectively).

Prognostic models are prone to over-fitting as they tend to deliver over-optimistic performance in the dataset from which they are initially developed (Steyerberg, 2009). We therefore assessed two cross-validation approaches for our models by: a) random sampling; and b) K-fold leave-one-out (LOO) cross-validation. Resampling approach was used to randomly split the data in two and test the calibration of our models by performing a GoF test on both subgroup samples. Although model performance outlined in Table 3.11 were comparable in datasets 1 (calibration sample) and 2 (testing sample) from the subgroups, there was evidence for over-fitting with several inconsistencies in the prediction of independent variables between them and CIs considerably widened (see Appendices 5-8), as well as the observation of a lack of fitness in the validation sample for 90-day recurrence and prolonged disease (Table 3.11). LOO cross validation tests indicated that there wasn't sufficient evidence to reject the prospect of these cross-validated models being generalisable to independent datasets (i.e. with GoF p-values being non-significant). However,

despite original AUC values already demonstrating room for improvement, noticeable dips in our overall performance of the models were observed, with AUC absolute values decreasing by 3, 7, 5 and 5% for 90-day recurrence, severe-complicated disease, 30-day mortality and prolonged disease, respectively (Table 3.12).

3.3.4 Assessment of existing clinical prediction rules

An overview of the systematic review process can be seen in Appendix 13.

3.3.4.1 90-day recurrence

Of the two selected CPRs focusing on disease recurrence and described in Table 3.4, we only had data available to assess one: D'Agostino *et al*. (D'Agostino *et al*., 2014) (Table 3.13). It is important to note that this CPR has not been validated in an independent cohort and evaluation using our cohort data was limited by the use of differing outcome definitions, with D'Agostino *et al*. defining recurrence cut-off time as 30 days post-completion of CDI therapy (D'Agostino *et al*., 2014) as opposed to our definition that used 90-day post-CDI diagnosis. We did, however, attempt to assess their CPR using both 30- and 90-day cut-off points following CDI diagnosis.

Although we achieved a similar overall AUC to D'Agostino *et al*., using both 30- and 90-day recurrence (0.64 versus 0.62, and 0.61, respectively; Table 3.13), the overall performance was not satisfactory. We observed that the optimum cut-off value in our analysis was a score of 4 points and above, however, whilst this delivered high specificity (>97%), sensitivity, PPV and NPV were all very poor. A direct comparison to D'Agostino *et al*. model was not possible since this information was not provided by their work.

A full breakdown of the scores within our cohort can be seen in Appendix 14.

Table 3.11 – Random sampling model validation across all disease outcomes

Outcome	Dataset 1 (Training)					Dataset 2 (Testing)				
	N	AUC	Correctly classified	Cragg & Uhler's R^2	Hosmer-Lemeshow P-value	N	AUC	Correctly classified	Cragg & Uhler's R^2	Hosmer-Lemeshow P-value
90-day recurrence	99	0.72	66%	0.19	0.42	91	0.78	71%	0.36	**0.04**
Severe-complicated disease	83	0.86	83%	0.42	0.66	100	0.86	90%	0.42	0.34
30-day mortality	141	0.82	94%	0.21	0.94	145	0.79	90%	0.17	0.15
Prolonged disease	122	0.72	72%	0.20	0.65	115	0.74	72%	0.23	**0.05**

***AUC**: Area under the curve; **N**: Number;*

Table 3.12 –Model statistics across all disease outcomes

Outcome	Original model: Overall data					Leave-one-out cross-validation	
	N (%)	AUC	Correctly classified	Cragg & Uhler's R^2	Hosmer-Lemeshow P-value	AUC	Hosmer-Lemeshow P-value
90-day recurrence	200/220 (91)	0.75	71%	0.24	0.62	0.72	0.16
Severe-complicated disease	183/256 (71)	0.86	86%	0.39	0.30	0.79	0.07
30-day mortality	286/305 (94)	0.78	92%	0.18	0.90	0.73	0.65
Prolonged disease	237/274 (86)	0.70	71%	0.17	0.52	0.65	0.53

AUC:** Area under the curve**; N:** Number**;

Table 3.13 – Assessment of existing clinical prediction rules for CDI recurrence

CPR	Data	Outcome definition	N	Prevalence of outcome (%)	Cut-off score	AUC[a]	Sensitivity	Specificity	PPV	NPV
D'Agostino *et al*, 2014[b]	Original	30-day (FT)	962	194 (20)	NP	0.64	-	-	-	-
	Current study	30-day (FD)	239	72 (30)	≥4[c]	0.62	0.10	0.97	0.58	0.71
		90-day (FD)		109 (46)		0.61	0.08	0.98	0.75	0.56

***AUC**: Area under the curve; **CPR**: Clinical prediction rule; **FD**: From diagnosis; **FT**: From treatment completion; **N**: Number; **NP**: Data not provided; **NPV**: Negative predictive value; **PPV**: Positive predictive value;*

[a] All AUCs in this table are overall values, and are not assessed using the cut-offs; [b] This study utilised data from two clinical trials and their outcome was not to assess prediction of recurrence but to in fact produce a risk of recurrence for two separate treatment choices (fidaxomicin and vancomycin) dependent upon defined clinical variables; [c] Cut-off suggested based on our data as no cut-off provided in the literature;

3.3.4.2 Severe-complicated disease

Of the five existing CPRs for prediction of severe-complicated disease, described in Table 3.4, we had data available to assess three of them (Table 3.14).

Firstly, we assessed the CPR of Drew *et al*. (Drew and Boyle, 2009) using their suggested cut off value of ≥4 points. Notably, our patient cohort was significantly larger (192 versus 58) and while our AUC was 0.60, their work did not provide that data, although they reported information on sensitivity, specificity, PPV and NPV (Table 3.14). Despite our larger sample size, our sensitivity was markedly lower (0.41 versus 0.80) even though specificity, PPV and NPV values were comparable.

Next the CPR by Lungulescu *et al*. (Lungulescu *et al*., 2011) was investigated using their suggested cut-off value of ≥2 points. Although we observed a slightly higher specificity than theirs (0.74 versus 0.65), all other statistics we generated were markedly inferior (Table 3.14), including AUC (0.56 versus 0.78).

Thirdly, we assessed the CPR of Hensgens *et al*. (Hensgens *et al*., 2014) using their suggested cut-off of ≥4. This CPR was the only one for the prediction of severe-complicated disease to have been validated in a separate cohort of patients. All statistics assessed for our cohort were markedly lower than those of the original derivation cohort and the subsequent validation cohort (Table 3.14), including AUC (0.51 versus 0.78 and 0.73, respectively).

A full breakdown of the scores within our cohort for each CPR can be seen in Appendices 15-17. Although definitions of severe-complicated disease differed slightly across all studies including our own, these normally included some form of attributable mortality, ICU admission and the need to undergo a related surgical procedure, such as colectomy. In general, the prevalence of severe-complicated disease was similar across all studies (Table 3.14).

Table 3.14 – Assessment of existing clinical prediction rules for severe-complicated *Clostridium difficile* infection

CPR	Data	Outcome definition	N	Prevalence of outcome (%)	Cut-off score	AUC[a]	Sensitivity	Specificity	PPV	NPV
Drew *et al*, 2009[b]	Original	28-day mortality (attributable, FD) ICU admission Colectomy Pancolitis	58	8 (14)	≥4	NP	0.80	0.77	0.25	0.98
	Current study	30-day mortality (attributable, FD) ICU/HDU admission Colectomy CT evidence of severe disease	192	34 (18)	≥4	0.60	0.41	0.79	0.30	0.86
Lungulescu *et al*, 2011[c]	Original	Inpatient mortality (attributable) Critical care monitoring and/or colectomy Attributable hospital stay >10 days	255	47 (18)	≥2	0.78	0.82	0.65	0.38	0.93
	Current study	See above	188	32 (17)	≥2	0.56	0.38	0.74	0.23	0.85
Hensgens *et al*, 2014	Original	30-day mortality (attributable, FD) ICU admission Colectomy	D: 395 V: 139	D: 47 (12) V: 7 (5)	≥4	D: 0.78 V: 0.73	D: 0.43 V: 0.43	D: 0.90 V: 0.92	D: 0.39 V: 0.21	D: 0.92 V: 0.97
	Current study	See above	234	29 (12)	≥4	0.51	0.14	0.78	0.15	0.77

***AUC**: Area under the curve; **CT**: Computed tomography; **D**: Derivation cohort; **FD**: From diagnosis; **HDU**: High dependency unit; **ICU**: Intensive care unit; **N**: Number; **NP**: Not provided; **NPV**: Negative predictive value; **PPV**: Positive predictive value; **V**: Validation cohort;*

[a] All AUC values here are based upon the cut-off threshold suggested by the publication in question; [b] Letter to the Editor; [c] Retrospectively derived;

3.3.4.3 30-day mortality

Of the four CPRs regarding mortality, described in Table 3.4, own data was available to assess three of them (Table 3.15), with only one of them being validated in a separate cohort of patients (Butt *et al*., 2013).

Within our cohort, we were able to calculate overall AUCs for the CPRs of Bhangu *et al*. and Welfare *et al*. (0.52 and 0.59, respectively; Table 3.15). However, these studies did not provide a reference value for comparison. Our AUC value for the CPR of Butt *et al*. was markedly lower than that of both the derivation and validation cohorts employed by them (0.51 versus 0.70 and 0.65, respectively; Table 3.15).

Both Welfare *et al*. and Butt *et al*. failed to inform cut-off values meaning that data regarding sensitivity, specificity, PPV and NPV was unavailable for comparison (Butt *et al*., 2013; Welfare *et al*., 2011). However, using suggested cut-offs of ≥6 and ≥2 points, respectively, derived from our own data we were able to simulate these (Table 3.15). Despite acceptable specificity and NPV, sensitivity and PPV were appreciably poor. Although Bhangu *et al*. did suggest a cut-off value (≥4 points) (Bhangu *et al*., 2010), we failed to identify any patients within our cohort above this threshold, thus limiting any assessment in our samples.

A full breakdown of the scores within our cohort for each CPR can be seen in Appendices 18-20. Although definitions of mortality were similar across all studies including our own (generally all cause within 30 days), it is important to note that the all-cause mortality rate in our cohort was markedly lower compared to the aforementioned studies (Table 3.15).

Table 3.15 – Assessment of existing clinical prediction rules for mortality in *Clostridium difficile* infection

CPR	Data	Outcome definition	N	Prevalence of outcome (%)	Cut-off score	AUC[a]	Sensitivity	Specificity	PPV	NPV
Bhangu *et al*, 2010	Original	30-day inpatient (all cause, FD)	158	60 (38)	≥4	NP	0.14	0.99	0.89	0.65
	Current study	30-day (all-cause, FD)	224	17 (8)	≥4	0.52	NA	NA	NA	NA
Welfare *et al*, 2011	Original	30-day (all cause, FD)	2761	835 (30)	NP	NP	-	-	-	-
	Current study	See above	306	26 (8)	≥6[b]	0.59	0.27	0.84	0.13	0.93
Butt *et al*, 2013	Original	Inpatient (all cause) or 30-day (all cause, FD1)	D: 213[c] V: 158	D: 51 (24) V: 60 (38)	NP	D: 0.70 V: 0.65	-	-	-	-
	Current study	See above	239	19 (8)	≥2[b]	0.51	0.11	0.83	0.05	0.91

***AUC**: Area under the curve; **D**: Derivation cohort; **FD**: From diagnosis; **FD1**: From discharge; **NA**: Not able to be assessed as limited/no patients within the category the original study used; **NP**: Data not provided; **NPV**: Negative predictive value; **PPV**: Positive predictive value; **V**: Validation cohort;*

[a] All AUCs in this table are overall values as cut-offs were either not provided or we had no patients above the suggested cut-off and therefore could not assess our cohort based on this; [b] Cut-off suggested based on our data as no cut-off provided in the literature; [c] Limited to a specialised C. diff cohort ward;

3.4 Discussion

Using a prospective cohort of well-phenotyped patients we have been able to identify a number of independent risk predictors for unfavourable CDI disease outcomes that are generally consistent with previous literature (Tables 3.7-3.10). However, subsequent statistical assessment and validation of our models identified that despite adequate overall performance, they were unstable and unlikely to be generalised and validated in external cohorts (Tables 3.11 and 3.12). Random sampling resulted in widening of confidence intervals and a number of inconsistencies were found between calibration and testing datasets, whereas LOO cross validation suggested a noticeable drop in performance of the cross validated models in relation to the original training counterparts (see Appendices 5-8). Our cohort was also used to assess existing CPRs for CDI disease outcomes. For all the CPRs that we were able to evaluate, performance was considered inadequate for the prediction of the selected primary outcome measures (Tables 3.13-3.15).

A number of statistical procedures have been undertaken in order to assess core characteristics and performance of our derived multivariable models, but whilst this constitutes an important exercise towards the standardisation and validation of results, similar measures have only been taken by a minority of studies (Bloomfield *et al*., 2013; Boone *et al*., 2014; Sailhamer *et al*., 2009) out a myriad of publications investigating risk factors for the prediction of CDI outcomes (Tables 3.1-3.3). Whilst the majority of studies simply reported univariate associations between potential risk predictors and the outcomes, this does not necessarily result in a clinically useful predictive risk capability. Perhaps unsurprisingly, only a handful of studies chose to progress towards CPR development (Table 3.4). Whilst the majority of these studies undertook more extensive statistical assessment, only 3 out of 11 studies reviewed have validated their CPR in an external cohort (Butt *et al*., 2013; Hensgens *et al*., 2014; Hu *et al*., 2009), with a further three incorporating some form of internal/cross validation (D'Agostino *et al*., 2014; Welfare *et al*., 2011; Zilberberg *et al*., 2009). In addition to the lack of literature, we were only able to

assess a limited number of CPRs mainly due to existence of overly complicated parameters, which are often not routine and are very difficult to derive. This is contradictory to a CPR premise which achieves optimum performance and widespread acceptance when it employs generalisable easy-to-obtain parameters and algorithms that are simple to implement without sacrificing accuracy (Gagliardi *et al*., 2011; Kastner *et al*., 2011). Furthermore, of the tools that could be assessed, the majority did not provide sufficient information to warrant a full comparison nor did such studies presented adequate statistical assessment.

The poor external validity demonstrated through CPR assessment is likely to be the result of a very large degree of heterogeneity observed across previous studies, especially in relation to the definition of primary outcomes, CDI diagnostic algorithms, clinical parameters collected, patient recruitment sources, small sample sizes, nature of study design and temporal distribution. Outcome definition is probably one of the most significant as this varies significantly across studies (Table 3.16). For recurrence, for example, the most common cut-off adopted has been both 60- and 90-days, but these were each present in only 38% of the studies reviewed, respectively. For mortality, 30-day all-cause death was the most commonly employed definition; again this time point was only adopted by 52% of studies. The severe-complicated disease definition is usually a composite of multiple variables, of which the major component was mortality (incorporated in 94% of studies) with 30-day attributable death being the most commonly parameter definition (43% of studies reviewed). Another point of intense debate is the lack of consensus regarding the baseline/starting point of events, with the majority defining it 'from CDI diagnosis'. This was present in 54, 57 and 65% of studies focusing upon recurrence, severe-complicated disease and mortality, respectively (Table 3.16). Of the 48 studies reviewed by this work, only nine studies used a two-step approach for ascertaining diagnosis and selecting their CDI cases, with another three studies using PCR as a stand-alone test. The majority of the remaining studies used toxin EIA alone (Table 3.16), which has been criticised due to its poor sensitivity performance and low NPV (type II error). As such, their cohorts

may have been skewed towards overly symptomatic cases, thus reducing representativeness of their results.

It is likely that the majority of CDI studies to date suffered from a lack of statistical power. Historically, previously derived CPRs for conditions such as heart disease and pneumonia have incorporated cohorts >30,000 patients (Auble *et al*., 2007; Fine *et al*., 1997), which is in stark contrast to the CDI-related CPRs outlined in Table 3.4: with ~50% of the cohorts totalling less than 200 patients and ~75% totalling less than 400 patients. Whilst meta-analysis across studies would be of potential benefit, this has seriously been limited by the heterogeneity of the literature. In this respect, unsurprisingly only one investigation has been conducted to date and that focused upon disease recurrence (Garey *et al*., 2008). Furthermore, the majority of previous studies included patients who have been retrospectively recruited. This type of study design is significantly more affected by missing data, making it less suitable for direct comparisons, such as in causal analysis for the estimation of the effect of risk factors in the original publications.

Our CDI cohort was recruited through a two-step process (toxin EIA followed by toxigenic culture) and our outcome definitions were consistent with those most commonly used in the literature. As described previously, our study also benefits from a prospective design and the assessment of variables was readily available at baseline. Furthermore, it is the first study to our knowledge to focus upon risk factors for prolonged disease and is one of the few to concurrently investigate multiple outcomes. Despite these advantages, this work is not without its limitations. Firstly, despite a sample size larger than or comparable to over 75% of the previous investigations across all outcomes, the study is likely to lack adequate power in order to draw further conclusions. Secondly, we did not search conference abstract databases and therefore may be missing out a small number of experimental CPRs that, despite not being published, may have fared well in our assessment. Thirdly, the use of a prospective recruitment strategy has meant that we were unable to recruit the most severe of CDI patients, particular those in life threatening conditions, and as such our cohort may not be fully representative of the entire disease spectrum, which may have

further biased our data analysis. For instance, this can be observed through our markedly lower all-cause 30-day mortality rate (8%) in comparison to some previous studies (Inns *et al*., 2013; Kenneally *et al*., 2007; Khan *et al*., 2012; Labbé *et al*., 2008; Solomon *et al*., 2013; Wilson *et al*., 2010; Zilberberg *et al*., 2009), despite using an identical definition. However, these figures may also have been confounded by the source of their patient recruitment (i.e. specialty ward), in which some studies displayed similar figures than ours. Altogether the above may have influenced on our ability to adequately assess mortality-focused CPRs.

The results presented here clearly demonstrate that whilst associations between readily available variables and CDI outcomes are possible to be identified, statistical assessment of their utility reveals that these may not always be clinically applicable and translated to the bedside. This is further highlighted by the poor utility of existing CPRs for the prediction of primary outcomes in our cohort. Before advancements can be made, there is a need for standardisation across multiple areas (see Table 3.16) in future studies and collaborative efforts for the recruitment of large, and well-defined prospective cohorts.

Table 3.16 – Overview of the three main criteria requiring standardisation across future studies for the identification of risk factors associated with poor disease outcomes in CDI

	Recurrence (n=13)		Severe-complicated (n=17)		Mortality (n=23)	
	Different options	**Most popular (%)**	**Different options**	**Most popular (%)**	**Different options**	**Most popular (%)**
Diagnostic testing method	Culture Cytotoxin assay Toxin EIA PCR GDH + Cytotoxin assay	Toxin EIA (46%)	Culture Cytotoxin assay Toxin EIA PCR Toxin EIA + culture	Toxin EIA (47%)	Cytotoxin assay Toxin EIA PCR Toxin EIA + cytotoxin Toxin EIA + culture	Toxin EIA (48%)
Initiation of outcome	From diagnosis From treatment initiation From treatment completion From cure From discharge	From diagnosis (54%)	From diarrhoea onset From diagnosis From treatment initiation Not applicable (e.g. inpatient) Not specified	From diagnosis (57%)	From hospitalisation From diagnosis From ICU admission From treatment completion Not applicable (e.g. inpatient)	From diagnosis (65%)
Outcome threshold	≥14 days 30 days 45 days 60 days 90 days	60 days (38%) 90 days (38%)	Inpatient 30 days 90 days Prior to treatment completion None specified	30 days (59%)	Inpatient 30 days 90 days 100 days None specified	30 days (65%)

***EIA**: Enzyme immunoassay; **ICU**: Intensive care unit; **PCR**: Polymerase chain reaction;*

Chapter 4

Is the interleukin-8 promoter polymorphism rs4073/-251T>A associated with *Clostridium difficile* infection?

Contents

4.1 Introduction

As described in Chapter 1 Section 1.3, the pathogenesis of CDI has been attributed to the two potent clostridial toxins, tcdA and tcdB (Babcock *et al*., 2006b; Lyras *et al*., 2009b), both of which are reported to damage the epithelial mucosa (Hatheway, 1990) and elicit a strong immunological response (Hippenstiel *et al*., 2000; Ishida *et al*., 2004). Our current understanding of factors that determine variability in patient response to CDI is limited (Kelly *et al*., 1994; McDonald *et al*., 2005b; Poxton *et al*., 2001).

It has been previously shown for various infectious diseases that variations in genes that encode molecules that mediate attachment, pathogen recognition, inflammatory cytokine response, and innate and acquired immunity can affect disease severity as well as determine susceptibility to specific pathogens and infectious diarrhea, including *STAT3* (Amre *et al*., 2010; Cenit *et al*., 2010; Ferguson *et al*., 2010), *JAK2* (Ferguson *et al*., 2010), *IL1RN* (Queiroz *et al*., 2009), *TNFA* (Queiroz *et al*., 2009), *IL10* (Flores *et al*., 2008), *NOD2* (Queiroz *et al*., 2009) and *IL8* (Jiang *et al*., 2003). As well as being associated with susceptibility to enteroaggregative *Escherichia coli* diarrhoea, a common polymorphism in the promoter region of *IL8* (rs4073, -251 T>A) has been investigated in CDI (Garey *et al*., 2010; Jiang *et al*., 2006; Jiang *et al*., 2007).

The pro-inflammatory cytokine IL-8 is one of the major mediators of the inflammatory response. Disruption of the actin cytoskeleton by *C. diff* toxins has been shown to coincide with p38 mitogen-activated protein kinase (p38 MAPK)-dependent and MAPK-activated protein kinase 2 (MK2)-dependent IL-8 release into the intestinal lumen during CDI (Bobo *et al*., 2013; Garey *et al*., 2010; Steiner *et al*., 1997). Higher levels of faecal IL-8 are commonly found in CDI subjects and the risk AA-genotype of an IL-8 gene promoter polymorphism (-251 T>A, rs4073) has been shown to increase the odds of both developing CDI and recurrent disease by at least 3-fold, as well as increasing IL-8 release in the intestine lumen (Garey *et al*., 2010; Jiang *et al*., 2006; Jiang *et al*., 2007). As a result IL-8 has been proposed as a potential biomarker by other groups (Garey

et al., 2010), though the genetic associations have not been independently replicated and its clinical validity has not been clearly demonstrated.

Using a prospectively recruited cohort of carefully phenotyped patients, this study aimed to evaluate the IL-8 variant with regards to the risk of CDI and recurrent disease, relate the variant to faecal IL-8 levels and undertake a meta-analysis using the available literature.

4.2 Methods

4.2.1 Study design

Using the criteria defined in Chapter 2, a discovery cohort (n=423) comprising 288 CDI cases and 135 controls with AAD, was recruited from July 2008 to November 2011 across two large hospital sites in Merseyside; RLBUHT and WTH (Figure 4.1). Blood and faecal samples were collected from all patients.

A retrospective replication cohort comprising 270 individuals (170 CDI cases & 100 healthy volunteers as controls) was used to confirm our genetic observations (Figure 4.1). Cases were recruited from RLBUHT between October 2000 and September 2001, whilst controls were healthy volunteers (staff and students) from the University of Liverpool.

Relevant information on demographics, admission and clinical history of CDI was collected for each patient and recorded in an anonymised case report proforma. Ethical approval for the study was obtained from the Liverpool Research and Ethical Committee under reference numbers 08/H1005/32 and 08/H1017/19, and each patient provided written informed consent prior to recruitment. All individuals were white Caucasians.

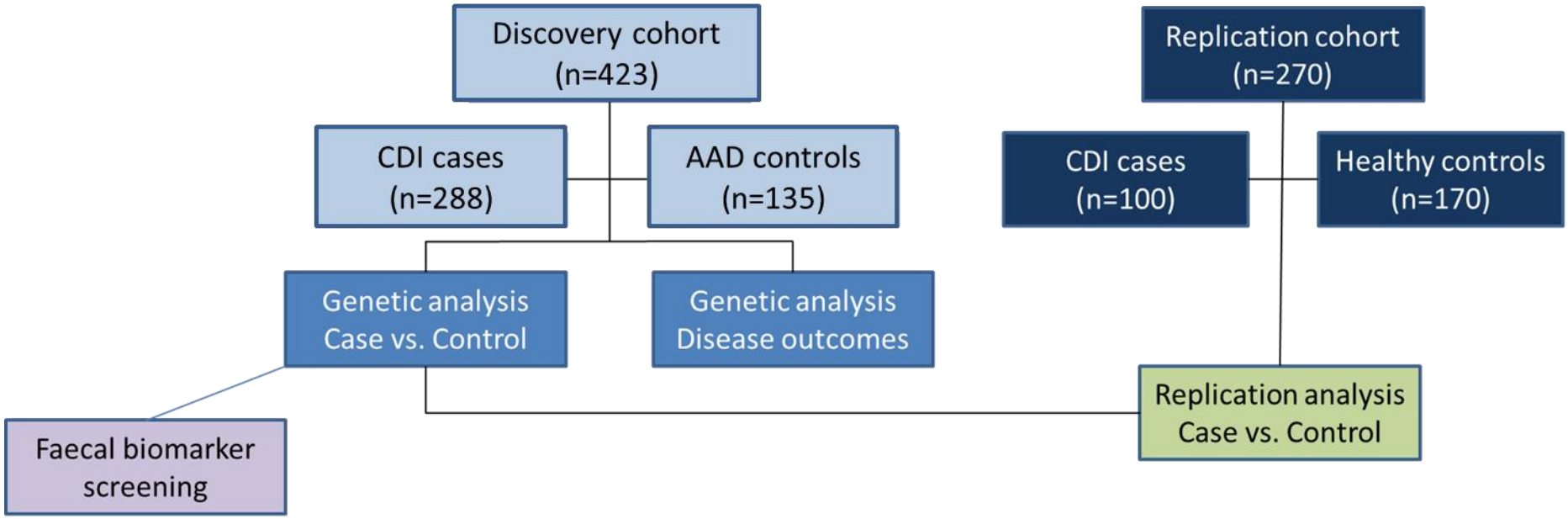


Figure 4.1 – Overview of study design

AAD: Antibiotic-associated diarrhea; CDI: Clostridium difficile infection; n: number;

4.2.2 Definition of outcomes

Cases and controls were defined as per Chapter 2. Disease outcomes studied by Garey *et al*. included recurrent CDI, refractory CDI and all-cause mortality rate, all assessed at 90 days (Garey *et al*., 2010). Recurrent disease was defined as the development of subsequent CDI episodes following treatment of the initial episode and refractory disease was considered if patients did not respond to the initial standard treatment and diarrhoea was not immediately resolved.

4.2.3 DNA isolation & genotyping

DNA was extracted from human blood samples using either Chemagen paramagnetic bead chemistry (Chemagen Biopolymer-Technologie AG; Baesweiler, Germany) or EZ 96 Total DNA Isolation Kit (Omega Biotek, Norcross, USA) following manufacturers' protocols.

IL-8 rs4073 (-251 T>A) was genotyped using an off-the-shelf TaqMan allelic discrimination genotyping assay (Life Technologies Ltd., Paisley, UK). Total reaction volume was 5 µl and consisted of: -

- 2 µl of 1x Taqman genotyping master mix
- 0.13 µl of 1x Taqman genotyping assay
- 2.87 µl of distilled water (dH_2O)
- 20 ng of dried genomic DNA

Reactions were run on the Applied Biosystems HT 7900 Fast Real-Time PCR system (Applied Biosystems, USA) using the following cycling conditions: -

- Stage 1: 50°C for 2 min
- Stage 2: 95°C for 10 min
- Stage 3: 50 cycles
    - 95°C for 15 s
    - 60° for 60 s

Genotype calls were made in accordance to the reference alleles and sequence orientation of dbSNP (NCBI build 37). Quality control measures included Hardy-Weinberg equilibrium (HWE, p>0.01), as well as the incorporation of repeat samples and blanks. Results were analysed using the provided SDS software (version 2.2).

4.2.4 Biomarker measurement in stools

In order to replicate previous findings of a direct link between IL-8 genotype and faecal IL-8 levels, a subset of patients was selected from the discovery cohort (76 CDI cases & 33 AAD controls), which was of an equivalent size of the original report (Jiang *et al*., 2006).

For stool testing, as per previous studies (Greenberg *et al*., 2002; Steiner *et al*., 1997), aliquots of neat stools were diluted 1:5 in phosphate buffer solution (PBS) supplemented with 2.5 μg/mL leupeptin, 11 μg/mL aprotinin, and 5 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride (Sigma, Haverhill, UK) and stored at −70°C until further use. A commercial ELISA kit was used to test the subset of patients for IL-8 (Quantikine, R&D systems, Abingdon, UK), with the minimum detectable dose (MDD) equal to 3.5 pg/ml. All procedures were carried out according to manufacturer's instructions, and a standard 4-parameter logistic nonlinear regression method was used to calculate protein concentrations.

4.2.5 Statistical analysis

Chi-squared test assuming a recessive mode of effect and test for allelic distribution assuming an additive effect (binary regression assuming one degree of freedom) were performed to ascertain differences in genotypic distribution between cases and control groups, as well as within cases for differences in genotypic distribution for detectable/undetectable levels of faecal IL-8 (defined as <3.5 pg/ml as per manufacturer's instructions) and the presence/absence of recurrent CDI, refractory CDI and mortality within 90 days. Our discovery and replication cohorts were analysed separately, with the replication cohort only used for genotypic distribution between cases and controls. All analyses were performed using Stata statistical package v.9.2

(StataCorp, College Station, USA) and StatsDirect v.2.7.9 (StatsDirect Ltd, Altrincham, UK).

Due to the relatively small sample sizes of both our own and the previous studies, meta-analyses were conducted as a way of increasing the statistical power to detect significant associations. Prior to meta-analysing, the data was analysed by two different statistical methods (assuming additive or recessive modes of effect) to account for the differing statistical methods of choice for both our own and the previous studies. For this purpose, cases from our discovery and replication cohorts were combined and, as with the previous studies, analysed against both AAD and healthy controls, respectively. Adjustment for potential confounders was lacking in the previous literature, meaning this could not be incorporated into the analysis here. Meta-analysis ORs and 95% CIs were generated based on a random effects model using the 'Metafor' package of 'R' v.2.15.2. Power calculations were simulated using nQuery Advisor and nTerim (Statistical Solutions Ltd., Cork, Ireland).

4.3 Results

4.3.1 Demographics

Outlined in Table 4.1, the discovery cohort comprised a total of 288 CDI cases and 135 controls. No significant differences were observed between CDI cases and AAD controls for gender (57% female versus 56% female, respectively; p=1.00) or median CCI score (1.0 versus 1.0; p=0.52). However, significant differences (p<0.01) were identified for mean age (70.6 versus 65.6 yrs), mean BMI (24.4 versus 26.9) and median time delay between testing positive and subsequent recruitment (3.0 versus 2.0 days). Furthermore, all-cause mortality within 90 days was significantly greater amongst CDI cases (21.1% versus 4.5%; p<0.01). 46% (89/192) of CDI cases assessed during recruitment experienced recurrence within 90 days. The prevalence of refractory CDI was 51% (108/211).

Table 4.1 – Demographics of discovery cohort

	CDI Cases (n=288)	AAD Controls (n=135)	P-value*
Patient's characteristics			
Gender = Female - n (%)	163/288 (57)	75/133 (56)	1.00
Age – Mean in years (SD)	70.6 (16.0)	65.6 (17.5)	<0.01
BMI – Mean (SD)	24.4 (6.2)	26.9 (7.0)	<0.01
CCI score** – Median (IQR)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.52
Time delay (testing/recruitment) – Median (IQR)	3.0 (2.0-4.0)	2.0 (2.0-3.0)	<0.01
Clinical Parameters			
All cause death within 90 days– n (%)	54/256[a] (21.1)	6/132[a] (4.5)	<0.01
Refractoriness within 90 days – n (%)	108/211[b] (51.2)	-	
Recurrence within 90 days – n (%)	89/192[b] (46.4)	-	
Presence of ribotype 027 – n (%)	86/266[c] (32.3)	-	

AAD: Antibiotic-associated diarrhoea; BMI: Body mass index; CCI: Charlson Comorbidity Index; CDI: Clostridium difficile infection; IQR: Interquartile range; n: number;

** Means for normally distributed, continuous variables were compared using Independent samples T-test for continuous, for non-normal distribution median values were compared using Mann Whitney U test. Categorical data was assessed using a Chi-squared test; **CCI score is calculated without accounting for age*

[a] Data regarding death within 90 days was unavailable for 32 of our cases and 3 of our controls; [b] Data regarding refractoriness and recurrence of disease within 90 days was unavailable for 47 and 66 of our cases, respectively. A further 30 cases died within the follow-up period prior to experiencing recurrent/refractory CDI and therefore could not be included in the final analysis; [c] Isolates were successfully recovered from 266/288 (92%) of our cases and thus ribotyping could not be done in 22 cases;

For the two sub-cohorts used in the measurement of faecal IL-8, no significant differences were observed between CDI cases (n=73) and AAD controls (n=39) for gender (55% female versus 72% female, respectively; p=0.10), median Charlson Comorbidity Index score (1.0 versus 1.0; p=0.90) or time delay (2.0 versus 1.0 days; p=0.18). However, significant differences were identified for median age (75.9 versus 64.9 yrs; p=0.01) and BMI (23.0 versus 28.3; p<0.01)

Furthermore, all-cause mortality within 90 days was significantly greater amongst CDI cases (23.3 versus 7.7%; p=0.04).

Our replication cohort consisted of 100 CDI cases, of which 52 (52%) were male and 48 (48%) female, with a combined median age of 71.3 yrs. The healthy control arm consisted of 70 males (41%) and 100 females (59%) with a combined median age of 29 yrs. This cohort was used solely for replication of our genetic analysis, and data on ribotyping or measurement of faecal IL-8 was not available for these patients.

4.3.2 Genotypic analysis

4.3.2.1 CDI cases versus AAD controls

Genotype call rate was >98% and all replicates showed concordant results. Genotypic distribution across all groups was in HWE and the observed minor allele frequency (MAF) was consistent with previous literature. Case-Control analysis in both cohorts showed no significant differences in rs4073 genotype distribution (p=0.84 and p=0.87; Table 4.2). We also failed to observe significant differences after combining all cases and comparing them against the AAD and healthy control groups using both recessive (p=0.63 and p=0.42; Table 4.3) and additive (p=0.89 and p=0.35; Table 4.4) models.

Table 4.2 - Genetic analysis of CDI cases versus controls across individual discovery and replication cohorts

rs number	Minor Allele		Genotype Counts (MAF)		Cases vs. Controls
Discovery Cohort		CDI Cases (n=286)	AAD Controls (n=135)	P-value	OR (95% CI)
rs4073	A	93/141/48 (0.42)	39/72/20 (0.43)	0.84	0.97 (0.71-1.31)
Replication cohort		CDI Cases (n=100)	Healthy Controls (n=170)	P-value	OR (95% CI)
rs4073	A	30/47/21 (0.45)	49/84/36 (0.46)	0.87	0.97 (0.68-1.38)

CI: Confidence Intervals; IL-8: Interleukin-8; MAF: Minor Allele Frequency; OR: Odds Ratio;

Table 4.3 - Genetic analysis of combined CDI cases versus individual control groups assuming a recessive mode of effect

	Genotype		n	P-value	OR (95% CI)
	AA	AT or TT			
CDI cases (combined)	71	315	519 (386 vs. 133)	0.63	1.14 (0.67-1.92)
AAD controls	22	111			
CDI cases (combined)	71	315	555 (386 vs. 169)	0.42	0.83 (0.53-1.31)
Healthy controls	36	133			

AAD: Antibiotic-associated diarrhoea; CI: Confidence Intervals; HWE: Hardy-Weinburg Equilibrium; n: number; OR: Odds Ratio;

Table 4.4 - Genetic analysis of combined CDI cases versus individual control groups assuming an additive mode of effect

	Genotype			HWE	n	P-value	OR (95% CI)
	AA	AT	TT				
CDI cases (combined)	71	191	124	0.87	519 (386 vs. 133)	0.89	0.98 (0.74-1.30)
AAD controls	22	72	39	0.24			
CDI cases (combined)	71	191	124	0.87	555 (386 vs. 169)	0.35	0.88 (0.68-1.14)
Healthy controls	36	84	49	1.00			

AAD: Antibiotic-associated diarrhoea; CI: Confidence Intervals; HWE: Hardy-Weinburg Equilibrium; n: number; OR: Odds Ratio;

4.3.2.2 Association with faecal IL-8 level

Faecal IL-8 concentrations across genotypes within both CDI cases and AAD controls are summarised in Table 4.5. We failed to replicate previous findings of a significant association between faecal IL-8 concentration and IL-8 rs4073 genotype in our CDI patients (p=0.28), despite a similar sample size to that of the original study (Jiang *et al*., 2006). We also failed to replicate these findings when analysing based on a cut-off of 3.5 pg/ml for detectable levels (as per the manufacturers information), using both recessive (p=0.73; Table 4.6) and additive (p=0.96; Table 4.7) modes of effect.

Table 4.5 - Faecal IL-8 production split per genotype group of the IL-8 -251 SNP (rs4073) in a subset of CDI cases and AAD subjects

CDI Cases (n=73; MAF=44.5%)			
T/T	21	172.5	87.6 - 601
T/A	39	29.3	5.4 - 569
A/A	13	257.1	<3.0 - 1,473.4
AAD Controls (n=39; MAF=44.9%)			
T/T	12	<3.0	<3.0 -<3.0
T/A	19	<3.0	<3.0 – 4.0
A/A	8	7.9	<3.0 - 22.7

IQR: Inter-quartile range; MAF: Minor Allele Frequency; N: Numbers; SE: Standard Error;

Table 4.6 – Analysis of detectable faecal IL-8 level versus IL-8 rs4073 genotype within CDI cases only, assuming a recessive mode of effect

	Genotype		n	P-value	OR (95% CI)
	AA	AT or TT			
Cases detectable for IL-8	9	53	74 (62 vs. 12)	0.73	0.84 (0.31-2.24)
Cases undetectable* for IL-8	4	8			

*CI: Confidence interval; IL-8: Interleukin-8; n: Number; OR: Odds ratio; *Defined as <3.5 pg/ml*

Table 4.7 - Analysis of detectable faecal IL-8 level versus IL-8 rs4073 genotype within CDI cases only, assuming an additive mode of effect

	Genotype			n	P-value	OR (95% CI)
	AA	AT	TT			
Cases detectable for IL-8	9	33	20	74 (62 vs. 12)	0.96	0.99 (0.56-1.74)
Cases undetectable* for IL-8	4	7	1			

*CI: Confidence interval; IL-8: Interleukin-8; n: Number; OR: Odds ratio; * Defined as <3.5 pg/ml*

4.3.2.3 CDI disease outcomes

A comparison between patients with 90-day recurrent CDI and those with a single episode in our discovery cohort did not reveal any differences in genotypic distribution under either recessive or additive inheritance modes (p=0.79 and p=0.75, respectively; Tables 4.8 & 4.9). Similarly, no genotypic distribution differences were observed when comparing patients suffering from 90-day refractory CDI against those having undergone successful treatment (p=0.70 and p=0.79, respectively; Tables 4.8 & 4.9) and when comparing patients suffering from 90-day mortality against those who had survived (p=0.61 and p=0.40, respectively; Tables 4.8 & 4.9). There was also no relationship with carriage of the 027 strain when analysed using either recessive (p=0.78) or additive (p=0.83) modes of effect.

Table 4.8 – IL-8 rs4073 versus CDI disease outcomes assessed at 90 days, using the discovery cohort only and assuming a recessive mode of effect

Disease outcome	Genotype		n	P-value	OR (95% CI)
	AA	AT or TT			
Recurrence	16	73	192 (89 vs. 103)	0.79	1.11 (0.52-2.35)
Non-recurrence	17	86			
Refractory	20	88	211 (108 vs. 103)	0.70	1.15 (0.56-2.34)
Non-refractory	17	86			
Mortality	11	43	256 (54 vs. 202)	0.61	1.22 (0.57-2.60)
Non-mortality	35	167			

CI: Confidence Intervals; n: number; OR: Odds Ratio;

Table 4.9 – IL-8 rs4073 versus CDI disease outcomes assessed at 90 days, using the discovery cohort only and assuming an additive mode of effect

Disease outcome	Genotype			n	P-value	OR (95% CI)
	AA	AT	TT			
Recurrence	16	46	27	192 (89 vs. 103)	0.75	1.07 (0.71-1.62)
Non-recurrence	17	53	33			
Refractory	20	54	34	211 (108 vs. 103)	0.79	1.06 (0.71-1.56)
Non-refractory	17	53	33			
Mortality	11	29	14	256 (54 vs. 202)	0.40	1.21 (0.78-1.87)
Non-mortality	35	103	64			

CI: Confidence Intervals; n: number; OR: Odds Ratio;

4.3.4 Meta-analysis & power calculations

Meta-analyses combining our data with those published in the literature using several phenotypes were also undertaken – the results are shown in Tables 4.10 & 4.11. Significant associations (OR (95% CIs)) identified by the previous literature for IL-8 rs4073 genotype in relation to CDI cases versus AAD controls (3.26 (1.09-9.71)), CDI cases versus healthy controls (3.37 (1.13-10.02)), faecal IL-8 levels (6.75 (1.43-31.90)) and 90-day recurrence (2.74 (1.01-7.40)) were no longer significant after meta-analysis (1.72 (0.63-4.71), 1.53 (0.39-5.94). 2,17 (0.28-16.64), & 1.64 (0.68-3.95), respectively). Non-significant associations identified by the previous literature remained so after meta-analysis.

Power calculations across the multiple outcome measures were based on odds ratio and effect sizes reported by Jiang *et al*. (2006) and Garey *et al*. (2010) and revealed that their power to detect true associations was limited and estimated to be 59% for case-control comparison, 72% for predicting faecal IL-8 levels, 52% for 90-day recurrence, 7% for 90-day refractory disease and 26% for 90-day mortality. A direct comparison with our cohorts produced estimates of around 99%, 97%, 68%, 9% and 45%, respectively.

Table 4.10 – Meta-analysis of IL-8 rs4073 relating to CDI susceptibility using data generated by this study and available literature (Garey *et al.*, 2010; Jiang *et al.*, 2006) *[CI: Confidence interval; IL-8: Interleukin-8; OR: Odds ratio]*

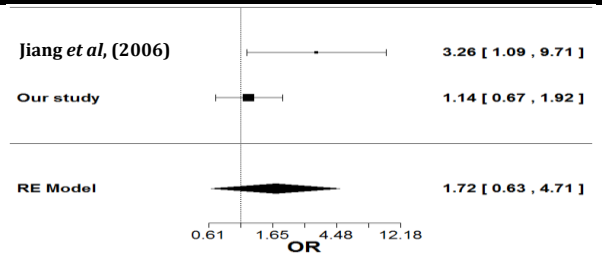
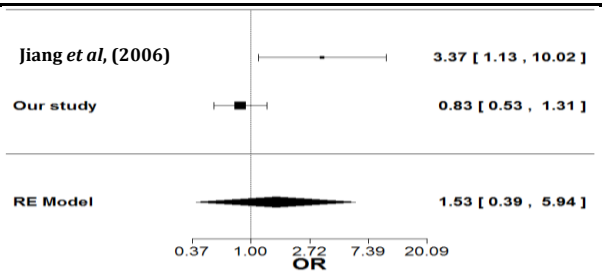
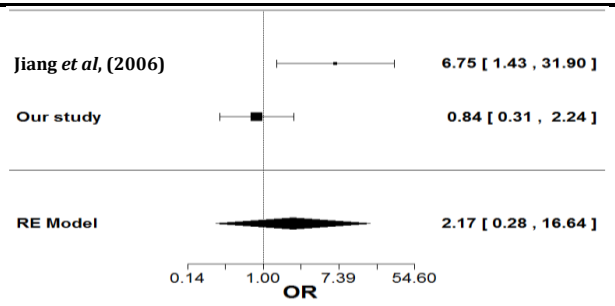
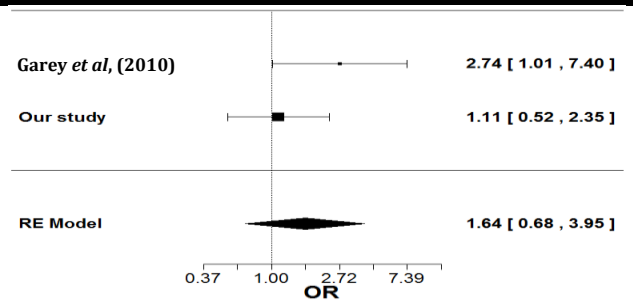
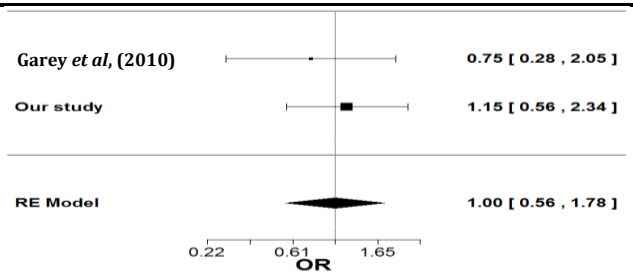
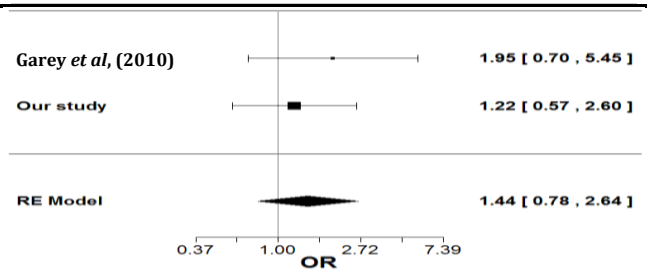
Outcome	Source	Chi² test assuming a recessive effect mode	Genotype AA	Genotype AT TT	OR (95% CIs) P-value	Meta-analysis plot for each study and combined data under a recessive mode of effect (OR, 95% CIs)
CASE-CONTROL COMPARISON VS. IL-8 RS4073 GENOTYPES	Jiang *et al.* 2006	CDI Cases (38) vs. Diarrhoea controls (36)	15	23	3.26 (1.09-9.71) **0.03**	
			6	30		
	This study	CDI cases (386) vs. Diarrhoea controls (133)	71	315	1.14 (0.67-1.92) 0.63	
			22	111		
	Jiang *et al.* 2006	Cases (38) vs. Healthy controls (37)	15	23	3.37 (1.13-10.02) **0.025**	
			6	31		
	This study	Combined cases (386) vs. Healthy controls (169)	71	315	0.83 (0.53-1.31) 0.42	
			36	133		
FAECAL IL-8 LEVELS VS. IL-8 RS4073 GENOTYPES (CASES ONLY)	Jiang *et al.* 2006	Cases detectable (13) vs. Cases undetectable (20)	9	4	6.75 (1.43-31.90) **0.01**	
			5	15		
	This study	Cases detectable (62) vs. Cases undetectable (12)	9	53	0.84 (0.31-2.24) 0.73	
			4	8		

Table 4.11 – Meta-analysis of IL-8 rs4073 relating to CDI disease outcomes using data generated by this study and available literature (Garey *et al.*, 2010; Jiang *et al.*, 2006) *[CI: Confidence interval; IL-8: Interleukin-8; OR: Odds ratio]*

Outcome	Source	Chi² test assuming a recessive effect mode	Genotype		OR (95% CIs) P-value	Meta-analysis plot for each study and combined data under a recessive mode of effect (OR, 95% CIs)
			AA	AT TT		
90-DAY RECURRENCE VS. IL-8 RS4073 GENOTYPES (CASES ONLY)	Garey *et al.* 2010	Recurrence (23) vs. Non-recurrence (73)	10	13	2.74 (1.01-7.40) **0.04**	
			16	57		
	This study	Recurrence (89) vs. Non-recurrence (103)	16	73	1.11 (0.52-2.35) 0.79	
			17	86		
90-DAY REFRACTORY DISEASE VS. IL-8 RS4073 GENOTYPES (CASES ONLY)	Garey *et al.* 2010	Refractory (30) vs. Non-refractory (66)	7	23	0.75 (0.28-2.05) 0.58	
			19	47		
	This study	Refractory (108) vs. Non-refractory (103)	20	88	1.15 (0.56-2.34) 0.70	
			17	86		
90-DAY MORTALITY VS. IL-8 RS4073 GENOTYPES (CASES ONLY)	Garey *et al.* 2010	Mortality (21) vs. Non-mortality (75)	8	13	1.95 (0.70-5.45) 0.20	
			18	57		
	This study	Mortality (54) vs. Non-mortality (202)	11	43	1.22 (0.57-2.60) 0.61	
			35	167		

4.4 Discussion

Acute CDI disease occurs as a result of an uncontrolled toxin-driven inflammatory response culminating in generalised colitis and colonic necrosis (Bobo *et al*., 2013), a process that is compounded by inter-individual variability in both CDI susceptibility and recurrence. IL-8 has previously been suggested as a potential biomarker for inter-individual variation in susceptibility (Garey *et al*., 2010; Greenberg *et al*., 2002; Jiang *et al*., 2006; Steiner *et al*., 1997).

Using a prospective cohort of well-characterised patients, we failed to replicate previous findings of an association between IL-8 rs4073 and an increased risk of developing CDI (Jiang *et al*., 2006) and experiencing recurrent disease (Garey *et al*., 2010), confirmed in a meta-analysis with the previously published data (Tables 4.10 & 4.11, respectively). This could well be due to unforeseen population stratification. Our patients were all Caucasian with observed MAF (43.5%) very similar to that reported in Caucasians by both the Hapmap and 1000 genomes projects (~40%). However, the previous studies in question did not undertake ethnically matched analyses and the prior knowledge that rs4073 allele frequency varies greatly across ethnic groups may explain why the genotypic distribution for their AAD controls differed significantly from HWE ($p<0.007$). Our power calculations suggest that previous studies have been significantly underpowered to detect true associations.

Although this polymorphism is located in a putative transcriptionally active domain, it remains uncertain whether it actually influences the secretion and overall release of IL-8 in the colon during CDI: we were unable to replicate a previous association between IL-8 levels and rs4073 genotype using a slightly larger cohort size, also confirmed in a meta-analysis with previously published data (Table 4.10). We also explored the possibility that the risk *AA* genotype would be more likely affected by NAP1/BI1/027 strains than other circulating strains, but our results showed no association (Chi-squared $p=0.78$).

While the findings of our study were generally negative, we have adopted stringent criteria in our approaches, employing a significantly larger sample size than previous studies as well as an ethnically matched group of Caucasian patients. Furthermore, we employed an independent set of individuals for the purpose of replication of our case-control findings.

There are a number of potential limitations in our study. Firstly, our study, as well as those conducted previously, did not carry out longitudinal sampling of stools to monitor the trajectory of faecal IL-8 over time. Secondly, we only used a single laboratory test (ELISA for CDT) for the primary identification of CDI cases. Although this is still a common procedure, modern algorithms currently make use of a more sensitive first step screening - based on either GDH, or a nucleic acid amplification test NAAT - to minimise the odds of reporting false negative results. Therefore it is possible that our cohort may have lacked a fully representative range of cases.

Thirdly, we have focused on only one SNP and therefore cannot exclude the possibility of other SNPs within the IL-8 gene having an effect on CDI susceptibility. However, there is a general lack of understanding of the genetic basis of inter-individual variability underlying both CDI susceptibility and recurrence. Although we evaluated two cohorts of patients, and our sample size, was larger than in the studies published previously, we cannot exclude a lower effect size of this SNP. For such a complex disease it is highly likely that various genes across various pathways will be involved, and the effects that variation in single genes have on disease susceptibility or severity will be modest at most (Flores and Okhuysen, 2009).

Biomarkers which can act as indicators of disease, disease relapse and disease stratification, are needed to direct CDI therapies more effectively. Further studies are certainly warranted to identify the genetic predisposition to CDI, and the adoption of systematic hypothesis-free methods using genome-wide coverage coupled with larger cohort sizes and a well-defined array of phenotypes will be pivotal for unveiling and validating true genetic susceptibility markers.

Chapter 5

Investigation of faecal lactoferrin and calprotectin in a prospective CDI cohort

Contents

5.1 Introduction

As outlined in Chapter 1 Section 1.3, pathogenesis of CDI is attributed to the two potent clostridial toxins, tcdA and tcdB (Babcock *et al*., 2006b; Lyras *et al*., 2009b). Their synergistic effects cause fluid accumulation and damage to the epithelial mucosa (Hatheway, 1990), further eliciting pro-inflammatory cytokine release (Hippenstiel *et al*., 2000; Ishida *et al*., 2004). Concurrent activation and recruitment of neutrophils results in an inflammatory response in the gastrointestinal tract of CDI patients, but this is variable, ranging from self-contained mild inflammation to severe pseudomembranous colitis (Kelly and Kyne, 2011; Savidge *et al*., 2003).

Toxins are the essential virulence factors accounting for CDI pathogenicity. Current diagnostic tools rely on their detection by either cytotoxin neutralisation or enzyme immunoassays. Multi-step algorithms have also been adopted in an attempt to improve sensitivity by combining toxin detection with sensitive screening of the presence of the organism by selective culture, GDH detection and/or NAAT of the PaLoc (Planche *et al*., 2013). These tests do not allow for stratification of disease severity and prognosis in patients with CDI. Validated non-invasive enteric markers for CDI that allow for better patient assessment and enable a more personalised approach to treatment would be valuable (Planche *et al*., 2013).

Faecal material represents a very complex and heterogeneous biological matrix. Candidate faecal biomarkers must possess properties that ensure reliability and reproducibility of results and they must be unaffected by extra-digestive processes. FL and FC, derived predominantly from activated neutrophils and unaffected by extra-digestive processes, have been extensively evaluated in IBD and infectious diarrhea (D'Inca *et al*., 2007; García-Sánchez *et al*., 2010; Jones *et al*., 2008; Langhorst *et al*., 2008; Schoepfer *et al*., 2009; Schoepfer *et al*., 2010; Schoepfer *et al*., 2008; Sipponen *et al*., 2008a; Sipponen *et al*., 2008b; Sipponen *et al*., 2008c; van Langenberg *et al*., 2010).

A small number of studies have investigated FL and FC in the context of CDI (Table 5.1). Some have shown an association of FC in several acute diarrhoeal diseases caused by bacteria, with the highest mean levels observed in patients with CDI (192 mg/l) (Shastri *et al.*, 2008). Others have shown a significant association when comparing FC levels in toxin positive and GDH positive plus tcdA/tcdB PCR confirmed patients to diarrhoea controls (Whitehead *et al.*, 2014). Similarly, FL has been shown to be elevated in patients with CDI (Archbald-Pannone *et al.*, 2010; LaSala *et al.*, 2013; Vaishnavi *et al.*, 2000; van Langenberg *et al.*, 2010) with more recent studies suggesting a positive correlation with disease severity (Boone *et al.*, 2013; El Feghaly *et al.*, 2013; Steiner *et al.*, 1997) and fluoroquinolone resistance (Pawlowski *et al.*, 2009). There are however limitations with the published studies: these include their retrospective nature, limited phenotype data, lack of matched controls, use of non-quantitative tests, and variations in the assessment of CDI outcome measures. Sample sizes have varied from 2 to 120, and none of the studies have compared FC and FL in the same patient groups.

In this study, we use a prospective design, a carefully phenotyped cohort and simultaneous evaluation of both faecal markers, to investigate whether FL and FC would have clinical value in patients suffering from CDI.

Table 5.1 – Overview of previous studies evaluating the role of lactoferrin and calprotectin in faeces in patients with *Clostridium difficile* infection

Study	Country	Healthcare setting	Paticipants	Measure used	Results	Associated outcomes (p-value)
Faecal Lactoferrin						
Steiner *et al*. (1997)	USA	Hospital	Mild CDI (n=6) Severe CDI (n=12)	Qualitative (positive/negative)	1/6 = positive 9/12 = positive	Disease severity* (P=0.021)
Vaishnavi *et al*. (2000)	India	Hospital	CDI cases (n=41) Diarrhoea controls (n=190)	Qualitative (positive/negative)	33/41 = positive 123/190 = negative	*C. diff* toxin positivity & negativity (P<0.001 for both)
Pawlowski *et al*. (2009)	USA	Hospital	CDI cases (n=34)	Cut-off (72.5 µg/g)	10 resistant = >72.5 µg/g 16 resistant = <72.5 µg/g 8 susceptible = <72.5 µg/g	Moxifloxacin resistance (P=0.041)
Archbald-Pannone *et al*. (2010)	USA	LTCF	CDI cases (n=2) Diarrhoea controls (n=22)	Continuous	134.1 µg/ml 28.8 µg/ml	*C. diff* colonisation (P=0.008)
Van Langenberg *et al*. (2010)	Australia	Hospital	CDI cases (n=8) Diarrhoea controls (n=334)	Continuous	33.3 µg/ml 22.6 µg/ml	*C. diff* positivity (P=0.017)
El Feghaly *et al*. (2013)	USA	Hospital	CDI cases (n=120)	Cut-off (7.25 µg/ml)	72/120 (60%) = >7.25 µg/ml (Outcome data not provided)	Severe HINES VA Score** (P=0.002)
Boone *et al*. (2013)	USA	Hospital & outpatients	Mild CDI (n=7) Moderate CDI (n=57) Severe CDI (n=21)	Continuous	73 µg/g 292 µg/g 961 µg/g (Ribotype data not provided)	Disease severity*** and Ribotype 027 (P = 0.0003 & P=0.012)
LaSala *et al*. (2013)	USA	Hospital	GDH neg (n=43) GDH positive/Tox neg/PCR neg (n=14) GDH positive/Tox positive (n=25) GDH positive/Tox neg/PCR positive (n=30)	Continuous	13 µg/ml 18 µg/ml 80 µg/ml 24 µg/ml	Toxin positivity (vs. GDH negative; p=0.006) (vs. GDH positive/CDT negative/PCR negative; p=0.002) (vs. GHD positive/CDT negative/PCR positive; p=0.015)

Table 5.1 (continued) - Overview of previous studies evaluating the role of lactoferrin and calprotectin in faeces in patients with *Clostridium difficile* infection

Study	Country	Healthcare setting	Paticipants	Measure used	Results	Associated outcomes (p-value)
Faecal Calprotectin						
Shastri *et al*. (2008)	Germany	Hospital	CDI cases (n=87) Healthy controls (n=200)	Continuous	192 mg/l 171/196 = <15 mg/l	-
Whitehead *et al*. (2014)	UK	Hospital	Tox positive (n=45) GDH positive/PCR positive (n=75) Diarrhoea controls (n=99)	Continuous	336 µg g-1 249 µg g-1 106 µg g-1	*C. diff* positivity (P<0.05)

**Disease was considered severe if any of the following was present: diarrhoea severe enough to produce clinical signs of volume depletion and to require hospitalisation, WBC count of >10,000/ml, or temperature of >38.3°C*

***Scoring system accounting for fever (>38°C), ileus (clinical or radiographic), systolic blood pressure (<100 mmHg), WBC (15000<WBC<30000 cells/µl) and CT scan findings (colonic wall thickening, colonic dilatation, ascites)*

****Automatically classified as severe if age ≥65 years, WBC >15 × 10^9/L, stool ≥10 per day, not able to tolerate oral intake, usually abdominal complaints, radiographic or peritoneal signs, multiple comorbidities including but not limited to renal failure and immunosuppression*

5.2 Methods

5.2.1 Study design

164 CDI cases and 52 AAD controls were recruited from RLBUHT between July 2008 and May 2010. Blood and faecal samples were collected from all patients. Recruitment criteria were defined as per Chapter 2. As well as case versus control analysis, we also investigated four primary CDI disease outcomes: 90-day recurrence, 30-day mortality and prolonged disease (defined as per Chapter 3 Section 3.2.1) and disease severity at baseline. The severity of CDI symptoms at baseline were assessed using the guidelines proposed by Public Health England (Public Health England, 2013), except for incorporation of a more stringent cut-off for WCC (>20 $x10^9$/L) and replacement of acute rising creatinine with an estimated Glomerular Filtration Rate (eGFR) of <30 ml/min/1.73m^2.

5.2.2 Biomarker measurement in stools

Aliquots of neat stools were prepared as per Chapter 2. Both FC and FL levels were measured using commercially available *in vitro* diagnostic (IVD) ELISA kits (Calpro, Lysaker, Norway; IBD Scan Techlab, Blacksburg, USA, respectively). All procedures were carried out according to manufacturer's instructions, with the exception of the FL sample preparation step, whereby an inoculation loop was used as an agitator during a 30 minute shaking step in order to ensure optimal recovery of proteins. Where necessary, further dilutions and extra points on the standard curve were included. A standard 4-parameter logistic nonlinear regression method was used to calculate faecal biomarker concentrations.

5.2.3 Statistical analysis

Levels of FL and FC were subject to 4-tier percentile categorisation (i.e. Low <25%, Medium-Low 25-50%, Medium-High 50-75% and High >75%). Univariate binary logistic regression was conducted for both case-control comparison and sub-group analysis of cases for the outcomes proposed above.

Covariates including age, gender, BMI, CCI score, presence of ribotype 027 and time delay between testing positive and subsequent recruitment were assessed. CCI was originally developed without adjustment for age (Charlson *et al*., 1987) and therefore as age was already included as an individual covariate, we calculated our CCI unadjusted for age, consistent with previous studies (Boone *et al*., 2014; Caplin *et al*., 2011; Daskivich *et al*., 2014), in order to avoid introducing an undesirable level of collinearity into our analysis. Although an outcome measure itself, severity of disease was also assessed as a covariate for all other CDI outcomes. Statistically significant covariates were added to the final regression model to produce adjusted p-values and ORs. A p-value of <0.05 was considered significant.

Retrospective power calculations were simulated using nQuery Advisor + nTerim 2.0 (Statistical Solutions Ltd., Cork, Ireland). The literature lacks reliable data for conducting a priori power calculation.

Receiver operating characteristic (ROC) curve analysis was conducted to identify optimal cut-off values for our CDI cohort and to compare these against the recommended kit values established for active intestinal inflammation. The Pearson correlation coefficient was employed to assess the relationship between the faecal markers.

5.3 Results

5.3.1 Demographics

Demographics of the patient cohort are summarised in Table 5.2. No significant differences were observed between CDI cases and AAD controls for mean age (70.2 versus 66.4 yrs; $p=0.13$), gender (58% female versus 67% female, respectively; $p=0.26$) or median CCI score (1.0 versus 1.0; $p=0.22$). However, significant differences were identified for mean BMI (24.6 versus 28.2; $p<0.01$) and the median time delay between testing positive and subsequent recruitment (3.0 days versus 2.0 days; $p<0.01$). *C. diff* isolates were successfully

recovered from 149 (91%) of the CDI cases, of which all were toxigenic and 72 (48%) had the ribotype 027.

The proportion of patients suffering from symptoms of 10 or more days was higher amongst CDI cases compared with controls (34.8 versus 18.2%, respectively; p=0.04). This difference was also significant when considering durations of symptoms as measured from initial onset of symptoms (57.2% versus 26.1%; p<0.01). Of the CDI cases, 37.2% (61/164) were assessed as having severe disease, while 36% (49/137) of cases experienced recurrent episodes during the 90-day follow-up period.

5.3.2 Comparative analysis

ROC case-control analysis of FL resulted in a cut-off value of 8.1 ng/µl with an AUC of 0.86 (95% CI 0.80-0.92), producing a sensitivity of 81.7% (75.8-87.6%), specificity of 76.9% (65.4-88.4%), PPV of 91.8% (87.3-96.3%) and NPV of 57.1% (45.5-68.7%) (Figure 5.1). This result is similar to the recommended kit cut-off point (7.25 ng/µl). For FC our optimal cut-off value differed from that proposed by the manufacturer (148 versus 50 mg/kg, respectively), suggesting that FC levels are elevated in the AAD group. ROC analysis resulted in an AUC of 0.86 (0.81-0.92), producing sensitivity of 81.8% (75.8-87.8%), specificity of 76.5% (64.9-88.1%), whilst PPV and NPV were 91.5% (86.9-96.1%) and 57.4% (45.6-69.2%), respectively (Figure 5.1). There was a high degree of correlation between FC and FL (r^2=0.74), consistent across all patient groups (Figure 5.2).

Table 5.2 –Demographics of discovery cohort

	CDI Cases (n=164)	AAD Controls (n=52)	P-value*
Patient's characteristics			
Gender – Female n (%)	95 (58)	35 (67)	0.26
Age – Mean in years (SD)	70.2 (15.9)	66.4 (15.8)	0.13
BMI – Mean (SD)	24.6 (6.4)	28.2 (6.9)	<0.01
CCI score** – Median (IQR)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.22
Time delay (testing/recruitment) – Median (IQR)	3.0 (2.0-4.8)	2.0 (2.0-3.0)	<0.01
Clinical Parameters			
Duration of symptoms – 10 days and over – n (%)	48/138 (34.8)	8/44 (18.2)	0.04
All cause death within 30 days – n (%)	14/164 (8.5)	1/52 (1.9)	0.13
Disease severity at baseline – n (%)	61/164 (37.2)	-	-
Recurrence within 90 days – n (%)	49/137[b] (35.8)	-	-
Frequency of ribotype 027 – n (%)	72/149[c] (48.3)	-	-

AAD: Antibiotic-associated diarrhoea; BMI: Body mass index; CCI: Charlson Comorbidity Index; CDI: Clostridium difficile infection; IQR: Interquartile range; n: number; SD: Standard deviation;

**Means for normally distributed, continuous variables were compared using Independent samples T-test for continuous, for non-normal distribution median values were compared using Mann Whitney U test. Categorical data was assessed using a Chi-squared test for all counts >5, and Fisher's Exact test for those <5;*

***CCI score is calculated without accounting for age (see section 3.2.3);*

[a] Data regarding duration of symptoms was unavailable for 26 of our cases and 8 of our controls; [b] Data regarding recurrence of disease within 90 days was unavailable for 11 of our cases. A further 16 cases died within the follow-up period prior to experiencing any recurrent symptoms and therefore could not be included in the final analysis; [c] Isolates were successfully recovered from 149/164 cases and thus ribotyping could not be done in 15 of our cases;

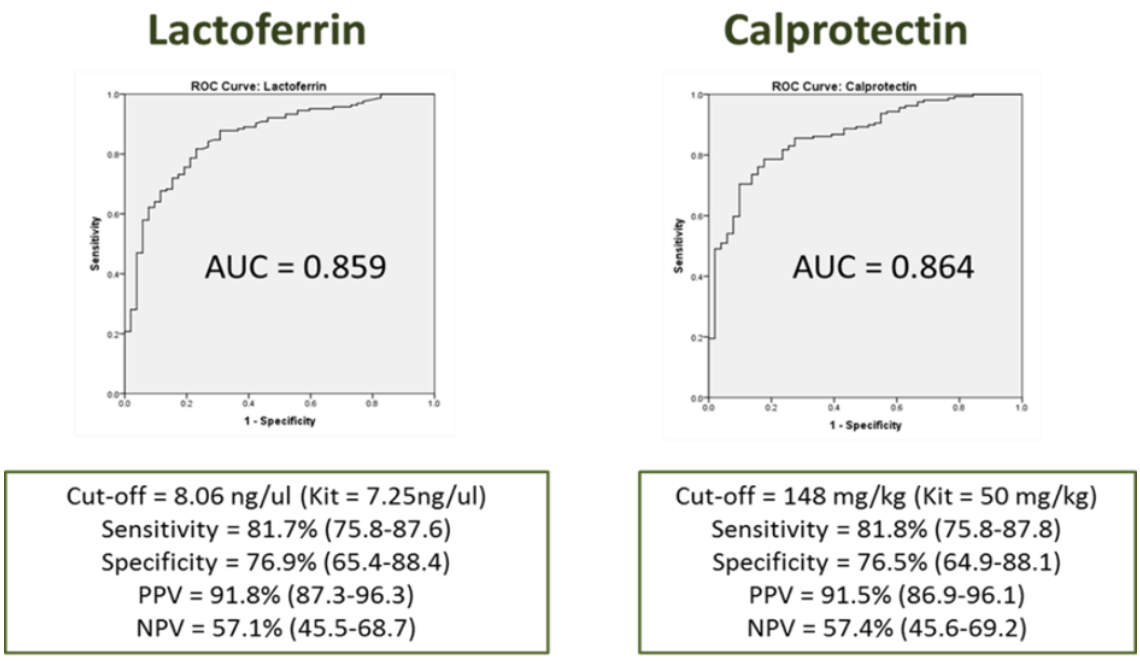


Figure 5.1 – ROC curve analyses of Faecal Lactoferrin and Faecal Calprotectin concentrations in *Clostridium difficile* infection cases (n=164) versus Antibiotic-associated diarrhoea controls (n=52)

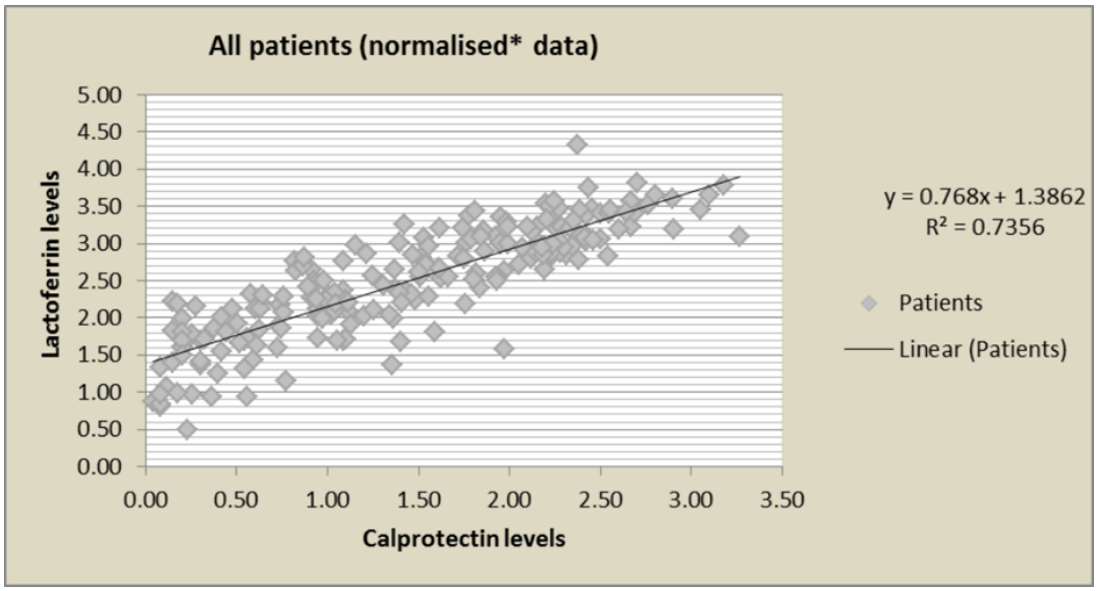


Figure 5.2 – Correlation plot of Faecal Lactoferrin and Faecal Calprotectin concentrations in all patients (cases and controls combined; n=210)

5.3.3 Faecal concentrations in relation to CDI

Median levels of both markers were significantly higher in CDI cases compared to AAD controls (57.9 versus 2.7 ng/µl and 684.8 vs. 66.5 mg/kg, respectively; Table 5.3), which was confirmed by percentile case-control analysis ($p<1 \times 10^{-5}$ for both; Table 5.4).

No significant associations were identified through sub-group percentile analysis for CDI disease outcomes (Tables 5.5-5.8). Although a marked increase was observed for both faecal markers in patients suffering from severe disease compared to their non-severe counterparts (FL: 104.6 versus 40.1 ng/µl; FC 969.3 versus 512.7 mg/kg; Table 5.3), these narrowly missed statistical significance (p=0.06 and p=0.26, respectively; Table 5.8). The lack of association identified with prolonged symptoms remaining when this outcome was alternatively measured from symptom onset.

Carriers of the ribotype 027 generally displayed higher median levels of FC and FL (1011 versus 658 mg/kg and 83.2 versus 51.0 ng/µl, respectively), but this was not statistically significant (p=0.09 and p=0.57, respectively). Median levels of both FC and FL were higher in culture positive compared with culture negative samples, but again this failed to reach statistical significance (712.2 versus 345.8 mg/kg, p=0.46 for FC; 63.5 versus 31.7 ng/µl, p=0.22 for FL). However, median levels of both FC and FL were significantly higher in culture negative patients compared to AAD controls (345.8 versus 66.5 mg/kg, p<0.01 for FC; 31.7 versus 2.7 ng/µl, p<0.001 for FL).

4.3.4 Power calculations

For both biomarkers, power to detect a significant difference was calculated as ≥99% for the majority of analyses (Table 5.9). However, we had inadequate power for analysis of 30-day mortality for both FL and FC.

Table 5.3 - Descriptive levels of faecal lactoferrin & calprotectin in relation to *Clostridium difficile* infection disease outcomes

	Lactoferrin			Calprotectin		
	N	Median, ng/µl (IQR)	Min. – Max.	N	Median, mg/kg (IQR)	Min. – Max.
Case versus Control						
CDI case	164	57.9 (11.4-177.5)	0.5-1,839.0	159	684.8 (203.7-1,581.0)	9.7-21,450.2
AAD control	52	2.7 (0.7-7.8)	0.1–203.5	51	66.5 (23.1-145.7)	3.1-1,810.9
Death within 30 days						
Death	14	62.4 (19.6-223.2)	0.9-1,250.0	14	543.8 (139.7-2,678.9)	38.1-4,418.0
Non-death	150	57.9 (11.0-174.5)	0.5-1,839.0	145	702.6 (203.9-1,549.1)	9.7-21,450.2
Duration ≥10 days						
Yes	48	59.0 (25.6-157.7)	0.5-1,839.0	46	737.2 (289.9-1,608.1)	9.7-21,450.2
No	90	48.3 (7.8-178.2)	0.6-1,510.0	88	581.5 (175.1-1,458.4)	20.9-6,415.4
Recurrence within 90 days						
Recurrence	49	83.6 (9.8-189.4)	0.6-1,839.0	46	744.1 (318.7-1,755.8)	22.9-5,660.6
Non-recurrence	88	55.7 (12.1 – 158.4)	0.5-1,510.0	87	627.9 (173.3-1,423.6)	9.7-21,450.2
Severity at baseline						
Severe	61	95.1 (16.3-187.6)	0.6-799.9	60	889.6 (275.2-1,876.2)	38.8-21,450.2
Non-severe	103	40.1 (10.2-176.9)	0.5-1,839.0	99	535.0 (173.3-1,541.0)	9.7-6,047.0

AAD: Antibiotic-associated diarrhoea; CDI: Clostridium difficile infection; IQR: interquartile range; Min: minimum; Max: maximum; n: number;

Table 5.4 – Faecal lactoferrin and calprotectin levels: CDI cases versus AAD controls

Faecal lactoferrin	CDI Cases (n=164)	AAD Controls (n=52)	Adjusted P-value[a]	Adjusted OR (95% CI)
Low (Comparator group)	20	33	-	-
Medium-Low	41	14	<0.0001	5.03 (2.05-12.34)
Medium-High	51	3	<0.0001	31.67 (8.14-123.26)
High	52	2	<0.0001	41.57 (8.55-202.10)
				Global p-value <1 x 10^{-5}
Faecal calprotectin	**CDI Cases (n=159)**	**AAD Controls (n=51)**	**Adjusted P-value[b]**	**Adjusted OR (95% CI)**
Low (Comparator group)	21	31	-	-
Medium-Low	38	15	0.02	3.03 (1.21-7.53)
Medium-High	49	4	<0.0001	21.82 (6.13-77.71)
High	51	1	<0.0001	85.87 (10.21-721.90)
				Global p-value <1 x 10^{-5}

AAD: Antibiotic-associated diarrhoea; CDI: Clostridium difficile infection; CI: Confidence interval; IQR: Interquartile range; n: number; OR: Odds ratios;

P-value was calculated using binary logistic regression with data grouped into percentiles. Analysis was adjusted for significant covariates: [a] BMI and time delay between testing positive and subsequent recruitment; [b] BMI, score on Charlson Comorbidity Index and time delay between testing positive & subsequent recruitment

Table 5.5 – Faecal lactoferrin and calprotectin levels: 30-day mortality

Faecal lactoferrin	**Death (n=14)**	**Survival (n=150)**	**Adjusted P-value[a]**	**Adjusted OR (95% CI)**
Low (Comparator group)	2	39	-	-
Medium-Low	5	36	0.22	3.00 (0.51-17.56)
Medium-High	3	38	0.28	2.90 (0.41-20.41)
High	4	37	0.25	2.91 (0.47-18.17)
				Global p-value = 0.63
Faecal calprotectin	**Death (n=14)**	**Survival (n=145)**	**Adjusted P-value[b]**	**Adjusted OR (95% CI)**
Low (Comparator group)	4	35	-	-
Medium-Low	4	36	0.89	0.90 (0.20-3.97)
Medium-High	1	39	0.25	0.27 (0.03-2.56)
High	5	35	0.61	1.46 (0.35-6.11)
				Global p-value = 0.51

AAD: Antibiotic-associated diarrhoea; CDI: Clostridium difficile infection; CI: Confidence interval; IQR: Interquartile range; n: number; OR: Odds ratios;

P-value was calculated using binary logistic regression with data grouped into percentiles. Analysis was adjusted for significant covariates: [a] Score on Charlson Comorbidity Index and disease severity at baseline; [b] Disease severity at baseline;

Table 5.6 – Faecal lactoferrin and calprotectin levels: Prolonged symptoms

Faecal lactoferrin	**≥10 days (n=48)**	**<10 days (n=90)**	**Adjusted P-value[a]**	**Adjusted OR (95% CI)**
Low (Comparator group)	7	29	-	-
Medium-Low	17	19	0.02	3.71 (1.29-10.63)
Medium-High	14	19	0.04	3.05 (1.04-8.95)
High	10	23	0.30	1.80 (0.59-5.47)
				Global p-value = 0.07
Faecal calprotectin	**≥10 days (n=46)**	**<10 days (n=88)**	**Adjusted P-value[a]**	**Adjusted OR (95% CI)**
Low (Comparator group)	10	25	-	-
Medium-Low	12	21	0.49	1.43 (0.52-3.96)
Medium-High	12	23	0.61	1.30 (0.47-3.59)
High	12	19	0.39	1.58 (0.56-4.42)
				Global p-value = 0.84

AAD: Antibiotic-associated diarrhoea; CDI: Clostridium difficile infection; CI: Confidence interval; IQR: Interquartile range; n: number; OR: Odds ratios;

P-value was calculated using binary logistic regression with data grouped into percentiles. Analysis was adjusted for significant covariates: [a] No adjustment occurred as no covariates were found to be significant;

Table 5.7 – Faecal lactoferrin and calprotectin levels: 90-day recurrence

Faecal lactoferrin	**Recurrence (n=49)**	**Non-recurrence (n=88)**	**Adjusted P-value[a]**	**Adjusted OR (95% CI)**
Low (Comparator group)	14	20	-	-
Medium-Low	9	25	0.33	0.55 (0.17-1.83)
Medium-High	13	22	0.61	0.75 (0.26-2.21)
High	13	21	0.91	1.07 (0.36-3.16)
				Global p-value = 0.69
Faecal calprotectin	**Recurrence (n=46)**	**Non-recurrence (n=87)**	**Adjusted P-value[b]**	**Adjusted OR (95% CI)**
Low (Comparator group)	7	24	-	-
Medium-Low	14	22	0.29	1.84 (0.60-5.60)
Medium-High	11	24	0.43	1.59 (0.51-4.98)
High	14	17	0.08	2.85 (0.90-9.05)
				Global p-value = 0.35

AAD: Antibiotic-associated diarrhoea; CDI: Clostridium difficile infection; CI: Confidence interval; IQR: Interquartile range; n: number; OR: Odds ratios;

P-value was calculated using binary logistic regression with data grouped into percentiles. Analysis was adjusted for significant covariates: [a]Age and presence of ribotype 027; [b] Age;

Table 5.8 – Faecal lactoferrin and calprotectin levels: Disease severity

Faecal lactoferrin	**Severe (n=61)**	**Non-severe (n=103)**	**Adjusted P-value[a]**	**Adjusted OR (95% CI)**
Low (Comparator group)	12	29	N/A	N/A
Medium-Low	11	30	0.81	0.89 (0.34-2.32)
Medium-High	22	19	0.03	2.80 (1.13-6.96)
High	16	25	0.35	1.55 (0.62-3.88)
				Global p-value = 0.06
Faecal calprotectin	**Severe (n=60)**	**Non-severe (n=99)**	**Adjusted P-value[a]**	**Adjusted OR (95% CI)**
Low (Comparator group)	13	26	N/A	N/A
Medium-Low	11	29	0.57	0.76 (0.29-1.99)
Medium-High	19	21	0.20	1.81 (0.73-4.50)
High	17	23	0.40	1.48 (0.59-3.69)
				Global p-value = 0.26

AAD: Antibiotic-associated diarrhoea; CDI: Clostridium difficile infection; CI: Confidence interval; IQR: Interquartile range; n: number; OR: Odds ratios;

P-value was calculated using binary logistic regression with data grouped into percentiles. Analysis was adjusted for significant covariates: [a] No adjustment occurred as no covariates were found to be significant;

Table 5.9 – Assessment of power across *Clostridium difficile* infection outcome analyses

Disease outcome	N	Power (%)	
		Faecal lactoferrin	Faecal calprotectin
Case vs. control	164 vs. 52	99	99
30-day mortality	14 vs. 150	6[a]	19[b]
Prolonged symptoms (test)	48 vs. 90	86	99
90-day recurrence	49 vs. 88	99	91
Disease severity (egfr)	61 vs. 103	99	99

a: To achieve 80% power we would require 1370 patients in both sample groups

b: To achieve 80% power we would require 167 patients in both sample groups

5.4 Discussion

FC and FL are derived from neutrophils in faecal material, and have been shown to correlate with the degree of inflammation in diseases such as IBD. Since CDI is also characterised histologically by intense neutrophilic infiltration (Price and Davies, 1977), FC and FL may represent potential biomarkers of disease activity. Using a prospective cohort of inpatient CDI cases and AAD controls, we confirmed previous findings that both FC and FL increase during CDI ($p<0.0001$) (Archbald-Pannone *et al*., 2010; Shastri *et al*., 2008; Vaishnavi *et al*., 2000; van Langenberg *et al*., 2010; Whitehead *et al*., 2014). There was a high degree of correlation between the two biomarkers, not surprising given their cellular origin. No previous CDI studies have evaluated both faecal biomarkers in the same patient group. These findings are consistent with those seen in IBD (D'Inca *et al*., 2007; Jones *et al*., 2008; Langhorst *et al*., 2008; Schoepfer *et al*., 2008; Sipponen *et al*., 2008a; Sipponen *et al*., 2008b; Sipponen *et al*., 2008c).

There are more studies on FL than FC for CDI (Table 5.1) but only a few have provided quantitative data. For FL, the reported mean/median values for CDI cases have differed markedly across studies (33-961 µg/ml; Table 5.1) (Archbald-Pannone *et al*., 2010; Boone *et al*., 2013; LaSala *et al*., 2013; van Langenberg *et al*., 2010). Our median value is at the lower end of this range (57.9 ng/µl). By contrast, our observed median level for FC was markedly higher than that in the two previous CDI studies (648.8 mg/kg versus 192 and 249-336 mg/kg) (Shastri *et al*., 2008; Whitehead *et al*., 2014). The median levels in our AAD controls were lower for both FL and FC (2.7 ng/µl versus 22.6-22.8 µg/ml and 66.5 mg/kg versus 106 µg/g, respectively; Table 5.1) than reported previously in the two FL studies and one FC study that included diarrhoea controls in their analysis (Archbald-Pannone *et al*., 2010; van Langenberg *et al*., 2010; Whitehead *et al*., 2014). Another study showed that 171 of 196 healthy controls (87%) had an FC level less than 15 mg/l (Shastri *et al*., 2008), a similar observation to that seen in our AAD controls (41/51; 80%). Considerable

variability was observed in different patients with CDI, which is consistent with data from IBD studies for both FL (4.34-179 μg/ml) (Langhorst *et al*., 2008; Langhorst *et al*., 2005; Schoepfer *et al*., 2008; Sipponen *et al*., 2008a; Sipponen *et al*., 2008b; Sipponen *et al*., 2008c) and FC (164-2171 mg/kg) (Costa *et al*., 2003; García-Sánchez *et al*., 2010; Langhorst *et al*., 2005; Schoepfer *et al*., 2009; Schoepfer *et al*., 2010; Sipponen *et al*., 2008a; Sipponen *et al*., 2008b; Sipponen *et al*., 2008c; Summerton *et al*., 2002; Tibble *et al*., 2000).

While our data show that FC and FL can differentiate between CDI and AAD, the use of these biomarkers for diagnosis per se would not add much value to the diagnostic paradigms currently in place. However, identification of patients with complicated CDI disease (for example disease leading to more prolonged symptoms and recurrent disease) would be useful. Despite marked increases in both faecal biomarkers in relation to disease severity, these failed to reach statistical significance and we observed no association with the other outcome measures evaluated. It is important to note that we had adequate statistical power to detect all of these outcomes except for 30-day mortality (Table 5.9).

Direct comparisons between this and other studies are limited by variability in methodologies adopted, the lack of quantitative data, and differences in the severity grading criteria (Boone *et al*., 2013; El Feghaly *et al*., 2013; Steiner *et al*., 1997). Another problem may result from the potential short-lived characteristics of the biomarkers, which may hamper the predictive power of these markers unless they are captured within specific timeframes. A longitudinal study of FL (Boone *et al*., 2012) suggested that FL could be used to monitor disease activity and response since FL tends to return to baseline very rapidly following remission (Boone *et al*., 2013; Boone *et al*., 2012).

Our study has limitations. Firstly, we only used a single laboratory test (ELISA for CDT) for the primary identification of CDI cases. Although this is still a common procedure, modern algorithms currently make use of a more sensitive first step screening - based on either GDH, or NAAT - to minimise the odds of

reporting false negative results. Therefore it is possible that our cohort may have lacked a fully representative range of cases. Furthermore, our AAD controls were not a homogenous group of patients and it is difficult to assess their fitness for this sort of analysis given that antimicrobials and/or PPIs may not be the sole underlying cause of their gastrointestinal tract dysbiosis.

Nevertheless, our data highlight the difficulties in using FL and FC as biomarkers for CDI. The variability observed would reduce predictive accuracy, part of which may be due to differences in laboratory methodology. The volume of diluent for specimen suspension, and laboratory handling can each influence results, and caution should be exercised in the interpretation of single results (Kopylov *et al*., 2014). Although serial testing may have some value, it would add to the cost, and may be challenging in diseases such as CDI, thus further reducing its utility. Furthermore, these biomarkers can be elevated due to other diseases (Kopylov *et al*., 2014), and this is particularly important for CDI where infected patients are usually elderly with multiple co-morbidities.

There are no guidelines concerning the use of faecal biomarkers for the classification of CDI cases. In IBD research, where faecal biomarkers constitute a potential non-invasive alternative to colonoscopy, the most recent diagnostics guidance by UK National Institute for Health and Care Excellence (NICE) (National Institute for Health and Care Excellence, October 2013) still recommends that further research is needed on the use and clinical utility of faecal marker testing. Biomarkers which can act as indicators of disease, disease relapse and disease stratification, are also needed to direct CDI therapies more effectively. Our results suggest that FC and FL have limited applicability in this role.

Chapter 6

Development of ECL assays to measure host immune response from serum during CDI

Contents

6.1 Biomarkers

In 1998, the Biomarker Definitions Working Group of the National Institutes of Health (NIH) defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Group., 2001; Strimbu and Tavel, 2010). The term biomarker is comprehensive and encompasses substances: -

A) That are introduced into an organism as a means to examine organ function or other aspects of health (e.g. rubidium chloride as a radioactive isotope to evaluate perfusion of heart muscle)
B) Whose detection indicates a particular disease state (e.g. the presence of a specific antibody may indicate an infection)

Biomarkers currently play a major role in medicinal biology but the term is thought to have first been coined as early as 1980 during the investigation of the role of serum UDP-galactosyl transferase in breast carcinoma (Paone *et al*., 1980). Biomarkers have been used across several scientific fields and are pivotal in clinical diagnosis and pre-clinical research as reliable indicators of biological state/status changes that indeed correlate with disease risk/susceptibility, progression or response to a treatment. The importance of biomarkers is emphasised by the significant budgets allocated by the NIH to support biomarker research: in 2008-2009, over $2.5 billion of the USA budget was awarded through research grants.

Clinical biomarkers aid healthcare professionals in several areas including drug target identification, drug response, early diagnosis, disease prevention and stratification of treatment. Drug-related biomarkers are traditionally inherent to early drug development studies and clinical trials, and employed as a means to investigate drug processing, metabolism, and effectiveness with the aim to

establish optimal doses and dosing regimen for future studies. This is often supplemented by the study of safety biomarkers, such as the ones used to monitor liver and cardiac function. Conversely disease-related biomarkers are used to assess a pre-existing disease-condition, the probable treatment response of the patient, and the individual disease progression with or without treatment (Sapsford *et al*., 2010). Biomarkers are commonly divided into three main classes, all of them being relevant to infectious disease research:

a) Risk indicators/predictive biomarkers
b) Diagnostic biomarkers
c) Prognostic biomarkers

The biomarker development process is briefly summarised in Figure 6.1.

Fig 6.1 Overview of biomarker development process

The use of laboratory-measured biomarkers in both pre-clinical and clinical research is still relatively modern, and therefore development and refinement of best practice is a continuous process (Strimbu and Tavel, 2010). This is important when considering the increased appeal offered by the use of molecular biomarkers as substitutes for rather subjective clinical parameters, which is still a widespread practice and largely employed for the management of various diseases (Biomarkers Definitions Working Group, 2001). Several molecular biomarker tests have become established in clinical care and are routinely used for the ascertainment of a patient's general health and

homeostasis. Attempts to develop clinical risk indices involving multiple mainstream markers are being pursued for the identification of patients at most risk of experiencing acute and fatal conditions, with exemplary approaches utilised for the assessment of cardiovascular diseases (Cross *et al*., 2012; Folsom, 2013; Macdonald *et al*., 2013), lymphoma (Katsuya *et al*., 2012; Perry *et al*., 2012), human immunodeficiency virus (HIV) infection (Tate *et al*., 2013), chronic obstructive pulmonary disease (COPD) (Motegi *et al*., 2013) and inflammation-related diseases such as Crohn's and CDI (Benitez *et al*., 2013; Bloomfield *et al*., 2013). However, validation of disease-specific markers is challenging and requires a deep knowledge of the pathogenesis and biological processes.

6.1.1 Biomarkers in CDI

Biomarker evidence for CDI has been limited, in part due to the lack of comprehensive studies and mechanistic understanding of the disease. Predictive biomarkers have not been validated for CDI yet and there is an imperative need for new biomarkers to stratify CDI patients. There is the question as to whether CDI biomarkers simply have yet to be discovered, or whether the current methodologies in place to measure existing parameters lack the necessary credentials to pursue validation, such as sensitivity, specificity, or reliability issues.

As outlined in Chapter 1 Section 1.3, both biochemical and molecular studies have shown that the major clinical signs and symptoms of CDI can be explained largely by the actions of two large, glucosylating toxins, namely tcdA and tcdB (Babcock *et al*., 2006a; Kim *et al*., 1987; Lyerly *et al*., 1985; Lyras *et al*., 2009b). Clinical manifestations range from asymptomatic carriage and mild diarrhoea to fulminant pseudomembranous colitis (Kelly *et al*., 1994; Kyne *et al*., 1999; McFarland *et al*., 1989; Rubin *et al*., 1995). It appears that host, rather than bacterial, factors determine these differences in clinical presentation (Cheng *et al*., 1997; McFarland *et al*., 1991).

Antibody responses to tcdA and tcdB are evident in approximately 60% of healthy individuals (Kelly *et al*., 1992; Viscidi *et al*., 1983), suggesting widespread exposure to *C. diff* in the environment. Furthermore, there is evidence that the toxin-specific immune response is important for both CDI pathogenesis and course of disease (Aronsson *et al*., 1985; Bauer *et al*., 2014; Drudy *et al*., 2004; Islam *et al*., 2014; Johnson *et al*., 1992; Katchar *et al*., 2007; Kyne *et al*., 2000a, 2001; Mulligan *et al*., 1993; Sanchez-Hurtado *et al*., 2008; Solomon *et al*., 2013; Warny *et al*., 1994). Studies having investigated immune response to *C. diff* are summarised in Appendix 21.

The recent emergence of hypervirulent epidemic *C. diff* resistant strains has warranted the development of novel, non-antibiotic based treatment regimes. These strains have been linked with increased morbidity, mortality and recurrence rates worldwide (McDonald *et al*., 2005b; Warny *et al*., 2005), largely because of their increased virulence and hyperproduction of tcdA and tcdB (Kuehne *et al*., 2010; Thelestam and Chaves-Olarte, 2000). However, only a fraction of individuals exposed develop infection and therefore it is important to understand inter-patient variability and the immune response patterns associated with CDI. It has been shown that antibody-mediated neutralisation of these toxins affords protection against CDI (Giannasca and Warny, 2004; Giannasca *et al*., 1999). This includes both anti-tcdA and anti-tcdB passive immunisation studies (Babcock *et al*., 2006a; Kink and Williams, 1998; Lowy *et al*., 2010; Lyerly *et al*., 1991; Roberts *et al*., 2012; van Dissel *et al*., 2005) and vaccines designed to evoke an effective neutralising immune anti-toxin response (Greenberg *et al*., 2012). As detailed in Chapter 1 Section 1.8, the current focus for vaccine development has been placed on the antibody response to *C. diff* toxins due to its closer relationship to CDI. Part of these efforts has been hampered by the lack of reliable methods to quantitate this immune response and to date no commercial assays have been validated. Previous research has been limited to in-house methods based on traditional ELISA and qualitative measurement of response against toxins A and B only.

Phylogenetic analysis has shown that the unprecedented wave of outbreaks witnessed in the last decade was a result of global spread of emerging strains originating from North America, most likely driven by increased resistance to several classes of antibiotics such as fluoroquinolones (He *et al*., 2013). Since then, the research landscape has focused on gaining a better understanding of the spread, evolution and establishment of the disease. For example, the demonstration that tcdB but not tcdA is necessary for full virulence of *C. diff* in experimental infection models (Lyras *et al*., 2009b) has called into question the importance of tcdA in disease pathogenesis since a number of pathogenic strains do not possess the encoding gene. Strains such as PCR-ribotype 027/NAP1/BI1, have also been found to express a third unrelated virulence factor in the form of a binary toxin complex (Barbut *et al*., 2005; Cartman *et al*., 2010; McDonald *et al*., 2005b), which is characterised by an enzymatically active "A" domain (cdtA) and a cell binding and translocation "B" component (cdtB) (Xie *et al*., 2014). Although the exact role of the binary toxin in the pathogenesis and development of CDI is still debatable, recent data on its mechanism of action and significant correlations of binary-producing strains with CDI progression suggest that further research is needed in this area.

Given all the uncertainties, there is a need to re-evaluate the role of the immune response to *C. diff* toxins. This study aimed to develop improved assays for quantification of immune response to tcdA and tcdB, as well as novel assays for quantification of CDT, something that has never been described in the literature.

6.2 Measurement of biomarkers

Various approaches currently exist for the measurement of proteomic, metabonomic, secretomic, genomic and transcriptomic biomarker targets. However, examination of any molecular biomarker is highly dependent upon its physico-chemical and biological properties as well as on the complexity of the biological matrix that is being examined.

Moreover, the protein content of the human serum/plasma proteome is made up of proteins across wide quantitative dynamic ranges; at the high-end, albumin (normal concentration range 35-50 mg/ml) and at the low-end, interleukin-6 (normal range 0-5 pg/ml) (Anderson and Anderson, 2002). Hence, discovery and validation per se of specific protein biomarkers is already confined to low abundance targets amongst a multitude of high abundance molecules such as albumin and as such, screening methodologies are required by default to deliver extremely high levels of sensitivity and specificity in order to be able to accurately measure them (Anderson and Anderson, 2002). Since one of the predominant features of CDI research involves a systemic, often acute, host-mediated response, the present work sought to develop a robust framework for the evaluation of disease-related protein biomarkers readily available from sera of CDI patients. Whilst microarray technology offers great potential for biomarker discovery, the primary focus of this work was to improve current methods for quantification of immune response markers and therefore focus was given to portable methods showing high translational potential, described below.

6.2.1 ELISA

ELISA is a popular protein analysis method to quantify levels of response markers, such as inflammatory cytokines and antibodies released as result of a disease or condition. The ELISA was first developed in the 1970s when PhD graduate student Eva Engvall, using the premise that specific enzymes in the presence of an appropriate substrate will react to induce a detectable colour change (Avrameas, 1969; Engvall *et al*., 1971; Nakane and Pierce, 1966, 1967). Based on this principle, the traditional ELISA is an absorbancy-based test that uses antibodies in which colour change is an indication of a positive reaction with a target protein. The ELISA became the preferred choice of immunoassays after the use of radio-immunoassays (RIAs), the then established method, was diminished due to the potential hazard posed by the handling of radioactive

products, which were employed for signaling the reaction of labeled antigens/antibodies with designated targets (Yalow and Berson, 1960) .

Various variants of ELISA are now in widespread use, such as the indirect ELISA, sandwich ELISA, competitive ELISA, and multiple and portable ELISA, with the most typical being the sandwich 'double-antibody' immunoassay. In such a scenario, primary antibody is coated onto well-bottoms of designed plates, typically in a 96-well format. A series of steps involving washing of wells and addition of sample results in the target analyte being bound to the primary capture antibody. A secondary enzyme-linked (typically horseradish peroxidase, HRP) antibody then binds to a separate region of the antigen. The appropriate substrate is then added, and the resultant colour change is detected using spectrophotometry.

ELISA is renowned for its simplicity and reproducibility. It has been employed in a broad range of applications within and outside human medicine, becoming a standard diagnostic tool for autoimmune diseases and other conditions such as HIV (Iweala, 2004; Le Pottier *et al*., 2009). In CDI, ELISAs were originally employed as a method of choice for the initial examination of antibody immune response to tcdA and tcdB (Aronsson *et al*., 1983; Jiang *et al*., 2007; Katchar *et al*., 2007; Kyne *et al*., 2000a, 2001; Leung *et al*., 1991; Sanchez-Hurtado *et al*., 2008). Such an extensive range of applications resulted in commercialisation of ELISA kits and related instruments, such as automated pipetting, multichannel, washing station and reader devices (Lequin, 2005). Furthermore, the increase in the commercial production of plate-based assays has led to the emergence of multiplex formats (Kingsmore, 2006; Lash *et al*., 2006; Mendoza *et al*., 1999). One of them, Pierce SearchLight (ThermoFisher, USA), relies on accurate pre-spotting of multiple capture antibodies and is essentially an enhanced ELISA array.

Despite their widespread uptake, ELISAs are not without their flaws. Seen as labour- and time-intensive, their performance is largely dependent on antibody

quality, the incubation periods and the selection of appropriate blocking and dilution buffers (Malekzadeh *et al*., 2012).

Furthermore, they are prone to both cross-reactivity and matrix interference (Malekzadeh *et al*., 2012), and have limited sensitivity and linear detection range for several applications. This is critical particularly for protein targets present at very low concentrations and for reliable analysis of different body fluids (Lequin, 2005). Ultrasensitive ELISA kits have been brought to the market in attempt to counteract this but are considerably more expensive and simply result in shifting and further narrowing the already restricted dynamic range (Malekzadeh *et al*., 2012).

Over the last decade there has been an increase in the popularity of enhanced platforms based on fluorescent and electrochemiluminescent (ECL) technologies. These newer ELISA-like multiplex-capable platforms propose replacement of the chromogenic technology, which the traditional ELISA relies upon. Their technologies allow the creation of quantifiable signals that benefit from higher sensitivities and a wider dynamic range, which alongside quicker turn-around times and reduction in sample and reagent consumptions make a compelling case for their adoption (Leng *et al*., 2008; Toedter *et al*., 2008). As they are linked to non-enzymatic reporters, they are not strictly ELISAs per se but are grouped with these due to similarities in general principles and laboratory practice.

6.2.2 Fluorescence versus Electrochemiluminescence (ECL)

Fluorescent immunoassays are typically bead-based, with notable platforms including Luminex (Luminex Corporation, Texas) and GyroLab (Gyros AB, Sweden). The fluidic-based Luminex platform is the most widely used incorporating fluorescent technology. Following the principles of a traditional sandwich immunoassay, a primary antibody is linked to a polystyrene bead, each of which is internally dyed with a red fluorophore. Antigen binding then

occurs by one of two methods; by a biotin-labelled secondary antibody or by directly labeled phycoerythrin (PE). The amount of protein bound is identified by the PE-emitted fluorescence. Luminex xMAP technology allows for recognition of up to 100 different beads identified by the fluorescence ratio of the internal red dye, and therefore up to 100 different target analytes can be screened concomitantly. The ability to perform multiplex analysis of several analytes in a single sample is xMAP's major selling point (Marchese *et al*., 2009). One major disadvantage of this platform is the potential structural damage sustained as a result of the conjugation chemistry required to covalently bind antibodies to the beads (Anderson *et al*., 2011; Marchese *et al*., 2009). GyroLab employs microfluidic technology involving a compact disc (CD) containing microchannels and a reaction chamber coated with streptavidin but is grouped with other bead-based platforms due to a lack of notable differences/technological advantages.

ECL-based techniques also provide a viable alternative to the colorimetric methods of the traditional ELISAs (Blackburn *et al*., 1991; Deaver, 1995; Guglielmo-Viret and Thullier, 2007), with a reported eight-fold increase in sensitivity (Guglielmo-Viret and Thullier, 2007). ECL-based platforms, such as that provided by Roche Diagnostics (Roche Diagnostics Ltd, UK) and Meso Scale Discovery (MSD, USA), provide high sensitivity levels and good reproducibility, combined with generally low levels of interference from epitopes in serum, plasma and other complex matrices (Blackburn *et al*., 1991; Guglielmo-Viret and Thullier, 2007; Swanson *et al*., 1999). ECL also reduces the potential need for retesting of high concentration samples due to a broader dynamic range (Marchese *et al*., 2009) and has further benefits such as speed of analysis and the absence of integrated fluids, which eliminate the clogging issues common to the bead-based systems discussed above (Marchese *et al*., 2009). ECL was initially adopted by Roche, whose assay involves separate cartridges and reading cells. MSD provides an advance by developing current generation

integrated to the analytical plates, which in turn maximises assay performance and throughput.

Owing the technical limitations of the traditional ELISA, it is apparent that there is marked room for improvement. The advantages of the emerging ECL platforms combined with the need for robust methods to quantitate host-mediated antibody responses to both tcdA and tcdB provide sufficient basis for both the improvement of existing methods and the development of novel strategies, including the development of novel assays for the detection of response to cdtA and cdtB.

Unlike ECL, bead-based methods do not offer enough flexibility for the customisation of these assays and the option for the ECL system (MSD) was made after conducting a thorough review of the available literature and following careful consideration of existing platforms in the market.

Furthermore, steady transition of existing ELISAs onto the ECL format is possible given the existence of transferrable information and in such a circumstance, provided a valuable basis for the development and typing of one of the candidate markers of this study (i.e. the immunity-linked protein MBL - see Chapter 7).

6.2.3 Meso Scale Discovery (MSD)

Meso Scale Discovery (MSD) technology revolves around microtitre plates with integrated carbon electrodes at the bottom of each well, to which biological reagents can be passively adsorbed whilst retaining a high level of biological activity. Detection antibodies are conjugated with electrochemiluminescent labels (MSD SULFO-TAG), which emit light when electrochemically stimulated. The detection process is initiated at the carbon electrodes whereby the necessary electrical stimulus causes the ruthenium label ($Ru(bpy)_3^{2+}$) in the detection tags to emit light at 620 nm. This redox reaction (illustrated in Figure

6.2) is enhanced by co-reactants, such as tripropylamine (TPA), present in the MSD read buffer, and multiple excitation cycles of each label amplify the signal to enhance light levels and improve sensitivity.

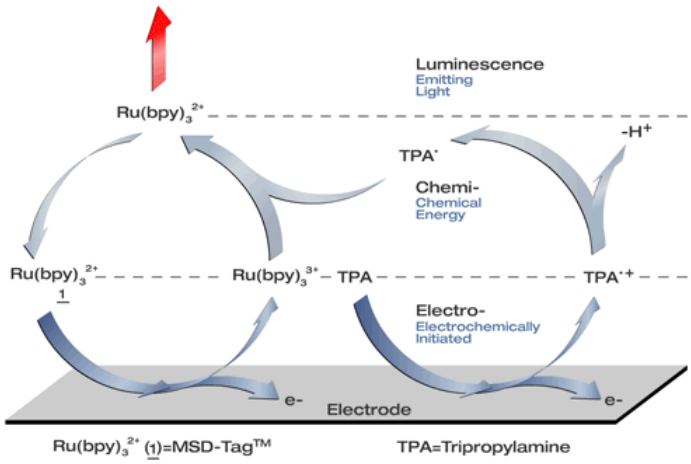


Figure 6.2 – Overview of redox reaction required for electrochemiluminescence

The necessary voltage is applied by the MSD imager that is also responsible for measuring the intensity of the emitted light, providing a quantitative measure of analyte in the sample. Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light). In addition to the separation-based ("wash") method employed by conventional ELISAs, MSD ECL assays have the potential to be used in a separation-free format ("no-wash"), where ready buffer is applied directly to the sample and well. The no-wash method may offer the advantage of removing a potential source of variation and reducing operating times at the cost of decreasing sensitivity (Thompson *et al.*, 2009). This is possible with MSD-ECL given the proximity-based nature of its signal generation where only labels near the electrode are excited and detected (Thompson *et al.*, 2009). Other technologies (fluorescent polarisation (FP)) and

platforms (AlphaLISA, PerkinElmer, USA; Quansys, Quansys Biosciences, USA; Pierce SearchLight, ThermoFisher, USA) based upon enhanced chemiluminescence, including some with bead-based formats, are comparable to MSD-ECL in terms of assay parameters but lack the potential for both multiplexing and development process of custom assays.

A recent study comparing four different ligand-binding assay technology platforms (ELISA, MSD, GyroLab and AlphaLISA) for measurement of a human IgG_1 MAb drug analyte in rat serum evaluated MSD and GyroLab as future default platforms for total MAb biotherapeutic assay development, based mainly on superior assay performance and parameters (Table 6.1 & Table 6.2) (Leary *et al*., 2013).

Table 6.1 – Summary of assay platforms and parameters for the human IgG generic (Fc-specific) assay (adapted from Leary *et al*. 2013)

Various assay parameters for the platform comparison are summarised in Table 5.1. The dynamic range and assay sensitivity for each platform are listed in 100% matrix; total assay time includes both estimated sample preparation time and incubation times

Platform	LBA format	Readout	Dynamic range (ng/ml)	Sensitivity (ng/ml)	Sample MRD	Required sample volume (µl)	Total assay time (h)
ELISA	Plate	Colorimetric	8.0-666	88.0	20	12	5
MSD	Plate	ECL	15.6-4000	15.6	4	25	2
Gyrolab	Bead	Fluorescence	10.5-6400	10.5	2	4	1.5
AlphaLISA	Bead	Luminescence	181-1097	181	N/A	2.5	2.5

LBA: Ligand-binding assay; MRD: Minimum required dilution

Table 6.2 – Additional platform selection considerations (adapted from Leary *et al*. 2013)

In addition to assay performance and parameters listed in Table 5.1, other factors considered for ligand-binding assay platform selection are summarised in Table 5.2. These factors include costs, special equipment needs and reagent requirements

Platform	Cost	Requires special buffers	Special equipment required	Reagent modifications required	Single vendor for technology
ELISA	+	No	Spectrophotometric plate reader	Labeled detector reagent (e.g. biotin, enzyme etc)	No
MSD	++	Read buffer (proprietary)	MSD plate reader	Biotin capture & Ru detector reagent	Yes
Gyrolab	++++	Sample & detection buffers (proprietary)	Gyrolab Instrument	Biotin capture and Alexa detector reagent	Yes
AlphaLISA	+++	No	Envision plate reader	Bead conjugation, biotinylation of capture	Yes

+: least expensive; ++++: most expensive; Ru: Ruthenium

The platform of choice for this study is justified on the grounds of three significant requisites, whereby MSD-ECL was superior: costs, degree of customisation and transferability. Costs were inclusive of the reading instrument itself as well as per-run cost for consumables, both of which were lower for the MSD-ECL platform (Leary *et al*., 2013). Crucially, the MSD-ECL platform offers flexibility for the customisation of both existing and novel biomarker immunoassays. Analogies in the fundamental basis of workflows with ELISA affords ECL a rapid and convenient transition from chemistries utilised in existing ELISAs to the MSD platform, thereby benefitting from several of its technical advantages over ELISA. In terms of transferability, this is a common issue due to each technology being only available from a single vendor. Hence, discontinuation of a platform would require transfer of assays to an alternate methodology, where compatible. Due to the plate-based nature of MSD assays, a hypothetical MSD-to-ELISA transfer would be relatively simple, as opposed to the moderate optimisation required for other formats such as the microfluidic Gyrolab platform (Leary *et al*., 2013).

The combination of ultra-low detection limits, wide dynamic ranges, minimal sample usage and decreased matrix effects have led the MSD plate-based format to become a widely adopted platform by research departments of the pharmaceutical sector (Myler *et al*., 2011). Given the reasons above, the MSD-ECL constitutes in the platform-of-choice for the assay development of candidate protein biomarkers of this thesis.

6.3 Assay development

6.3.1 Overview

Different sandwich immunoassays formats can be developed using MSD plates; typical immunoassays involve those using antibodies as capture tags (Figure 6.3A). Non-antibody molecules (such as proteins/antigens, carbohydrates,

virus-like particles, membranes and cells) can also be used as capture reagents (Figure 6.3B). For this work toxin proteins (tcdA, tcdB and cdtA/B) have especially been utilised as epitopes for the detection of specific antibodies (circled).

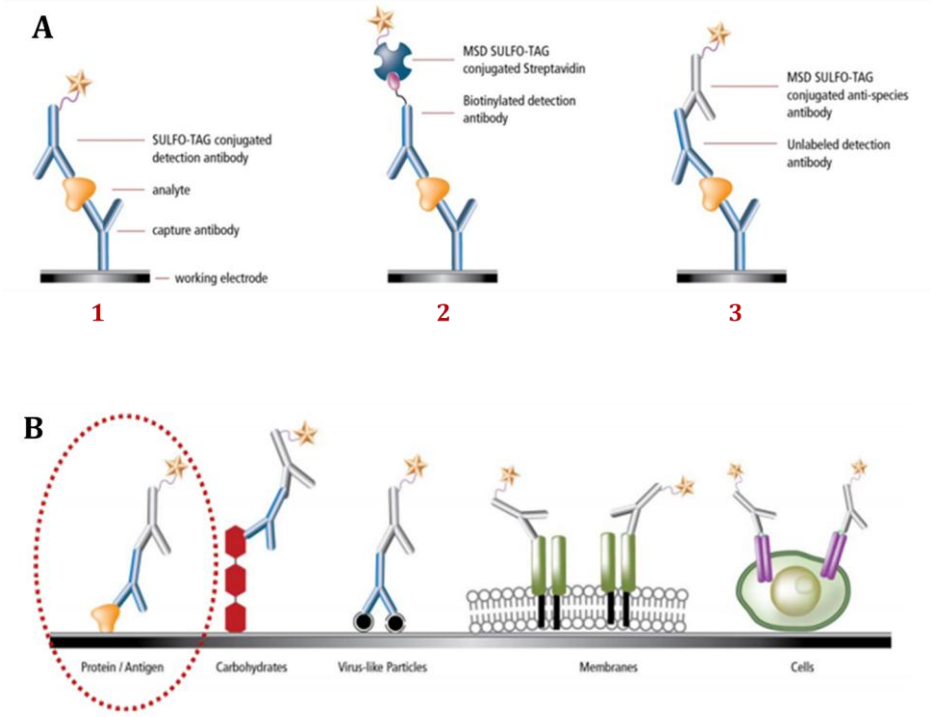


Figure 6.3 – Overview of the different sandwich immunoassay formats that may be developed using MSD plates (Meso Scale Discovery, 2013): (A) Typical immunoassays using capture antibodies; (B) Immunoassays using non-antibody capture reagents

Typical immunoassays*: The graphic provides examples of different assay formats that are possible using antibodies as capture reagents on MSD plates. (1) MSD SULFO-TAG is directly conjugated to the detection antibody. (2) Biotinylated detection antibody binds to SULFO-TAG Streptavidin. (3) Detection antibody binds to SULFO-TAG-conjugated anti-species antibody.* ***Immunoassays using non-antibody capture reagents****: Capture materials, such as peptides antigens, carbohydrates, lysates, cells, membranes, and virus-like particles, can be directly immobilised on MSD plates.*

ECL assay development needs to follow several optimisation procedures and ultimately comply with a number of performance requisites. MSD provides plates with two different surface types: High Bind (HB) plates have a hydrophilic surface and can facilitate the quantification of analytes at higher concentrations, Standard Bind (SB) plates have a hydrophobic surface and tend to offer higher sensitivity whilst frequently exhibiting lower non-specific binding, especially with complex sample matrices. Both plate types are the primary initial choices recommended by the manufacturer for the assay development process. Other variables associated with the optimisation are coating concentration of epitopes, type of coating buffer and blocking buffer, dilution factor of samples and concentration of detection antibodies, amongst others. The objectives of successful assay development are: -

- High signal-to-background ratio (SBR)
- Sensitivity
- Specificity
- Reproducibility
- Wide linear dynamic range

Signals were determined using a proprietary SECTOR Imager and should be directly proportional to the amount of analyte present in the samples. This section provides a detailed description of all optimisation steps leading to assay completion, summarised in Figure 6.4.

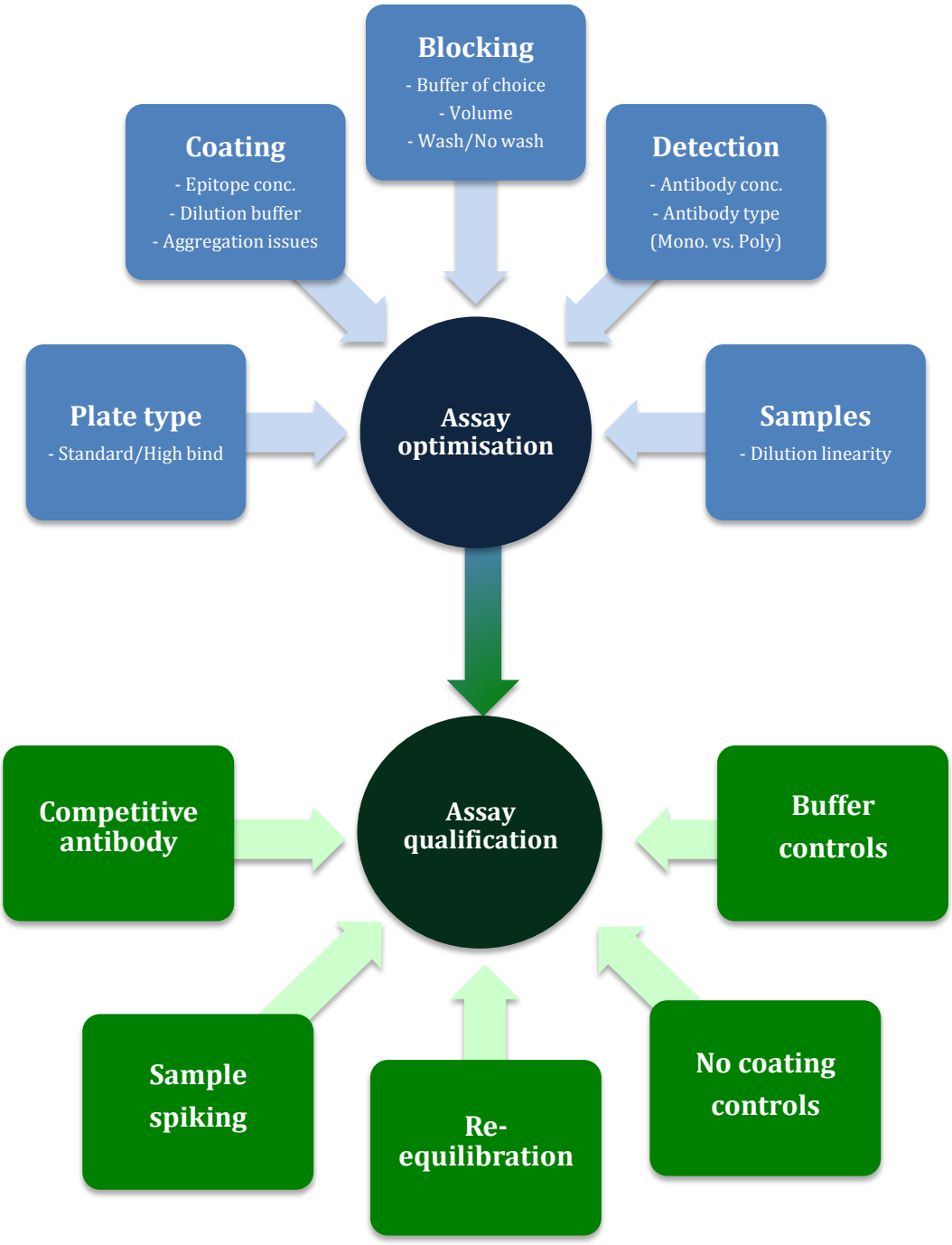


Figure 6.4 – Overview of steps involved in assay development pathway: assay optimisation and qualification

6.3.2 Biological Materials

6.3.2.1 Capture

Purified aliquots of native tcdA and tcdB, derived from reference strain vpi10463, were obtained through collaboration with Dr. Clifford Shone from Public Health England in Porton (formerly Health Protection Agency).

cdtA and cdtB (both precursor (cdtB-pre) and active (cdtB-act) forms), from strain 196, were produced recombinantly through the expression in *Bacillus megaterium* and were obtained from the research group of Professor Klaus Aktories (University of Freiburg, Germany).

6.3.2.2 Detection

Monoclonal IgG and IgM antibodies were ordered from Hybridoma Reagent Laboratory (HRL) (Stratech Ltd, UK).

Polyclonal IgG and IgM antibodies were ordered from Jackson ImmunoResearch Laboratories Inc. (Pennsylvania, USA). These were sulfo-tagged on site.

6.3.2.3 Samples

Patient recruitment and sampling is described in detail in Chapter 2. Human sera were used across all assays, isolated from whole blood via centrifugation (2600g for 20 min), aliquoted and stored at -80°C prior to use.

6.4 Toxin A & B assays

The first four assays developed involved measurement of both IgG and IgM response to major *C. diff* toxins tcdA and tcdB, based upon the sandwich assay ELISA format using directly labeled antibodies.

6.4.1 Determination of initial working conditions

6.4.1.1 Selection of the type of ECL bind plate and initial run

Using manufacturer's guidelines, the following fixed conditions were included for the selection of either plate types (SB versus HB): 1x PBS as coating buffer, 1x PBS + 0.05% Tween20 (PBST) as washing buffer, PBST + 5% Foetal Bovine Serum (FBS) as blocking buffer and detection antibody concentration at 1 µg/ml (diluted in blocking buffer). Varying conditions were assessed and optimised as follow: toxin coating concentration (0-5 µg/ml) and sample dilution factor (neat to 1:625).

A generalised MSD assay protocol is as follows: -

1. *Dilute capture in coating buffer. Add 25 µl to each well. Tap plate to ensure even coverage. Seal and incubate overnight at 4°C*
2. *Wash 3 x 150 µl with washing buffer*
3. *Add 150 µl blocking buffer and seal. Incubate for 1 h at room temperature with shaking*
4. *Wash 3 x 150 µl with washing buffer*
5. *Add 25 µl sample, diluted to appropriate factor in blocking buffer, and seal. Incubate for 2 h at room temperature with shaking*
6. *Wash 3 x 150 µl with washing buffer*
7. *Add 25 µl of detection (anti-isotype antibody plus streptavidin Sulfo-Tag diluted to appropriate concentration in blocking buffer) and seal. Incubate for 1 h at room temperature with shaking*
8. *Wash 3 x 150 µl with washing buffer*
9. *Add 150 µl of MSD Read Buffer (diluted to 2x working mixture with distilled water)*
10. *Read plate within 15 min*

The initial optimisation runs suggested a flat response with the use of HB plates with no meaningful differences in relation to baseline signal (zero coating and buffer only conditions), irrespective of the analyte (toxins), antibody isotypes or test samples employed (see Table 6.2 for IgG response to tcdA). Conversely, data generated using SB plates demonstrated a continuous trend in signal intensity (highlighted in red) as toxin coating concentrations increased and this was particularly marked for IgG response to both tcdA and tcdB (Table 6.2 for IgG response to tcdA). Hence, only SB plates were taken forward for full optimisation and development. Further tables can be viewed in Appendices 22-24.

Table 6.2 – Comparison of standard- and high-bind plates for IgG response to tcdA

Sample code	Plate type	Coating conc. (µg/ml)	Fold Dilution					
			Buffer	625	125	25	5	Neat
A3X	HB	0.0	136	123	156	211	162	227
		2.5	125	115	137	177	141	214
		5.0	126	128	155	181	155	223
		10.0	129	121	169	165	117	208
	SB	0.0	121	105	117	171	165	125
		2.5	95	104	157	167	158	148
		5.0	87	373	**132**	**209**	**215**	**209**
		10.0	414	201	**333**	**346**	**387**	**361**
PHR	HB	0.0	110	118	157	217	194	948
		2.5	107	116	146	189	158	940
		5.0	107	122	149	241	168	920
		10.0	111	116	153	192	149	924
	SB	0.0	95	102	150	272	353	248
		2.5	116	117	161	306	358	305
		5.0	101	**121**	**172**	**391**	**441**	**386**
		10.0	223	**279**	**265**	**635**	**541**	**490**

HB: High bind; SB: Standard bind;

6.4.1.2 Determination of coating concentration and selection of blocking buffer

For practical reasons, tcdA was employed for the initial determination of coating concentrations. Given the high homology between the two proteins, the optimum settings could then be successfully transferred to the tcdB assays. Further fixed conditions included in this step were plate type (SB) and detection antibody concentration (1 µg/ml). Varying conditions were blocking buffer (PBST + 5% FBS versus PBST + 1% Bovine serum albumin (BSA) + 1% milk powder), coating buffer (PBS, PBS + 0.1% sodium dodecyl sulfate (SDS) versus PBS + 0.1% Tween20 (PBST-coat)), toxin coating concentration (0-50 µg/ml) and sample dilution factor (neat to 1:25).

A high SBR combined with a steady signal increase at a particular dilution range would deliver optimum results. By focusing on SBR, it was possible to determine an optimal coating concentration (25 µg/ml). For IgM, the SBR tended to fall at high coating concentrations (i.e. 50 µg/ml), suggesting that concentrations above 25 µg/ml are not recommended (Table 6.4). Compared to PBST + 5% FBS, PBST + 1% BSA + 1% milk powder blocking buffer (Blocker 1) delivered superior performance and required a lower concentration of toxin coating in order to achieve an acceptable SBR (Tables 6.3 & 6.4 illustrate this with the PBS coating). PBST + 5% FBS also showed a higher background for IgG response (Table 6.3). Further tables can be viewed in Appendices 25-28.

Table 6.3 - Comparison of blocking buffers for IgG response to tcdA across varying PBS coat concentrations

Coat	Block	Coating conc. (µg/ml)	Sample dilution			Buffer	SBR
			Neat	1:5	1:25		
PBS	Blocker 1	0	101	92	108	111	0.9
		5	197	92	107	91	2.2
		25	**243**	**110**	**109**	**93**	**2.6**
		50	185	109	107	100	1.9
	PBST + 5% FBS	0	76	79	89	95	0.8
		5	133	106	123	106	1.3
		25	191	120	158	128	1.5
		50	233	170	174	119	2.0

FBS: Foetal bovine serum; PBS: Phosphate buffer solution; PBST: 1x phosphate buffer solution + 0.05% Tween20; SBR: Signal-to-background ratio;

Table 6.4 - Comparison of blocking buffers for IgM response to tcdA across varying PBS coat concentrations

Coat	Block	Coating conc. (µg/ml)	Sample dilution			Buffer	SBR
			Neat	1:5	1:25		
PBS	Blocker 1	0	125	119	113	114	1.1
		5	**502**	**1513**	**346**	**132**	**3.8**
		25	**463**	**387**	**211**	**94**	**4.9**
		50	187	189	145	95	2.0
	PBST + 5% FBS	0	89	82	89	96	0.9
		5	166	105	91	85	2.0
		25	**401**	**247**	**189**	**110**	**3.6**
		50	**338**	**168**	**108**	**82**	**4.1**

FBS: Foetal bovine serum; PBS: Phosphate buffer solution; PBST: 1x phosphate buffer solution + 0.05% Tween20; SBR: Signal-to-background ratio;

6.4.1.3 Determination of coating buffer and efficacy of toxin sonication

As described above, three coating buffers were tested with various concentrations of capture epitopes. PBS and PBS + 0.1% SDS provided a significantly better SBR in comparison to PBST-coat as a coating solution (Tables 6.3 & 6.4; Appendices 24-28), and thus the latter was not included for further testing. Again for practical reasons, the initial assessment of both IgG and IgM response was conducted with tcdA and optimum conditions were then replicated with tcdB. In addition, coating images during this process suggested a certain degree of toxin aggregation (Figure 6.5A). In order to assess the impact of this on assay performance, a 5-minute sonication step was included prior to the coating steps with either PBS alone, or PBS + 0.1% SDS. Sonication led to a decrease in observed toxin aggregation (Figure 6.5B) with either coating buffers. As previous, the use of PBS alone delivered higher overall signals and a significantly better SBR than those observed when using PBS + 0.1% SDS (Table 6.5). PBS alone was therefore selected as the primary choice of coating vehicle for the downstream steps with both tcdA and tcdB. Further tables can be viewed in Appendices 29 & 30.

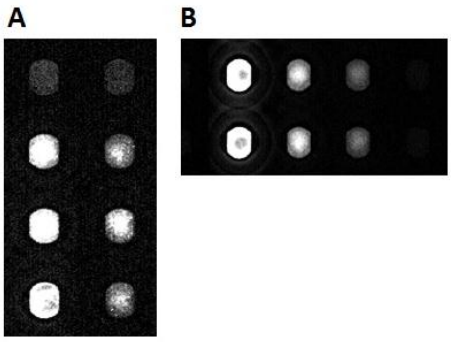


Figure 6.5 –Toxin coating diluted in PBS

A: Aggregation observed using unsonicated toxin coating; B: Aggregation combatted by sonication of toxin prior to coating

Table 6.5 – Electrochemiluminescence signal comparison for IgG response to tcdA across varying blocking buffers

Antibody	Analyte	Sample	Dilution	Mean signal	
				PBS (SBR)	PBS + 0.1% SDS (SBR)
IgG	tcdA	A3X	Buffer	81	74
			Neat	1,173 (14.5)	924 (12.5)
			5	703 (8.7)	343 (4.6)
			25	449 (5.5)	337 (4.6)
		PHR	Buffer	72	71
			Neat	4,427 (61.5)	4,126 (58.1)
			5	1,397 (19.4)	1,255 (17.7)
			25	613 (8.5)	559 (7.9)

IgG: Immunoglobulin G; PBS: Phosphate buffer solution; SBR: Signal-to-background ratio; SDS: Sodium dodecyl sulfate; tcdA: Clostridium difficile toxin A;

6.4.1.4 Determination of polyclonal IgG and IgM detection antibodies and optimal IgM sample dilution

Despite the acceptable development progress, signal spectra observed were modest, and did not significantly improve during the initial optimisation process, and were irrespective of sample dilution factor adopted. Hence, further optimisation was pursued and polyclonal IgG and IgM antibodies were tested as equivalent replacements to their respective monoclonal IgG and IgM versions. In order to ensure steady progress of the assay development, this optimisation made use of the optimal conditions previously identified and an additional 8 clinical samples (CDA 1004, CDA 1022, CDN 0529, CDN 0538, CDN 0574, CDP 0022, CDP 0054 and CDP 0057) were tested to compare results between monoclonal versus polyclonal antibodies. Since the objective here was rapid assessment of the potential benefits of the polyclonal antibodies, samples were run at a single dilution factor (1:5 for measuring IgG response and 1:2 for IgM)

Both polyclonal IgG and IgM antibodies significantly improved the signal and differentiation of samples over their monoclonal counterparts. Using correlation analysis, it was possible to determine that sample rank order was also preserved: $R^2 = 0.946$ and $R^2 = 0.999$, respectively (Figure 6.6 for IgM; for IgG see Appendix 31). Sample dilution at 1:2 for IgM was satisfactory for all samples. There was no evidence for assay saturation and no further optimisation was necessary. In contrast, IgG assays produced an extremely wide signal range and signal saturation was observed in a number of cases. Therefore dilution linearity was performed for all IgG assays (tcdA and tcdB) in order to identify the optimal conditions.

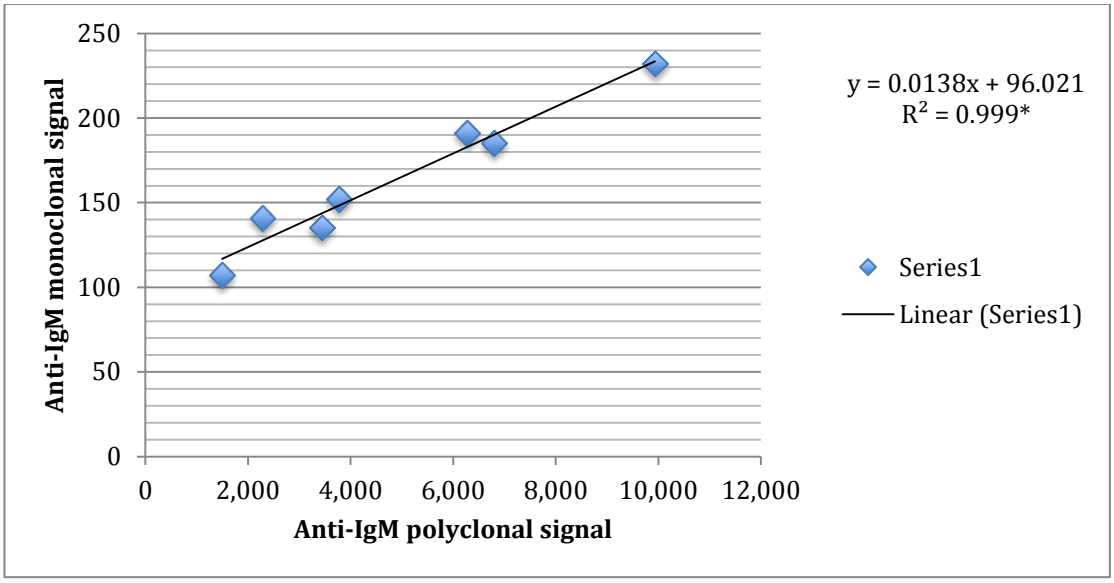


Figure 6.6 – Signal correlation across two different anti-IgM antibodies

**Correlation value is inclusive of sample outlier, which is not shown in the figure.*

6.4.1.5 Determination of optimal sample dilution with IgG polyclonal antibodies

Two coded clinical samples previously tested (CDA 1004 and CDN 0538) were subject to varying sample dilutions ranging from 1:5 to 1:160. These had previously given high and low signals for IgG response with monoclonal antibodies, respectively.

For the high signal intensity sample (CDA 1004), linearity was only reached after 1:40 dilution (Table 6.6, Figure 6.7), with a variation of 0.7% between the 1:40 and 1:80 dilutions. For the low intensity sample (CDN 0538), linearity was observed between the 1:20 and 1:40 dilutions (Appendices 32 & 33), but as dilution further increased this tended to be less consistent. This is explained by the fact that as samples were diluted further, their signal intensity levels fast approached background levels, and therefore background noise accounted for a larger proportion of the overall signal. Based on the above, a dilution factor of 1:40 was proposed for both IgG assays (tcdA and tcdB) as opposed to the 1:2 dilution factor employed for the IgM assays. With IgG known to account for a large percentage of total antibodies in the serum, it was anticipated that signal intensity for the IgG assays would be considerably higher than IgM assays.

Table 6.6 – Dilution linearity for IgG response to tcdB in a high response sample (CDA 1004)

Sample	Sample dilution	Absolute signal (ECL units)	Signal factored for dilution
CDA 1004	1:5	1,383,713	6,918,565
	1:10	1,357,893	13,578,930
	1:20	1,098,406	21,968,120
	1:40	701,970	28,078,800
	1:80	348,661	27,892,880
	1:160	189,475	30,316,000

ECL: Electrochemiluminescence;

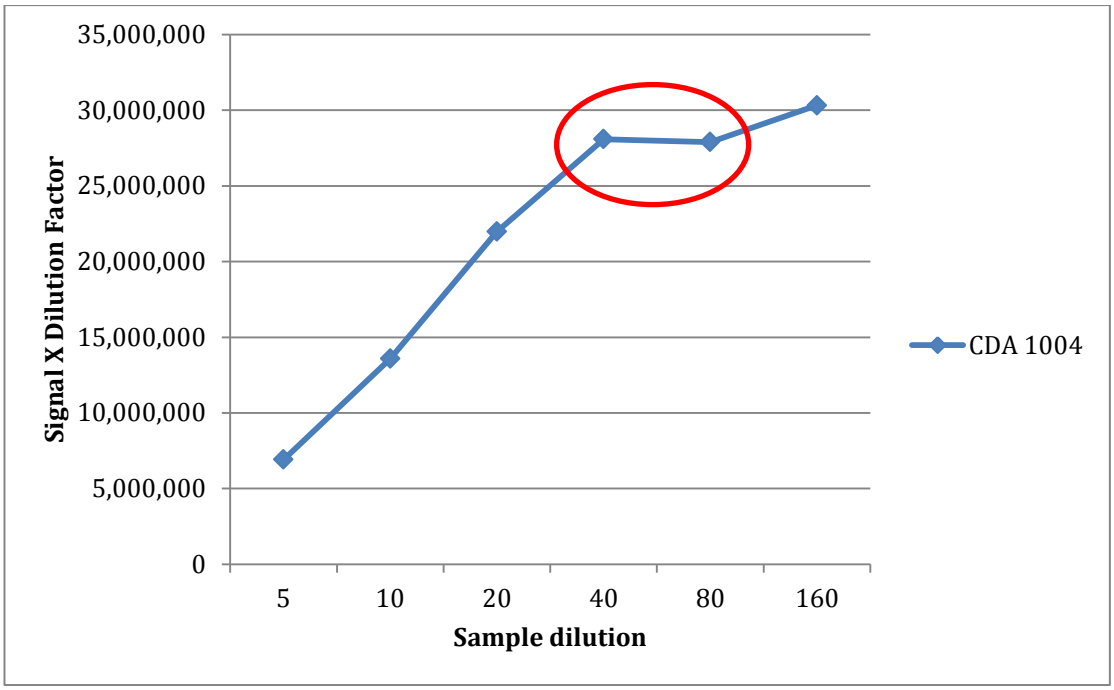


Figure 6.7 – Dilution linearity for IgG assay with tcdB in a high response sample (CDA 1004)

6.4.2 Assay qualification

In order to ensure reproducibility, specificity and sensitivity of the assays, a number of quality control measures were undertaken.

6.4.2.1 Addition of competitive unconjugated goat anti-rabbit antibodies to assess potential interference caused by non-specific binding of the detection antibodies

High background could be the result of non-specific binding of the Fc (Fragment, crystallisable) region of the detection labelled antibodies to the biological matrix, analyte or plate. Fc regions are important for modulating immune cell activity and therefore are able to interact with serological proteins as well as with specific Fc receptors (FcR) present in leukocytes. To address this

possibility, an extra step involving the addition of a competitive unconjugated goat anti-rabbit antibody - similar in characteristics in the Fc region to the detection antibody - was added between the addition of samples and incubation with detection antibody. If the Fc fragment was indeed a key factor for causing background noise, then a decrease in the signal would be expected following the addition of the extra antibody step. Clearly, no variation in signal was found (Table 6.7), indicating that the background noise observed in the runs was not due to non-specific binding of the goat Fc fragment of the sulfo-tagged detection antibody.

Table 6.7 – Addition of extra antibody

Antibody	Analyte	Sample	Signal		CV
			Extra antibody	No extra antibody	
IgG	tcdA	C1	43,015	43,170	0.3
		C2	58,197	57,028	1.4
		C3	48,621	47,470	1.7
		C4	42,067	40,762	2.2
		CDN 0529	147,396	144,621	0.4
		CDN 0538	10,062	10,296	1.6
		CDP 0057	39,708	41,399	2.9
	tcdB	C1	20,097	19,988	0.4
		C2	24,144	24,153	0.0
		C3	20,548	20,468	0.3
		C4	42,664	42,375	0.5
		CDN 0529	232,613	219,165	4.2
		CDN 0538	1,988	1,978	0.4
		CDP 0057	8,294	8,525	1.9

CV: Coefficient of variation; IgG: Immunoglobulin G; tcdA: Clostridium difficile toxin A; tcdB: Clostridium difficile B;

6.4.2.2 Sample spiking and re-equilibration

Sample Spiking

Serial spiking of samples with free toxin was employed as a means of quality control, in which signal inhibition would be indicative of the specificity of the assay. Rather than binding to the toxin coated onto the plate, specific response antibodies present in sera, can instead bind to the circulating free toxin. This will then prevent the immunoglobulins to form a complex attached to the plate and generate a signal. Therefore, by adding controlled amounts of epitope (toxins) to the sample, a proportional decrease in the signal level would then be expected until a point of signal inhibition close to the background noise was reached (point of saturation). If the signal originally observed was a result of non-specific binding, then toxin spiking would be unlikely to affect this process.

a) IgM assay:

Three samples (CDA 1026, CDN 0511 and CDN 0516) with high intensity response to the toxins (ECL units) were pooled together to be spiked with toxin. In the same way, two samples (CDA 1022 and CDP 0022) with low intensity responses were combined for this purpose. Strong inhibition was achieved across both the high and low intensity pooled samples at the optimal 1:2 sample dilution. Optimal inhibition would result in a decreased signal approaching background levels; here, SBR for un-spiked samples versus the samples spiked with the highest toxin concentration (12,800 pg/ml) resulted in a dramatic decrease (Table 6.8; 330.6 versus 23.7 and 21.0 versus 4.8, respectively).

b) IgG assay:

Since the baseline IgG responses were considerably higher than the corresponding IgM counterparts, it was necessary to assess inhibition in samples displaying higher intensity responses: PHR and CDA 1004 (medium and high intensity responses, respectively). Although not as effective as the IgM assays, significant inhibition was observed using a top spike of 3,200 pg/ml at

the optimal 1:40 sample dilution (Appendices 34 & 35). The experiment suggested that a much larger amount of toxin was needed in order to demonstrate complete inhibition, which would become impractical for the purpose of this investigation given the restricted availability of these toxins, especially in a native presentation.

Table 6.8 – Spiking results for IgM response to tcdB using pooled samples

Toxin (pg/ml)	Signal (1:2 dilution)	
	Pooled high response (SBR)	Pooled low response (SBR)
Buffer	75	77
Unspiked	24,800 (330.6)	1,618 (21.0)
200	23,762 (316.8)	827 (10.7)
800	18,725 (249.7)	754 (9.8)
1,600	15,267 (203.6)	675 (8.8)
3,200	12,519 (166.9)	611 (7.9)
6,400	8,335 (111.1)	492 (6.4)
12,800	1,781 (23.7)	366 (4.8)

SBR: Signal-to-background ratio;

Re-equilibration of antibodies

IgGs are smaller molecules than IgMs and incomplete inhibition is potentially due to the re-equilibration of response antibodies from the spiked free toxin and subsequent binding to the analyte on the plate. Re-equilibration implies that the longer the spikes samples are incubated, the greater the chances of dissociation of the antibodies, thus allowing them to generate a signal. As spiked samples were originally incubated for 30 minutes, incubation times were stratified with a range of incubation periods between 5-30 minutes and examined for the IgG assay using the same two samples (PHR and CDA 1004). Interestingly, the highest degree of signal inhibition was observed for the 5 min incubation period, with inhibition decreasing as time increased (Table 6.9). This

indicates that re-equilibration is present and occurs rapidly, suggesting that the inability to attain complete inhibition through antigen spiking is at least in part due to re-equilibration of the antigen-antibody complex. Other technical issues such as the large quantities of the analytes may also have prevented demonstration of complete signal inhibition. As such, the combined spiking data indicates that both IgG and IgM assays have a satisfactory level of specificity. As a further precaution, fixed controls were included for all downstream steps in order to account for background effects and inter-plate variability.

Table 6.9 – Re-equilibration results for IgG response to tcdB

Analyte	Sample	Spike conc. (pg/ml)	Incubation time (min)	Mean signal
tcdB	CDA 1004	3,200	5	20,436
			10	36,560
			20	65,550
			30	57,950
		Unspiked	30	357,380
	PHR	3,200	5	9,851
			10	11,303
			20	17,947
			30	21,909
		Unspiked	30	57,595

tcdB: Clostridium difficile toxin B;

6.4.2.3 No-coating analyte and buffer controls

Including uncoated wells (zero coating) allows the investigation of non-specific events, such as binding of serum proteins and/or labelled antibodies directly to the surface of the ECL plate. Signals levels in the absence of analyte varied across samples but were generally low. Conversely, the presence of toxin coating accounted for a substantial rise in SBR for all samples tested, which indicated that the assay had good sensitivity (Table 6.10). The inclusion of buffer controls (analyte coating + blank/PBS (no sample) + detection antibody)

allowed observation of the level of non-specific binding of the detection antibody directly to the analyte and/or to the surface of the plate in the absence of sample. Buffer signals were extremely low throughout the entire assay optimisation process and no interference was noticed.

Table 6.10 – Overview of results for no coating control

Antibody	Analyte	Sample	Coat	No coat
IgG	tcdA	C2	17,802	2,396
		WP 0018	263,574	8,345
		WP 0019	43,016	9,403
		WP 0020	77,240	8,663
	tcdB	C2	13,891	2,460
		WP 0018	48,778	9,490
		WP 0019	34,657	8,378
		WP 0020	25,029	8,949
IgM	tcdA	C2	17,067	391
	tcdB		7,838	699

IgG: Immunoglobulin G; IgM: Immunoglobulin M; tcdA: Clostridium difficile toxin A; tcdB: Clostridium difficile toxin B;

6.4.2.4 Plate controls

Once initial conditions were optimised, 3 sample controls (CDA 1004, PHR and C1) were used throughout the development process to account for inter-plate variability. For each control sample, a correction factor was derived from the overall plate runs and then applied across individual plates. Furthermore, of the 88 clinical samples tested on each plate, 8 samples were tested in duplicate in order to monitor intra-plate variability.

6.5 Binary toxin assays

Optimal settings obtained from the development of the tcdA and tcdB assays were applied for the initial development of the *C. diff* binary toxin assays based on IgG and IgM responses to: a) cdtA alone (cdtA); b) cdtB-activated alone (cdtB-act); c) cdtB-precursor alone (cdtB-pre); d) combined cdtA + cdtB-activated (cdtA+cdtB-act).

The binary toxin epitopes have differing molecular weights (50, 100 and 75 kDa for cdtA, cdtB-pre and cdtB-act, respectively), confirmed by in-house data (Figure 6.8). Figure 6.9 shows that cdtB-act forms oligomers that are stable in favorable conditions (e.g. PBS and SDS) but get dissociated with heat and other denaturing buffers, further indicating that these epitopes are reliable, match the proposed molecular weight and have the expected properties. Therefore it was proposed to test them in isolation, and also combined; cdtB-act, but not cdtB-pre, form hepatmers and it is thought that approximately 2-3 cdtAs will enter a cell with one cdtB-act heptamer (data unpublished). As such, this study incubated 1 cdtA molecule with 2 cdtB-act molecules to form the activated complex.

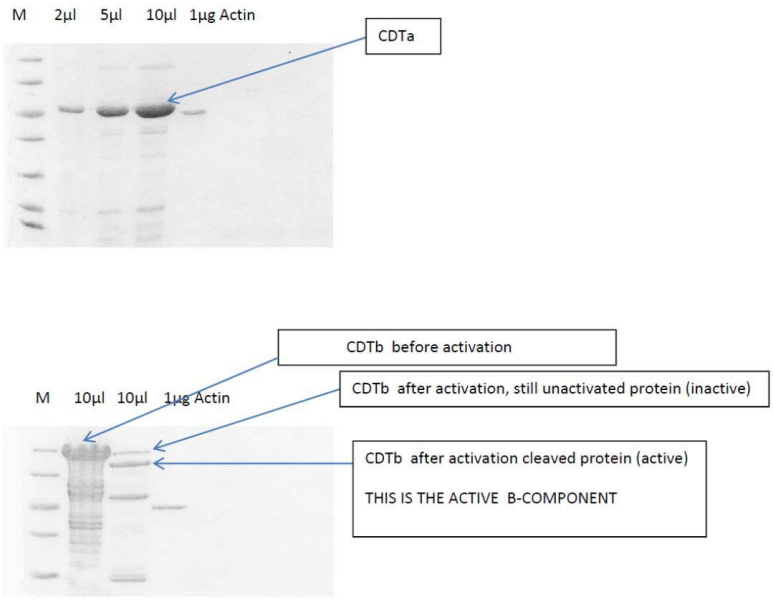


Figure 6.8 – Confirmation of binary toxin analytes via gel electrophoresis

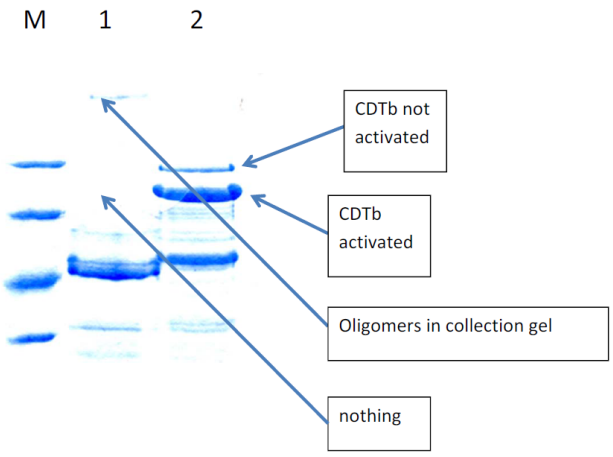


Figure 6.9 – Assessing the stability of binary toxin analytes via gel electrophoresis

Both lanes show equal amounts of cdtB from the same prep. Gel is Coomassie stained. ***Lane 1****: cdtB after activation in Lammli-Buffer (SDS) – NOT boiled.* ***Lane 2****: cdtB after activation in Lammli-Buffer (SDS) – Boiled*

6.5.1 Identification of initial working conditions

6.5.1.1 Determination of coating & detection antibody concentrations for IgG response to cdtB-act, cdtB-pre and cdtA+cdtB-act

This step focused primarily on the optimisation of IgG assays for all four binary toxin analytes. As with the original toxin assays, non-variable conditions were PBS coating buffer, SB plate type, Blocker 1 as blocking buffer and 1 µg/ml detection as the selected antibody concentration. Varying conditions comprised coating concentration (4 & 16 µg/ml) and sample dilution factor (1:5 & 1:10). Three samples were used: two from patients who had CDI caused by a binary toxin-producing strain (ribotypes 027 & 078), which also provided overall high signals for the tcdA and tcdB assays (CDA 1130 & CDN 0552), and PHR, a control individual who had continuous exposure to *C. diff* strains.

Initial testing conditions delivered particularly high signals meaning that samples required further dilution (Table 6.11). Despite this, the majority of samples showed an adequate coefficient of variation (CV <20) between analyte coating concentrations of 4 and 16 µg/ml (Table 6.11; highlighted in orange). The lower threshold of 4 µg/ml of either toxin was thus selected as the concentration of choice for the development process.

6.5.1.2 Adjustment of sample dilution for IgG response to cdtA, cdtB-act, cdtB-pre and cdtA+cdtB-act

Given the saturation observed with the initial optimisation run, serial dilution between 1:50 and 1:500 was attempted for all combinations using a strategy similar to step 1, except that a fixed analyte coating concentration of 4 µg/ml was adopted throughout.

Good linearity was observed for three analytes (cdtB-act, cdtB-pre and cdtA+B-act) (Figure 6.10). The 1:200 dilution was selected as optimal as it afforded a large signal window of detection, whilst maintaining linearity and sensitivity. In

contrast, linearity was not achieved for the IgG assay with cdtA (Figure 6.11) and therefore required further work. Further figures can be viewed in Appendices 36 & 37.

Table 6.11 – Overly high signals observed during initial binary toxin optimisation

Analyte	Sample	Blocker	Sample dilution	Signal		CV
				4 µg/ml coat	16 µg/ml coat	
cdtA	A1130	Blocker 1	1:5	254,340	348,505	22.1
			1:10	145,804	231,356	32.1
	N552		1:5	245,056	299,822	14.2
			1:10	193,859	236,424	14.0
	PHR		1:5	344,903	399,004	10.3
			1:10	238,062	268,967	8.6
cdtB-act	A1130		1:5	1,361,890	1,325,172	1.9
			1:10	1,263,441	1,073,002	11.5
	N552		1:5	1,420,483	1,402,840	0.9
			1:10	1,400,590	1,367,432	1.7
	PHR		1:5	552,921	459,209	13.1
			1:10	331,897	296,178	8.0
cdtB-pre	A1130		1:5	1,361,138	1,352,364	0.5
			1:10	1,315,927	1,288,236	1.5
	N552		1:5	1,415,749	1,412,210	0.2
			1:10	1,379,213	1,359,311	1.0
	PHR		1:5	639,693	578,503	7.1
			1:10	395,303	340,103	10.6
cdtA+cdtB-act	A1130		1:5	1,367,318	1,350,041	0.9
			1:10	1,228,036	1,290,665	3.5
	N552		1:5	1,398,775	1,385,480	0.7
			1:10	1,378,744	1,355,331	1.2
	PHR		1:5	698,381	634,508	6.8
			1:10	421,545	426,227	0.8

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component – precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; CV: Coefficient of variation;*

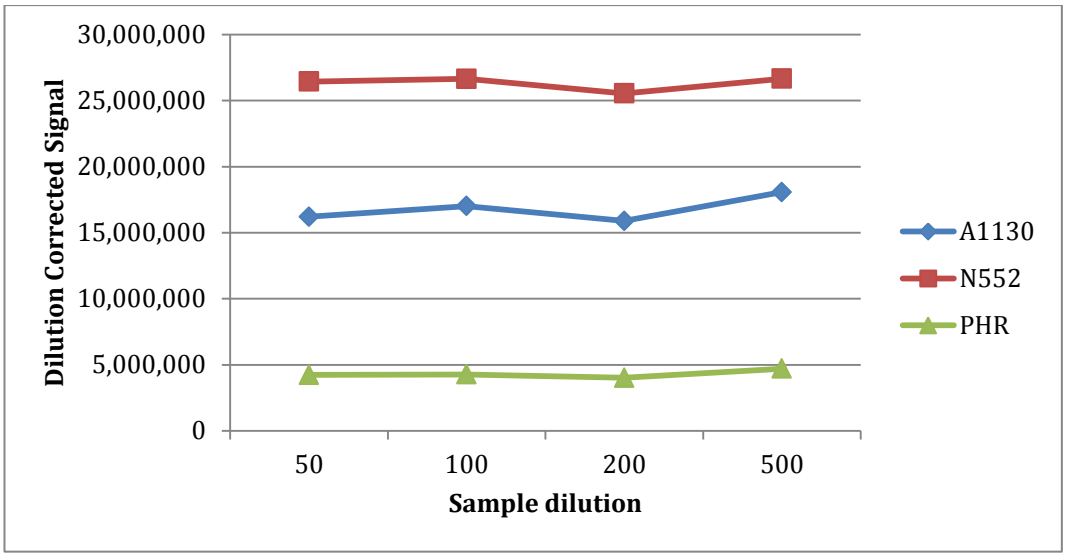


Figure 6.10 – Dilution linearity plot for the IgG assay with the combined analyte cdtA+cdtB-act using Blocker 1

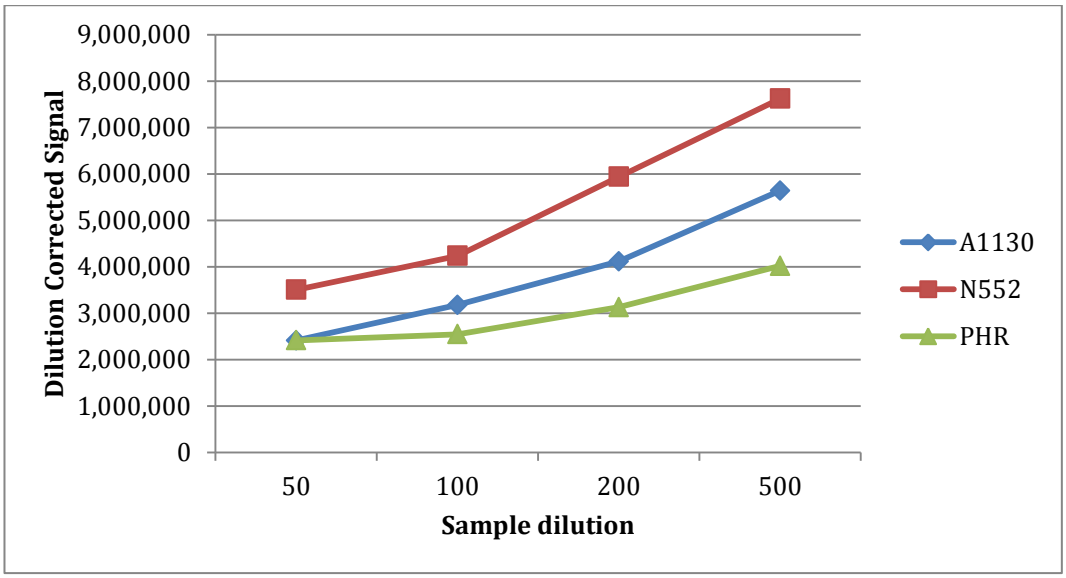


Figure 6.11 – Dilution linearity plot for IgG assay with cdtA using Blocker 1

6.5.1.3 Further adjustment of analyte coating concentration and detection antibody concentration for the IgG assay with cdtA

In addition to further sample dilution, it is possible that the lack of linearity observed with the IgG assay with cdtA was the result of suboptimal settings for the analyte coating and the detection antibody concentrations. It was therefore hypothesised that if the signal intensity can be decreased by adjusting these conditions, then improved linearity may be observed for the cdtA assay. Concentrations for the analyte coating were further reduced from 4 and 16 µg/ml to 3.2 and 1.6 µg/ml, whilst for the detection antibodies this was brought down from 1.0 µg/ml to 0.8 and 0.4 µg/ml. Other conditions were as previously described.

The combination of 1.6 µg/ml analyte coating with both 0.4 and 0.8 µg/ml detection antibody concentrations delivered the highest SBRs at all dilutions (dilutions 1:50 and 1:500 illustrated in Tables 6.12 & 6.13). The slight improvement in overall performance observed with the 0.8 µg/ml detection antibody resulted in the 1.6 µg/ml analyte coating with 0.8 µg/ml detection antibody being taken forward. However, dilution linearity was still unsatisfactory with differences in the corrected signals becoming more apparent as samples were further diluted to 1:500 (Figure 6.12). Further figures relating to other concentrations can be viewed in Appendices 38-40.

Table 6.12 – ECL signal values for the IgG assay with cdtA using varying combinations of coating and detection concentrations

Sample	3.2C/0.8D	3.2C/0.4D	1.6C/0.8D	1.6C/0.4D
Buffer	455	431	181	157
Signal at 1:50 dilution				
CDA 1130	47,060	40,542	45,073	39,020
CDN 0552	67,853	53,977	66,563	54,616
PHR	53,534	44,479	60,206	51,613
Signal at 1:500 dilution				
CDA 1130	9,968	8,585	8,678	7,480
CDN 0552	13,337	12,179	14,183	13,040
PHR	6,823	6,245	7,348	6,379

C: Coating, in µg/ml; D: Detection, in µg/ml;

Table 6.13 – SBR for the IgG assay with cdtA using varying combinations of coating and detection concentrations

Sample	3.2C/0.8D	3.2C/0.4D	1.6C/0.8D	1.6C/0.4D
SBR at 1:50 dilution				
CDA 1130	103.4	94.1	249.0	248.5
CDN 0552	149.1	125.2	367.8	347.9
PHR	117.7	103.2	332.6	328.7
SBR at 1:500 dilution				
CDA 1130	21.9	19.9	47.9	47.6
CDN 0552	29.3	28.3	78.4	83.1
PHR	15.0	14.5	40.6	40.6

C: Coating, in µg/ml; D: Detection, in µg/ml;

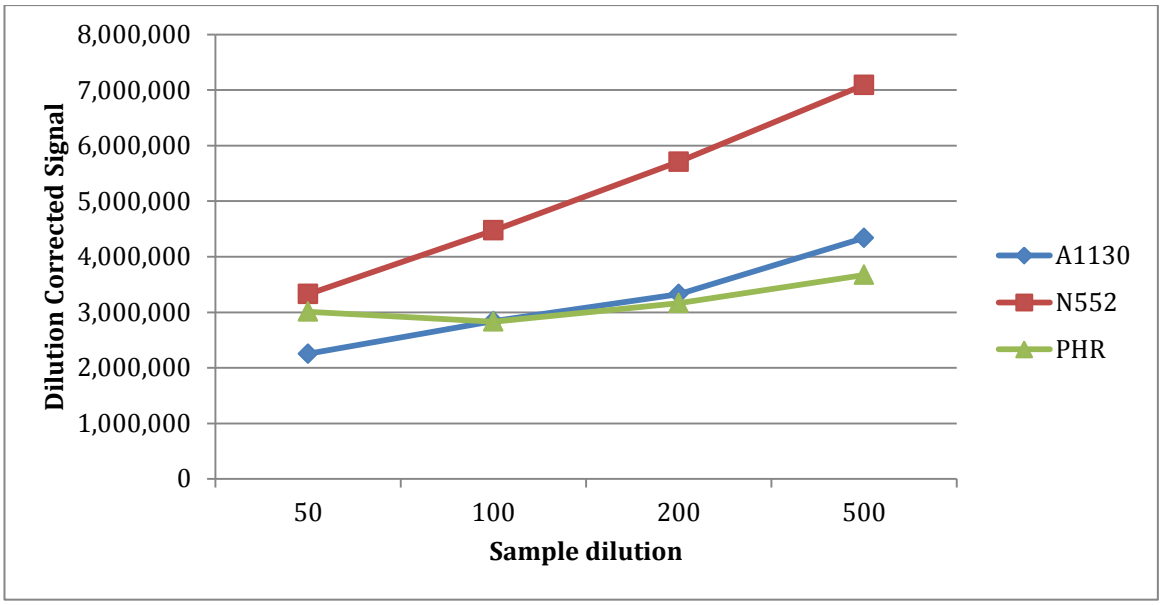


Figure 6.12 – Dilution linearity for IgG response to cdtA using a combination of 1.6 µg/ml coating and 0.8 µg/ml detection

The lack of dilution linearity was particularly evident for one of the test samples (CDN 0552), suggesting the presence of a significant level of matrix interference. Since the observed blank (zero sample) signal was very modest, this allowed assessment of a further increase in sample dilution (ranging from 1:500 to 1:4,000) in order to address the issue. Two additional randomly selected samples (CDN 0545 and CDN 0555) were also included for this purpose.

These changes delivered more consistent results with improved linearity being observed across all samples (Figure 6.13). For the 1:1,000 dilution, linearity had only just begun to be achieved and therefore the 1:2,000 sample dilution factor was taken forward as it maintained both suitable linearity combined with a reasonable SBR (Table 6.14; highlighted in orange).

Table 6.14 - Signal to background ratio for IgG response to cdtA at 1:2,000 dilution using a combination of 1.6 µg/ml coating and 0.8 µg/ml detection

Sample	Dilution	Coat signal	SBR
No coat buffer control	**-**	**No coat signal = 77**	**-**
CDA 1130	500	8,827	114.6
	1000	4,828	62.7
	2000	3,030	39.4
	4000	1,390	18.1
CDN 0545	500	12,687	164.8
	1000	6,848	88.9
	2000	3,453	44.8
	4000	1,767	22.9
CDN 0552	500	14,239	184.9
	1000	10,418	135.3
	2000	5,262	68.3
	4000	2,924	38.0
CDN 0555	500	5,157	67.0
	1000	3,060	39.7
	2000	1,513	19.6
	4000	850	11.0
PHR	500	9,096	118.1
	1000	4,336	56.3
	2000	2,602	33.8
	4000	1,529	19.9

SBR: Signal-to-background ratio;

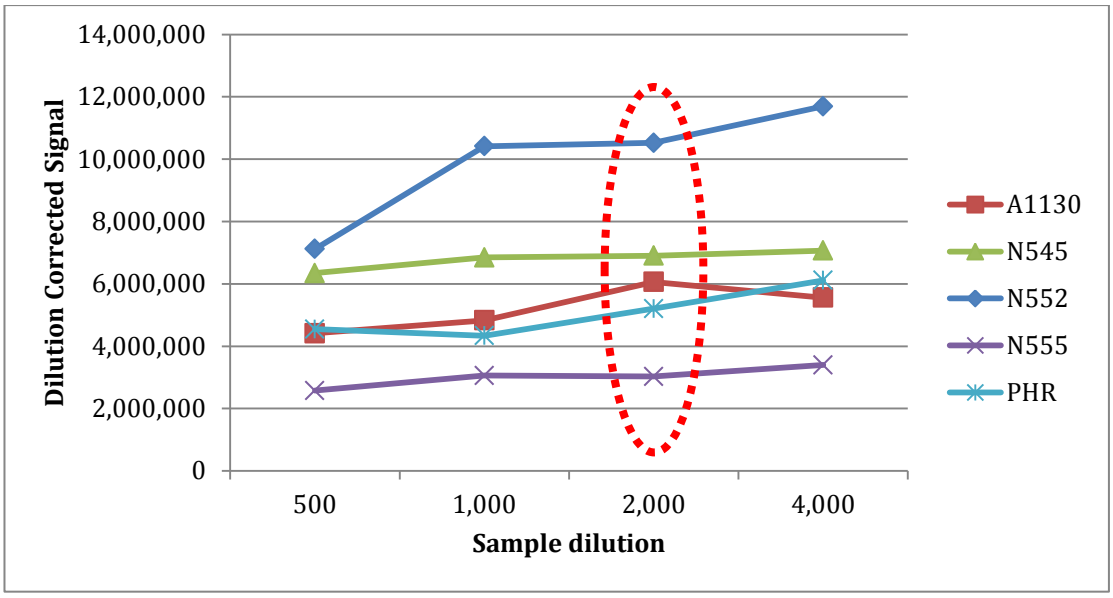


Figure 6.13 – Dilution linearity plot for IgG response to cdtA using a combination of 1.6 µg/ml coating and 0.8 µg/ml detection antibody with sample dilutions ranging from 1:500 to 1:4,000

6.5.1.4 Determination of optimal assay conditions for IgM assay with all binary toxin epitopes

Compared to the IgG assays with either tcdA or tcdB analytes, it was previously identified that the detection range for the IgM assays were narrower and a much lower dilution factor was required. Hence, this information served as the basis for the initial development of the IgM assays with the binary toxin analytes. Four samples, which overlapped with the ones employed in the previous steps, were utilised (CDA 1130, CDN 0545, CDN 0552 and PHR) and sample dilutions ranged between 1:5 and 1:100. All other settings were transferred from the optimal conditions obtained with the development of the IgG assays using the binary toxin analytes (Blocker 1; 1.6 µg/ml coating and 0.8 µg/ml detection for cdtA; 4 µg/ml coating and 1.0 µg/ml detection for all other binary epitopes).

From the initial run, it was possible to infer that linearity was suboptimal for all analytes tested (Figure 6.14; Appendices 41-43). As observed in Table 6.15, SBR steadily moved closer to background levels and as such, diluting the samples beyond 1:100 would have hampered the sensitivity of the assay by compressing the detection window. A trade-off between linearity and signal detection range was therefore necessary and a dilution factor of 1:50 was selected for further development.

Table 6.15 – Overview of SBR at increasing dilution for all binary toxin analytes

Analyte	Sample	SBR across dilutions			
		1:5	1:10	1:50	1:100
cdtA	CDA 1130	163.8	105.8	25.7	16.3
	CDN 0545	536.5	348.3	96.5	55.4
	CDN 0552	111.4	79.9	29.7	19.4
	PHR	78.1	61.2	26.3	16.3
cdtB-act	CDA 1130	162.3	66.1	14.8	9.5
	CDN 0545	115.8	77.3	24.2	15.3
	CDN 0552	280.3	80.0	17.2	12.3
	PHR	238.0	141.7	36.4	21.2
cdtB-pre	CDA 1130	120.1	69.7	22.0	16.2
	CDN 0545	462.3	268.8	74.8	42.7
	CDN 0552	352.4	104.6	34.7	24.6
	PHR	60.0	48.3	22.1	13.6
cdtA+cdtB-act	CDA 1130	261.7	131.5	28.4	19.1
	CDN 0545	533.0	303.1	76.2	42.5
	CDN 0552	237.3	138.6	29.9	20.5
	PHR	246.5	171.1	47.2	30.8

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component – precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; SBR: Signal-to-background ratio;*

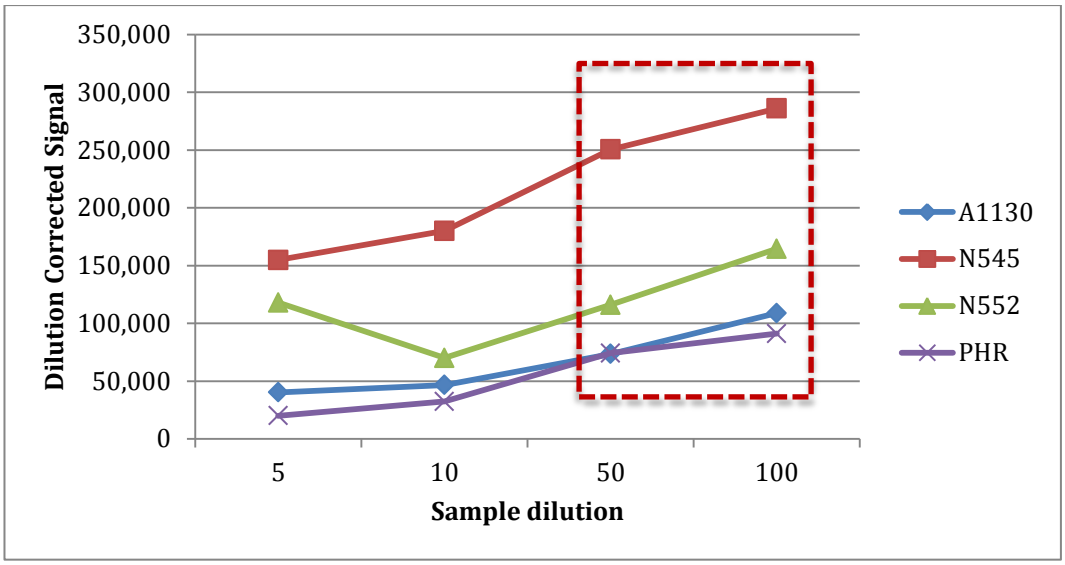


Figure 6.14 – Dilution linearity plot for IgM response to cdtB-pre

6.5.1.5 Use of no-coat sample controls for the determination of sample background noise

Following the examination of the initial conditions set for the measurement of both IgG and IgM responses to the binary toxin analytes, a no-coating optimisation was conducted in order to evaluate biological matrix interference present in an individual sample (CDN 0527).

Compared to the no-coat buffer sample control, the results indicated that a significant proportion of the background noise was due to biological matrix interference. This was observed with both IgG and IgM assays across all binary toxin analytes (Table 6.16). To address this problem, a further optimisation with an additional blocking buffer was undertaken.

Table 6.16 – No coat controls for the IgG and IgM assay with the binary toxin analytes using Blocker 1 and sample CDN 0527

Antibody	Binary toxin component	Detection conc. (µg/ml)	Dilution	Signal
IgG	No coat	0.8	1:2,000	1,330
	cdtA			2,919
	No coat	1.0	1:200	19,684
	cdtB-act			25,048
	cdtB-pre			33,936
	cdtA+cdtB-act			26,073
IgM	No coat	0.8	1:50	1,434
	cdtA			2,581
	No coat	1.0		1,589
	cdtB-act			2,494
	cdtB-pre			2,763
	cdtA+cdtB-act			2,522

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component – precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; IgG: Immunoglobulin G; IgM: Immunoglobulin M;*

6.5.1.6 Selection of 1x Tris Buffered Saline with 1% Casein as blocking buffer for comparison against Blocker 1

Given the high degree of background observed in the previous optimisation condition with Blocker 1, the use of 1x Tris Buffered Saline with 1% Casein (Casein Blocker) was considered as an alternative blocking agent. Comparison with Blocker 1 blocking buffer was therefore conducted with two samples, including CDN 0527 that was used in the previous no-coating control step.

Again, data was analysed based upon the percentage of total signal represented by the background of the no-coat control samples. Results indicated that the proportion of signal accounted by the no-coat control was significantly lower with the Casein Blocker compared to Blocker 1. This was true for both the IgG (Figure 6.15) and IgM assays (Appendix 44). Furthermore, the rank order of

samples for coating signal before and after subtraction of the no coat background signal was better conserved in the presence of Casein Blocker compared to Blocker 1, particularly for IgM (Table 6.17; mismatching ranks highlighted in orange)

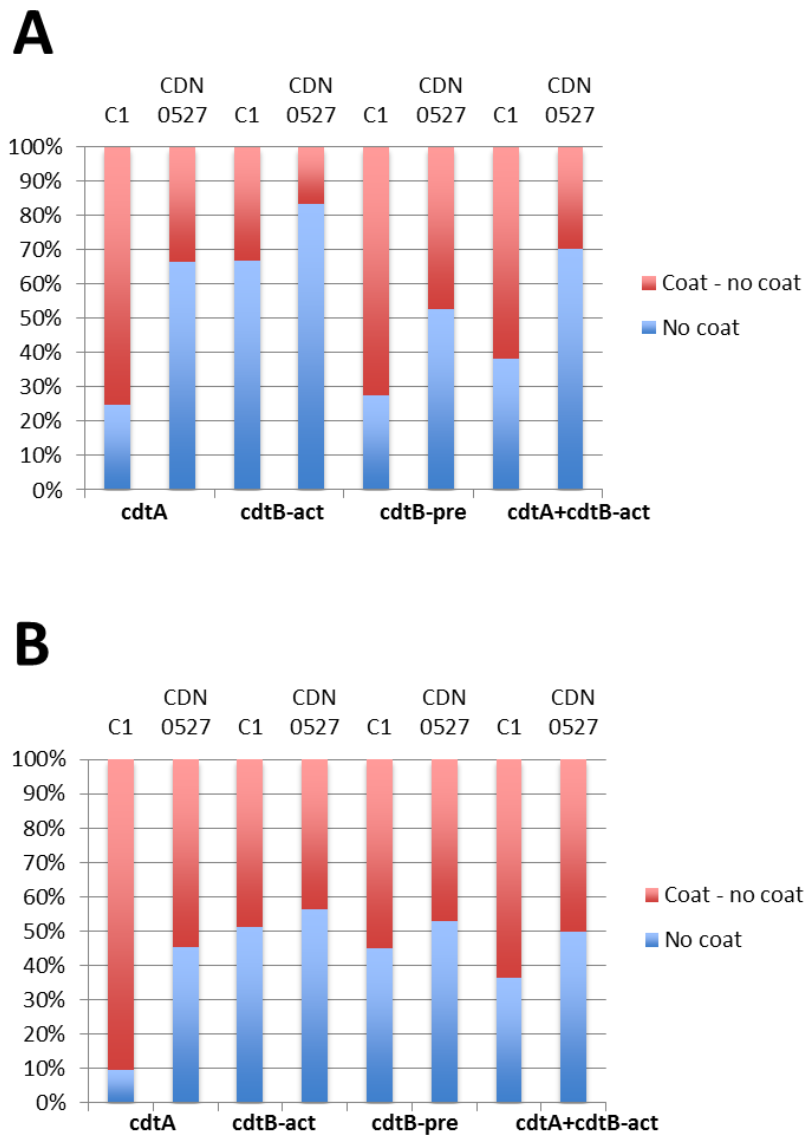


Figure 6.15 – Percentage of overall signal accounted by background noise (no coat sample data) for the IgG assay using all binary toxin analytes with Blocker 1 (A) and Casein Blocker (B)

Table 6.17 – No coat data for IgG & IgM assays using all binary toxin analytes with Blocker 1 or Casein blocker

Epitope	Blocker	Antibody	Dilution	Sample	Signal			Rank order	
					Coat	No coat	Coat minus no coat	Coat	Coat minus no coat
cdtA	Blocker 1	IgG	1:2,000	C1	1,611	399	1,212	8	7
				CDN 0527	3,233	2,156	1,077	7	8
cdtB-act			1:200	C1	5,964	3,986	1,978	6	6
				CDN 0527	27,599	22,994	4,605	3	5
cdtB-pre				C1	14,380	3,956	10,424	4	3
				CDN 0527	46,063	24,292	21,771	1	1
cdtA+cdtB-act				C1	10,967	4,189	6,778	5	4
				CDN 0527	35,185	24,705	10,480	2	2
cdtA		IgM	1:50	C1	2,754	1,423	1,331	3	1
				CDN 0527	2,983	1,686	1,297	1	2
cdtB-act				C1	2,393	1,164	1,229	6	4
				CDN 0527	2,204	1,595	609	7	8
cdtB-pre				C1	2,042	1,272	770	8	7
				CDN 0527	2,797	1,628	1,169	2	5
cdtA+cdtB-act				C1	2,547	1,292	1,255	5	3
				CDN 0527	2,668	1,623	1,045	4	6

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component – precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; IgG: Immunoglobulin G; IgM: Immunoglobulin M;*

Table 6.17 (continued) – No coat data for IgG & IgM assays using all binary toxin analytes with Blocker 1 or Casein blocker

Epitope	Blocker	Antibody	Dilution	Sample	Signal			Rank order	
					Coat	No coat	Coat minus no coat	Coat	Coat minus no coat
cdtA	Casein Blocker	IgG	1:2,000	C1	2,089	200	1,889	7	5
				CDN 0527	848	385	463	8	8
cdtB-act			1:200	C1	2,702	1,379	1,323	6	7
				CDN 0527	5,125	2,882	2,243	3	4
cdtB-pre				C1	2,979	1,338	1,641	5	6
				CDN 0527	10,984	5,814	5,170	1	1
cdtA+cdtB-act				C1	3,965	1,437	2,528	4	3
				CDN 0527	6,129	3,063	3,066	2	2
cdtA		IgM	1:50	C1	953	259	694	2	2
				CDN 0527	1,007	251	756	1	1
cdtB-act				C1	437	258	179	8	7
				CDN 0527	520	347	173	7	8
cdtB-pre				C1	776	266	510	4	4
				CDN 0527	947	323	624	3	3
cdtA+cdtB-act				C1	662	295	367	6	6
				CDN 0527	679	290	389	5	5

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component – precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; IgG: Immunoglobulin G; IgM: Immunoglobulin M;*

6.5.1.7 Adoption of a no-wash blocking step using Casein Blocker and dilution linearity for both IgG and IgM assays

No-wash blocking

Although the use of Casein Blocker resulted in a significant improvement over the previously adopted procedure, matrix interference was still present, particularly for IgG. The current procedure adopted had involved an incubation of 150 µl of blocking buffer for 1 h followed by washing of the wells and addition of samples. To bolster blockade of non-specific binding, the incubation step was modified such that a reduced volume of 25 µl of Casein Blocker was added to the wells and no wash was performed prior to the addition of samples. This resulted in greater suppression of the no-coat signal for all binary toxin analytes across the samples tested. For IgG, the highest signal values obtained were now ≤1,000, which was considered to be reasonable, with a decrease observed in the overall percentage of signal accounted for by the background no coat signal (Table 6.18; decreases highlighted in orange). Furthermore, the rank order of coated signals, as well as overall signals for no coat signals subtracted from coated signals, remained unaltered (Table 6.19; mismatching ranks highlighted in orange). This was not the case when using the previous 150 µl blocking/wash step (Table 6.17). For IgM, initial matrix interference and the rank order of samples were already satisfactory, and these observations remained consistent after introduction of the no-wash blocking step.

Table 6.18 – No coat data for IgG assay using all binary toxin analytes, Casein Blocker & two blocking protocols

Epitope	Antibody	Dilution	Sample	Percentage of signal attributed to background		Difference
				150 µl/Wash	25 µl/No wash	
cdtA	IgG	1:2,000	C1	9.6	31.4	21.8
			CDN 0527	45.4	40.8	-4.6
cdtB-act		1:200	C1	51.0	46.4	-4.6
			CDN 0527	56.2	62.9	6.6
cdtB-pre			C1	44.9	14.8	-30.1
			CDN 0527	52.9	25.3	-27.6
cdtA+cdtB-act			C1	36.2	25.0	-11.2
			CDN 0527	50.0	39.6	-10.4

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component – precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; IgG: Immunoglobulin G;*

Table 6.19 – No coat data for IgG assay using all binary toxin analytes and Casein Blocker

Epitope	Antibody	Dilution	Sample	Signal			Rank order	
				Coat	No coat	Coat minus no coat	Coat	Coat minus no coat
cdtA	IgG	1:2,000	C1	239	75	164	8	8
			CDN 0527	306	125	181	7	7
cdtB-act		1:200	C1	857	398	459	6	6
			CDN 0527	1,723	1,083	640	4	5
cdtB-pre			C1	2,715	402	2,313	2	2
			CDN 0527	4,279	1,082	3,197	1	1
cdtA+cdtB-act			C1	1,524	381	1,143	5	4
			CDN 0527	2,451	971	1,480	3	3

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component – precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; IgG: Immunoglobulin G;*

Dilution linearity

As optimal sample dilution had previously been defined using Blocker 1, it was important to ascertain this in the presence of the new blocking protocol (25 µl of Casein Blocker, no wash step). Sample CDN 0529 was also included to ensure sample CDN 0527 was representative. All other settings were consistent with those previously employed with Blocker 1.

For the IgM assay, dilution linearity was assessed from 1:5-1:100. Results were comparable to the ones found with Blocker 1 and dilution linearity was again suboptimal. However, at the 1:50 and 1:100 dilutions, no difference was observed between the no coat signals for both samples compared to the large differences observed at the 1:5 and 1:10 dilutions (Table 6.20). When combined with overall SBR achieved (Table 6.21), a sample dilution factor of 1:50 was confirmed as the preferred choice for the IgM assay using Casein Blocker. For the IgG assay, dilution linearity was assessed between 1:500-1:4,000. Previous IgG dilution linearity in the presence of blocker 1 was satisfactory; however, in the presence of Casein Blocker, as with IgM, dilution linearity was suboptimal. Furthermore, large differences were observed between the no coat signals for both samples across all dilutions (Table 6.20), and as a result, required further attention.

Table 6.20 – Differences in no coat signal between samples CDN 0527 and CDN 0529 across varying dilutions for the IgG and IgM assays using Casein Blocker

Binary condition	Block buffer	Antibody	Detection conc. (ug/ml)	Dilution	No coat signal		
					CDN 0527	CDN 0529	CDN 0527/ CDN 0529
No coat	Casein Blocker	IgG	0.8	500	542	127	4.3
				1000	294	99	3.0
				2000	188	90	2.1
				4000	118	66	1.8
			1	100	3194	627	5.1
				200	1532	291	5.3
				500	571	164	3.5
				1000	335	83	4.0
		IgM	0.8	5	2962	977	3.0
				10	849	614	1.4
				50	234	207	1.1
				100	128	146	0.9
			1	5	3060	1139	2.7
				10	879	686	1.3
				50	234	214	1.1
				100	138	131	1.1

IgG: Immunoglobulin G; IgM: Immunoglobulin M;

Table 6.21 – Dilution linearity SBR values in two samples for the IgM assay with binary toxin analytes using Casein Blocker

Binary condition	Block buffer	Dilution	CDN 0527			CDN 0529		
			Signal		SBR	Signal		SBR
			No coat	Coat		No coat	Coat	
cdtA	Casein Blocker	5	2,962	5,428	1.8	977	13,965	14.3
		10	849	2,311	2.7	614	7,393	12.0
		50	234	958	4.1	207	1,545	7.5
		100	128	422	3.3	146	836	5.7
cdtB-act		5	3,060	3,266	1.1	1,139	4,772	4.2
		10	879	980	1.1	686	3,461	5.0
		50	234	255	1.1	214	1,300	6.1
		100	138	233	1.7	131	755	5.8
cdtB-pre		5	3,060	5,375	1.8	1,139	3,851	3.4
		10	879	2,470	2.8	686	2,107	3.1
		50	234	749	3.2	214	615	2.9
		100	138	443	3.2	131	336	2.6
cdtA+cdtB-act		5	3,060	4,677	1.5	1,139	14,956	13.1
		10	879	1,781	2.0	686	7,707	11.2
		50	234	498	2.1	214	1,692	7.9
		100	138	282	2.0	131	870	6.6

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component – precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; IgG: Immunoglobulin G; IgM: Immunoglobulin M; SBR: Signal-to-background ratio;*

6.5.1.8 Detection antibody concentrations for IgG assay: all binary toxin analytes

Due to the lack of linearity observed in the IgG assay in the previous optimisation, detection antibody concentration was decreased from 1.0 to 0.4 and 0.1 µg/ml. An optimum detection antibody concentration of 0.1 µg/ml using a sample dilution factor of 1:500 delivered the highest SBR (Table 6.22; highlighted in orange). No apparent benefits were found with the use of the intermediate 0.4 µg/ml concentration, with varying dilutions delivering the highest SBR (Table 6.22; highlighted in purple).

6.5.2 Assay qualification

In order to ensure that observations using samples CDN 0527 and CDN 0529 would be representative of the study cohort, and to ensure that the set conditions were sufficiently robust and reproducible for the downstream steps, an additional run with 20 clinical samples was conducted using the optimum conditions achieved for both IgG and IgM assays with the binary toxin analytes.

All samples provided minimum no-coat signals across both IgG and IgM assays (Table 6.23). For the calculation of general no-coat background levels the mean signal was taken for each assay (125 ECL units for IgG with all binary toxin analytes, 120 ECL units for IgM with cdtA and 119 ECL units for IgM with all other binary toxin analytes, respectively), to which was added 3 standard deviation units (SD = 114, 63 and 69, respectively), giving hypothetical cut-off points of 467, 308 and 325 ECL units, respectively. Therefore, any samples whose raw signals were below these hypothetical cut-off points would not be treated as meaningful for the analysis (Table 6.23; highlighted in orange), whereas signal intensities higher than that would be assumed to be representative of a positive response to the analyte. Our rate was ~8%, which is consistent with that expected when assuming a Gaussian distribution (5%).

Table 6.22 – Overview of SBR values for IgG assay with all binary toxin analytes using detection antibody concentrations of 0.4 and 0.1 µg/ml

Binary epitope	Coating conc. (ug/ml)	Detection conc. (ug/ml)	Dilution	Signal		SBR
				No coat	Coat	
cdtA	0.04	0.1	500	455	1,348	3.0
			1,000	239	573	2.4
			2,000	153	336	2.2
			4,000	107	197	1.8
		0.4	500	466	1,174	2.5
			1,000	236	548	2.3
			2,000	167	339	2.0
			4,000	111	191	1.7
cdtB-act	0.1	0.1	100	1,790	2,502	1.4
			200	933	1,267	1.4
			500	388	569	1.5
			1,000	232	301	1.3
		0.4	100	2,763	3,406	1.2
			200	1,030	1,547	1.5
			500	466	660	1.4
			1,000	236	344	1.5
cdtB-pre		0.1	100	1,790	5,691	3.2
			200	933	2,954	3.2
			500	388	1,474	3.8
			1,000	232	655	2.8
		0.4	100	2,763	8,866	3.2
			200	1,030	3,852	3.7
			500	466	1,790	3.8
			1,000	236	807	3.4
cdtA+cdtB-act		0.1	100	1,790	2,991	1.7
			200	933	1,444	1.5
			500	388	665	1.7
			1,000	232	376	1.6
		0.4	100	2,763	4,624	1.7
			200	1,030	2,146	2.1
			500	466	867	1.9
			1,000	236	458	1.9

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component–precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; SBR: Signal-to-background ratio;*

Table 6.23 – Overview of samples in relation to hypothetical cut-off points across all binary toxin analyte assays

Sample	IgG					IgM					
	No coat	cdtA	cdtB-act	cdtB-pre	cdtA+cdtB-act	No coat (cdtA)	No coat (other)	cdtA	cdtB-act	cdtB-pre	cdtA+cdtB-act
CDN 0589	78	637	388	2,371	900	53	56	217	130	353	222
CDN 0594	109	472	226	665	294	153	61	331	133	382	235
CDP 0001	239	5,404	267	1334	529	124	143	1,117	309	907	552
CDP 0022	164	563	16,101	11,920	15,197	66	63	305	977	1,095	1,333
CDP 0006	128	641	250	1,472	5,421	115	126	212	166	296	202
CDP 0069	168	553	327	903	420	299	315	733	397	767	521
CDA 1128	65	617	189	1,028	491	66	73	561	150	487	394
CDA 1129	69	218	242	545	235	89	104	282	156	318	219
CDA 1131	63	675	454	931	802	59	59	575	166	372	479
CDA 1132	112	1,360	1,475	4,543	791	140	140	472	342	705	293
CDA 1133	91	797	185	772	304	102	115	425	181	355	295
CDA 1134	65	480	399	796	496	131	68	722	258	995	439
CDP 0065	570	976	1,119	2,309	1,229	123	119	435	261	574	423
CDP 0014	89	2,788	419	3,994	1,892	100	108	1,461	675	1,558	1,287
CDP 0016	76	1,148	740	2,191	1,078	92	94	1,003	299	1,055	536
CDP 0017	67	1,006	480	1,829	724	78	82	657	186	720	389
CDP 0019	77	480	239	986	434	131	133	606	219	669	508
CDP 0023	99	652	4,396	5,613	3,663	232	252	574	703	960	774
CDP 0024	67	318	282	898	289	58	62	322	128	387	204
CDP 0030	108	2,225	706	2,946	1,409	197	208	3,217	501	3,717	2,755

cdtA: Clostridium difficile binary toxin A; cdtB-act: Active Clostridium difficile binary toxin B; cdtB-pre: Clostridium difficile binary toxin B component – precursor; cdtA+cdtB-act: *Clostridium difficile binary toxin A plus active Clostridium difficile binary toxin B; IgG: Immunoglobulin G; IgM: Immunoglobulin M;*

6.5.3 Typing of samples from the entire patient cohort

Although four combinations of candidate epitopes were tested, only two were selected for typing of the cohort: cdtA and precursor cdtB. *C. diff* binary toxins require the coupling of the catalytic cdtA component with its mature and larger cdtB counterpart in order to become functional. cdtB is the crucial binding domain and is activated by enzymes present in the gut environment shortly before its connection to cdtA and to the host target receptor (lipolysis-stimulated lipoprotein receptor; LSR) (Papatheodorou *et al*., 2011). However, freely available activated cdtB is thought to be short-lived and therefore less immunogenic than precursor cdtB. Furthermore, the manipulation of *C. diff* binary toxin complex is still an evolving field and a difficult procedure to control *in vitro* as the optimum proportion of cdtA binding in relation to cdtB is not well defined.

Consistent with the information described above, our optimisation results illustrated in Table 6.23 demonstrate that higher percentage of samples in the cdtA and cdtB-pre assays are able to be distinguished from the hypothetical calculated cut-off. Hence, the selection of cdtA and precursor cdtB as preferred analytes, as opposed to activated cdtB or the combination of activated cdtB + cdtA, was justified for the typing of the cohort.

Final assay protocols can be seen in Appendix 45. As with the tcdA and tcdB assay, 3 sample controls (CDA 1004, PHR and C1) were used throughout the development process to account for inter-plate variability. For each control sample, a correction factor was derived from the overall plate runs and then applied across the individual plates. Furthermore, of the 88 clinical samples tested on each plate, 8 samples were tested in duplicate in order to monitor intra-plate variability.

6.6 Conclusions

There is a substantial need for novel and better assays that are able to quantify host response against *C. diff* toxins. Using the advanced technologies of the MSD ECL platform combined with a systematic development protocol and the use of fully characterised epitopes, some of them uniquely sourced, enabled the successful development of enhanced quantitative assays for the candidate epitopes tested. Notably, it was also possible to deliver novel assays to measure total IgG and IgM responses to *C. diff* binary toxins, something that has never been attempted.

Only one commercially available assay exists on the market (tgcBIOMICS GmbH, Bingen, Germany), but a major methodological limitation of this and in-house developed formats, including those from this study, has been the lack of validated humanised standards, both positive and negative, for the development of standard curves, ascertainment of antibody concentrations and accurate control of non-specific binding events. Hence, this restricts transferability of the assay and it is possible that the results of this work may also have been biased by the presence of some samples with a high degree of biological matrix interference, leading to relative ECL analysis being conducted on the basis of percentile categorisation.

The use of in-house standards by previous studies that allowed the absolute quantification of specific antibody responses were limited due to their reliance upon ELISA technology as opposed to the increased sensitivity associated with MSD's ECL technology employed here. The toxin spiking strategy was introduced with the aim of assessing assay specificity. While this was a worthwhile exercise, results were not fully conclusive due to the fact that spiking did not bring signals down to background levels (especially for the IgG assay with tcdA and tcdB). Accurate determination of matrix background levels of an assay can only be undertaken through the use of true negative controls free from anti-toxin antibodies. In this sense it is understood that most individuals at some point in their lives would have been exposed to *C. diff* and its toxins and therefore some degree of response would be expected. Therefore,

selective immunodepletion of a human serum pool using the toxin epitopes would constitute a potential strategy to address this problem but not without its drawbacks, such as the need for unattainable quantities of material (both samples and toxin epitopes) and the development of a lengthy methodological protocol. Through collaborative efforts, we are currently obtaining complimentary data regarding neutralisation of tcdA and tcdB. Samples with high anti-toxin titres would be expected to neutralise the toxins, whilst samples with low titres may not achieve neutralisation. Whilst research is still ongoing, initial results are promising.

There is a great application potential of these assays for a more accurate investigation of the host immune response to *C. diff* toxins during both symptomatic and asymptomatic phases. As long as the general limitations of the methodology and clinical background are understood, this work provides a robust platform for generating further research opportunities, directly benefiting both observational and interventional studies, including monitoring of CDI patients during vaccine trials.

Details on typing of the patient cohort using the developed assays described above can be found in Chapter 8.

Chapter 7

Investigating the role of mannose-binding lectin in *Clostridium difficile* infection

Contents

7.1 Introduction

The initiation and propagation of inflammatory cascades is an essential housekeeping property of the innate immune response during infections. The success of the immune response depends upon a network of cellular and humoral factors including effector mechanisms that range from innate immune cells (e.g. epithelial cells and phagocytes) to innate immune soluble factors (e.g. cytokines, coagulation factors and soluble pattern recognition molecules). MBL, a key pattern recognition molecule, activates the lectin-complement pathway of innate immunity through binding to repetitive sugar arrays on microbial surfaces (Turner, 2003). MBL is also a potent regulator of inflammatory pathways: it can modulate phagocyte interaction with mucosal organisms at the site of infection (Super *et al*., 1989), and interacts with other components of the innate immune system such as toll-like receptors (Wang *et al*., 2011).

Low MBL concentrations have been associated with increased susceptibility to infections in both animal models and humans (Møller-Kristensen *et al*., 2006; Shi *et al*., 2004), as well as with poor disease prognosis (Turner, 2003). The modulation of disease severity is partly thought to be through a complex, dose-dependent influence on cytokine production (Jack *et al*., 2001a). Serum MBL concentrations range from negligible to as high as 10,000 ng/ml (Madsen *et al*., 1998; Osthoff and Trendelenburg, 2013; Steffensen *et al*., 2000); this varies with ethnicity and with the screening method adopted (Harrison *et al*., 2012). Within healthy Whites the median concentration has been shown to be 800-1,000 ng/ml (Hansen *et al*., 2003; Steffensen *et al*., 2000).

Despite the existence of two sets of MBL genes in the mammalian genome (*MBL1* and *MBL2*), only *MBL2* remains functional in humans and the implications of this evolutionary loss are unclear (Sastry *et al*., 1989). MBL secretion in humans is dependent on the *MBL2* genetic architecture (Guo *et al*., 1998; Sastry *et al*., 1995). To date, 57 genetic variants have been identified within the entire *MBL2* gene (SNP database, Build 140), with only six of them known to affect secretion and/or function of the encoded protein (Figure 7.1) (Madsen *et al*., 1995; Madsen *et al*., 1998).

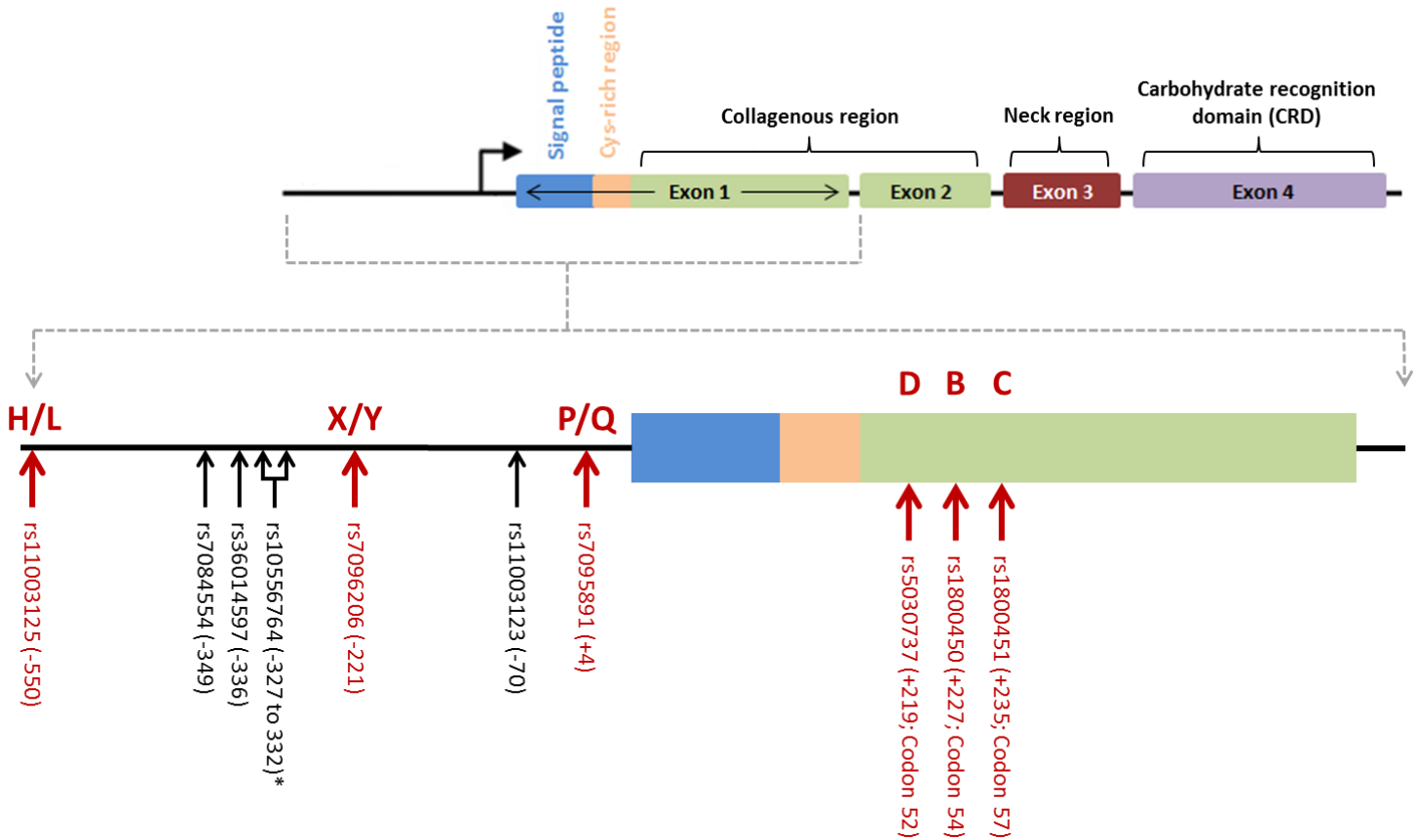


Figure 7.1 – Schematic representation of the major *MBL2* isoform and genetic polymorphisms.

*Polymorphisms responsible for the haplotypes that ultimately determine MBL expression levels are indicated by the red arrows. [*In this study, rs10556764 (6 bp deletion) was used as a proxy SNP for rs7095891]*

The mutated alleles *B*, *C* or *D* are collectively termed *O* and their correspondent wild-type alleles are jointly referred to as variant *A*, with the presence of any given *O* variant (in either the heterozygous or homozygous state) resulting in MBL deficiency (Madsen *et al*., 1995; Madsen *et al*., 1998). The existence of strong linkage disequilibrium (LD) between the promoter and structural gene variants means that only seven common haplotypes (out of a possible 64) have been described: *HYPA*, *LYQA*, *LYPA*, *LXPA*, *HYPD*, *LYPB* and *LYQC* (Bernig *et al*., 2004; Garred *et al*., 2006). *HYPD*, *LYPB* and *LYQC* lead to the production of unstable ligands with shorter half-lives that are easily degraded to lower oligomeric forms. Studies that have evaluated both genetic mutations and serum concentrations in White adults are summarised in Table 7.1.

Clostridium difficile is an opportunistic spore-forming bacterium that can effectively colonise the intestinal tract following antibiotic-driven dysbiosis. *Clostridium difficile* infection (CDI) is the result of intense colonic inflammation caused by the release of potent enterotoxins. Though research into both diagnostic/prognostic biomarkers for CDI is limited, investigations have largely focused on mediators of inflammation in the gut such as faecal interleukin-8 (El Feghaly *et al*., 2013), lactoferrin (El Feghaly *et al*., 2013) and calprotectin (Shastri *et al*., 2008), and linked them with disease severity (El Feghaly *et al*., 2013; Rao *et al*., 2014). More recently, both serum interleukin-23 and procalcitonin have also been proposed as potential biomarkers for CDI severity (Buonomo *et al*., 2013; Rao *et al*., 2013). However, the role of these biomarkers in the stratification of problematic CDI patients remains unclear, and thus remains an important area of research.

To date, there have been no studies on the role of either MBL levels or *MBL2* genetic variants with CDI, possibly because MBL is not thought to bind to the surface of *C. difficile* (Townsend *et al*., 2001). However, there is growing evidence for an association between MBL and major modulators of inflammation, such as toll-like receptors and CRP, both of which have been associated with CDI (Eyre *et al*., 2012b; Ryan *et al*., 2011). Therefore, we sought to investigate the role of MBL in a prospective cohort of CDI cases and inpatient controls.

Table 7.1 – Disease-related studies investigating both *MBL2* genotypic data and protein concentrations of MBL in White adults

Study	Country	Disease	n*	Association with outcome
Infection-related conditions				
Garred *et al*. 1997	Denmark	HIV	96	Yes (G)
Garred *et al*. 1999	Denmark	Infection in SLE patients	91	Yes (G)
Soborg *et al*. 2003	Denmark	Tuberculosis	59	Yes (G)
Yang *et al*. 2003	Australia	Chronic obstructive pulmonary disease	82	Yes (G)
Bouwman *et al*. 2005	Netherlands	Severe infection post-liver transplant	49	Yes (G)
Druszczyńska *et al*. 2006	Poland	Tuberculosis	108	Yes (P)
Eisen *et al*. 2006	Australia	Sepsis	170	Yes (G+P)
Gordon *et al*. 2006	United Kingdom	Sepsis	80	Yes (G+P)
Perez-Castellano *et al*. 2006	Spain	Community-acquired pneumonia	97	No
Louropoulou *et al*. 2008	Netherlands	Periodontitis	92	No
Van Till *et al*. 2008	Netherlands	AYI in secondary peritonitis patients	88	Yes (G+P)
Ampel *et al*. 2009	USA	Coccidioidomyosis	38	Yes (P)
Harrison *et al*. 2012	United Kingdom	Aspergillosis	108	Yes (G+P)
Navratilova *et al*. 2012	Czech Republic	Prosthetic Joint Infection	92	Yes (G)
Wong *et al*. 2012	Sweden	Infection in neutropaenic chemotherapy patients	108	No
Chalmers *et al*. 2013	United Kingdom	Infection in bronchiecstasis patients	470	Yes (G+P)
Osthoff *et al*. 2013	Australia	*Staphylococcus Aureus* infection	70	Yes (G+P)

Table 7.1 (continued) – Disease-related studies investigating both *MBL2* genotypic data and protein concentrations of MBL in White adults

Study	Country	Disease	n*	Association with outcome
Other conditions				
Garred *et al*. 2000	Denmark	Rheumatoid arthritis	189	Yes (G)
Megia *et al*. 2004	Spain	Gestational diabetes mellitus	105	Yes (G+P)
Seibold *et al*. 2004	Switzerland	Inflammatory bowel disease	76	Yes (G+P)
Kamesh *et al*. 2007	United Kingdom	ANCA-associated vessel vasculitis	137	No
Nielsen *et al*. 2007	Denmark	Crohn's disease	171	No
Swierzko *et al*. 2007	Poland	Reproductive tumours	183	No
Christiansen *et al*. 2009	Denmark	Recurrent late pregnancy loss	75	Yes (G)
Kaunisto *et al*. 2009	Finland	Diabetes	1064	No
Hoffmann *et al*. 2010	Germany	Inflammatory bowel disease	181	No
Troelsen *et al*. 2010a	Denmark	SLE	41	Yes (G+P)
Troelsen *et al*. 2010b	Denmark	Rheumatoid arthritis	114	Yes (P)
Troelsen *et al*. 2010c	Denmark	Rheumatoid arthritis	229	Yes (G+P)
Kiseljaković *et al*. 2014	Bosnia-Herzegovina	Postmenopausal osteoporosis	37	No

n: number; HIV: Human Immunodeficiency virus; SLE: Systemic lupus erythematosus; AYI: Abdominal yeast infection; ANCA: Anti-neutrophil cytoplasmic antibody; G: Genetic; P: protein

**This refers to total number of White patients differs with both serum concentration & genotypic data and therefore may differ from total study number*

7.2 Methods

7.2.1 Study design

Recruitment of patients was conducted using the criteria defined in Chapter 2. 308 CDI cases and 145 AAD controls were recruited from July 2008 to March 2012, of whom 98% were Whites. As well as case versus control analysis, four primary CDI disease outcomes were also investigated: 90-day recurrence, 30-day mortality and prolonged disease (defined as per Chapter 3 Section 3.2.1) and disease severity at baseline (defined as per Chapter 4 Section 4.2.1).

7.2.2 Determination of MBL serum concentrations

Serum was isolated from whole blood via centrifugation at 2,600 g for 20 minutes and then stored at -80°C until further use.

A commercially-available IVD ELISA kit (Sanquin Blood Supply; Amsterdam, Netherlands) was transferred onto the MSD ECL-based platform, undergoing appropriate optimisation prior to use (see Appendix 46), which adhered to the standards outlined in Chapter 6. The MBL kit control was used across all plates to determine inter-plate variability and subsequent correction factor used for each plate. Final minimum detection level (lower limit of detection; LLOD) and minimum quantification level (lower limit of quantification; LLOQ) were calculated by taking the mean values across all plates. The mean LLOD and LLOQ across all plates were 11.3 and 11.0 ng/µl, respectively.

Signal values ranged from only 50-500 ECL units, which denote a compressed signal range inherent with the assay. To counteract this effect, an attempt was made to increase the upper range of the standard curve and subsequently adopt a lower sample dilution. However, this proved unfeasible due to the unavailability of higher standards and technical impracticalities of using standalone recombinant MBL protein. Since this may have potentially limited discrimination of the quantitative values, data were subject to binary categorisation based on three previously used deficiency cut-offs: 50, 100 and

500 ng/ml (Eisen *et al*., 2008; Gröndahl-Yli-Hannuksela *et al*., 2013; Seibold *et al*., 2004).

7.2.3 Determination of *MBL2* variants

DNA was extracted from human blood samples using Chemagen paramagnetic bead chemistry (Chemagen Biopolymer-Technologie AG; Baesweiler, Germany) according to manufacturers' protocol. A total of nine variants lying in the promoter and exon 1 were typed (Figure 7.1) by either pyrosequencing (PyroMark Q96 custom assays, Qiagen; rs36014597, rs7084554, rs1800451, rs1800450, rs5030737 and rs10556764) or Taqman SNP genotyping (Applied Biosystems; rs7096206, rs11003125 and rs11003123). The variants rs1800451 (*C*), rs1800450 (*B*), rs5030737 (*D*), rs7096206 (*X/Y*) and rs11003125 (*H/L*) were used for haplotype determination, whilst rs10556764, a 6bp Ins/Del in complete linkage disequilibrium with rs7095891 *(P/Q),* was used as a proxy. Another recognised tagging marker for *P/Q* (rs11003123) was independently typed to evaluate the accuracy of the pyrosequencing assays.

7.2.3.1 Pyrosequencing

PyroMark Assay Design software v.2.0 was used to design our pyrosequencing assays. Assay 1 targeted rs1800451, rs1800450 and rs5030737 (exonic mutations). Assay 2 targeted the deletion, rs10556764, as well as rs36014597 and rs7084554. Primers and probes are summarised in Table 7.2.

PCR optimisation was conducted using 20 ng genomic DNA and temperature gradients following standard guidelines, with PCR products assessed using an Agilent Bioanalyser 2100 (Agilent Technologies; Berkshire, UK). Optimised PCR reactions for both Assays 1 and 2 comprised 15 µl as follows: -

- 2x PCR mastermix – 7.5 µl
- 10 x Primer set – 1.5 µl
- Distilled water – 5 µl
- DNA - 1µl (at 20 ng/µl)

Final cycling conditions were as follows: -

- 95°C for 3 mins
- 40 cycles
    - 95°C for 20 s
    - Assay 1: 58°C for 30 s/Assay 2: 61°C for 30 s
    - 72°C for 30 s
- 72°C for 5 mins

Optimised products were then run on a PyroMark Q96 ID following the recommended assay protocol. Repeat samples and blanks were included for quality control (QC) purposes and results were analysed using PyroMark Q96 v.2.5.8 software.

7.2.3.2 Taqman genotyping

rs7096206 and rs11003123 were genotyped using off-the-shelf SNP genotyping assays, whilst rs11003125 was genotyped using a custom SNP genotyping assay (Applied Biosystems, USA) as per Michaud *et al*. (Michaud *et al*., 2013). The primers and probes are summarised in Table 7.2. Final reactions for all three SNPs comprised 6 µl as follows: -

- 2 µl of 1x Taqman genotyping master mix
- 0.13 µl of 1x Taqman genotyping assay
- 2.87 µl of distilled water (dH_2O)
- 20 ng of dried genomic DNA

Reactions were run on the Applied Biosystems HT 7900 Fast Real-Time PCR system (Applied Biosystems, USA) using the following cycling conditions: -

- Stage 1: 50°C for 2 min
- Stage 2: 95°C for 10 min
- Stage 3: 45 cycles
    - 95°C for 15 s
    - 60° for 60 s

Repeat samples and blanks were incorporated for QC purposes, and results were analysed using SDS software (version 2.2; Applied Biosystems, USA).

Table 7.2 –Primers and probes used for determination of *MBL2* variants

Pyrosequencing	
Assay 1	Forward: 5'-ATGGTGGCAGCGTCTTACTC-3'
	Reverse: 5'-Biotin- ACAGAACAGCCCAACACGTA – 3'
	Sequencing primer: 5'-TTCCCAGGCAAAGAT-3'
Assay 2	Forward: 5′-Biotin- TCAGCTGCCCAGATACAAAGATG-3'
	Reverse: 5′-AATGAGTGGAAACCCAGGTGTCT-3'
	Sequencing primer: 5'- CCCAGGTGTCTGTAGG -3'
Taqman SNP genotyping	
rs11003125	Forward: GGAGTTTGCTTCCCCTTGGT
	Reverse: GGGCCAACGTAGTAAGAAATTTCCA
	Reporter 1 (VIC): CAAGCCTGTGTAAAAC
	Reporter 2 (FAM): CAAGCCTGTCTAAAAC

7.2.4 Statistical analysis

Median MBL serum concentrations were compared for individual SNPs and haplotypes by the Mann-Whitney U-test, and then subjected to stratification based upon previously used two-marker grouping profiles termed high- (*YA/YA* & *XA/YA*), intermediate- (*XA/XA* & *YA/YO)* and low-expressing (*XA/YO* & *YO/YO)* genotypes (Chalmers *et al*., 2013; Eisen *et al*., 2008).

The effect of both *MBL2* genetics (based on stratified expression genotypes) and serum MBL concentrations (based upon deficiency cut-offs) were individually taken forward for case-control comparison and sub-group analysis of cases. For the latter, this included logistic regression for the following outcome measures: A) severity of disease, B) duration of symptoms longer than 10 days, C) 90-day recurrence, and D) 30-day mortality. Covariates including demographic variables, the presence of PCR ribotype 027/NAP/BI1 and potential

confounders (immunosuppressive therapy, renal disease and diabetes, CCI score and time delay between sample testing positive and recruitment) were individually assessed. CCI was originally developed without adjustment for age (Charlson *et al*, 1987), therefore as age was already included as an individual covariate in our analysis, we calculated our CCI unadjusted for age in order to avoid introducing an undesirable level of collinearity into our analysis. This is consistent with previous studies (Boone *et al*., 2014; Caplin *et al*., 2011; Daskivich *et al*., 2014). Although an outcome measure itself, severity of disease was also assessed as a covariate for all other CDI outcomes. Statistically significant covariates were added to the final regression model to produce adjusted P-values, odds ratios and 95% confidence intervals. All analyses were carried out using SPSS v.20. Retrospective power calculations were simulated using nQuery Advisor + nTerim 2.0 (Statistical Solutions Ltd., Cork, Ireland).

7.3 Results

7.3.1 Patient demographics

Demographics of the patient cohort are summarised in Table 7.3. No significant differences were observed between CDI cases and AAD controls for gender (57% female for both; p=1.00), presence of immunosuppression (17 versus 24%; p=0.07), renal comorbidity (51 versus 57%; p=0.27) or diabetes (19 versus 27%; p=0.06), and mean CCI score (1.0 versus 1.0; p=0.44). However, significant differences were identified for mean age (70.1 versus 65.0 years; p<0.01), mean BMI (24.6 versus 26.9; p<0.01) and median time delay between testing positive and recruitment (3.0 versus 2.0 days; p<0.01). *C. diff* isolates were successfully recovered from 283 (92%) of the CDI cases, of which all were toxigenic and 89 (31%) had the ribotype 027.

The proportion of patients suffering from symptoms of 10 or more days was higher amongst CDI cases compared with controls (39 versus 12%; p<0.01). This difference was also significant when considering durations of symptoms as measured from initial onset of symptoms (60 versus 24%; p<0.01). Of the CDI cases, 41% (127/308) were assessed as having severe disease, while 38%

(83/220) of cases experienced recurrent episodes during the 90-day follow-up period.

Table 7.3 – Demographics of patients with *Clostridium difficile* infection (CDI) and antibiotic-associated diarrhea (AAD)

Patient's characteristics	CDI Cases (n=308)	AAD Controls (n=145)	P-value*
Gender – Female, n (%)	177/308 (57)	81/142 (57)	1.00
Age – Mean in years (SD)	70.1 (16.4)	65.0 (17.6)	<0.01
BMI – Mean (SD)	24.6 (6.8)	26.9 (6.9)	<0.01
Presence of immunosuppression – n (%)	52/307 (17)	35/144 (24)	0.07
Presence of renal comorbidity – n (%)	157/307 (51)	82/144 (57)	0.27
Presence of diabetes – n (%)	58/307 (19)	39/144 (27)	0.06
CCI score – Median (IQR)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.44
Time delay (testing/recruitment) – Median (IQR)	3.0 (2.0-4.0)	2.0 (2.0-3.0)	<0.01
Clinical Parameters			
Duration of symptoms ≥10 days – n (%)[a]	107/271 (39)	15/125 (12)	<0.01
All-cause mortality within 30 days – n (%)	26/305 (9)	5/142 (4)	0.07
Disease severity at baseline – n (%)	127/308 (41)	-	-
Recurrence within 90 days – n (%)[b]	83/220 (38)	-	-
Frequency of ribotype 027 – n (%)[c]	89/283 (31)	-	-

%: percentage; AAD: Antibiotic-associated diarrhea; BMI: body mass index; CCI: Charlson Comorbidity Index; CDI: Clostridium difficile infection; IQR: Interquartile range; n: number; SD: Standard deviation;

**Means for normally distributed, continuous variables were compared using Independent samples T-test for continuous, for non-normal distribution median values were compared using Mann Whitney U test. Categorical data was assessed using a Chi-squared test for all counts >5, and Fisher's Exact test for those <5;*

[a] Data regarding duration of symptoms was unavailable for 37 of our cases and 20 of our controls; [b] Data regarding recurrence of disease within 90 days was unavailable for 60 of our cases. A further 28 cases died within the follow-up period prior to experiencing any recurrent symptoms and therefore could not be included in the final analysis; [c] Isolates were successfully recovered from 283/308 cases and thus ribotyping could not be done in 25 of our cases;

7.3.2 Power calculations

Power to detect a significant difference was calculated as ≥99% for Case versus control and 90-day recurrence analyses. However, for analysis of 30-day mortality, prolonged symptoms and disease severity at baseline we had inadequate power (67, 78 & 75%, respectively; Table 7.4).

Table 7.4 - Assessment of power across clinical outcome associated with *Clostridium difficile* infection

Disease outcome	Number	Percentage power achieved
Case versus control	305 vs. 142	99
30-day mortality	26 vs. 276	67[a]
Prolonged symptoms	107 vs. 161	78[b]
90-day recurrence	81 vs. 136	99
Disease severity	125 vs. 180	75[c]

Achieving 80% power would require the following number of patients in each group: [a] 66; [b] 134; [c] 166;

7.3.3 Relationship of genotype with serum MBL concentrations

Where appropriate, genotype frequency data were compared against those of a recent genome-wide association study (GWAS) involving a subset of these patients to identify any potential discrepancies. All duplicates were found to be concordant within and across genotyping platforms. A selection of pyrosequencing outputs can be seen across Appendices 47-51.

Of the 9 variants typed in the CDI cases and AAD controls, 3 were excluded: 1 SNP (rs7084554) deviated from Hardy-Weinberg Equilibrium (HWE <0.001); rs11003123 was deemed redundant due to complete LD with the INS/DEL polymorphism (rs10556764); and rs36014597 was also in complete LD with

both rs10556764 and rs11003123. As mentioned previously, rs11003123 was employed as a QC proxy for the pyrosequencing of the deletion polymorphism and therefore complete LD was expected. Conversely, rs36014597 had not been confirmed to be in LD with rs10556764, nor *P/Q*. Of the 6 polymorphisms analysed, genotyping success rate was ≥95%. Their minor allele frequencies were in line with those reported in the literature (Table 7.5).

For both groups, seven common haplotypes were derived from the 6 polymorphisms (Figure 7.2), which is consistent with other previous studies in Whites (Table 7.6) (Adamek *et al*., 2013; Steffensen *et al*., 2000). Presence of the mutant allele for all individual *MBL2* variants had a significant influence on serum MBL concentration across all patients, except for the *X* allele encoded by rs7096206 (p=0.30; Table 7.5). All the assembled *MBL2* haplotypes also significantly impacted on serum concentrations, except for haplotype *LXPA* where there was no difference compared with the overall median value (p=0.34; Table 7.6). Genotypic and haplotypic analyses demonstrated that the presence of a variant allele for any of the three exonic variants (rs1800451, rs1800450 and rs5030737) were the major contributing factors for lower MBL concentration (Tables 7.5 & 7.6).

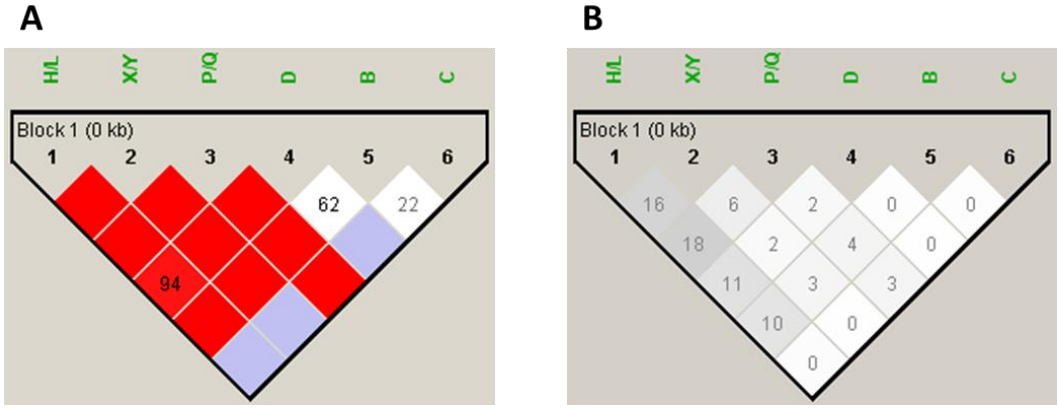


Figure 7.2 – Linkage disequilibrium plots, detailing D' (A) and R^2 (B), for the 6 *MBL2* polymorphisms known to affect protein expression levels.

Here, P/Q refers to the 6 bp deletion (rs10556764) used as a proxy SNP in this study.

Table 7.5 – Overview of the six *MBL2* variants employed for the haplotype construction and association with MBL concentrations

	rs11003125	**rs10556764**	**rs7096206**	**rs5030737**	**rs1800450**	**rs1800451**
Nucleotide change	-550 G>C	-327 to -332 CTCTTT/-	-221 G>C	+219 G>A	+227 C>T	+235 C>T
Haplotype component	*H/L*	Ins/Del (*P/Q* proxy)	*X/Y*	Codon 52 (*D*)	Codon 54 (*B*)	Codon 57 (*C*)
Minor allele	*H*	Del	*Y*	*D*	*B*	*C*
MAF	0.36	0.20	0.23	0.07	0.14	0.01
Median for presence of minor variant: ng/ml (n)	537.5 (262)	503.2 (158)	396.1 (185)	158.3 (58)	73.8 (113)	51.0 (12)
Median for absence of minor variant: ng/ml (n)	223.1 (180)	330.6 (265)	376.8 (256)	483.8 (373)	578.5 (315)	419.7 (419)
P-value*	<0.001	<0.001	0.30	<0.001	<0.001	<0.001

*MAF = Minor Allele Frequency; n: Number; *P-value was calculated using a Mann-Whitney U test comparing median concentrations for presence versus absence of the minor variant of each individual SNP, across all patients (cases and controls combined);*

Table 7.6 – MBL serum concentrations across *MBL2* haplotypes in patients with *Clostridium difficile* infection and antibiotic-associated diarrhea

	HYPA	LYPA	LYQA	LXPA	HYPD	LYPB	LYQC
Presence of haplotype							
n (% frequency)	213 (29)	44 (6)	143 (19)	170 (23)	55 (7)	108 (15)	11 (1)
Median, ng/ml	612	587	529	428	157	73	48
(Range)	(17 - 3,981)	(0 - 2,500)	(0 - 3,981)	(0 - 2,968)	(0 - 815)	(0 - 637)	(0 - 492)
Absence of haplotype							
n (% frequency)	198 (9)	367 (17)	268 (13)	241 (11)	356 (17)	303 (14)	400 (19)
Median: Absence, ng/ml	171	388	324	377	484	568	420
(Range)	(0 – 2,374)	(0 - 3,981)	(0 - 2,968)	(0 - 3,981)	(0 - 3,981)	(0 - 3,981)	(0 - 3,981)
P-value*	<0.001	0.04	<0.001	0.34	<0.001	<0.001	0.001

*n: number; % freq.: Percentage frequency; *P-values were calculated using a Mann-Whitney test comparing MBL serum concentrations against the presence/absence of each individual haplotype;*

Patients with high-expressing genotypes had a median serum MBL concentration of 714 ng/ml, compared with 190 ng/ml with intermediate-expressing genotypes, and 32 ng/ml with low-expressing genotypes ($p<0.001$; Table 7.7; Figure 7.3A). The contribution of the *X* allele, seemingly insignificant when evaluated on an individual basis (Table 7.5), became apparent with a gradual decrease when compared with the equivalent genotypes containing the Y allele in the rank order: *XA/YA < YA/YA; XA/XA < XA/YA*, and *XA/YO < YA/YO* (Table 7.7; Figure 7.3B).

Table 7.7 – Median serum MBL concentrations across previously defined expression genotype groups*

MBL expression group	Genotype	n	Median (ng/ml)	Combined median (ng/ml)
High	YA/YA	124	854	714
	XA/YA	113	561	
Intermediate	XA/XA	16	270	190
	YA/YO	91	175	
Low	XA/YO	41	32	32
	YO/YO	26	31	

**Expression groups defined according to Eisen et al. 2008 (Eisen et al., 2008)*

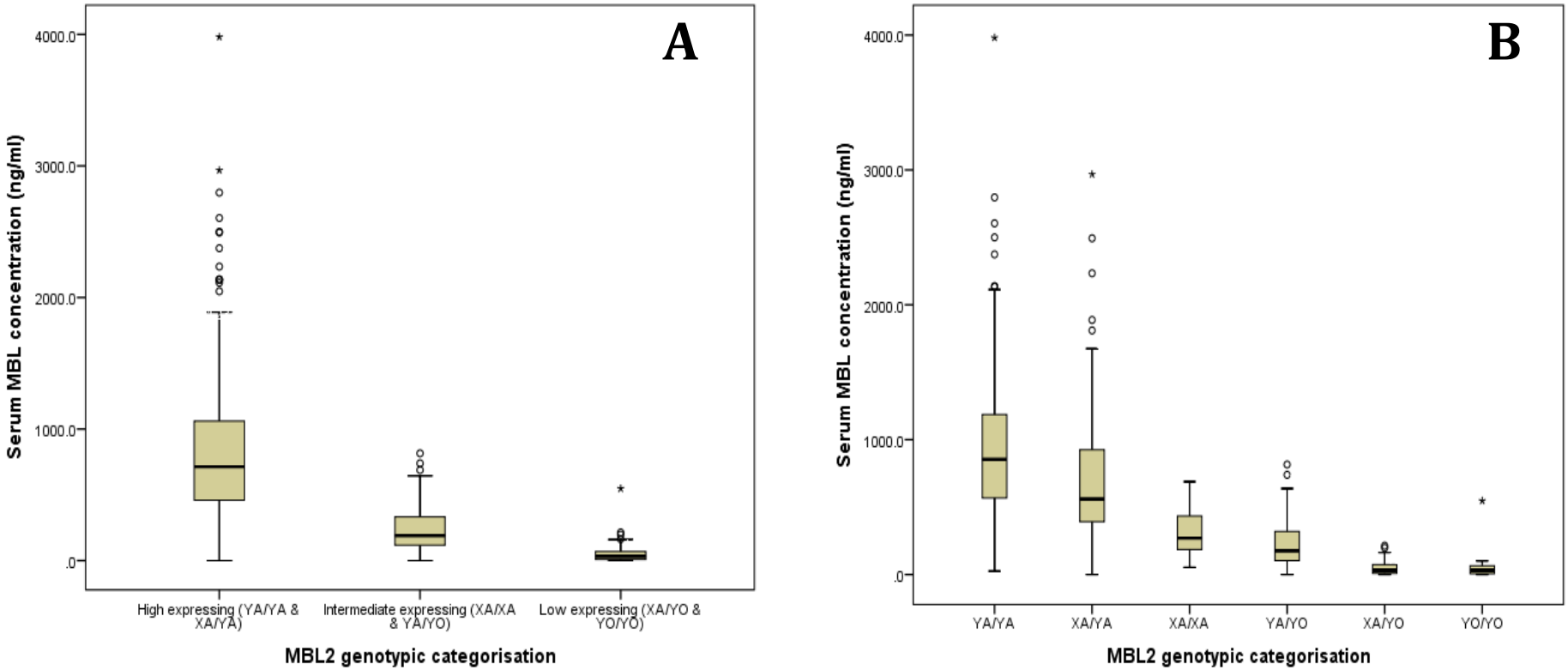


Figure 7.3 – Median serum MBL concentrations in relation to: (A) 3-tier grouping based on proposed expression profiles; and (B) individual genotypic groups within proposed expression profiles

7.3.4 MBL deficiency cut-off points in relation to haplotype groups

In total 59 (13%), 93 (21%) and 258 (58%) patients had serum MBL concentrations below 50, 100 and 500 ng/ml, respectively. When these data were compared with the "expressing" genotype groups, 78% (42/54) and 68% (59/87) of those with concentrations below 50 and 100 ng/ml, respectively, were low expressors, compared to 28% (66/236) of those with a concentration less than 500 ng/ml (Table 7.8). The corresponding figures for high expressors were 4% (2/54), 6% (5/87) and 30% (70/236), respectively. Similarly, 96% (52/54) and 93% (81/87) of those with concentrations below 50 and 100 ng/ml, respectively, carried the deficient (*O*) haplotypes, compared to 65% (153/236) of those with a concentration less than 500 ng/ml (Table 7.8). Based on the results above, only the 50 and 100 ng/ml cut-offs were taken forward for further analysis, which is consistent with previous literature (Gröndahl-Yli-Hannuksela *et al*., 2013; Seibold *et al*., 2004).

7.3.5 Comparison of MBL levels versus CDI disease outcomes

Serum MBL concentrations are shown in Table 7.9. Analysis using both <50 and <100 ng/ml as cut-off points to signify deficiency identified no significant differences between CDI cases and AAD controls (p=0.79 and p=0.09, respectively) (Table 7.10). Evaluation of the clinical outcomes in CDI cases showed a significant association with CDI recurrence (p<0.01 for both; Table 7.10) with odds ratios of 3.18 and 2.61 at the <50 and <100 ng/ml cut-off points, respectively. No association was identified with any of the other outcomes including prolonged symptoms, 30-day mortality and disease severity at baseline (Table 7.10).

In order to ensure our association for recurrence holds true for early versus late recurrence, we assessed median MBL levels across three different recurrent groups using 2-way comparisons as follows: (1) 0-30 days versus 31-90 days (2) 0-60 days versus 61-90 days. Median MBL levels did not differ significantly across comparisons (p=1.00 & p=0.37, respectively), which suggests that the association of MBL with recurrence has not been biased, by either early or late

time-points, and holds true for overall recurrence. Furthermore, although the majority of our recurrent patients were recruited as an initial infection (81%), a small number (19%) had experienced at least one episode of CDI prior to the current episode for which they were recruited; median MBL levels did not differ significantly between these two groups of recurrent patients (p=0.64).

Table 7.8 – Distribution of expression genotypes and deficiency haplotypes across three different serum MBL deficiency cut-offs*

Cut-off (ng/ml)	n (%)			
	High expressors	Intermediate expressors	Low expressors	
50 (n=54)	2 (4)	10 (18)	42 (78)	
100 (n=87)	5 (6)	23 (26)	59 (68)	
500 (n=236)	70 (30)	100 (42)	66 (28)	
	Homozygous non-deficient haplotypes	Heterozygous deficient haplotypes	Homozygous deficient haplotypes	Dominant model[a]
50 (n=54)	2 (4)	34 (63)	18 (33)	52 (96)
100 (n=87)	6 (7)	57 (65)	24 (28)	81 (93)
500 (n=236)	83 (35)	128 (54)	25 (11)	153 (65)

n: number; [a] Dominant model refers to the presence of ≥1 deficiency haplotype across both the maternal and paternal haplotypes of each patient

**The number of patients deemed high (YA/YA & XA/YA), intermediate (XA/XA & YA/YO) and low (XA/YO & YO/YO) expressors, plus the number of patients carrying either deficient (O) or non-deficient (A) haplotypes, was assessed across three individual serum MBL deficiency cut-offs.*

Table 7.9 - Descriptive MBL serum concentrations in relation to *Clostridium difficile* infection disease outcomes

	Median, ng/ml (IQR)
Case versus Control	
Case (n=308)	361.8 (128.3-747.7)
Control (n=145)	491.9 (160.0-856.0)
Death within 30 days	
Death (n=26)	330.3 (115.9-673.0)
Non-death (n=279)	372.9 (128.4-754.2)
Duration ≥10 days	
Yes (n=107)	372.1 (83.7-728.9)
No (n=161)	392.9 (148.7-819.7)
Recurrence within 90 days	
Recurrence (n=83)	196.7 (60.9-570.4)
Non-recurrence (n=137)	452.1 (169.6-844.5)
Severity at baseline	
Severe (n=127)	372.1 (128.3-728.2)
Non-severe (n=181)	354.6 (128.0-787.1)

n: number; IQR: interquartile range; Min: minimum; Max: maximum;

Table 7.10 – Analysis of *Clostridium difficile* infection disease outcomes versus serum MBL concentration based on deficiency cut-offs of 50 and 100 ng/ml

	Case (n=308)	Control (n=145)	P-value	OR (95% CI)
<50 ng/ml	41 (13%)	18 (12%)	0.79[a]	1.09 (0.58-2.06)
<100 ng/ml	70 (23%)	23 (16%)	0.09[b]	1.61 (0.93-2.79)
	Death (n=26)	**Survival (n=276)**	**P-value**	**OR (95% CI)**
<50 ng/ml	3 (12%)	37 (13%)	0.78[c]	1.22 (0.31-4.82)
<100 ng/ml	5 (19%)	64 (23%)	0.84[c]	0.88 (0.27-2.89)
	≥10 days (n=107)	**<10 days (n=161)**	**P-value**	**OR (95% CI)**
<50 ng/ml	16 (15%)	19 (12%)	0.45[d]	1.31 (0.64-2.69)
<100 ng/ml	29 (27%)	32 (20%)	0.17[d]	1.50 (0.84-2.67)
	Recurrence (n=81)	**Non-recurrence (n=136)**	**P-value**	**OR (95% CI)**
<50 ng/ml	18 (22%)	13 (10%)	<0.01[e]	3.18 (1.40-7.24)
<100 ng/ml	29 (36%)	24 (18%)	<0.01[e]	2.61 (1.35-5.04)
	Severe (n=125)	**Non-severe (n=180)**	**P-value**	**OR (95% CI)**
<50 ng/ml	16 (13%)	25 (14%)	0.78[d]	0.91 (0.46-1.79)
<100 ng/ml	29 (23%)	41 (23%)	0.93[d]	1.02 (0.60-1.76)

n: number; OR: odds ratio; CI: confidence interval;

P-values & ORs were calculated using univariate logistic regression and adjusted for the presence of significant covariates: [a] Age, BMI, time delay between testing positive and recruitment & the presence of diabetes; [b] Age, BMI, time delay between testing positive and recruitment & the presence of diabetes and immunosuppressive therapy; [c] Age, BMI, score on Charlson Comorbidity Index and disease severity at baseline; [d] No covariates were found to be significant & therefore P-value remains unadjusted; [e] Age;

Despite the strong correlation observed between genotypes/haplotypes and serum MBL concentrations in this cohort, no significant associations were identified between high-, intermediate- and low-expressing genotypes and CDI disease outcomes (Table 7.11). There was an inverse correlation between MBL and CRP serum concentrations (Pearson's Correlation Coefficient R^2=-0.16, p=0.001; Figure 7.4). No significant correlation was identified with white cell count (R^2=-0.04, p=0.44).

Table 7.11 – Analysis of *Clostridium difficile* infection disease outcomes versus high, intermediate and low expressing *MBL2* genotypes

	Case (n=308)	**Control (n=145)**	**P-value[a]**	**OR (95% CI)**
High expressing group (comparator)	165	73	0.86	-
Intermediate expressing group	75	34	0.75	0.92 (0.55-1.55)
Low expressing group	44	24	0.61	0.85 (0.47-1.56)
	Death (n=26)	Survival (n=276)	P-value[b]	OR (95% CI)
High expressing group (comparator)	14	150	0.77	-
Intermediate expressing group	8	67	0.47	1.49 (0.50-4.43)
Low expressing group	3	40	0.75	1.26 (0.31-5.03)
	≥10 days (n=102)	<10 days (n=149)	P-value[c]	OR (95% CI)
High expressing group (comparator)	60	84	0.89	-
Intermediate expressing group	27	40	0.85	0.95 (0.52-1.71)
Low expressing group	15	25	0.64	0.84 (0.41-1.73)
	Recurrence (n=78)	Non-recurrence (n=133)	P-value[d]	OR (95% CI)
High expressing group (comparator)	42	83	0.46	-
Intermediate expressing group	22	31	0.32	1.43 (0.71-2.86)
Low expressing group	14	19	0.33	1.50 (0.66-3.39)
	Severe (n=120)	Non-severe (n=185)	P-value[c]	OR (95% CI)
High expressing group (comparator)	64	101	0.33	
Intermediate expressing group	36	39	0.18	1.46 (0.84-2.53)
Low expressing group	16	28	0.77	0.90 (0.45-1.80)

n: number; OR: odds ratio; CI: confidence interval;

P-values & ORs were calculated using univariate logistic regression and adjusted for the presence of significant covariates: [a] Age, BMI, time delay between testing positive and recruitment & the presence of diabetes; [b] Age, BMI, score on Charlson Comorbidity Index and disease severity at baseline; [c] No covariates were found to be significant & therefore P-value remains unadjusted; [d] Age;

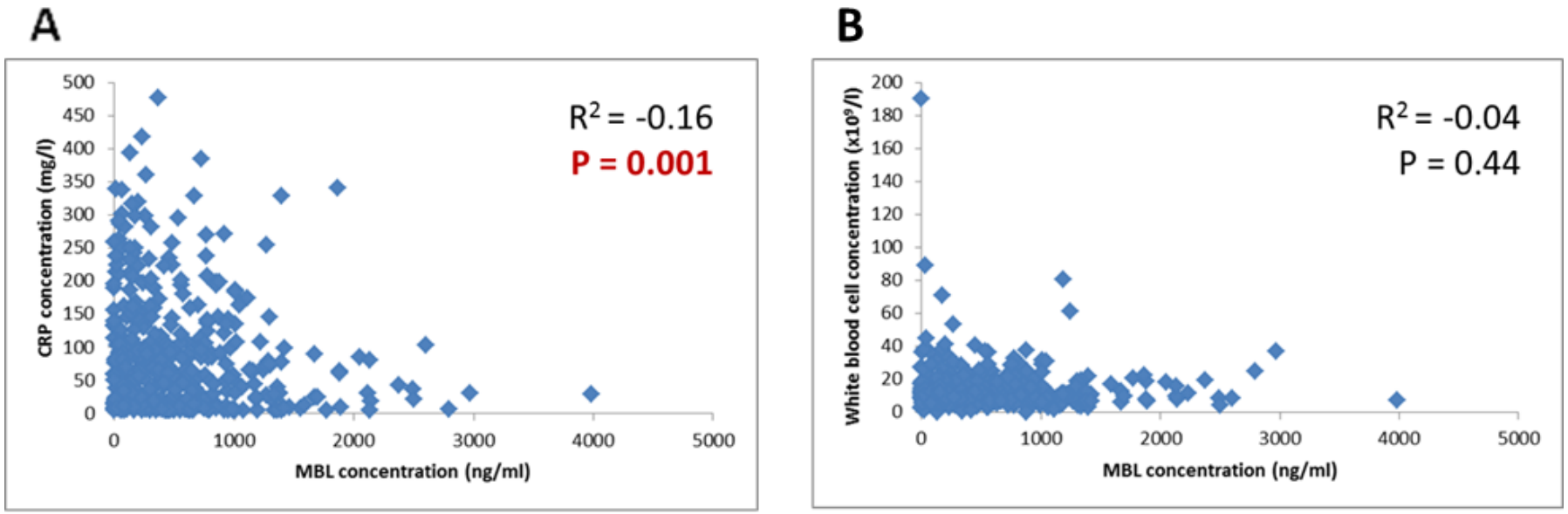


Figure 7.4 – Correlation plots comparing MBL concentrations against routine blood markers (A) CRP and (B) White blood cells

7.4 Discussion

Studies evaluating the role of MBL in infectious and immune diseases have focused on either genotype, phenotype, or occasionally on both parameters. The latter approach is preferred as it can show discordance between genotype and phenotype. This study is one of the larger disease-related studies concurrently investigating both genotypic/haplotypic variants and serum concentrations in Whites (Table 7.1) and is the first to demonstrate an association between serum MBL concentrations, but not genotype, and recurrence of CDI within 90 days using two distinct cut-off values for MBL deficiency. It was further established that this association has not been biased, by either early or late outcome onset, and holds true for overall recurrence.

The mechanistic basis of the association is unclear. With other bacterial and viral infections, MBL is thought to be capable of binding to the cell surfaces of invasive pathogens thereby stimulating a downstream immune response. However, this does not seem to be the case with *C. difficile* where binding of MBL has been shown to be low (Townsend *et al*., 2001). This suggests that MBL deficiency does not *per se* predispose to CDI and is consistent with the observed lack of difference in circulating concentrations of MBL between CDI cases and AAD controls. MBL has other functions including modulation of inflammation and clearance of apoptotic cells (Dommett *et al*., 2006). The former may be relevant to CDI, where MBL may be acting as a modulator of the disease. Consistent with this, clinical manifestations of MBL deficiency appear to be of more relevance either in infants when the immune system is still maturing or in susceptible groups when there is an associated immunodeficiency (Koch *et al*., 2001), such as in hospitalised elderly patients or following major clinical interventions. However, these are hypotheses that need further investigation.

Although MBL concentrations remain relatively constant in individuals due to genetic determinants, MBL is known to be a relatively modest acute phase reactant (Dean *et al*., 2005). This is in sharp contrast to other acute phase proteins such as CRP whose concentrations can increase sharply by 10 to 1,000-fold during acute inflammation (Ip *et al*., 2009). Elevated CRP concentrations

have previously been shown to be associated with various CDI outcomes including disease severity and recurrence (Eyre *et al*., 2012b; Khanafer *et al*., 2013). Consistent with this, low MBL concentrations have been associated with an increase in the level of CRP (Garred *et al*., 2002), and with our findings of the association with CDI recurrence and inverse correlation with CRP. In keeping with the immunomodulatory effect of MBL, it is known that low concentrations lead to increased secretion of the pro-inflammatory cytokines interleukin-6, interleukin 1-beta and TNF alpha (Garred *et al*., 2002; Jack *et al*., 2001b), all of which have also been shown to be elevated in response to CDI (Hirota *et al*., 2012; Vohra and Poxton, 2012).

The genetic architecture of the *MBL2* gene is complex (Figure 7.1) with the existence of numerous common functional polymorphisms and haplotypes (Figure 7.1, Tables 7.5-7.7). *MBL2* haplotype frequencies and the corresponding impact on serum MBL concentrations were in line with those previously reported (Madsen *et al*., 1995; Steffensen *et al*., 2000) (Table 7.6). This was also evident after stratification of MBL haplotypes based on previously defined expression genotypes (Chalmers *et al*., 2013; Eisen *et al*., 2008) with carriers of low-expressing genotypes showing much lower serum MBL concentrations than both intermediate- and high-expressing genotypes (32 ng/ml versus 190 and 714 ng/ml, respectively; Table 7.7). Despite the strong association observed between *MBL2* genotypes and serum MBL concentrations, and the association between MBL concentrations and CDI recurrence, there was no association between *MBL* genotype and CDI outcomes. Other studies have also identified associations with protein levels, but not with genotype (Table 7.1), highlighting the need to evaluate both MBL genotype and phenotype in infection and other immune conditions. The lack of association between MBL genotype and disease outcome may be due to the incomplete genetic penetrance of *MBL* genetic variation on phenotype. In this study, only 78% and 68% of the low-expressing genotypes accounted for deficient serum levels using the cut-off values of <50 and <100 ng/ml, respectively (Table 7.8). Genetic heterogeneity due to functionally related genes such as L-ficolin, MASP2, and surfactant proteins may also play a role, but this needs further investigation.

Our study sought to adhere to a stringent methodology through the use of a relatively large cohort size and extensive QC, but it is not without its limitations. Although there is less chance of MBL concentrations being confounded by infection-related events when compared to other response markers, one of the clear drawbacks of this work is the lack of longitudinal measurements, which is now being addressed in a new prospective study. The effect of proteins functionally related to MBL, and other markers of inflammation, and the relative roles they play in disease modulation needs further investigation. Previous studies have used various definitions for MBL deficiency, with commonly used cut-offs ranging from 50 (Gröndahl-Yli-Hannuksela *et al*., 2013) to 500 ng/ml (Eisen *et al*., 2008). It is thus difficult to compare results across different study groups given the heterogeneity of platforms, profile of cohorts and standards adopted for the measurement of MBL. Discrepancies between studies could be due to low sample sizes, poor assay performance and differences in techniques adopted by laboratories. We have tried to overcome some of these limitations by evaluating a number of cut-off levels but there is a need for international consensus and harmonisation in this area.

In conclusion, our data suggest that low serum MBL concentrations may act as a predictor of CDI recurrence. Further work is needed to validate these findings in an independent cohort of patients and to evaluate the mechanistic basis of this association. This area of research would also be advanced through consensus on definitions of deficiency, standardisation of methods employed for measurement of serum concentrations, and further evaluation of the genotype-phenotype relationships.

Chapter 8

Analysis of host immune response in relation to CDI primary outcomes

Contents

8.1 Introduction

The hypothesis behind the work detailed here is described in Chapter 6 Section 6.1.1. This study aimed to investigate the role of IgG and IgM responses to both tcdA and tcdB, as well as the previously uninvestigated CDT, as predictors of poor CDI outcomes using newly developed assays.

8.2 Methods

8.2.1 Study design

Recruitment of patients was conducted using the criteria defined in Chapter 2. As well as case versus control analysis, four primary CDI disease outcomes were also investigated: 90-day recurrence, 30-day mortality and prolonged disease (defined as per Chapter 3 Section 3.2.1) and disease severity at baseline (defined as per Chapter 4 Section 4.2.1).

8.2.2 Assay development & serum typing

The majority of previous studies that have quantified the serum immune response to *C. diff* toxins utilised an ELISA-based method from 1992 (Kelly *et al*., 1992). Due to the limitations surrounding ELISA-based methods, assays with increased sensitivity were developed for quantification in our patient cohort, using MSD's ECL technology (described in detail in Chapter 6). Finalised assays targeted IgG and IgM responses to tcdA and tcdB, as well as cdtA and cdtB-pre. Serum was isolated from whole blood via centrifugation at 2600 g for 20 mins and stored at -80°C until further use. Controls were included across plates to determine inter-plate variability and subsequent correction factor for each plate. Of the 88 clinical samples tested on each plate, 8 control samples were tested in duplicate to monitor intra-plate variability.

8.2.3 Statistical analysis

Toxin-specific antibody response levels were subject to 4-tier percentile categorisation (Low <25%, Medium-Low 25-50%, Medium-High 50-75% and

High >75%). Sub-group analysis was carried out based on a number of outcomes: severity of disease, 90-day recurrence, duration of symptoms and 30-day mortality, using univariate binary logistic regression. Covariates including gender, age, BMI and the presence of PCR-ribotype 027 were individually assessed via univariate binary logistic regression, as well as potential confounders of the host immune response such as immunosuppressive and antineoplastic therapy (immunosuppressants, corticosteroids and chemotherapy drugs), presence of renal disease or diabetes, time delay between sample testing positive and recruitment, and CCI score. CCI was originally developed without adjustment for age (Charlson *et al*., 1987) and therefore as age was already included as an individual covariate, CCI was calculated without adjustment for age to avoid the introduction of an undesirable level of collinearity into the analysis. This is consistent with previous studies (Boone *et al*., 2014; Caplin *et al*., 2011; Daskivich *et al*., 2011; Daskivich *et al*., 2014). Although an outcome measure itself, severity of disease was also assessed as a covariate for all other CDI outcomes.

Statistically significant covariates were added to the final regression model to produce adjusted P-values, ORs and 95% CIs. All analyses were carried out using SPSS v.20. Retrospective power calculations were simulated using nQuery Advisor + nTerim 2.0 (Statistical Solutions Ltd., Cork, Ireland). The literature lacks reliable data for conducting a priori power calculation.

8.3 Results

8.3.1 Patient demographics

Patient demographics are described in Chapter 7: Section 7.3.1 and Table 7.1.

8.3.2 Antibody response to *C. diff* toxins versus disease outcomes

Median anti-toxin IgG and IgM serum concentrations across all disease outcomes are summarised in Tables 8.2 & 8.3.

Table 8.2 – Descriptive antibody response levels to toxins A and B versus multiple CDI outcomes

	IgG/tcdA[1]		IgG/tcdB[1]		IgM/tcdA[2]		IgM/tcdB[2]	
	N	Median, ng/ml (IQR)	N	Median, ng/ml (IQR)	N	Median, ng/ml (IQR)	N	Median, ng/ml (IQR)
Case versus Control								
Case	306	43,207 (24,758-75,371)	306	18,942 (10,924-57,843)	300	21,508 (12,341-37,505)	300	5,260 (2,910-12,165)
Control	142	39,084 (23,215-66,748)	142	19,032 (11,314-38,081)	136	24,085 (15,002-42,391)	137	5,229 (2,560-8,865)
30-day mortality								
Death	26	40,834 (25,499-79,546)	26	21,456 (14,033-85,624)	26	18,785 (10,243-23,699)	26	4,580 (2,671-9,956)
Non-death	277	43,747 (24,948-72,445)	277	18,839 (10,668-52,453)	271	21,732 (13,027-38,597)	271	5,347 (3,006-12,793)
Duration of symptoms								
≥10 days	106	34,089 (23,043-57,595)	106	16,875 (9,725-40,435)	104	19,966 (11,658-32,574)	103	4,633 (2,684-12,954)
≤9 days	163	46,215 (26,825-78,806)	163	19,200 (11,518-57,751)	159	22,092 (12,188-38,339)	160	5,375 (3,141-10,711)
90-day recurrence								
Recurrence	82	39,468 (24,996-67,139)	82	19,070 (10,581-51,904)	77	21,521 (11,845-34,103)	77	4,625 (2,600-12,125)
Non-recurrence	136	47,812 (25,676-80,636)	136	21,129 (11,590-66,397)	135	22,108 (13,819-43,470)	135	5,653 (3,435-12,988)
Baseline severity								
Severe	126	31,615 (22,991-58,849)	126	16,051 (9,798-35,973)	125	20,218 (11,332-31,681)	125	4,940 (2,682-12,885)
Non-severe	180	49,031 (27,674-90,978)	180	21,313 (12,355-68,078)	175	22,617 (13,331-42,635)	175	5,424 (3,007-11,859)

IgG: Immunoglobulin G; IgM: Immunoglobulin M; IQR: Interquartile range; n: number; tcdA: Clostridium difficile toxin A; tcdB: Clostridium difficile toxin B; 1: Coating = 25.0 ug/ml; Detection = 1.0 ug/ml; Sample dilution = 1:40; 2. Coating = 25.0 ug/ml; Detection = 1.0 ug/ml; Sample dilution = 1:2;

Table 8.3 – Descriptive antibody response levels to binary toxin analytes versus multiple CDI outcomes

	IgG/cdtA[1]		IgG/cdtB-pre[2]		IgM/cdtA[3]		IgM/cdtB-pre[4]	
	N	Median, ng/ml (IQR)	N	Median, ng/ml (IQR)	N	Median, ng/ml (IQR)	N	Median, ng/ml (IQR)
Case versus Control								
Case	289	668 (386-1,086)	293	1,693 (1,077-2,805)	289	401 (246-670)	293	552 (356-949)
Control	132	592 (373-952)	134	1,755 (1,078-2,514)	132	407 (260-627)	134	553 (370-879)
30-day mortality								
Death	25	668 (395-960)	25	2,288 (1,211-3,105)	25	343 (202-681)	25	509 (229-666)
Non-death	261	668 (387-1,112)	265	1,670 (1,077-2,770)	261	408 (251-681)	265	566 (367-975)
Duration of symptoms								
≥10 days	102	699 (343-1,123)	101	1,603 (804-2,439)	102	367 (219-578)	101	477 (316-790)
≤9 days	150	668 (387-1,058)	156	1,717 (1,153-2,962)	150	442 (272-739)	156	693 (369-1,000)
90-day recurrence								
Recurrence	77	600 (389-970)	77	1,778 (1,208-2,645)	77	368 (214-583)	77	542 (344-860)
Non-recurrence	126	712 (395-1,180)	129	1,693 (1,131-2,895)	126	445 (272-701)	129	619 (381-975)
Baseline severity								
Severe	116	624 (340-881)	119	1,603 (1,047-2,739)	116	384 (247-606)	119	516 (313-874)
Non-severe	173	712 (387-1,171)	174	1,784 (1,099-2,931)	173	404 (243-702)	174	589 (364-992)

cdtA: Clostridium difficile binary toxin A; cdtB-pre: Clostridium difficile binary toxin B precursor; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IQR: Interquartile range; n: number; 1: Coating = 1.6 ug/ml; Detection = 0.1 ug/ml; Sample dilution = 1:500; 2. Coating = 4.0 ug/ml; Detection = 0.1 ug/ml; Sample dilution = 1:500; 3. Coating = 1.6 ug/ml; Detection = 0.8 ug/ml; Sample dilution = 1:50; 4. Coating = 4.0 ug/ml; Detection = 1.0 ug/ml; Sample dilution = 1:50;

Median serum concentrations of IgG against tcdA and tcdB were found to be significantly lower in patients suffering from severe CDI compared to those with mild disease (31,615 versus 49,031 ECL units (p<0.01) & 16,051 versus 21,313 ECL units (p=0.04), respectively; Tables 8.2 & 8.4). Similarly, medium serum concentration of IgM against cdtB-pre was significantly lower in patients whose symptoms lasted ≥10 days compared to those patients with symptoms ≤9 days (503 versus 696 ECL units (p=0.01); Tables 8.3 & 8.5). In relation to disease recurrence, median serum concentration of IgM against tcdB was significantly lower in patients experiencing disease recurrence within 90 days compared to non-recurrent patients (4,625 versus 5,653 ECL units (p=0.04); Tables 8.2 & 8.6).

Table 8.4 - Percentile analysis of immunoglobulin G response to *Clostridium difficile* toxins A and B: severe versus non-severe cases

IgG/tcdA	Severe (n=126)	Non-severe (n=180)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	37	39	-	-
Medium-High	22	54	0.52	1.25 (0.63-2.48)
Medium-Low	26	51	<0.01	2.80 (1.43-5.45)
Low	41	36	0.01	2.33 (1.19-4.55)
				Global p-value < 0.01
IgG/tcdB	**Severe (n=126)**	**Non-severe (n=180)**	**Adjusted P-value[a]**	**Adjusted OR (95% CI)**
High (Comparator)	40	35	-	-
Medium-High	24	52	0.53	1.24 (0.63-2.42)
Medium-Low	28	49	0.13	1.67 (0.87-3.24)
Low	34	44	0.01	2.48 (1.28-4.81)
				Global p-value = 0.04

n: number; OR: odds ratio; CI: confidence interval; IgG: Immunoglobulin G; tcdA: toxin A; tcdB: toxin B; [a] P-value and ORs remain unadjusted as no assessed covariates were found to be significant

Table 8.5 - Percentile analysis of immunoglobulin M response to *Clostridium difficile* binary toxin B-precursor: ≥10 versus ≤9 days of symptoms

IgM/cdtB-pre	≥10 days (n=164)	≤9 days (n=111)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	36	30	-	-
Medium-High	33	38	0.35	0.72 (0.37-1.42)
Medium-Low	48	20	0.06	2.00 (0.98-4.08)
Low	47	23	0.13	1.70 (0.85-3.41)
				Global p-value = 0.01

n: number; OR: odds ratio; CI: confidence interval; IgM: Immunoglobulin M; cdtB-pre: binary toxin B-precursor; [a] P-values and ORs were unadjusted as no assessed covariates were found to be significant;

Table 8.6 - Percentile analysis of immunoglobulin M response to *Clostridium difficile* toxin B: recurrence within 90 days versus non-recurrence

IgM/tcdB	Recurrence (n=77)	Non-recurrence (n=135)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	19	36	-	-
Medium-High	15	38	0.97	0.98 (0.42-2.31)
Medium-Low	16	36	0.71	0.85 (0.36-1.98)
Low	27	25	0.03	2.48 (1.09-5.61)
				Global p-value = 0.04

n: number; OR: odds ratio; CI: confidence interval; IgM: Immunoglobulin M; tcdB: toxin B; [a] P-values and ORs were all adjusted for age;

Although median serum concentrations of both IgG and IgM against all tested toxin epitopes were generally lower in those patients experiencing an unfavourable CDI outcome compared to those not, no further associations were identified (Tables 8.7-8.14). Furthermore, no significant differences in median levels were identified between CDI cases and non-infected AAD controls across any of the toxin epitopes tested (Tables 8.15 & 8.16).

Table 8.7 - Percentile analysis of immune response to *C. diff* toxins A and B: severe versus non-severe cases

IgM/tcdA	Severe (n=125)	Non-severe (n=175)	Adjusted P-value	Adjusted OR (95% CI)
High (Comparator)	34	41	-	-
Medium-High	26	49	0.32	1.40 (0.73-2.71)
Medium-Low	32	43	0.24	1.48 (0.77-2.86)
Low	33	42	0.18	1.56 (0.81-3.02)
				Global p-value = 0.55
IgM/tcdB	**Severe (n=125)**	**Non-severe (n=175)**	**Adjusted P-value**	**Adjusted OR (95% CI)**
High (Comparator)	34	41	-	-
Medium-High	32	43	0.40	0.76 (0.39-1.46)
Medium-Low	27	48	1.00	1.00 (0.52-1.91)
Low	32	43	0.74	1.11 (0.59-2.12)
				Global p-value = 0.69

CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio; tcdA: toxin A; tcdB: toxin B;

P-value and ORs remain unadjusted as no assessed covariates were found to be significant

Table 8.8 - Percentile analysis of immune response to *C. diff* binary toxin analytes A and B-precursor: severe versus non-severe cases

IgG/cdtA	Severe (n=116)	Non-severe (n=173)	Adjusted P-value	Adjusted OR (95% CI)
High (Comparator)	31	40	-	-
Medium-High	20	52	0.04	2.09 (1.05-4.17)
Medium-Low	33	41	0.04	2.08 (1.04-4.17)
Low	32	40	0.05	2.02 (1.00-4.05)
				Global p-value = 0.11

IgG/cdtB-pre	Severe (n=119)	Non-severe (n=174)	Adjusted P-value	Adjusted OR (95% CI)
High (Comparator)	31	42	-	-
Medium-High	28	45	0.56	0.82 (0.42-1.61)
Medium-Low	25	49	0.24	1.48 (0.77-2.86)
Low	35	38	0.61	1.19 (0.61-2.30)
				Global p-value = 0.35

IgM/cdtA	Severe (n=116)	Non-severe (n=173)	Adjusted P-value	Adjusted OR (95% CI)
High (Comparator)	28	43	-	-
Medium-High	25	48	0.21	1.54 (0.79-3.00)
Medium-Low	32	40	0.31	1.42 (0.73-2.77)
Low	31	42	0.52	1.25 (0.63-2.46)
				Global p-value = 0.62

IgM/cdtB-pre	Severe (n=119)	Non-severe (n=174)	Adjusted P-value	Adjusted OR (95% CI)
High (Comparator)	32	41	-	-
Medium-High	26	47	0.54	1.23 (0.63-2.40)
Medium-Low	30	44	0.39	1.33 (0.69-2.60)
Low	31	42	0.31	1.41 (0.73-2.75)
				Global p-value = 0.76

cdtA: binary toxin A; cdtB-pre: binary toxin B-precursor; CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio;

P-value and OR remain unadjusted as no assessed covariates were found to be significant

Table 8.9 - Percentile analysis of immune response to *C. diff* toxins A and B: duration of symptoms ≥10 days versus ≤9 days

IgG/tcdA	≥10 days (n=174)	≤9 days (n=114)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	38	31	-	-
Medium-High	42	32	0.84	1.07 (0.55-2.07)
Medium-Low	47	27	0.31	1.42 (0.73-2.78)
Low	47	24	0.18	1.60 (0.81-3.16)
				Global p-value = 0.47

IgG/tcdB	≥10 days (n=174)	≤9 days (n=114)	Adjusted P-value	Adjusted OR (95% CI)
High (Comparator)	41	27	-	-
Medium-High	45	30	0.97	0.99 (0.51-1.93)
Medium-Low	46	31	0.95	0.98 (0.50-1.90)
Low	42	26	0.86	1.06 (0.53-2.12)
				Global p-value = 1.00

IgM/tcdA	≥10 days (n=169)	≤9 days (n=113)	Adjusted P-value	Adjusted OR (95% CI)
High (Comparator)	39	29	-	-
Medium-High	45	30	0.75	1.12 (0.57-2.17)
Medium-Low	41	27	0.73	1.13 (0.57-2.24)
Low	44	27	0.58	1.21 (0.62-2.39)
				Global p-value = 0.96

IgM/tcdB	≥10 days (n=168)	≤9 days (n=114)	Adjusted P-value	Adjusted OR (95% CI)
High (Comparator)	41	25	-	-
Medium-High	42	30	0.65	0.85 (0.43-1.69)
Medium-Low	40	32	0.43	0.76 (0.39-1.51)
Low	45	27	0.96	1.02 (0.51-2.03)
				Global p-value = 0.81

cdtA: binary toxin A; cdtB-pre: binary toxin B-precursor; CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio;

P-values and ORs remain unadjusted as no assessed covariates were found to be significant

Table 8.10 - Percentile analysis of immune response to *C. diff* binary toxin analytes A and B-precursor: duration of symptoms ≥10 days versus ≤9 days

IgG/cdtA	≥10 days (n=163)	≤9 days (n=108)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	42	26	-	-
Medium-High	37	30	0.34	0.71 (0.36-1.43)
Medium-Low	40	27	0.62	0.84 (0.41-1.69)
Low	44	25	0.98	1.01 (0.50-2.04)
				Global p-value = 0.73
IgG/cdtB-pre	**≥10 days (n=164)**	**≤9 days (n=111)**	**Adjusted P-value[b]**	**Adjusted OR (95% CI)**
High (Comparator)	31	31	-	-
Medium-High	44	27	0.08	1.95 (0.93-4.06)
Medium-Low	45	27	0.05	2.08 (1.00-4.34)
Low	44	26	0.10	1.86 (0.89-3.86)
				Global p-value = 0.19
IgM/cdtA	**≥10 days (n=163)**	**≤9 days (n=108)**	**Adjusted P-value[c]**	**Adjusted OR (95% CI)**
High (Comparator)	37	31	-	-
Medium-High	42	27	0.45	1.30 (0.66-2.57)
Medium-Low	40	30	0.75	1.12 (0.57-2.19)
Low	44	20	0.09	1.84 (0.90-3.76)
				Global p-value = 0.37

cdtA: binary toxin A; cdtB-pre: binary toxin B-precursor; CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio;

P-values and ORs were adjusted for: [a] Age; [b] Presence of ribotype 027; [c] Unadjusted as no assessed covariates were found to be significant;

Table 8.11 - Percentile analysis of immune response to *C. diff* toxins A and B: recurrence within 90 days versus non-recurrence

IgG/tcdA	Recurrence (n=82)	Non-recurrence (n=136)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	19	36	-	-
Medium-High	19	39	0.95	1.03 (0.46-2.31)
Medium-Low	24	29	0.23	1.65 (0.74-3.71)
Low	20	32	0.38	1.45 (0.64-3.31)
				Global p-value = 0.53

IgG/tcdB	Recurrence (n=82)	Non-recurrence (n=136)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	19	40	-	-
Medium-High	23	33	0.16	1.78 (0.80-3.96)
Medium-Low	18	37	0.59	1.25 (0.55-2.84)
Low	22	26	0.06	2.21 (0.96-5.11)
				Global p-value = 0.24

IgM/tcdA	Recurrence (n=77)	Non-recurrence (n=135)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	15	44	-	-
Medium-High	24	27	0.03	2.49 (1.08-5.74)
Medium-Low	17	39	0.95	1.03 (0.44-2.39)
Low	21	25	0.13	1.92 (0.82-4.51)
				Global p-value = 0.07

CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio; tcdA: toxin A; tcdB: toxin B;

P-values and ORs were adjusted for [a] age;

Table 8.12 - Percentile analysis of immune response to *C. diff* binary toxin A and B-precursor: recurrence within 90 days versus non-recurrence

IgG/cdtA	Recurrence (n=77)	Non-recurrence (n=126)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	16	36	-	-
Medium-High	17	34	0.88	0.94 (0.40-2.22)
Medium-Low	26	29	0.22	1.68 (0.73-3.85)
Low	18	27	0.44	1.41 (0.59-3.37)
				Global p-value = 0.45

IgG/cdtB-pre	Recurrence (n=77)	Non-recurrence (n=129)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	18	32	-	-
Medium-High	22	33	0.63	1.22 (0.54-2.79)
Medium-Low	23	33	0.61	1.24 (0.55-2.81)
Low	14	31	0.76	0.87 (0.36-2.11)
				Global p-value = 0.82

IgM/cdtA	Recurrence (n=77)	Non-recurrence (n=126)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	14	36	-	-
Medium-High	20	36	0.47	1.37 (0.58-3.24)
Medium-Low	21	28	0.44	1.41 (0.59-3.38)
Low	22	26	0.26	1.65 (0.69-3.94)
				Global p-value = 0.73

IgM/cdtB-pre	Recurrence (n=77)	Non-recurrence (n=129)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	16	35	-	-
Medium-High	21	34	0.34	1.50 (0.65-3.48)
Medium-Low	20	35	0.91	1.05 (0.45-2.43)
Low	20	25	0.49	1.35 (0.57-3.21)
				Global p-value = 0.73

cdtA: binary toxin A; cdtB-pre: binary toxin B-precursor; CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio;

P-values and ORs were adjusted for [a] age;

Table 8.13 - Percentile analysis of immune response to *C. diff* toxins A and B: death within 30 days versus survival

IgG/tcdA	Death (n=26)	Survival (n=277)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	7	68	-	-
Medium-High	5	72	0.79	0.84 (0.22-3.20)
Medium-Low	8	69	0.69	1.29 (0.37-4.51)
Low	6	68	0.81	1.17 (0.34-4.05)
				Global p-value = 0.93

IgG/tcdB	Death (n=26)	Survival (n=277)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	8	67	-	-
Medium-High	6	71	0.62	0.71 (0.19-2.72)
Medium-Low	9	69	0.53	1.45 (0.45-4.66)
Low	3	70	0.60	0.67 (0.16-2.94)
				Global p-value = 0.65

IgM/tcdA	Death (n=26)	Survival (n=271)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	3	72	-	-
Medium-High	8	66	0.16	2.84 (0.67-12.14)
Medium-Low	7	68	0.94	1.07 (0.21-5.54)
Low	8	65	0.17	2.73 (0.64-11.59)
				Global p-value = 0.28

IgM/tcdB	Death (n=26)	Survival (n=271)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	5	70	-	-
Medium-High	6	69	0.43	1.77 (0.43-7.29)
Medium-Low	8	67	0.34	2.02 (0.48-8.46)
Low	7	65	0.16	2.72 (0.67-11.07)
				Global p-value = 0.58

CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio; tcdA: toxin A; tcdB: toxin B;

P-values and ORs were adjusted for [a] age, body mass index, score on the Charlson Comorbidity Index (exclusive of age) and disease severity at baseline

Table 8.14 - Percentile analysis of immune response to *C. diff* binary toxin A and B-precursor: death within 30 days versus non-death

IgG/cdtA	Death (n=25)	Non-death (n=261)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	4	67	-	-
Medium-High	9	65	0.42	1.76 (0.45-6.95)
Medium-Low	6	66	0.96	1.04 (0.24-4.52)
Low	6	63	0.48	1.64 (0.42-6.50)
				Global p-value = 0.78

IgG/cdtB-pre	Death (n=25)	Non-death (n=265)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	9	63	-	-
Medium-High	7	67	0.76	0.83 (0.25-2.76)
Medium-Low	4	69	0.51	0.63 (0.16-2.46)
Low	5	66	0.49	0.62 (0.16-2.39)
				Global p-value = 0.88

IgM/cdtA	Death (n=25)	Non-death (n=261)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	6	67	-	-
Medium-High	5	66	0.76	1.25 (0.30-5.20)
Medium-Low	6	67	0.91	1.08 (0.26-4.51)
Low	8	61	0.51	1.59 (0.40-6.28)
				Global p-value = 0.90

IgM/cdtB-pre	Death (n=25)	Non-death (n=265)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	4	69	-	-
Medium-High	6	67	0.32	2.17 (0.47-9.99)
Medium-Low	7	66	0.22	2.53 (0.58-11.01)
Low	8	63	0.35	2.01 (0.46-8.75)
				Global p-value = 0.66

cdtA: binary toxin A; cdtB-pre: binary toxin B-precursor; CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio;

P-values and ORs were adjusted for [a] age, body mass index, score on the Charlson Comorbidity Index (exclusive of age) and disease severity at baseline

Table 8.15 - Percentile analysis of immune response to *C. diff* toxins A and B: CDI cases versus non-colonised AAD controls

IgG/tcdA	CDI cases (n=306)	AAD controls (n=142)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	78	34	-	-
Medium-High	78	34	0.89	1.04 (0.57-1.90)
Medium-Low	76	36	0.60	0.85 (0.47-1.55)
Low	74	38	0.50	0.82 (0.45-1.47)
				Global p-value = 0.82

IgG/tcdB	CDI cases (n=306)	AAD controls (n=142)	Adjusted P-value[b]	Adjusted OR (95% CI)
High (Comparator)	83	29	-	-
Medium-High	70	42	0.05	0.55 (0.30-1.01)
Medium-Low	74	38	0.22	0.68 (0.37-1.25)
Low	79	33	0.63	0.86 (0.46-1.59)
				Global p-value = 0.23

IgM/tcdA	CDI cases (n=300)	AAD controls (n=136)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	70	39	-	-
Medium-High	73	36	0.99	1.01 (0.55-1.83)
Medium-Low	77	32	0.62	1.17 (0.64-2.14)
Low	80	29	0.33	1.36 (0.73-2.53)
				Global p-value = 0.75

IgM/tcdB	CDI cases (n=300)	AAD controls (n=137)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	82	27	-	-
Medium-High	69	41	0.12	0.62 (0.34-1.14)
Medium-Low	77	32	0.68	0.88 (0.47-1.65)
Low	72	37	0.31	0.73 (0.39-1.35)
				Global p-value = 0.43

AAD: Antibiotic-associated diarrhea; CDI: Clostridium difficile infection; CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio; tcdA: toxin A; tcdB: toxin B;

P-values and ORs were all adjusted for: [a] age, body mass index, time delay between testing positive & recruitment and presence of diabetes; [b] age, body mass index, time delay between testing positive & recruitment and presence of diabetes and immunosuppression;

Table 8.16 - Percentile analysis of immune response to *C. diff* binary toxin A and B-precursor: CDI cases versus non-colonised AAD controls

IgG/cdtA	CDI cases (n=289)	AAD controls (n=132)	Adjusted P-value[a]	Adjusted OR (95% CI)
High (Comparator)	80	25	-	-
Medium-High	71	35	0.10	0.58 (0.31-1.10)
Medium-Low	67	38	0.04	0.51 (0.27-0.96)
Low	71	34	0.20	0.66 (0.35-1.25)
				Global p-value = 0.19
IgG/cdtB-pre	**CDI cases (n=293)**	**AAD controls (n=134)**	**Adjusted P-value[b]**	**Adjusted OR (95% CI)**
High (Comparator)	78	29	-	-
Medium-High	66	41	0.13	0.66 (0.34-1.15)
Medium-Low	76	31	0.88	0.95 (0.51-1.78)
Low	73	33	0.70	0.89 (0.47-1.65)
				Global p-value = 0.39
IgM/cdtA	**CDI cases (n=289)**	**AAD controls (n=132)**	**Adjusted P-value[a]**	**Adjusted OR (95% CI)**
High (Comparator)	76	29	-	-
Medium-High	67	39	0.36	0.75 (0.40-1.39)
Medium-Low	72	33	0.43	0.77 (0.41-1.47)
Low	74	31	0.71	0.88 (0.46-1.67)
				Global p-value = 0.79
IgM/cdtB-pre	**CDI cases (n=293)**	**AAD controls (n=134)**	**Adjusted P-value[b]**	**Adjusted OR (95% CI)**
High (Comparator)	78	29	-	-
Medium-High	69	38	0.41	0.77 (0.42-1.42)
Medium-Low	72	36	0.41	0.77 (0.42-1.43)
Low	74	31	0.63	0.86 (0.45-1.62)
				Global p-value = 0.82

AAD: Antibiotic-associated diarrhea; CDI: Clostridium difficile infection; cdtA: binary toxin A; cdtB-pre: binary toxin B-precursor; CI: confidence interval; IgG: Immunoglobulin G; IgM: Immunoglobulin M; n: number; OR: odds ratio;

P-values and ORs were all adjusted for: [a] age, body mass index and time delay between testing positive and subsequent recruitment; [b] age, body mass index, time delay between testing positive and subsequent recruitment and diabetes;

Interestingly, the presence/absence of a binary toxin-producing strain produced no significant effects on immune response (Table 8.17). Advanced age, using a previously defined cut-off of ≥65 years (Bauer *et al*., 2011; Kyne *et al*., 2001; Pepin *et al*., 2005), displayed a moderate effect on overall response levels, however the trend was not consistent for all measures (Table 8.17).

After adjusting data for differences in coating, detection and sample dilution factors, observed signals were still significantly higher for IgG compared to IgM, and this was consistent across all tested toxin components. In general, antibody response to tcdA was higher than that of tcdB, and a high degree of correlation was observed between their antibody responses (Figure 8.1). For the tested binary toxin components, IgG response was comparable though measurements for IgM response was slightly higher against cdtA, and as with the tcdA and tcdB toxins there was a significant degree of concurrence between the antibody responses to both cdtA and cdtB-pre (Figure 8.1). Correlation of cdtA and cdtB with either tcdA or tcdB toxins was less pronounced and was not consistent across all combinations (Figure 8.1).

Table 8.17 - Assessing the impact of increased age and presence of a binary toxin-producing strain on immune response to *C. diff* toxins

	Age ≥65 yrs			**Binary toxin-producing strain**		
	No (n=97)	Yes (n=210)	P-value	No (n=159)	Yes (n=122)	P-value
IgG/tcdA	44,571	42,305	0.60	38,890	44,607	0.95
IgG/tcdB	16,900	20,280	0.29	19,286	17,718	0.66
IgM/tcdA	26,844	18,830	<0.01	22,092	20,124	0.87
IgM/tcdB	5,280	5,158	0.97	4,655	5,609	0.28
IgG/cdtA	806	626	0.03	700	626	0.69
IgG/cdtB-pre	1,626	1,778	0.39	1,658	1,778	0.44
IgM/cdtA	548	343	<0.01	404	403	0.58
IgM/cdtB-pre	746	504	<0.01	554	557	0.75

cdtA: binary toxin A; cdtB-pre: binary toxin B-precursor; IgG: Immunoglobulin G; IgM: Immunoglobulin M; tcdA: toxin A; tcdB: toxin B;

	IgG/tcdA	IgG/tcdB	IgM/tcdA	IgM/tcdB	IgG/cdtA	IgG/cdtB-pre	IgM/cdtA	IgM/cdtB-pre
IgG/tcdA								
IgG/tcdB	R = 0.45 P < 0.01							
IgM/tcdA	R = 0.29 P < 0.01	R = 0.18 P < 0.01						
IgM/tcdB	R = 0.21 P < 0.01	R = 0.52 P < 0.01	R = 0.34 P < 0.01					
IgG/cdtA	R = 0.19 P < 0.01	R = 0.01 P = 0.86	R = 0.18 P < 0.01	R = 0.02 P = 0.76				
IgG/cdtB-pre	R = 0.44 P < 0.01	R = 0.24 P < 0.01	R = 0.06 P = 0.34	R = 0.14 P < 0.05	R = 0.45 P < 0.01			
IgM/cdtA	R = 0.22 P < 0.01	R = 0.09 P = 0.15	R = 0.39 P < 0.01	R = 0.07 P = 0.24	R = 0.29 P < 0.01	R = 0.23 P < 0.01		
IgM/cdtB-pre	R = 0.06 P = 0.34	R = 0.05 P = 0.43	R = 0.14 P < 0.05	R = 0.08 P = 0.17	R = 0.53 P < 0.01	R = 0.73 P < 0.01	R = 0.37 P < 0.01	

Figure 8.1 – Overview of correlation across *Clostridium difficile* toxin epitopes

cdtA: C. diff binary toxin A; cdtB-pre: C. diff binary toxin B precursor; IgG: Immunoglobulin G; IgM: Immunoglobulin M; tcdA: C. diff toxin A; tcdB: C. diff toxin B; Correlation calculated using Pearsons Correlation Coefficient (R); Colour coding: Darker green = Positive correlation within toxin group; Lighter green = Positive correlation across toxin groups: Pale blue = Weak correlation

8.3.3 Power calculations

Across all toxin epitopes and disease outcomes assessed, power to detect a significant difference was calculated as ≥90% for 30/40 (75%) of the analyses (Table 8.18). Inadequate power (<80%) was observed in 7/40 (18%), the majority of which (4/7; 57%) arose from Case versus Control analyses.

Table 8.18 - Assessment of power across outcome analyses

Disease outcome	Percentage power achieved (Number needed in each group to achieve 80% power)							
	tcdA IgG	tcdB IgG	tcdA IgM	tcdB IgM	cdtA IgG	cdtB-pre IgG	cdtA IgM	cdtB-pre IgM
Case versus control	99	6[a]	99	8[b]	99	99	47[c]	5[d]
30-day mortality	40[e]	22[f]	99	89	*	99	98	96
Prolonged symptoms	99	99	99	99	92	99	99	99
90-day recurrence	99	84	32[g]	99	99	93	99	99
Disease severity	99	99	99	99	99	99	99	99

*cdtA: binary toxin A; cdtB-pre: binary toxin B-precursor; IgG: Immunoglobulin G; IgM: Immunoglobulin M; tcdA: toxin A; tcdB: toxin B; * No difference was observed between the two median values and therefore retrospective power could not be estimated;*

In order to achieve 80% power, the following number of patients would be needed in each group: [a] 13,345; [b] 5,290; [c] 127; [d] 27,692; [e] 264; [f] 394; [g] 333;

8.3.4 Time delay stratification

Though the median time delay between testing positive and subsequent recruitment across our CDI patients was 3.0 days (Chapter 7: Section 7.3.1), a small number (n=18) were recruited ≥7 days post-testing positive for *C. diff*. These patients were found to have an increased IgG and IgM response to all *C. diff* toxin epitopes compared to those with a time delay <7 days (n=290), which reached significance ($p<0.05$) for IgG response against tcdB and both IgG and IgM responses against cdtB-pre; Figures 8.2 & 8.3).

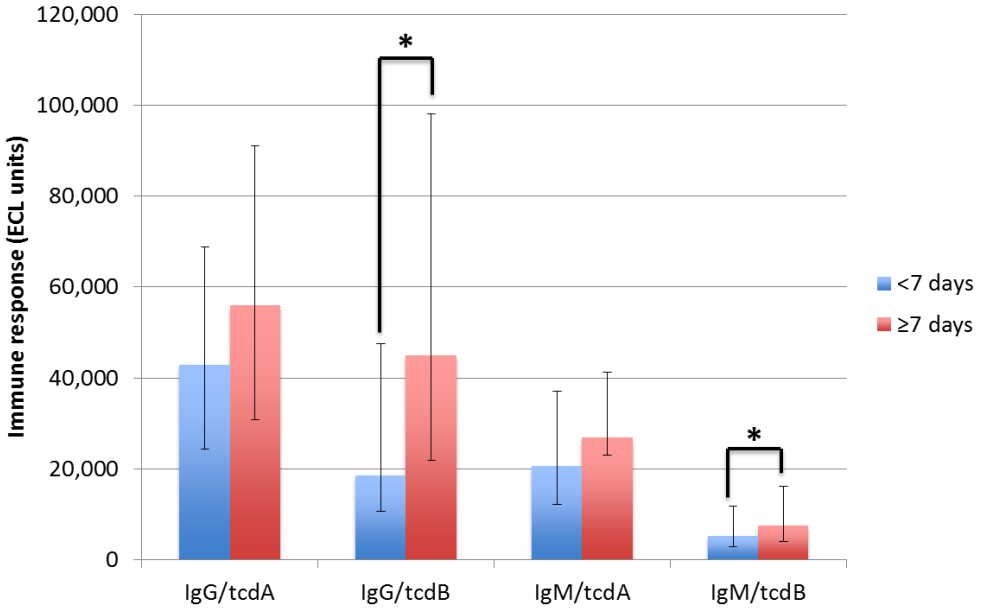


Figure 8.2 – Immune response levels to *C. diff* tcdA and tcdB toxins after time delay stratification (* = P<0.05)

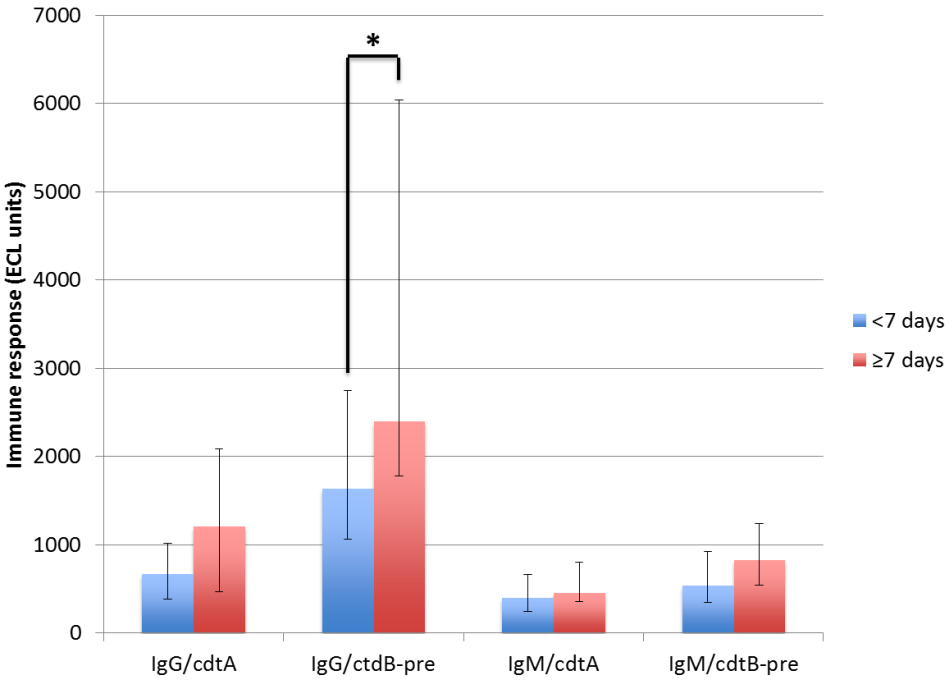


Figure 8.3 – Immune response levels to *C. diff* binary toxin components after time delay stratification (* = P<0.05)

8.4 Discussion

This study represents the first to have quantified the immune response to both the glucosylating toxins and binary toxin of *C. diff*, using a large, well-characterised and stringently-phenotyped set of individuals. Immune response was quantified using a novel in-house ECL-based method (described in detail in Chapter 6), which attempted to provide more clarity where previously used ELISA-based methods were lacking. Furthermore, stringent analysis methodology was employed to assess multiple covariates across all CDI patients for each outcome analysis.

Anti-toxin immune response in a non-colonised control group suffering from antibiotic-associated diarrhoea was found to be very similar to that of the CDI patients, which is in line with previous research (Jiang *et al*., 2007; Viscidi *et al*., 1983; Warny *et al*., 1994) and indicates a significant level of prior exposure. It is therefore likely that the immune response being measured here relates predominantly to pre-existing immunity mounted over time rather than a sole result from the current infectious episode. Conversely, associations were identified between lower anti-tcdA and anti-tcdB IgG titres and severe disease (assessed at baseline), which is consistent with previous research demonstrating a protective role of high serum anti-toxin IgG in severe CDI in animals (Johnson, 2012; Steele *et al*., 2012). Minor associations were also identified between lower anti-tcdB & anti-cdtB-pre IgM titres and disease recurrence & prolonged disease, respectively.

Although anti-toxin immune titres were generally lower in patients experiencing an unfavourable CDI outcome compared to those not, no further associations were identified across all investigated disease outcomes and toxin epitopes. Therefore, despite the minor associations highlighted above, this study failed to confirm previous claims for associations between anti-toxin immune response and either disease recurrence (Aronsson *et al*., 1985; Bauer *et al*., 2014; Kyne *et al*., 2001; Warny *et al*., 1994), prolonged symptoms (Warny *et al*., 1994), or mortality (Solomon *et al*., 2013). It is important to note that previous findings were identified using longitudinal sampling with samples

taken either at regimented post-diagnosis time points, or during allegedly acute (typically within 1-2 weeks post-diagnosis) and/or convalescent phases (anything thereafter). Consistent with our findings using only one baseline time-point, these previous studies also failed to identify any significant associations using baseline/acute samples only. The sole study to date to identify an association using baseline sampling did so by stratifying based upon IgG antibody sub-classes (Katchar *et al*., 2007).

It can therefore be implied that a lack of longitudinal sampling has resulted in the failure to detect potential milestones in patients' immune response trajectory resulting from their current infection. This concept has been strengthened based on examination of immune responses following stratification for the time delay, which occurred between positive diagnosis and patient recruitment/sampling, whereby those patients with a significantly longer time delay (≥7 days; n=18) displayed considerably higher IgG and IgM anti-toxin levels than patients with a time delay inferior to 7 days (Figures 8.2 & 8.3). This suggests the existence of a boost in the immune response arising from current infection, thus lending further weight for the undertaking of longitudinal sampling. Notably, the elevation in the anti-toxin IgG & IgM responses was not observed following data analysis of the mannose-binding lectin (MBL) assay, described in Chapter 6. Therefore it is possible this rise may be restricted to the adaptive immune system rather than the innate immune system.

This study also suffers from further limitations. The lack of an appropriate control group comprising asymptomatic carriers meant that it was not possible to investigate the role of the immune response in disease susceptibility due to the complexity of selectively recruiting these individuals. Secondly, for ethical reasons, patients that were medically very unwell could not be approached to join the study and as such it is possible that the immune response of these individuals would have differed from that found in our recruits. Thirdly, this study did not investigate the role of non-toxin antigens, antibody sub-classes, nor immunoglobulin A (IgA) response. Previous research has demonstrated that host response to non-toxin antigens may play a comparable role to the ones

induced by the toxins (Drudy *et al*., 2004; Péchiné *et al*., 2007) and that associations can be stratified based on specific antibody sub-classes (Katchar *et al*., 2007). Furthermore, despite initial flat findings for IgA, a recent study identified an association between disease susceptibility and lower pre-existing IgA titres against tcdB (Islam *et al*., 2014). Fourthly, the purified tcdA and tcdB aliquots were derived from reference strain vpi10463, and owing to probable functional differences specific to strain families (Lanis *et al*., 2010), it is possible that antibody levels measured using tcdB derived from vpi10463 may not reproduce accurate antibody measurements against the actual infecting strain. Unlike for tcdA and tcdB, there are currently no established methods to purify binary toxin from *C. diff* bacterial culture. Therefore recombinant binary toxin was used, which may or may not have the same biological properties as native binary toxins. Finally, despite employing the largest cohort size to date, the large degree of heterogeneity present within CDI-suffering patients means it is likely our study suffers from a lack of a power.

The importance of anti-toxin antibodies in regulating disease susceptibility, severity and poor disease outcome is highlighted by the number of experimental CDI vaccines under development. The finding that lower anti-toxin IgG titres result in severe disease advocates a prominent need for mounting a competent immune response to these toxins, in order to deter disease progression. Furthermore, it supports the use of active and passive immunotherapies for CDI management, whilst emphasising that neither toxin A nor toxin B can be downgraded in terms of importance. The finding that a lower IgM titre to the binary toxin precursor component B results in prolonged disease provides further evidence for the importance of binary toxin as an adjuvant virulence factor of *C. diff*, consistent with previous research demonstrating CDT-induced formation of microtubule-based protrusions that increase adherence of bacteria (Schwan *et al*., 2009). Pharmaceutical companies have been considering a potential need for its incorporation into novel therapeutic research.

Future work should focus on further improvements of the quantification methodology with stratification for further immunoglobulin sub-classes,

combined with longitudinal sampling of larger, well-phenotyped patient cohorts to gain further understanding of the role of the adaptive immune system in mediating susceptibility and modulation of CDI. The use of hypothesis-free approaches for the identification of non-toxin immunogenic candidates is also warranted.

Chapter 9

Final discussion

CDI is now regarded as the major cause of PMC, accounting for 15-39% of all cases of AAD (Dubberke and Wertheimer, 2009; McFarland, 2009; Viswanathan *et al*., 2010). Symptomatic patients exhibit a broad range of clinical manifestations, from mild, watery diarrhoea to life-threatening fulminant PMC that can lead to severe complications, including toxic megacolon, septic shock and death (Rupnik *et al*., 2009). The disease continues to be a major burden on healthcare facilities worldwide (Ghantoji *et al*., 2010; Wiegand *et al*., 2012). A significant proportion of patients (5-50%) suffer from disease recurrence post-treatment (Aslam *et al*., 2005), and the pooled attributable mortality has risen from 3.64% to 8.03% since the emergence of the last wave of epidemic strains between 2000-2009 (Dubberke *et al*., 2008; Karas *et al*., 2010; Kuijper *et al*., 2006). More worryingly, patient recovery can be a very slow process for a significant proportion of patients, until the balance of their gut microbiota has been re-established. Hospitals and healthcare systems have begun adopting specific policies and treatment regimens, and distinct standards exist for community identified cases (see Chapter 1 Section 1.8).

Affected patients generally display a range of similar features, including advanced age, polypharmacy and comorbidities, as well as a number of overlapping symptoms that makes stratification of patients at the start of the disease process more complex. Certainly the determination of highly predictive disease parameters at an early stage would bring enormous benefits for the management of CDI and more cost-effective strategies for tackling recurrence and deterring transmission (Johnson, 2009; Louie *et al*., 2011). It would also increase confidence in implementing more aggressive treatment regimens for patients that are likely to develop clinical complications (Bauer *et al*., 2009; Cohen *et al*., 2010), as well as identify cases that may benefit from alternative therapies such as fibre supplementation, probiotics and faecal transplantation.

Although a myriad of clinical variables have been implicated with poor CDI outcomes, the majority of previous studies on risk factors did not assess the robustness and performance of their models. Moreover, only a small number of CPRs have been developed to date (see Chapter 1 Section 1.9) and given the

heterogeneity that is evident across studies and the lack of external validation, it is not surprise that to date no CPR has gained widespread clinical acceptance.

In order to identify independent risk predictors for CDI disease outcomes in our cohort, data for multiple clinical variables were derived and analysed (Chapter 3) statistically to assess their performance. Using commonly adopted disease outcome measures (recurrence, severe-complicated disease and mortality) and a previously uninvestigated outcome (prolonged disease), we identified several significant predictors at univariate level, which overlapped with the current literature. However, statistical assessment of our models revealed that despite their acceptable predictive accuracy, the models were unstable and poorly validated in external cohorts. This was denoted by the large confidence intervals observed and fluctuations of independent predictors when cross-validation approaches were employed. As a comparative exercise, I also used data from the cohort to assess existing CPRs for prediction of CDI disease outcomes. For the CPRs where equivalent information was extractable, it was not possible to replicate the findings, despite our sample size being comparable with the majority of studies (Bhangu *et al*., 2010; Butt *et al*., 2013; Drew and Boyle, 2009; Hensgens *et al*., 2014; Lungulescu *et al*., 2011). This further emphasises the poor external validity of CPRs, and further work, with much larger patient cohorts are needed.

Despite several technological advances, the diagnosis of CDI is still challenging because of the broad spectrum of disease presentation. Historically, the diagnosis of nosocomial CDI cases has been made through an initial screening using *C. diff* toxin EIAs, which, despite their high PPV, tend to bias the identification towards overly symptomatic patients due to their limited sensitivity (see Chapter 1 Section 1.6.2). In particular, mild and incipient forms of the disease are less likely to be successfully diagnosed. In order to address the issue, modern guidelines are now endorsing the use of multi-step diagnostic algorithms, through the introduction of more sensitive first stage tests, such as the enzymatic detection of GDH and direct PCR from selected *C. diff* genomic targets (see Chapter 1 Section 1.6.6). Their increased sensitivity however also means that they cannot be used to definitively establish the diagnosis, since a

positive test does not necessarily imply CDI. Thus, a more specific second test is necessary to rule out colonisation by *C. diff* or asymptomatic carriage. The scenario is further compounded by the fact that the vast majority of clinical specimens referred for CDI testing come from patients suffering from some sort of diarrhoea, which makes an accurate differentiation between true incipient cases from simple carriage/colonisation even more difficult. Clearly, the existence of selection bias due to the limitations of CDI diagnostic standards has had a downstream impact on the recruitment of patients for this, and for the majority of previous studies, thereby adding an extra layer of complexity for drawing definitive comparisons and replicating findings.

Much larger sample sizes are clearly required to further elucidate the multifactorial basis of CDI. Therefore, a meta-analysis of previous studies would be an extremely valid attempt but this has been severely limited due to the considerable heterogeneity observed across studies (Abou Chakra *et al*., 2014), which stems from inconsistent outcome definitions, different follow-up periods, diagnostic testing methods employed and variables examined. A potential robust alternative would be to obtain original/raw data from previous studies to facilitate a larger and extensive Individual Patient Data (IPD) meta-analysis. This would reduce bias and improve the accuracy and quality of the previously reported associations. In addition, this would allow some standardisation in definitions of the outcome measures used, and the follow-up periods. However, this would be a major undertaking and relies on the original investigators providing the individual patient data and whether they are contactable.

Biomarker evidence for the outcomes associated with CDI is certainly scarce, which is in part due to the lack of comprehensive studies and mechanistic understanding of the disease. There is therefore a need to identify robust biomarkers that can help in stratifying patients in terms of potential clinical outcome, allowing the clinician to define at the beginning the type and intensity of treatment to be utilised. Pro-inflammatory cytokines constitute plausible candidates given that they can be released by toxin exposure (Hippenstiel *et al*., 2000; Ishida *et al*., 2004). The SNP rs4073/-251T>A within the gene encoding

pro-inflammatory cytokine IL-8 is the only genetic association with CDI that has been reported to date, with the AA genotype being shown to increase the odds of developing CDI, as well as experiencing recurrent disease, by at least 3-fold (Garey *et al*., 2010; Jiang *et al*., 2006; Jiang *et al*., 2007). A replication of this finding was undertaken as part of this thesis (Chapter 4). Our data using a larger sample size than the original studies, but similar clinical outcome definitions, failed to show an association with the IL8 SNP. A meta-analysis combining our data with the published data (Chapter 4), also failed to replicate the association. It therefore seems unlikely that this polymorphism plays a major role in CDI risk and recurrence, and the genetic effect size (if any) is substantially smaller than previously anticipated.

Since the increase in secretion of pro-inflammatory cytokines results in augmented intestinal inflammation, this study also sought to investigate the role of both faecal lactoferrin and calprotectin in CDI. Tests for FC and FL are quite well established in that they are two of the most widely investigated faecal biomarkers in IBD. Given the analogous conditions found in CDI, several authors have pursued this area with a number of positive association being reported for either marker individually (Archbald-Pannone *et al*., 2010; Boone *et al*., 2013; El Feghaly *et al*., 2013; LaSala *et al*., 2013; Shastri *et al*., 2008; Vaishnavi *et al*., 2000; Whitehead *et al*., 2014). Our work focused on simultaneous evaluation of both FL and FC, with the aim of assessing their clinical importance in patients already suffering from CDI (Chapter 5). Both FL and FC were good at differentiating between CDI cases and AAD controls in our cohort. However, there was no association with disease outcomes, indicating that both faecal biomarkers have limited applicability for disease stratification. Although it is apparent that host intrinsic factors play a major role in both inter-patient variability and susceptibility to CDI, it is pivotal that future studies adopt systematic approaches, larger cohort sizes and a well-defined array of phenotypes for the elucidation of mechanistic biomarkers to enable accurate prediction of unfavourable clinical outcomes so that more personalised interventional strategies can be developed and implemented. Recently, CDI clinical complications have been linked to the cytokine IL-23 (Buonomo *et al*.,

2013) and the peptide precursor to calcitonin PCT (Rao *et al*., 2013). However both have yet to undergo independent replication.

As outlined in Chapter 1 Section 1.3, both biochemical and molecular studies have shown that the major clinical signs and symptoms of CDI are largely explained by the detrimental actions of tcdA and tcdB (Babcock *et al*., 2006a; Kim *et al*., 1987; Lyerly *et al*., 1985; Lyras *et al*., 2009a). Neutralising them, either by natural or interventional resources, is therefore important for ameliorating symptoms. Indeed, there is growing evidence which shows that an adequate anti-toxin immunological response is important in reducing complications from the disease (Aronsson *et al*., 1985; Bauer *et al*., 2014; Drudy *et al*., 2004; Islam *et al*., 2014; Johnson *et al*., 1992; Katchar *et al*., 2007; Kyne *et al*., 2000a, 2001; Mulligan *et al*., 1993; Sanchez-Hurtado *et al*., 2008; Solomon *et al*., 2013; Warny *et al*., 1994). The recent emergence of so-called "hypervirulent" epidemic *C. diff* strains has accelerated the development of novel non-antibiotic based treatment regimes, and led to a stronger emphasis being placed on vaccine development and on immunoglobulin therapy against CDI. However, this area of research has been hampered by the lack of reliable methods to quantitate the pattern of specific immune responses to CDI, especially against tcdA and tcdB. This is consistent with ongoing discussions in the international community as to whether CDI specific biomarkers simply have yet to be discovered, or whether the current methodologies lack the necessary robustness for the validation of existing parameters due to their lack of fitness surrounding sensitivity, specificity, or reliability. Remarkably, no commercial assays are available for the quantitation of the anti-toxin immune response with previous research focusing on in-house methods based on traditional ELISA, which has several technical limitations. Therefore, one of the aims of the work pursued was to develop enhanced quantification assays, through the use of an ECL-based platform and a multi-step optimisation process, for measuring both IgG and IgM responses to tcdA and tcdB. In addition, novel quantitation assays for multiple CDT epitopes were also explored (Chapter 6). The assays developed were then taken forward for the evaluation of the IgG and IgM immune responses in our cohort of patients, which showed that lower anti-tcdA

and -tcdB IgG titres at baseline resulted in more severe disease (Chapter 8). This suggests that the the host needs to mount a competent immune response to these toxins in order to prevent disease progression. In addition, *in situ* detection of the *C. diff* binary toxin (cdtA/cdtB) has been attempted (Carman *et al*., 2011) but to the best of our knowledge this is the first study to focus on the antibody response to either cdtA, or cdtB. The binary toxin is an adjuvant virulence factor expressed by selected toxigenic strains. Our finding that a lower IgM titre to the binary toxin precursor component B predicted a prolonged disease course further emphasises the need to better understand the biology of this protein, and hence its clinical importance. Despite the novelty and potential benefits of the assays which were developed, it is important to emphasise that given the high homology between the toxin epitopes tested (especially tcdA and tcdB), it was not possible to rule out cross-reactivity between the assays, and how much of the measured response in each assay was indeed specific for each tested epitope and isotype. Indeed, since an individual antibody response to several antigens tends to correlate with the overall immune competence of the host, it is therefore not surprising that we have detected a degree of correlation in signal levels across our assays. Given the flexibility offered by our development strategy, further customisation is also possible, for example, to investigate antibody sub-classes that may offer increased predictability. In this respect, it is interesting to note that one of the few associations reported between CDI and immune response using baseline samples was achieved through the evaluation of different IgG sub-classes (Katchar *et al*., 2007). Our inability to replicate associations that have been reported previously linking anti-toxin host response with disease recurrence, mortality and prolonged disease may be related to the limited predictive power of these markers, and the fact that we did not have samples at different time points during the course of the illness in the patients. In order to further verify our assays and monitor longitudinal changes in the humoral immune response, a follow-on study is currently on-going, where patients have sample collections at baseline, 2 weeks and 6 weeks.

In order to identify other biomarkers, we went onto investigate MBL, a potentially interesting CDI biomarker that has been associated with susceptibility to several infectious diseases (Chapter 7). MBL is a key pattern recognition molecule of the complement system that binds to repetitive sugar arrays on several microbial surfaces, albeit not to *C. diff* (Townsend *et al*., 2001). Low MBL concentrations have been linked with increased susceptibility to infections in both animal models and humans (Møller-Kristensen *et al*., 2006; Shi *et al*., 2004). Equally important are the immunomodulatory properties of MBL, in which it acts in concert with major modulators of inflammation, such as toll-like receptors and CRP, both of which have been implicated in CDI (Eyre *et al*., 2012b; Ryan *et al*., 2011). One of the difficulties in this work was the lack of clinical definition surrounding MBL deficiency, where previous studies evaluating had differed significantly in both the criteria employed for ascertaining genetic and serological deficiency, which resulted in heterogeneity across studies. Therefore, we chose to investigate both genotype and phenotype, as this approach can identify potential discordance between the two. By transferring an existing MBL assay from an ELISA standard onto a more robust ECL-based platform, we also sought to maximise serological assay performance while at the same time employing a well-established method for screening for functional MBL2 genetic polymorphisms (see Appendix 45). We were able to demonstrate a significant association between low levels of MBL and CDI recurrence, but not with MBL genetic variation (Chapter 7). Other studies have also identified associations with protein levels in other infections and immune conditions (Chapter 7, Table 7.1) but it has not been possible to infer definitive conclusions due to the aforementioned heterogeneity in standards across studies. The mechanistic bases for this association is intriguing because MBL does not bind to the surface of *C. diff* (Townsend *et al*., 2001) and thus MBL deficiency *per se* is unlikely to directly underpin CDI predisposition. However, MBL does appear to have other relevant functions that can modulate the disease, such as through immunomodulation of inflammation and clearance of apoptotic cells (Dommett *et al*., 2006). In addition, low MBL concentrations lead to increased secretion of pro-inflammatory cytokines (Garred *et al*., 2002; Jack *et al*., 2001b), which have been shown to be elevated

in response to CDI (Hirota *et al*., 2012; Vohra and Poxton, 2012). We have also confirmed an inverse correlation between MBL and CRP levels. Since elevated CRP concentrations have previously been associated with various CDI outcomes, including disease severity and recurrence (Eyre *et al*., 2012b; Khanafer *et al*., 2013), our findings further endorse a mechanistic relationship between MBL and acute conditions.

It is well known that due to the upsurge of CDI cases in the last decade, stringent infection control measures have been introduced, such as revised antibiotic prescription policies, opening of isolation wards and improved hygiene and cleanliness. While these measures have had a positive effect and must continue, clearly without a better comprehension of the disease pathogenesis, evolution and inter-individual variability (both at the host and bacterial levels), progress in this area will be hampered. One major factor limiting the majority of studies to date is the lack of study power. This has also been a major limitation for the work conducted in this thesis. A lack of adequate study power means we may have been unable to detect smaller effect sizes, something which is extremely important given the large degree of heterogeneity present within CDI-suffering patients and the seemingly multi-factorial nature of the disease. Whilst we were able to demonstrate adequate statistical power using a post-hoc power analysis, this technique is seen as controversial and can result in uninformative and misleading values: post-hoc power in its simplest form is a one-to-one function of the p-value attained, and it has been demonstrated that all post-hoc power analyses suffer from the "power approach paradox". The challenging nature of prospective CDI recruitment meant we were unable to meet our proposed recruitment targets, although these were likely an underestimation. Whilst we employ one of the largest prospectively recruited cohorts to date, study power for the clinical risk factor work conducted in Chapter 3 could have been achieved through the use of retrospective recruitment resulting in a larger patient cohort, although this would not have been a possibility for extending study power in the remaining experimental chapters pertaining to biomarker assessment. As well as lacking study power, our cohort may not be fully representative of the entire disease

spectrum, which may have further biased our data analysis. Our prospective recruitment method meant that we were unable to recruit the most severe of CDI patients, particular those in life threatening conditions, and we only used a single laboratory test (ELISA for CDT) for the primary identification of CDI cases. Although this is still a common procedure, modern algorithms currently make use of a more sensitive first step screening - based on either GDH, or a nucleic acid amplification test NAAT - to minimise the odds of reporting false negative results.

A limited understanding of the disease across the research field has also produced several negative facets, especially in relation to the lack of standardised disease sub-phenotypes. This has hampered validation of existing findings and prevented systematic comparisons across studies. There has also been little progress in determining the prognosis of CDI; reliable predictive tools that allow for the stratification of patients are needed in order to personalise the type and intensity of treatment. Some biomarkers have been successfully employed for the categorisation of other clinical conditions, but this seems a long way off for CDI. It is extremely clear that the recruitment of extremely large patient cohorts using standardised phenotypes is essential for future studies to provide more definitive answers, something which will only be achieved through multi-centre international collaboration.

In summary, the thesis has evaluated some of the clinical and biological features associated with CDI, which is a continuing public health problem affecting both primary and secondary care. The genomic technological revolution has helped in understanding the disease from the point of view of the organism. For example, some units are beginning to undertake sequence based typing of *C. diff.* However, genomics and other 'omics' technologies have not really impacted on the effect of infection on the host. The thesis has evaluated some of these issues ranging from the heterogeneity in prediction rules to biological factors associated with the host immune response. There is a need for further study in this area, and the underlying message is that these studies need to be conducted with greater attention to the sample size and phenotyping of the patients recruited to these cohorts, underpinned by mechanistic investigations.

Appendices

Contents

1: Characteristics of CDI patients by recurrent versus non-recurrent disease

	N (%) or Median (IQR)		P-value	OR
	Recurrence (n=83)	Non-recurrence (n=137)		(95% CIs)
Demographics				
Age at baseline: per decade	77.5 (66.4-84.7)	69.6 (56.0-77.9)	**<0.01**	1.44 (1.19-1.76)
Gender: Female	45/83 (54)	80/137 (58)	0.54	0.84 (0.49-1.46)
Smoking pack years: per year increase	5.1 (0.0-35.0)	8.5 (0.0-33.0)	0.66	1.00 (0.99-1.01)
Body Mass Index	22.4 (19.4-27.4)	24.7 (21.0-28.3)	0.60	0.99 (0.95-1.03)
Medication information				
Number of co-medications at baseline	3.0 (2.0-5.0)	3.0 (2.0-4.0)	0.64	1.04 (0.89-1.20)
Taking fluoroquinolones prior to CDI	40/83 (48)	42/137 (31)	**0.01**	2.10 (1.20-3.70)
Taking PPIs prior to CDI	56/82 (68)	88/136 (65)	0.59	1.17 (0.66-2.10)
Concomitant antibiotics	44/83 (53)	67/137 (49)	0.56	1.18 (0.68-2.03)
Concomitant immunosuppressants	17/82 (21)	20/137 (15)	0.24	1.53 (0.75-3.12)
Clinical characteristics & underlying comorbidities				
Number of stools at baseline	5.0 (3.0-6.0)	4.0 (3.0-6.0)	0.77	0.99 (0.90-1.08)
Fever (≥36.8°C)	5/78 (6)	9/133 (7)	0.92	0.94 (0.30-2.92)
Charlson Comorbidity Index score	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.84	0.98 (0.81-1.19)
Diabetes	16/82 (20)	24/137 (18)	0.71	1.14 (0.57-2.30)
Hypotension	9/79 (11)	16/135 (12)	0.92	0.96 (0.40-2.28)
Current malignancy	2/83 (2)	3/137 (2)	0.92	1.10 (0.18-6.74)
Respiratory comorbidities at baseline	52/82 (63)	71/137 (52)	0.10	1.61 (0.92-2.82)
GI comorbidities at baseline	47/83 (57)	87/136 (64)	0.28	0.74 (0.42-1.28)
Recent abdominal surgery (90 days)	3/77 (4)	23/133 (17)	**0.01**	0.19 (0.06-0.67)

1 (continued): Characteristics of CDI patients by recurrent versus non-recurrent disease

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	Recurrence (n=83)	Non-recurrence (n=137)		
Laboratory results at baseline				
Hb: per unit (mmol/L) decrease	10.9 (9.8-12.0)	10.8 (9.6-11.9)	0.69	0.97 (0.83-1.13)
WCC: per 10^9/L increase	13.1 (10.0-21.3)	10.6 (7.6-15.5)	**0.05**	1.02 (1.00-1.05)
Neutrophils: per 10^9/L increase	10.1 (7.5-16.9)	8.0 (5.4-12.6)	**0.03**	1.03 (1.00-1.06)
Platelets: per 10^9/L increase	293.0 (198.0-395.0)	300.0 (218.0-392.0)	0.77	1.00 (1.00-1.00)
C-reactive protein: per ten unit (mg/L) increase	76.0 (28.0-156.0)	65.5 (27.0-130.0)	0.29	1.02 (0.99-1.05)
Creatinine: per unit (mmol/L) increase	95.5 (62.0-152.0)	71.5 (58.0-113.0)	**0.04**	1.00 (1.00-1.01)
eGFR: per ten unit (ml/min/1.73m^2) decrease	58.5 (32.6-88.4)	76.2 (46.6-102.6)	**0.02**	1.08 (1.01-1.16)
Albumin: per unit (g/L) decrease	29.0 (25.0-34.0)	31.0 (26.0-35.0)	0.23	1.03 (0.98-1.08)
Sodium: per unit (mmol/L) increase	135.0 (132.0-138.0)	135.0 (133.0-137.0)	0.70	0.99 (0.93-1.05)
Potassium: per unit (mmol/L) increase	3.8 (3.4-4.2)	3.8 (3.4-4.2)	0.65	0.90 (0.57-1.43)
Urea: per unit (mmol/L) increase	7.6 (5.2-12.5)	5.3 (3.7-8.0)	**<0.01**	1.08 (1.03-1.14)
Microbiological information				
Presence of faecal leukocytes	45/80 (56)	67/135 (50)	0.35	1.30 (0.75-2.27)
Toxin OD	1.6 (0.6-3.0)	2.6 (0.6-3.0)	0.22	0.86 (0.68-1.09)
Presence of PCR ribotype 027	33/75 (44)	43/126 (34)	0.16	1.52 (0.84-2.73)
Current admission information				
Length of hospitalisation prior to diagnosis, days	10.0 (1.0-30.0)	7.0 (1.0-19.0)	0.12	1.01 (1.00-1.02)
Admitted via an emergency ward	59/83 (71)	83/136 (61)	0.13	1.57 (0.87-2.82)
Admitted with diarrhoea	33/83 (40)	48/137 (35)	0.48	1.22 (0.70-2.15)
Suffered from previous CDI	14/76 (18)	18/131 (14)	0.37	1.42 (0.66-3.04)

1 (continued): Characteristics of CDI patients by 90-day recurrence versus non-recurrence

	N (%) or Median (IQR)		P-value	OR
	Recurrence (n=83)	Non-recurrence (n=137)		(95% CIs)
Current admission information (continued)				
Recent CDI infection (90 days)	11/81 (14)	9/137 (7)	0.09	2.23 (0.88-5.65)
Recent ICU/HDU admission (90 days)	16/79 (20)	27/134 (20)	0.99	1.01 (0.50-2.01)
Nosocomial admission	52/82 (63)	89/136 (65)	0.76	0.92 (0.52-1.62)
Duration of symptoms prior to diagnosis, days	2.0 (0.0-7.0)	1.0 (0.0-4.0)	0.09	1.03 (1.00-1.06)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **eGFR**: Estimated glomerular filtration rate; **GI:** Gastrointestinal; **Hb**: Haemoglobin; **HDU**: High dependency unit; **ICU**: Intensive care unit; **IQR**: Interquartile range; **N**: Number; **OD**: Optical density; **OR**: Odds ratio; **PPI**: Proton pump inhibitor; **WCC**: White cell count;*

2: Characteristics of CDI patients by severe-complicated versus mild disease

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	Severe-complicated (n=43)	Mild disease (n=213)		
Demographics				
Age at baseline: per decade	71.2 (58.1-78.7)	74.7 (61.2-80.9)	0.33	0.91 (0.76-1.10)
Gender: Female	30/43 (70)	121/213 (57)	0.12	1.75 (0.87-3.55)
Smoking pack years: per year increase	15.5 (0.0-30.0)	6.5 (0.0-35.0)	0.66	1.00 (0.98-1.01)
Body Mass Index	21.5 (18.1-28.5)	23.7 (20.5-27.6)	0.63	0.99 (0.94-1.04)
Medication information				
Number of co-medications at baseline	2.0 (0.0-4.0)	3.0 (2.0-4.0)	**<0.01**	0.76 (0.63-0.91)
Taken fluoroquinolones prior to CDI	13/43 (30)	79/213 (37)	0.39	0.74 (0.36-1.49)
Taken PPIs prior to CDI	21/42 (50)	150/212 (71)	**0.01**	0.41 (0.21-0.81)
Concomitant antibiotics	22/43 (51)	107/213 (50)	0.91	1.04 (0.54-2.00)
Concomitant immunosuppressants	11/43 (26)	38/212 (18)	0.25	1.57 (0.73-3.40)
Clinical characteristics & underlying comorbidities				
Number of stools at baseline	5.0 (3.0-7.0)	5.0 (3.0-6.0)	0.34	1.05 (0.95-1.17)
Fever (≥36.8°C)	5/41 (12)	11/202 (5)	0.12	2.41 (0.79-7.36)
Charlson Comorbidity Index score	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.51	0.93 (0.75-1.15)
Diabetes	8/43 (19)	36/212 (17)	0.80	1.12 (0.48-2.61)
Hypotension	9/38 (24)	21/208 (10)	**0.02**	2.76 (1.15-6.62)
Current malignancy	3/43 (7)	5/213 (2)	0.13	3.12 (0.72-13.58)
Respiratory comorbidities at baseline	29/43 (67)	115/212 (54)	0.11	1.75 (0.87-3.49)
GI comorbidities at baseline	33/43 (77)	122/212 (58)	**0.02**	2.43 (1.14-5.20)
Recent abdominal surgery (90 days)	7/42 (17)	24/210 (11)	0.35	1.55 (0.62-3.87)

2 (continued): Characteristics of CDI patients by severe-complicated versus mild disease

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	Severe-complicated (n=43)	Mild disease (n=213)		
Laboratory results at baseline				
Hb: per unit (mmol/L) decrease	9.9 (8.9-11.4)	10.9 (9.8-12.0)	**0.03**	1.26 (1.02-1.55)
WCC: per 10^9/L increase	15.2 (10.1-25.4)	11.0 (8.1-16.9)	0.16	1.01 (0.99-1.03)
Neutrophils: per 10^9/L increase	12.7 (8.0-21.6)	8.7 (5.6-13.1)	**0.01**	1.04 (1.01-1.07)
Platelets: per 10^9/L increase	275.0 (218.0-407.0)	296.0 (209.0-387.0)	0.38	1.00 (1.00-1.00)
C-reactive protein: per ten unit (mg/L) increase	108.5 (46.0-203.0)	66.0 (29.0-126.0)	**0.01**	1.05 (1.01-1.08)
Creatinine: per unit (mmol/L) increase	85.5 (53.0-143.0)	80.5 (59.0-128.0)	0.59	1.00 (1.00-1.00)
eGFR: per ten unit (ml/min/1.73m^2) decrease	59.1 (41.0-115.9)	69.1 (42.4-97.5)	0.27	0.96 (0.90-1.03)
Albumin: per unit (g/L) decrease	27.0 (23.5-30.0)	31.0 (26.0-35.5)	**<0.01**	1.11 (1.05-1.19)
Sodium: per unit (mmol/L) increase	134.0 (133.0-137.0)	135.0 (132.0-137.0)	0.88	1.01 (0.93-1.09)
Potassium: per unit (mmol/L) increase	4.0 (3.6-4.4)	3.7 (3.4-4.1)	0.15	1.50 (0.87-2.59)
Urea: per unit (mmol/L) increase	8.2 (4.8-13.0)	6.0 (4.0-9.6)	**0.02**	1.06 (1.01-1.11)
Microbiological information				
Presence of faecal leukocytes	18/43 (42)	117/213 (55)	0.12	0.59 (0.30-1.15)
Toxin OD	2.2 (1.0-3.0)	2.5 (0.6-3.0)	0.26	1.16 (0.90-1.51)
Presence of PCR ribotype 027	9/35 (26)	75/199 (38)	0.18	0.57 (0.25-1.29)
Current admission information				
Length of hospitalisation prior to diagnosis, days	7.0 (2.0-19.0)	8.0 (1.0-20.0)	0.69	1.00 (0.99-1.02)
Admitted via an emergency ward	22/33 (67)	137/212 (65)	0.82	1.09 (0.50-2.38)
Admitted with diarrhoea	17/43 (40)	82/213 (39)	0.90	1.04 (0.53-2.04)

2 (continued): Characteristics of CDI patients by severe-complicated versus mild disease

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	Severe-complicated (n=43)	Mild disease (n=213)		
Current admission information (continued)				
Suffered from previous CDI	9/39 (23)	32/207 (15)	0.25	1.64 (0.71-3.78)
Recent CDI infection (90 days)	6/39 (15)	20/211 (9)	0.27	1.74 (0.65-4.65)
Recent ICU/HDU admission (90 days)	8/33 (24)	36/213 (17)	0.31	1.57 (0.66-3.77)
Nosocomial admission	29/43 (67)	135/211 (64)	0.67	1.17 (0.58-2.34)
Duration of symptoms prior to diagnosis, days	1.0 (0.0-6.0)	2.0 (0.0-5.0)	0.89	1.00 (0.97-1.03)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **eGFR**: Estimated glomerular filtration rate; **GI:** Gastrointestinal; **Hb**: Haemoglobin; **HDU**: High dependency unit; **ICU**: Intensive care unit; **IQR**: Interquartile range; **N**: Number; **OD**: Optical density; **OR**: Odds ratio; **PPI**: Proton pump inhibitor; **WCC**: White cell count;*

3: Characteristics of CDI patients by 30-day mortality versus survival

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	Mortality (n=26)	Survival (n=279)		
Demographics				
Age at baseline: per decade	79.4 (72.2-85.9)	74.4 (60.4-80.8)	**0.02**	1.49 (1.07-2.07)
Gender: Female	14/26 (54)	161/279 (58)	0.70	0.86 (0.38-1.92)
Smoking pack years: per year increase	22.3 (7.4-41.6)	7.5 (0.0-35.0)	0.18	1.01 (1.00-1.02)
Body Mass Index	20.6 (18.2-23.4)	23.9 (20.5-28.1)	**0.01**	0.88 (0.81-0.96)
Medication information				
Number of co-medications at baseline	2.0 (0.0-4.0)	3.0 (0.0-4.0)	0.32	0.90 (0.74-1.11)
Taking fluoroquinolones prior to CDI	11/26 (42)	95/279 (34)	0.40	1.42 (0.63-3.21)
Taking PPIs prior to CDI	18/26 (69)	188/277 (68)	0.89	1.07 (0.45-2.54)
Concomitant antibiotics	15/26 (58)	135/279 (48)	0.37	1.45 (0.65-3.28)
Concomitant immunosuppressants	2/26 (8)	50/278 (18)	0.20	0.38 (0.09-1.66)
Clinical characteristics & underlying comorbidities				
Number of stools at baseline	4.0 (3.0-5.0)	5.0 (3.0-6.0)	0.51	0.95 (0.81-1.11)
Fever (≥36.8°C)	1/24 (4)	17/266 (6)	0.67	0.64 (0.08-5.00)
Charlson Comorbidity Index score	2.0 (0.0-4.0)	1.0 (0.0-2.0)	**0.01**	1.31 (1.07-1.61)
Diabetes	5/26 (19)	53/278 (19)	0.98	1.01 (0.36-2.80)
Hypotension	4/25 (16)	32/269 (12)	0.55	1.41 (0.46-4.37)
Current malignancy	2/26 (8)	6/279 (2)	0.11	3.79 (0.73-19.82)
Respiratory comorbidities at baseline	20/26 (77)	147/278 (53)	**0.02**	2.97 (1.16-7.62)
GI comorbidities at baseline	14/25 (56)	163/279 (58)	0.81	0.91 (0.40-2.07)
Recent abdominal surgery (90 days)	1/25 (4)	32/270 (12)	0.26	0.31 (0.04-2.37)

3 (continued): Characteristics of CDI patients by 30-day mortality versus survival

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	Mortality (n=26)	Survival (n=279)		
Laboratory results at baseline				
Hb: per unit (mmol/L) decrease	10.5 (9.9-11.9)	10.8 (9.6-11.9)	0.76	0.96 (0.76-1.22)
WCC: per 10^9/L increase	12.2 (10.0-15.6)	11.7 (8.1-17.7)	0.53	0.99 (0.94-1.03)
Neutrophils: per 10^9/L increase	10.0 (7.1-13.1)	9.0 (6.0-14.8)	0.81	0.99 (0.95-1.04)
Platelets: per 10^9/L increase	222.0 (184.0-342.0)	295.5 (213.0-395.0)	0.12	1.00 (0.99-1.00)
C-reactive protein: per ten unit (mg/L) increase	73.0 (45.0-131.0)	70.0 (29.0-140.0)	0.68	0.99 (0.94-1.04)
Creatinine: per unit (mmol/L) increase	89.0 (59.0-107.0)	81.0 (59.0-138.0)	0.52	1.00 (0.99-1.00)
eGFR: per ten unit (ml/min/1.73m^2) decrease	72.8 (44.6-114.4)	67.4 (41.4-99.9)	0.13	0.94 (0.88-1.02)
Albumin: per unit (g/L) decrease	30.0 (24.5-33.5)	30.0 (25.0-34.0)	0.79	1.01 (0.94-1.08)
Sodium: per unit (mmol/L) increase	134.0 (133.0-138.0)	135.0 (132.0-138.0)	0.81	1.01 (0.92-1.12)
Potassium: per unit (mmol/L) increase	3.7 (3.4-4.3)	3.8 (3.4-4.2)	0.82	0.92 (0.46-1.85)
Urea: per unit (mmol/L) increase	6.6 (5.0-11.6)	6.2 (4.0-10.5)	0.45	1.02 (0.97-1.08)
Microbiological information				
Presence of faecal leukocytes	11/25 (44)	145/275 (53)	0.41	0.70 (0.31-1.61)
Toxin OD	3.0 (0.8-3.0)	2.4 (0.6-3.0)	0.64	1.07 (0.80-1.44)
Presence of PCR ribotype 027	8/24 (33)	81/256 (32)	0.87	1.08 (0.44-2.63)
Current admission information				
Length of hospitalisation prior to diagnosis, days	11.0 (4.0-20.0)	8.0 (1.0-19.0)	0.38	0.99 (0.97-1.01)
Admitted via an emergency ward	17/21 (81)	150/235 (64)	0.12	2.41 (0.78-7.39)
Admitted with diarrhoea	8/26 (31)	105/279 (38)	0.49	0.74 (0.31-1.75)

3 (continued): Characteristics of CDI patients by 30-day mortality versus survival

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	Mortality (n=26)	Survival (n=279)		
Current admission information (continued)				
Suffered from previous CDI	4/25 (16)	42/256 (16)	0.96	0.97 (0.32-2.97)
Recent CDI infection (90 days)	1/24 (4)	27/265 (10)	0.36	0.38 (0.05-2.95)
Recent ICU/HDU admission (90 days)	1/20 (5)	45/230 (20)	0.14	0.22 (0.03-1.66)
Nosocomial admission	20/26 (77)	181/277 (65)	0.24	1.77 (0.69-4.55)
Duration of symptoms prior to diagnosis, days	1.0 (0.0-6.0)	2.0 (0.0-5.0)	0.93	1.00 (0.96-1.04)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **eGFR**: Estimated glomerular filtration rate; **GI:** Gastrointestinal; **Hb**: Haemoglobin; **HDU**: High dependency unit; **ICU**: Intensive care unit; **IQR**: Interquartile range; **N**: Number; **OD**: Optical density; **OR**: Odds ratio; **PPI**: Proton pump inhibitor; **WCC**: White cell count;*

4: Characteristics of CDI patients by symptoms ≥10 days versus <10 days

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	≥10 days (n=109)	<10 days (n=165)		
Demographics				
Age at baseline: per decade	76.9 (62.8-83.5)	72.7 (60.0-79.0)	0.06	1.16 (0.99-1.35)
Gender: Female	62/109 (57)	96/165 (58)	0.83	0.95 (0.58-1.55)
Smoking pack years: per year increase	11.9 (0.0-32.4)	7.9 (0.0-35.0)	0.84	1.00 (0.99-1.01)
Body Mass Index	22.9 (19.8-27.5)	23.9 (20.6-28.3)	0.55	0.99 (0.95-1.03)
Medication information				
Number of co-medications at baseline	2.0 (0.0-4.0)	3.0 (0.0-4.0)	0.39	0.95 (0.84-1.07)
Taking fluoroquinolones prior to CDI	39/109 (36)	54/165 (33)	0.60	1.15 (0.69-1.91)
Taking PPIs prior to CDI	69/108 (64)	112/164 (68)	0.45	0.82 (0.49-1.37)
Concomitant antibiotics	50/109 (46)	84/165 (51)	0.41	0.82 (0.50-1.33)
Concomitant immunosuppressants	20/109 (18)	25/164 (15)	0.50	1.25 (0.66-2.38)
Clinical characteristics & underlying comorbidities				
Number of stools at baseline	5.0 (3.0-7.0)	4.0 (3.0-6.0)	0.20	1.05 (0.97-1.14)
Fever (≥36.8°C)	10/106 (9)	6/159 (4)	0.07	2.66 (0.94-7.54)
Charlson Comorbidity Index score	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.21	0.90 (0.77-1.06)
Diabetes	20/108 (19)	32/165 (19)	0.86	0.94 (0.51-1.76)
Hypotension	14/103 (14)	17/161 (11)	0.46	1.33 (0.63-2.84)
Current malignancy	4/109 (4)	3/165 (2)	0.35	2.06 (0.45-9.38)
Respiratory comorbidities at baseline	59/108 (55)	85/165 (52)	0.61	1.13 (0.70-1.84)
GI comorbidities at baseline	70/108 (65)	90/165 (55)	0.09	1.54 (0.93-2.53)
Recent abdominal surgery (90 days)	11/105 (10)	20/161 (12)	0.63	0.83 (0.38-1.80)

4 (continued): Characteristics of CDI patients by symptoms ≥10 days versus <10 days

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	≥10 days (n=109)	<10 days (n=165)		
Laboratory results at baseline				
Hb: per unit (mmol/L) decrease	10.3 (9.3-11.8)	10.9 (9.8-12.0)	**0.03**	1.18 (1.02-1.36)
WCC: per 10^9/L increase	12.4 (9.2-19.8)	10.9 (7.9-15.5)	0.70	1.00 (0.99-1.02)
Neutrophils: per 10^9/L increase	10.3 (7.0-17.4)	8.6 (5.5-12.6)	**0.05**	1.03 (1.00-1.05)
Platelets: per 10^9/L increase	287.0 (213.0-354.0)	289.0 (208.5-394.0)	0.91	1.00 (1.00-1.00)
C-reactive protein: per ten unit (mg/L) increase	90.5 (39.0-164.0)	61.0 (29.0-106.0)	**0.01**	1.04 (1.01-1.07)
Creatinine: per unit (mmol/L) increase	81.0 (59.0-141.0)	78.0 (58.0-129.0)	0.47	1.00 (1.00-1.00)
eGFR: per ten unit (ml/min/1.73m^2) decrease	62.0 (40.8-99.4)	73.7 (43.9-102.1)	0.44	1.02 (0.97-1.07)
Albumin: per unit (g/L) decrease	29.0 (25.0-35.0)	31.0 (25.0-35.0)	0.06	1.04 (1.00-1.08)
Sodium: per unit (mmol/L) increase	135.0 (132.0-138.0)	136.0 (133.0-138.0)	0.42	0.98 (0.92-1.03)
Potassium: per unit (mmol/L) increase	3.7 (3.4-4.2)	3.8 (3.4-4.2)	0.80	0.95 (0.63-1.42)
Urea: per unit (mmol/L) increase	6.8 (4.3-10.5)	5.7 (3.9-9.4)	0.06	1.04 (1.00-1.08)
Microbiological information				
Presence of faecal leukocytes	58/108 (54)	79/162 (49)	0.43	1.22 (0.75-1.99)
Toxin OD	2.4 (0.7-3.0)	2.5 (0.5-3.0)	0.57	0.95 (0.79-1.14)
Presence of PCR ribotype 027	34/95 (36)	43/155 (28)	0.18	1.45 (0.84-2.51)
Current admission information				
Length of hospitalisation prior to diagnosis, days	8.5 (1.0-28.5)	7.0 (1.0-17.0)	0.06	1.01 (1.00-1.02)
Admitted via an emergency ward	56/87 (64)	86/135 (64)	0.92	1.03 (0.59-1.81)
Admitted with diarrhoea	42/109 (39)	63/165 (38)	0.95	1.01 (0.62-1.67)

4 (continued): Characteristics of CDI patients by symptoms ≥10 days versus <10 days

	N (%) or Median (IQR)		P-value	OR (95% CIs)
	≥10 days (n=109)	<10 days (n=165)		
Current admission information (continued)				
Suffered from previous CDI	19/97 (20)	22/155 (14)	0.26	1.47 (0.75-2.89)
Recent CDI infection (90 days)	12/102 (12)	11/156 (7)	0.20	1.76 (0.74-4.15)
Recent ICU/HDU admission (90 days)	15/84 (18)	23/133 (17)	0.92	1.04 (0.51-2.13)
Nosocomial admission	71/109 (65)	106/164 (65)	0.93	1.02 (0.62-1.70)
Duration of symptoms prior to diagnosis, days	2.0 (1.0-6.0)	1.0 (0.0-4.0)	**0.01**	1.04 (1.01-1.07)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **eGFR**: Estimated glomerular filtration rate; **GI:** Gastrointestinal; **Hb**: Haemoglobin; **HDU**: High dependency unit; **ICU**: Intensive care unit; **IQR**: Interquartile range; **N**: Number; **OD**: Optical density; **OR**: Odds ratio; **PPI**: Proton pump inhibitor; **WCC**: White cell count;*

5: Random sampling model validation for 90-day recurrence

Variable	P-value	OR (95% CIs)
Initial model (Dataset 1; n=88)		
Age at baseline: per decade	0.20	1.22 (0.90-1.66)
Taken fluoroquinolones prior to CDI	0.06	2.91 (0.97-8.67)
Respiratory comorbidities at baseline	0.15	2.22 (0.75-6.60)
Neutrophils: per 10^9/L increase	0.46	1.02 (0.97-1.07)
eGFR: per ten unit (ml/min/1.73m^2) decrease	0.81	1.02 (0.87-1.19)
Urea: per unit (mmol/L) increase	0.86	0.99 (0.90-1.09)
Recent abdominal surgery (90 days)	0.95	0.94 (0.16-5.43)
Presence of PCR ribotype 027	0.81	0.88 (0.32-2.45)
Length of hospitalisation prior to diagnosis, days	0.47	1.01 (0.99-1.03)
Admitted via an emergency ward	0.41	0.61 (0.19-1.95)
Recent CDI infection (90 days)	0.71	1.45 (0.21-9.88)
Duration of symptoms prior to diagnosis, days	0.26	1.04 (0.97-1.11)
Reduced model (Dataset 1; n=99)		
Age at baseline: per decade	0.03	1.36 (1.03-1.78)
Taken fluoroquinolones prior to CDI	0.03	2.86 (1.10-7.44)
Recent abdominal surgery (90 days)	0.69	0.73 (0.15-3.52)
Neutrophils: per 10^9/L increase	0.23	1.02 (0.99-1.06)
Length of hospitalisation prior to diagnosis, days	0.43	1.01 (0.99-1.03)
Duration of symptoms prior to diagnosis, days	0.34	1.03 (0.97-1.10)
Reduced model (Dataset 2; n=91)		
Age at baseline: per decade	<0.01	2.35 (1.47-3.76)
Taken fluoroquinolones prior to CDI	0.22	1.94 (0.68-5.54)
Neutrophils: per 10^9/L increase	0.87	0.99 (0.94-1.06)
Length of hospitalisation prior to diagnosis, days	0.01	1.03 (1.01-1.05)
Duration of symptoms prior to diagnosis, days	0.22	1.04 (0.98-1.10)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **eGFR**: Estimated glomerular filtration rate; **OR**: Odds ratio; **PCR**: Polymerase chain reaction;*

6: Random sampling model validation for severe-complicated disease

Variable	P-value	OR (95% CIs)
Initial model (Dataset 1; n=67)		
GI comorbidities at baseline	0.08	14.72 (0.75-289.25)
Hb: per unit (mmol/L) decrease	0.21	1.45 (0.81-2.61)
Neutrophils: per 10^9/L increase	0.97	1.00 (0.88-1.13)
Albumin: per unit (g/L) decrease	0.14	1.22 (0.94-1.58)
C-reactive protein: per ten unit (mg/L) increase	0.97	1.00 (0.88-1.15)
Urea: per unit (mmol/L) increase	0.11	1.13 (0.97-1.32)
Potassium: per unit (mmol/L) increase	0.59	1.91 (0.18-19.91)
Number of co-medications at baseline	0.14	0.53 (0.23-1.24)
Taking PPIs prior to CDI	0.33	0.31 (0.03-3.25)
Respiratory comorbidities at baseline	0.99	0.99 (0.07-14.53)
Gender: Female	0.05	38.11 (1.06-1367.33)
Presence of faecal leukocytes	0.58	0.59 (0.09-3.88)
Presence of PCR ribotype 027	0.73	1.69 (0.09-31.17)
Fever (≥38.6°C)	0.39	0.15 (0.00-11.30)
Current malignancy	0.85	1.96 (0.00-2429.33)
Hypotension	0.66	1.76 (0.14-22.51)

6 (continued): Random sampling model validation for severe-complicated disease

Variable	P-value	OR (95% CIs)
Reduced model (Dataset 1; n=83)		
GI comorbidities at baseline	0.08	4.98 (0.84-29.33)
Hb: per unit (mmol/L) decrease	0.21	1.36 (0.84-2.20)
Albumin: per unit (g/L) decrease	0.02	1.21 (1.02-.42)
Urea: per unit (mmol/L) increase	0.40	1.04 (0.95-1.14)
Potassium: per unit (mmol/L) increase	0.35	1.87 (0.51-6.87)
Taking PPIs prior to CDI	0.27	0.45 (0.11-1.86)
Gender: Female	0.03	7.40 (1.29-42.45)
Fever (≥38.6°C)	0.84	1.30 (0.11-15.32)
Current malignancy	0.65	2.11 (0.08-56.16)
Hypotension	0.44	2.11 (0.32-13.83)
Reduced model (Dataset 2; n=100)		
GI comorbidities at baseline	0.47	1.75 (0.38-8.01)
Hb: per unit (mmol/L) decrease	0.19	1.33 (0.87-2.04)
Albumin: per unit (g/L) decrease	0.21	1.08 (0.96-1.23)
Urea: per unit (mmol/L) increase	0.10	1.14 (0.98-1.33)
Potassium: per unit (mmol/L) increase	0.26	1.90 (0.63-5.72)
Taking PPIs prior to CDI	0.04	0.18 (0.03-0.93)
Gender: Female	0.40	2.08 (0.38-11.27)
Fever (≥38.6°C)	0.02	25.81 (1.74-383.88)
Hypotension	0.44	2.41 (0.26-22.77)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **GI**: Gastrointestinal; **Hb**: Haemoglobin; **OR**: Odds ratio; **PCR**: Polymerase chain reaction; **PPI**: Proton pump inhibitor;*

7: Random sampling model validation for 30-day mortality

Variable	P-value	OR (95% CIs)
Initial model (Dataset 1; n=68)		
Age at baseline: per decade	0.99	1.01 (0.43-2.35)
Body Mass Index	0.09	0.82 (0.66-1.03)
Charlson Comorbidity Index score	0.03	2.77 (1.13-6.77)
Smoking pack years: per year increase	0.29	1.02 (0.98-1.06)
Platelets: per 10^9/L increase	0.63	0.98 (0.91-1.06)
Respiratory comorbidities at baseline	0.50	0.32 (0.01-8.71)
Admitted via an emergency ward	0.18	13.12 (0.30-582.62)
Reduced model (Dataset 1; n=141)		
Age at baseline: per decade	0.20	1.42 (0.83-2.43)
Body Mass Index	0.09	0.87 (0.73-1.02)
Platelets: per 10^9/L increase	0.07	0.94 (0.87-1.01)
Charlson Comorbidity Index score	0.14	1.40 (0.89-2.22)
Reduced model (Dataset 2; n=145)		
Age at baseline: per decade	0.11	1.48 (0.92-2.39)
Body Mass Index	0.03	0.86 (0.75-0.98)
Platelets: per 10^9/L increase	0.52	0.98 (0.94-1.03)
Charlson Comorbidity Index score	0.16	1.23 (0.92-1.65)

***CI**: Confidence interval; **OR**: Odds ratio;*

8: Random sampling model validation for prolonged disease

Variable	P-value	OR (95% CIs)
Initial model (Dataset 1; n=87)		
Age at baseline: per decade	0.68	1.07 (0.76-1.51)
GI comorbidities at baseline	0.95	1.03 (0.37-2.93)
Duration of symptoms prior to diagnosis, days	0.14	1.07 (0.98-1.16)
Neutrophils: per 10^9/L increase	0.04	1.09 (1.00-1.18)
Albumin: per unit (g/L) decrease	0.72	1.02 (0.93-1.11)
Length of hospitalisation prior to diagnosis, days	0.73	1.00 (0.98-1.03)
Hb: per unit (mmol/L) decrease	0.32	1.17 (0.86-1.59)
Urea: per unit (mmol/L) increase	0.96	1.00 (0.91-1.11)
Presence of PCR ribotype 027	0.71	1.25 (0.39-4.07)
C-reactive protein: per ten unit (mg/L) increase	0.84	0.99 (0.92-1.07)
Fever (≥38.6°C)	0.49	1.91 (0.30-12.19)
Recent CDI infection (90 days)	0.88	1.13 (0.22-5.91)
Number of stools at baseline	0.60	0.95 (0.79-1.15)
Reduced model (Dataset 1; n=122)		
Age at baseline: per decade	0.32	1.15 (0.88-1.50)
Duration of symptoms prior to diagnosis, days	0.19	1.05 (0.98-1.12)
Presence of PCR ribotype 027	0.32	1.56 (0.65-3.70)
Neutrophils: per 10^9/L increase	0.01	1.08 (1.02-1.14)
Urea: per unit (mmol/L) increase	0.84	0.99 (0.92-1.07)
Length of hospitalisation prior to diagnosis, days	0.31	1.01 (0.99-1.03)
Hb: per unit (mmol/L) decrease	0.21	1.16 (0.92-1.46)
Reduced model (Dataset 2; n=115)		
Age at baseline: per decade	0.40	1.13 (0.85-1.52)
Duration of symptoms prior to diagnosis, days	0.03	1.05 (1.00-1.10)
Presence of PCR ribotype 027	0.17	1.89 (0.76-4.71)
Neutrophils: per 10^9/L increase	0.73	1.01 (0.97-1.04)
Urea: per unit (mmol/L) increase	0.02	1.08 (1.01-1.15)
Length of hospitalisation prior to diagnosis, days	0.06	1.02 (1.00-1.05)
Hb: per unit (mmol/L) decrease	0.13	1.23 (0.94-1.61)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **GI**: Gastrointestinal; **Hb**: Haemoglobin; **OR**: Odds ratio; **PCR**: Polymerase chain reaction;*

9: Imputed multivariate model for predicting disease recurrence (n=200)

Variable	P-value	OR (95% CIs)
Age at baseline: per decade	<0.01	1.46 (1.19-1.80)
Taken fluoroquinolones prior to CDI	0.02	2.10 (1.13-3.93)
Recent abdominal surgery (90 days)	0.03	0.21 (0.05-0.82)
Neutrophils: per 10^9/L increase	0.07	1.03 (1.00-1.06)
Length of hospitalisation prior to diagnosis, days	0.03	1.01 (1.00-1.03)
Duration of symptoms prior to diagnosis, days	0.15	1.03 (0.99-1.06)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **OR**: Odds ratio;*

10: Imputed multivariate model for predicting severe-complicated disease (n=183)

Variable	P-value	OR (95% CIs)
GI comorbidities at baseline	0.03	2.52 (1.08-5.87)
Hb: per unit (mmol/L) decrease	0.20	1.18 (0.92-1.53)
Albumin: per unit (g/L) decrease	0.03	1.09 (1.01-1.18)
Urea: per unit (mmol/L) increase	0.06	1.06 (1.00-1.13)
Potassium: per unit (mmol/L) increase	0.23	1.50 (0.78-2.89)
Taking PPIs prior to CDI	<0.01	0.17 (0.12-0.61)
Gender: Female	0.04	2.47 (1.07-5.74)
Fever (≥38.6°C)	0.10	3.16 (0.82-12.20)
Current malignancy	0.17	3.24 (0.60-17.47)
Hypotension	0.28	1.86 (0.60-5.77)

***CDI**: Clostridium difficile infection; **CI**: Confidence interval; **GI**: Gastrointestinal; **Hb**: Haemoglobin; **OR**: Odds ratio; **PPI**: Proton pump inhibitor;*

11: Imputed multivariate model for predicting mortality (n=286)

Variable	P-value	OR (95% CIs)
Age at baseline: per decade	0.03	1.45 (1.04-2.03)
Body Mass Index	<0.01	0.86 (0.78-0.95)
Platelets: per 10^9/L increase	0.09	0.97 (0.93-1.01)
Charlson Comorbidity Index score	0.02	1.32 (1.05-1.66)

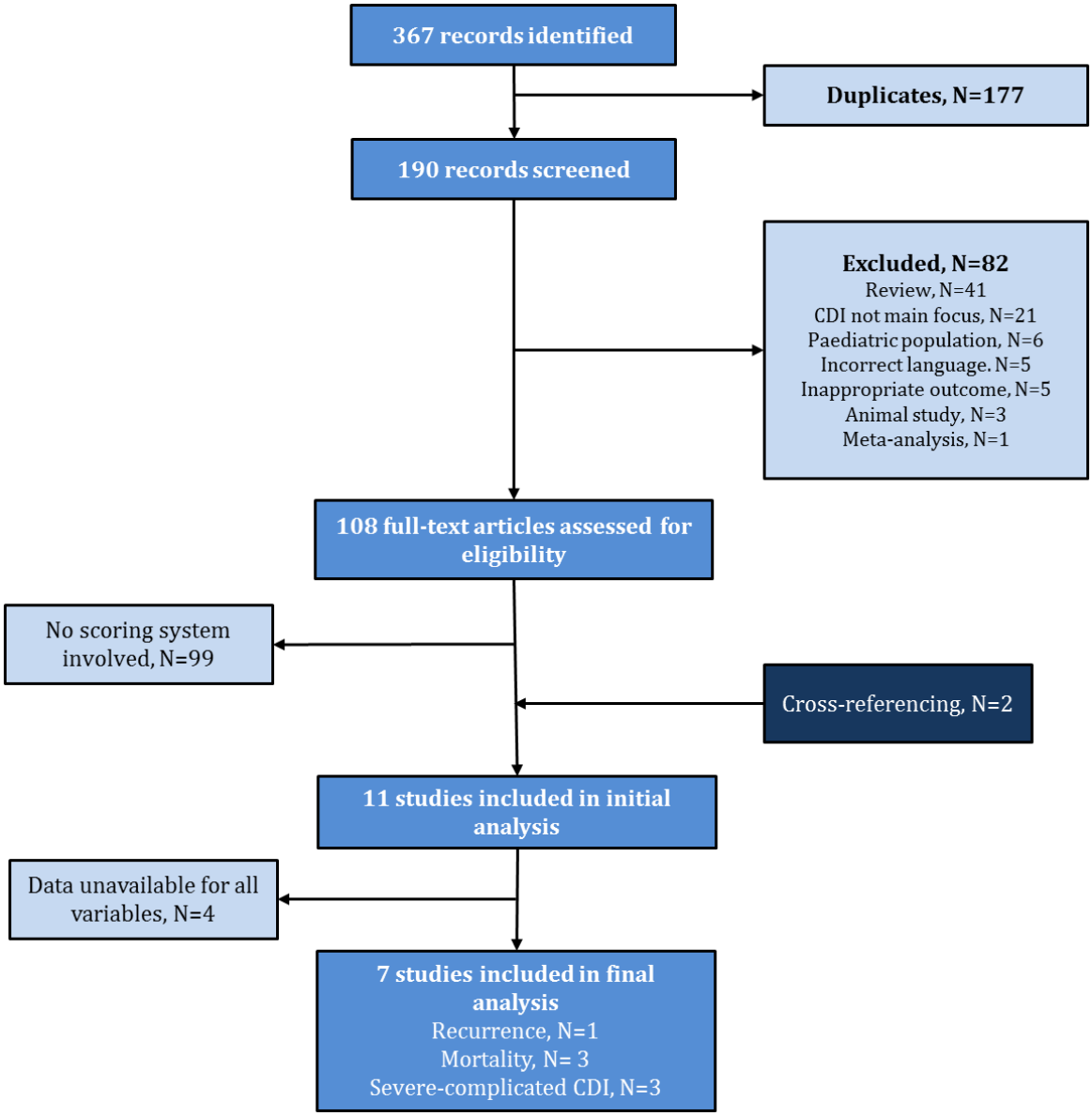
***CI**: Confidence interval; **OR**: Odds ratio;*

12: Imputed multivariate model for predicting prolonged disease (n=237)

Variable	P-value	OR (95% CIs)
Age at baseline: per decade	0.30	1.09 (0.92-1.29)
Duration of symptoms prior to diagnosis, days	0.01	1.05 (1.01-1.08)
Presence of PCR ribotype 027	0.24	1.42 (0.79-2.57)
Neutrophils: per 10^9/L increase	0.08	1.02 (1.00-1.05)
Urea: per unit (mmol/L) increase	0.11	1.04 (0.99-1.08)
Length of hospitalisation prior to diagnosis, days	0.08	1.01 (1.00-1.02)
Hb: per unit (mmol/L) decrease	0.04	1.18 (1.01-1.39)

***CI**: Confidence interval; **Hb**: Haemoglobin; **OR**: Odds ratio; **PCR**: Polymerase chain reaction;*

13: Overview of systematic review process



14: Recurrence CPR assessment: D'Agostino *et al*. 2014

Score	Our study: 30-day (Overall prevalence = 30%)		Our study: 90-day (Overall prevalence = 46%)		D'Agostino: 30-day (Overall prevalence = 20%)	
	Outcome prevalence (%)	Prevalence based on cut-off (%)	Outcome prevalence (%)	Prevalence based on cut-off (%)	Outcome prevalence (%)	Prevalence based on cut-off (%)
0	16/77 (21)	65/227 (29)	24/77	100/227 (44)	NP (NP)	No cut-off suggested
1	14/57 (25)		28/57		NP (NP)	
2	19/49 (39)		28/49		NP (NP)	
3	16/44 (36)		20/44		NP (NP)	
4	6/11 (55)	7/12 (58)	8/11	9/12 (75)	NP (NP)	
5	1/1 (100)		1/1		NP (NP)	

*NP: Data not provided; * No cut-off was suggested by the authors therefore in order to produce descriptive stats for our cohort we decided upon a cut-off of ≥4;*

15: Severe-complicated CPR assessment: Drew *et al*. 2009

Score	Our study (Overall prevalence = 18%)		Drew *et al** (Overall prevalence = 14%)	
	Outcome prevalence (%)	Prevalence based on cut-off (%)	Outcome prevalence (%)	Prevalence based on cut-off (%)
0	1/21 (5)	20/145 (14)	NP (NP)	NP (NP)
1	3/50 (2)		NP (NP)	
2	7/42 (17)		NP (NP)	
3	9/32 (28)		NP (NP)	
4	3/17 (18)	15/46 (30)	NP (NP)	NP (NP)
5	5/15 (33)		NP (NP)	
6	1/4 (25)		NP (NP)	
7	2/6 (33)		NP (NP)	
8	1/2 (50)		NP (NP)	
9	2/3 (67)		NP (NP)	

*NP: Data not provided; * Letter to the Editor;*

16: Severe-complicated CPR assessment: Lungulescu *et al*. 2011

Score	Our study (Overall prevalence = 17%)		Lungulescu *et al* (Overall prevalence = 21%)	
	Outcome prevalence (%)	Prevalence based on cut-off (%)	Outcome prevalence (%)	Prevalence based on cut-off (%)
0	7/63 (11)	20/136 (15)	0/29 (0)	6/89 (7)
1	13/73 (18)		6/60 (10)	
2	8/43 (19)	12/52 (23)	18/53 (34)	28/73 (38)
3	3/8 (38)		8/15 (53)	
4	1/1 (100)		2/5 (40)	

17: Severe-complicated CPR assessment: Hensgens *et al*. 2014

Score	Our study (Overall prevalence = 12%)		Hensgens *et al*: Derivation (Overall prevalence = 12%)		Hensgens *et al*: Validation (Overall prevalence = 5%)	
	Outcome prevalence (%)	Prevalence based on cut-off (%)	Outcome prevalence (%)	Prevalence based on cut-off (%)	Outcome prevalence (%)	Prevalence based on cut-off (%)
-3	0/1 (0)	25/207 (12)	0/15 (0)	28/340 (8)	NP (NP)	4/125 (3)
-2	2/14 (14)		0/40 (0)		NP (NP)	
-1	1/2 (50)		0/7 (0)		NP (NP)	
0	1/22 (5)		2/65 (3)		NP (NP)	
1	6/71 (9)		6/92 (7)		NP (NP)	
2	3/12 (25)		3/26 (11)		NP (NP)	
3	12/85 (14)		17/95 (18)		NP (NP)	
4	1/4 (25)	4/27 (15)	2/7 (34)	21/55 (39)	NP (NP)	3/14 (21)
5	3/21 (14)		11/35 (32)		NP (NP)	
6	No patients		1/3 (33)		NP (NP)	
7	0/2 (0)		4/6 (63)		NP (NP)	
8	No patients		3/3 (100)		NP (NP)	

NP: Data not provided;

18: Mortality CPR assessment: Bhangu *et al*. 2010

Score	Our study (Overall prevalence = 8%)		Bhangu *et al* (Overall prevalence = 38%)	
	Outcome prevalence (%)	Prevalence based on cut-off (%)	Outcome prevalence (%)	Prevalence based on cut-off (%)
0	7/92 (8)	17/224 (8)	3/22 (14)	50/142 (35)
1	6/85 (7)		16/64 (25)	
2	2/39 (5)		19/35 (54)	
3	2/8 (25)		12/21 (57)	
4	No patients	No patients	6/7 (86)	8/9 (89)
5	No patients		2/2 (100)	

19: Mortality CPR assessment: Welfare *et al*. 2011

Score	Our study (Overall prevalence = 9%)			Welfare *et al* (Overall prevalence = 30%)		
	Outcome prevalence (%)	Prevalence based on cut-off (%)*		Outcome prevalence (%)	Prevalence based on cut-off (%)	
0	2/40 (5)	8/138 (6)	19/254 (7)	NP (NP)	NP (<22)	No binary cut-off suggested
2	1/31 (3)			NP (NP)		
3	5/67 (8)			NP (NP)		
4	5/38 (13)	17/166 (10)		NP (NP)	NP (32-48)	
5	6/78 (8)			NP (NP)		
6	6/49 (12)		7/52 (13)	NP (NP)		
7	0/1 (0)			NP (NP)		
8	1/2 (50)	1/2 (50)		NP (NP)	NP (66)	

*NP: Data not provided; *No binary cut-off was suggested by the authors therefore in order to produce descriptive stats for our cohort we decided upon a cut-off of ≥6;*

20: Mortality CPR assessment: Butt *et al*. 2013

Score	Our study (Overall prevalence = 8%)		Butt *et al*: Derivation (Overall prevalence = 21%)		Butt *et al*: Validation (Overall prevalence = 38%)	
	Outcome prevalence (%)	Prevalence based on cut-off (%)*	Outcome prevalence (%)	Prevalence based on cut-off (%)	Outcome prevalence (%)	Prevalence based on cut-off (%)
0	6/93 (7)	17/199 (9)	13/125 (10)	No cut-off suggested	9/43 (21)	No cut-off suggested
1	11/106 (10)		20/86 (23)		23/62 (37)	
2	2/34 (6)	2/40 (5)	12/28 (43)		25/46 (54)	
3	0/6 (0)		5/5 (100)		2/3 (67)	

**No cut-off was suggested by the authors therefore in order to produce descriptive stats for our cohort we decided upon a cut-off of ≥2;*

21: Summary of previous studies investigating serum immune response to *Clostridium difficile* toxins and non-toxin antigens

Study	Immunoglobulin	Protein	n	Outcome	Association
Aronsson *et al.* (1985)	IgA IgG IgM	tcdA tcdB	61 CDI cases	Recurrence (FT)	Anti-tcdB IgG
Johnson *et al.* (1992)	IgA IgG	tcdA	21 CDI cases 9 Asymptomatic carriers 10 Controls	Disease susceptibility	Anti-tcdA IgA Anti-tcdA IgG
				Asymptomatic carriage	None
Mulligan *et al.* (1993)	IgA IgM Polyvalent[a]	SCAs	5 CDI cases 21 Asymptomatic carriers 26 Controls	Disease susceptibility	None
				Asymptomatic carriage	Anti-SCA IgA Anti-SCA IgM Anti-SCA Polyvalent
Warny *et al.* (1994)	Serum IgA Serum IgG	tcdA	40 CDI cases 280 Controls	Disease susceptibility	None
				60-day recurrence (FT)	Anti-tcdA IgG
				Duration of symptoms	Anti-tcdA IgG
Kyne *et al.* (2000)	IgA IgG IgM	tcdA tcdB NTAs	47 CDI cases 37 Asymptomatic carriers 187 Controls	Colonisation	None
				Asymptomatic carriage	Anti-tcdA IgG
Kyne *et al.* (2001)	IgA IgG IgM	tcdA tcdB NTAs	63 CDI cases	60-day recurrence (FD)	Anti-tcdA IgG Anti-tcdA IgM
Drudy *et al.* (2004)	IgA IgG IgM	SLPs	55 CDI cases 34 Asymptomatic carriers 57 Controls	Disease susceptibility	None
				Asymptomatic carriage	None
				60-day recurrence (FT)	Anti-SLP IgM
Jiang *et al.* (2007)	IgG	tcdA	24 CDI cases 20 Controls	Disease susceptibility	None[b]
Katchar *et al.* (2007)	IgA IgG (total) IgG (subclasses 1-4)	tcdA tcdB	26 CDI cases[c]	60-day recurrence (FT)	Anti-tcdA IgG2 Anti-tcdA IgG3

21 (continued): Summary of previous studies investigating serum immune response to *Clostridium difficile* toxins and non-toxin antigens

Study	Immunoglobulin	Protein	n	Outcome	Association
Sanchez-Hurtado *et al*. (2008)	IgG IgM	Crude toxin[d] tcdA EDTA extraction[e] SLPs LC	21 CDI cases 21 Asymptomatic controls 26 Controls	Disease susceptibility	Anti-crude toxin IgG Anti-SLP IgG
				Asymptomatic carriage	None
Solomon *et al*. (2013)	IgG IgM	tcdA tcdB	150 CDI cases	30-day all-cause mortality	Anti-tcdA IgG
				60-day recurrence (FS)	None
Bauer *et al*. (2014)	IgA IgG	tcdA tcdB Non-toxin CSAs	120 CDI cases[f]	60-day recurrence (FT)	Anti-tcdA IgA Anti-tcdA IgG Anti-tcdB IgG
Islam *et al*. (2014)	IgA IgG IgM	tcdA tcdB	20 CDI cases (Brighton) 18 Controls	Disease susceptibility	Anti-tcdB Pooled antibody Anti-tcdB IgA
			20 CDI cases (Michigan) 20 Controls		None

CSA: Cell surface antigen: EDTA: Ethylenediaminetetraacetic acid; FD: From discharge; FS: From symptom resolution; FT: From treatment completion; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IgM: Immunoglobulin M; LC: Lipocarbohydrate; NTA: Non-toxin antigen; SCA: Somatic cell antigen; SLP: Surface layer protein; tcdA: Clostridium difficile toxin A; tcdB: Clostridium difficile toxin B;

[a] IgA-, IgG- and IgM-specific; [b] Only identified a significant association when stratifying immune response based upon IL-8 rs4073 genotype; [c] 13 recurrent CDI cases versus 13 non-recurrent matched controls; [d] Dialysis culture supernatant containing toxins A and B together with other extracellular products, including surface-layer proteins; [e] Contained cell-surface proteins and carbohydrates; [f] Samples were taken during a prospective cohort study into the safety and efficacy of a whey protein concentrate to prevent recurrences after successful antibiotic treatment of CDI;

22: Comparison of standard- and high-bind plates for IgG response to tcdB

Sample code	Plate type	Coating conc. (µg/ml)	Fold Dilution					
			Buffer	625	125	25	5	Neat
A3X	HB	0.0	115	118	147	187	133	167
		2.5	122	119	142	153	105	167
		5.0	114	116	139	142	105	158
		10.0	115	118	140	142	96	142
	SB	0.0	98	97	97	161	150	109
		2.5	91	94	94	127	120	99
		5.0	91	96	96	126	117	112
		10.0	99	100	100	147	139	119
PHR	HB	0.0	105	115	115	207	162	772
		2.5	109	112	112	213	189	773
		5.0	101	112	112	211	184	862
		10.0	261	118	118	217	220	735
	SB	0.0	90	98	98	279	316	195
		2.5	93	113	113	405	521	415
		5.0	91	114	114	374	532	509
		10.0	86	119	119	404	656	768

HB: High bind; SB: Standard bind;

23: Comparison of standard- and high-bind plates for IgM response to tcdA

Sample code	Plate type	Coating conc. (µg/ml)	Fold Dilution					
			Buffer	625	125	25	5	Neat
A3X	HB	0.0	99	89	89	82	88	160
		2.5	111	89	89	81	84	152
		5.0	101	93	93	84	79	152
		10.0	103	86	88	84	80	146
	SB	0.0	89	95	335	95	116	140
		2.5	89	94	89	91	108	140
		5.0	279	89	97	99	136	171
		10.0	77	90	93	107	137	270
PHR	HB	0.0	87	89	87	83	80	118
		2.5	90	90	84	79	80	107
		5.0	90	88	86	81	82	103
		10.0	92	90	85	82	79	108
	SB	0.0	101	102	103	113	107	109
		2.5	92	103	100	111	95	101
		5.0	106	96	98	100	93	104
		10.0	92	94	93	102	91	101

HB: High bind; SB: Standard bind;

24: Comparison of standard- and high-bind plates for IgM response to tcdB

Sample code	Plate type	Coating conc. (µg/ml)	Fold Dilution					
			Buffer	625	125	25	5	Neat
A3X	HB	0.0	95	90	89	80	77	1296
		2.5	104	87	90	108	77	135
		5.0	87	85	87	77	77	132
		10.0	97	87	85	80	69	131
	SB	0.0	93	96	94	98	107	114
		2.5	88	91	92	93	98	111
		5.0	88	91	95	94	95	129
		10.0	101	90	91	1164	101	109
PHR	HB	0.0	87	86	83	79	83	110
		2.5	86	85	87	79	79	95
		5.0	84	85	81	76	77	92
		10.0	89	85	85	78	78	91
	SB	0.0	91	91	96	104	90	95
		2.5	89	95	94	100	90	91
		5.0	92	92	97	104	91	95
		10.0	87	95	90	97	91	95

HB: High bind; SB: Standard bind;

25: Block buffer comparison for IgG response to tcdA across varying coat concentrations

Coat	Block	Coating conc. (µg/ml)	Sample dilution			Buffer	SBR
			Neat	1:5	1:25		
PBS + 0.1% SDS	Blocker 1	0	107	135	140	97	1.1
		5	197	178	409	93	2.1
		25	202	226	772	86	2.3
		50	199	370	361	83	2.4
	PBST + 5% FBS	0	103	135	122	94	1.1
		5	486	795	640	275	1.8
		25	469	882	825	440	1.1
		50	645	440	815	251	2.6

FBS: Foetal bovine serum; PBS: Phosphate buffer solution; PBST: 1x phosphate buffer solution + 0.05% Tween20; SBR: Signal-to-background ratio; SDS: Sodium dodecyl sulfate;

26: Block buffer comparison for IgG response to tcdA across varying coat concentrations

Coat	Block	Coating conc. (µg/ml)	Sample dilution			Buffer	SBR
			Neat	1:5	1:25		
PBST-coat	Blocker 1	0	98	128	138	123	0.8
		5	91	106	118	112	0.8
		25	98	107	137	112	0.9
		50	118	104	170	105	1.1
	PBST + 5% FBS	0	84	97	97	109	0.8
		5	86	85	99	117	0.7
		25	81	82	104	102	0.8
		50	83	83	103	106	0.8

FBS: Foetal bovine serum; PBS: Phosphate buffer solution; PBST: 1x phosphate buffer solution + 0.05% Tween20; PBST-coat: 1x Phosphate buffer solution + 0.1% Tween20; SBR: Signal-to-background ratio;

27: Block buffer comparison for IgM response to tcdA across varying coat concentrations

Coat	Block	Coating conc. (µg/ml)	Sample dilution			Buffer	SBR
			Neat	1:5	1:25		
PBS + 0.1% SDS	Blocker 1	0	91	103	104	88	1.0
		5	125	121	120	78	1.6
		25	171	171	153	77	2.2
		50	184	209	194	78	2.4
	PBST + 5% FBS	0	74	76	73	73	1.0
		5	169	96	97	74	2.3
		25	1431	111	100	71	20.2
		50	263	153	115	77	3.4

FBS: Foetal bovine serum; PBS: Phosphate buffer solution; PBST: 1x phosphate buffer solution + 0.05% Tween20; SBR: Signal-to-background ratio; SDS: Sodium dodecyl sulfate;

28: Block buffer comparison for IgM response to tcdA across varying coat concentrations

Coat	Block	Coating conc. (µg/ml)	Sample dilution			Buffer	SBR
			Neat	1:5	1:25		
PBST-coat	Blocker 1	0	122	119	122	109	1.1
		5	401	435	1894	106	3.8
		25	180	161	142	124	1.5
		50	119	123	128	113	1.1
	PBST + 5% FBS	0	84	80	88	95	0.9
		5	97	88	88	95	1.0
		25	405	363	502	239	1.7
		50	104	93	103	97	1.1

FBS: Foetal bovine serum; PBS: Phosphate buffer solution; PBST: 1x phosphate buffer solution + 0.05% Tween20; PBST-coat: 1x phosphate buffer solution + 0.1% Tween20; SBR: Signal-to-background ratio;

29: ECL signal comparison for IgG response to tcdB across varying block buffers

Antibody	Analyte	Sample	Dilution	Mean signal	
				PBS (SBR)	PBS + 0.1% SDS (SBR)
IgG	tcdB	A3X	Buffer	82	94
			Neat	641 (7.8)	579 (6.2)
			5	300 (3.7)	232 (2.5)
			25	130 (1.6)	153 (1.6)
			125	97 (1.2)	120 (1.3)
		PHR	Buffer	89	94
			Neat	2,017 (22.7)	3,622 (38.5)
			5	1,202 (13.5)	1,463 (15.6)
			25	389 (4.4)	506 (5.4)
			124	133 (1.5)	195 (2.1)

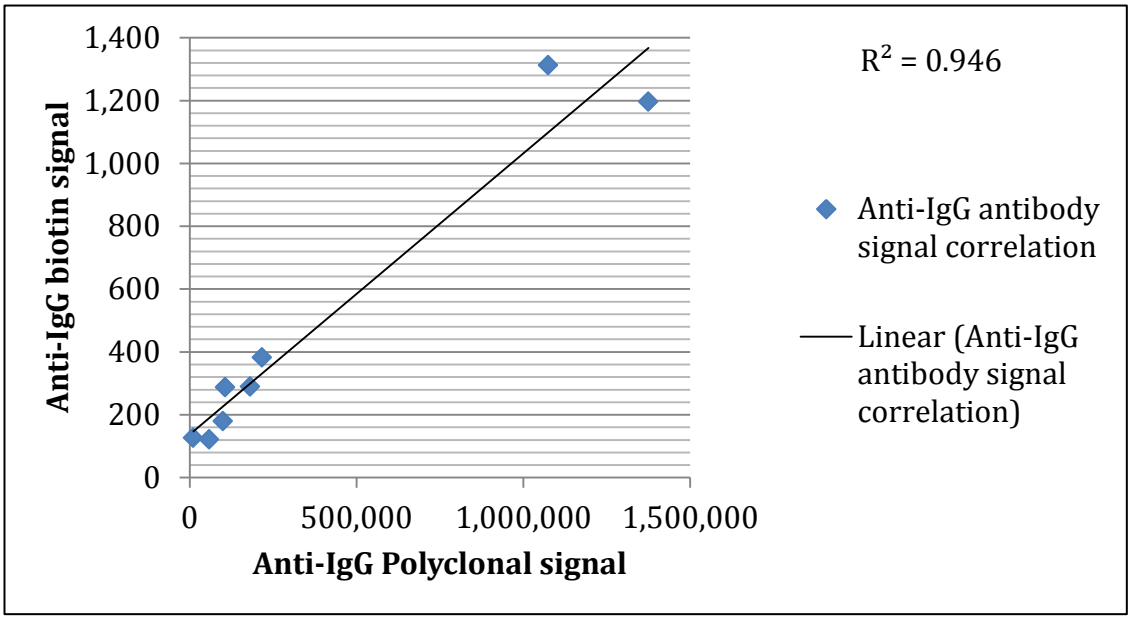
IgG: Immunoglobulin G; PBS: Phosphate buffer solution; SBR: Signal-to-background ratio; SDS: Sodium dodecyl sulfate; tcdB: Clostridium difficile toxin B;

30: ECL signal comparison for IgM response to tcdB across varying block buffers

Antibody	Analyte	Sample	Dilution	Mean signal	
				PBS (SBR)	PBS + 0.1% SDS (SBR)
IgM	tcdB	A3X	Buffer	79	81
			Neat	89 (1.1)	82 (1.0)
			5	92 (1.2)	79 (1.0)
		PHR	Buffer	86	82
			Neat	86 (1.0)	86 (1.0)
			5	82 (1.0)	83 (1.0)

IgM: Immunoglobulin M; PBS: Phosphate buffer solution; SBR: Signal-to-background ratio; SDS: Sodium dodecyl sulfate; tcdA: Clostridium difficile toxin B;

31: Signal correlation across two different anti-IgG antibodies

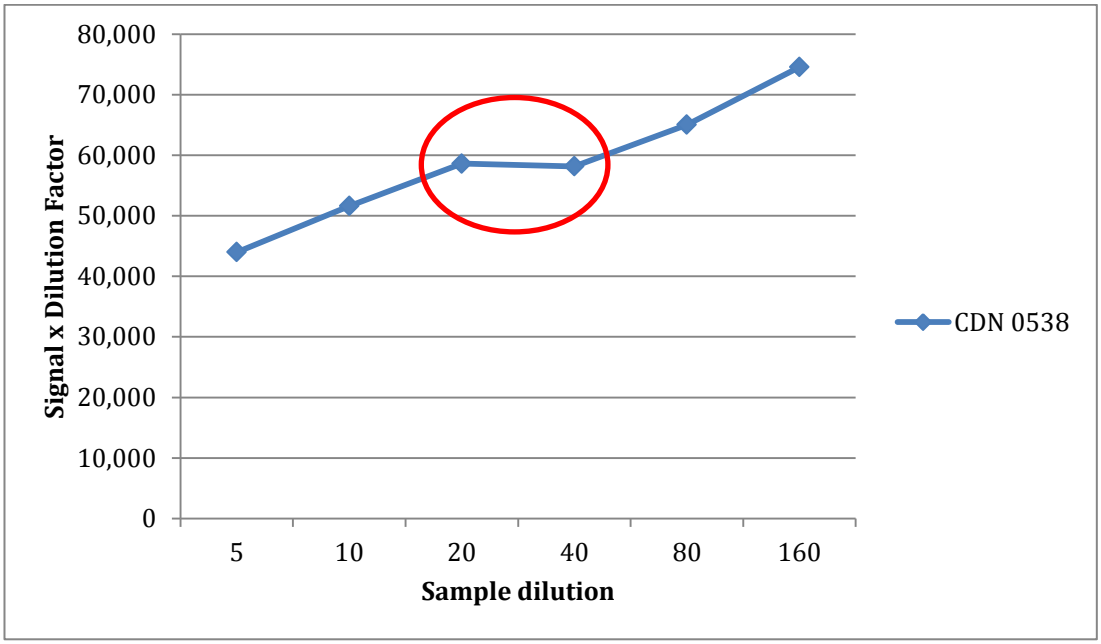


32: Dilution linearity for IgG response to tcdB in a low response sample

Sample	Sample dilution	Absolute signal (ECL units)	Signal factored for dilution
CDN 0538	1:5	8,799	43,995
	1:10	5,161	51,610
	1:20	2,931	58,620
	1:40	1,454	58,160
	1:80	813	65,040
	1:160	466	74,560

ECL: Electrochemiluminescence;

33: Dilution linearity for IgG assay with tcdB in a low response sample



34: Spiking results for IgG response to tcdA

Toxin (pg/ml)	Signal (1:40 dilution)	
	Pooled high response (SBR)	Pooled medium response (SBR)
Buffer	133	133
Unspiked	344,805 (2,593)	56,038 (421)
12.5	195,207 (1,468)	26,338 (198)
50	173,173 (1,302)	23,102 (174)
200	162,583 (1,222)	23,500 (177)
800	122,257 (919)	19,581 (147)
1,600	115,547 (869)	21,633 (163)
3,200	99,041 (745)	16,631 (125)

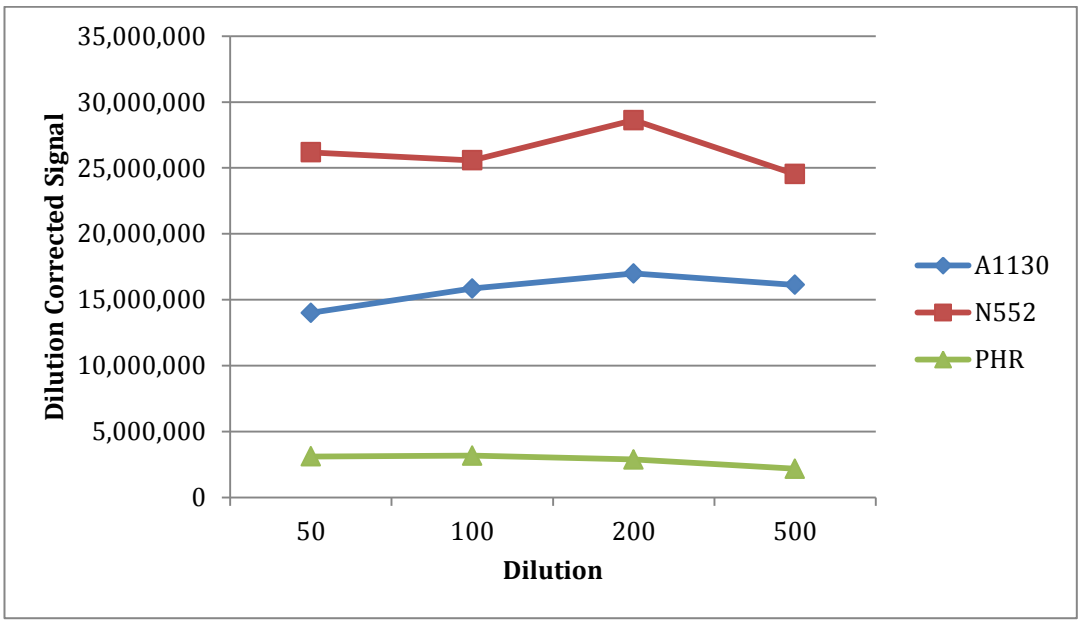
SBR: Signal-to-background ratio;

35: Spiking results for IgG response to tcdB

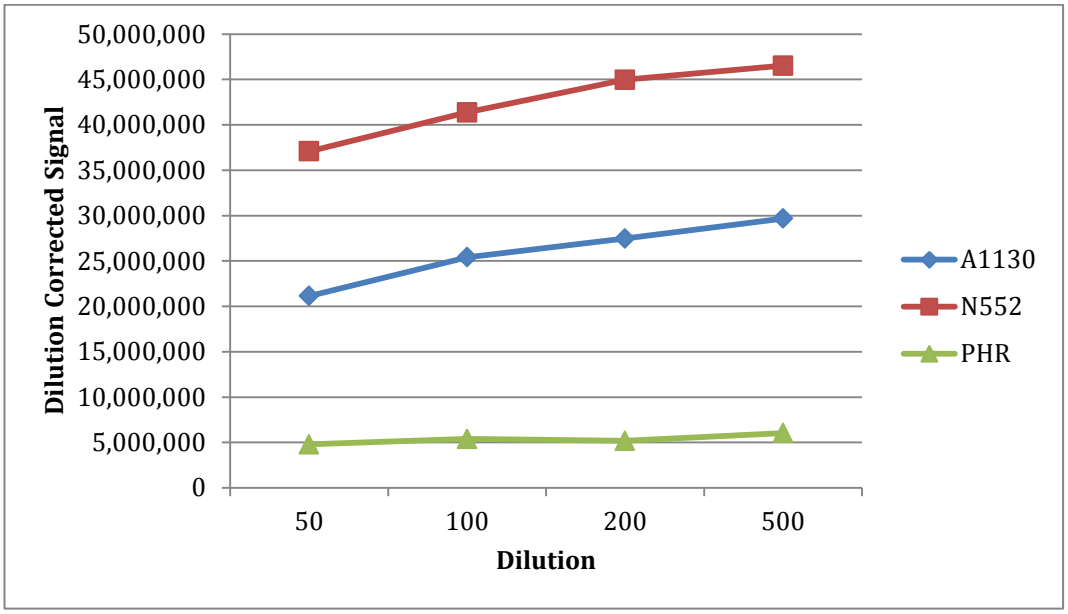
Toxin (pg/ml)	Signal (1:2 dilution)	
	Pooled high response (SBR)	Pooled low response (SBR)
Buffer	111	111
Unspiked	443,284 (3,994)	52,181 (470)
12.5	294,203 (2,650)	23,006 (207)
50	259,571 (2,338)	21,030 (189)
200	160,707 (1,448)	16,239 (146)
800	78,944 (711)	12,899 (116)
1,600	64,061 (577)	10,611 (96)
3,200	57,896 (522)	8,495 (77)

SBR: Signal-to-background ratio;

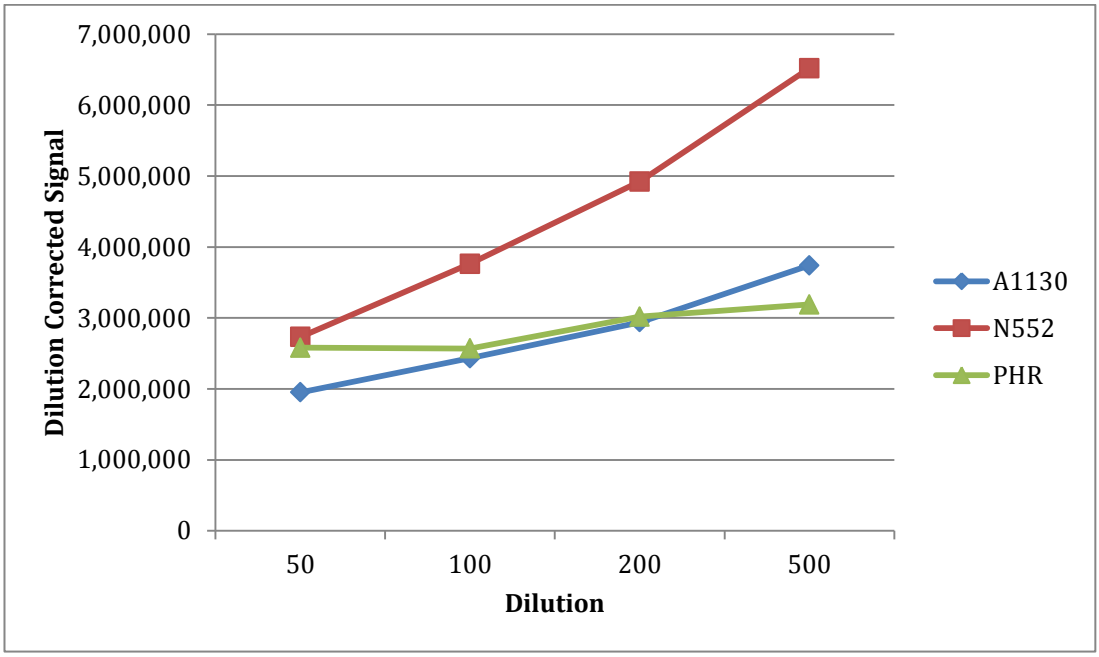
36: Dilution linearity plot for the IgG assay with cdtB-act using Blocker 1



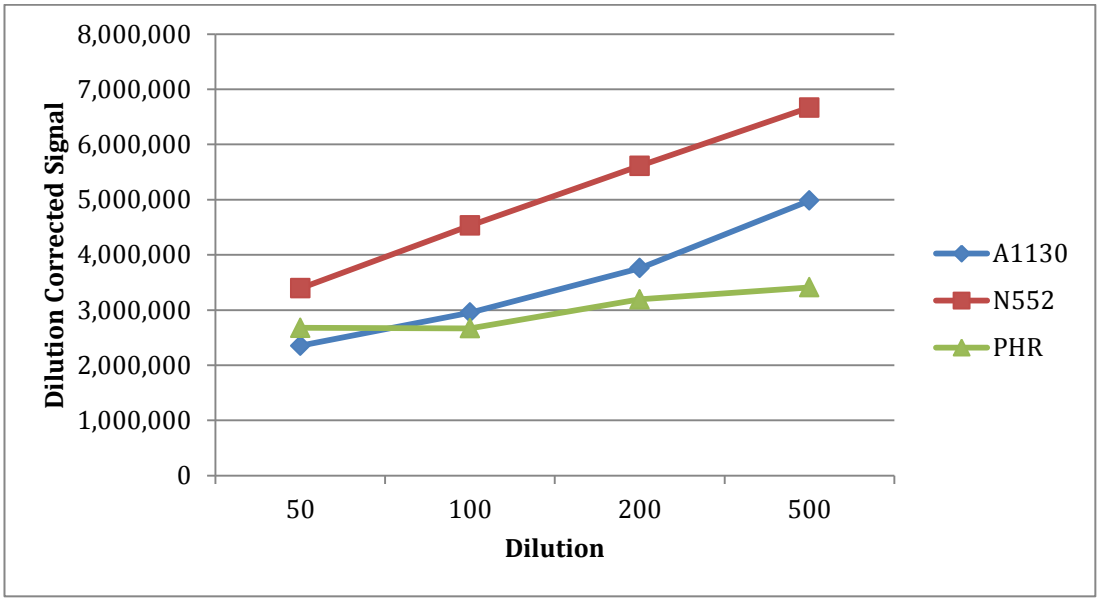
37: Dilution linearity plot for the IgG assay with cdtB-pre using Blocker 1



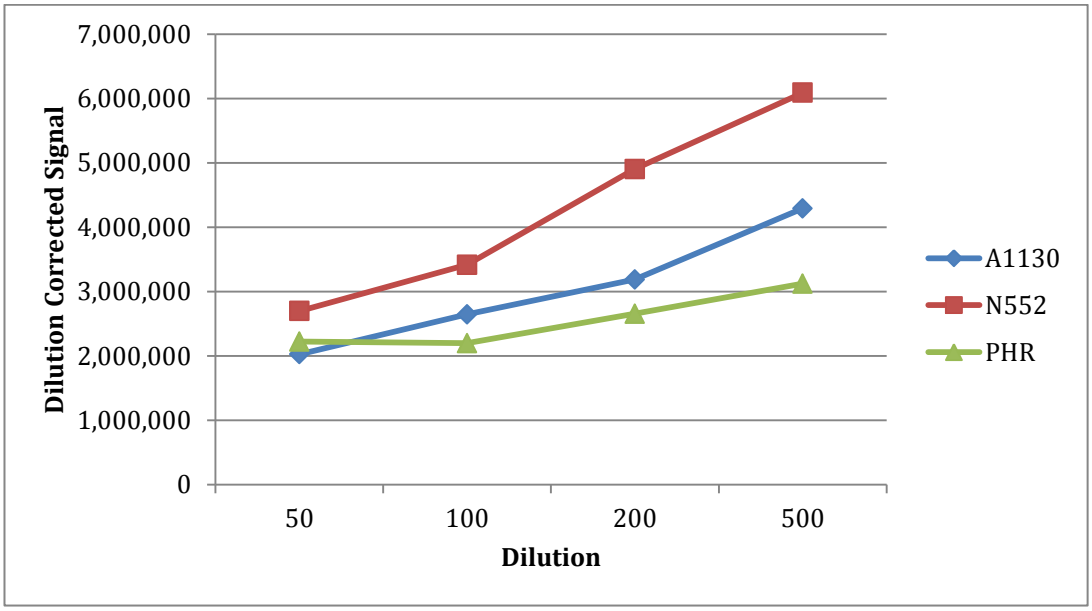
38: Linearity for IgG response to cdtA with 1.6 µg/ml coat & 0.4 µg/ml detection



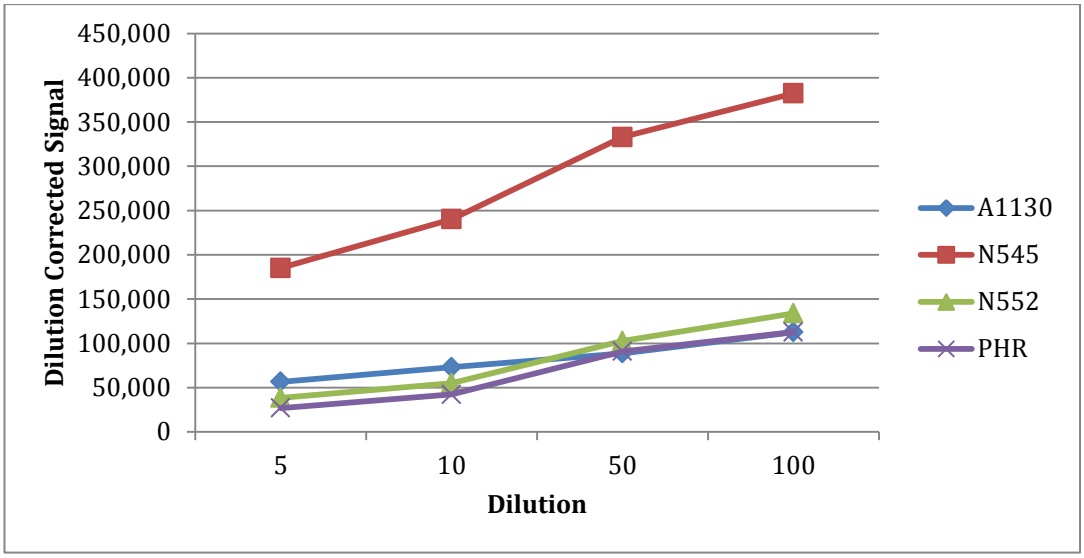
39: Linearity for IgG response to cdtA with 3.2 µg/ml coat & 0.8 µg/ml detection



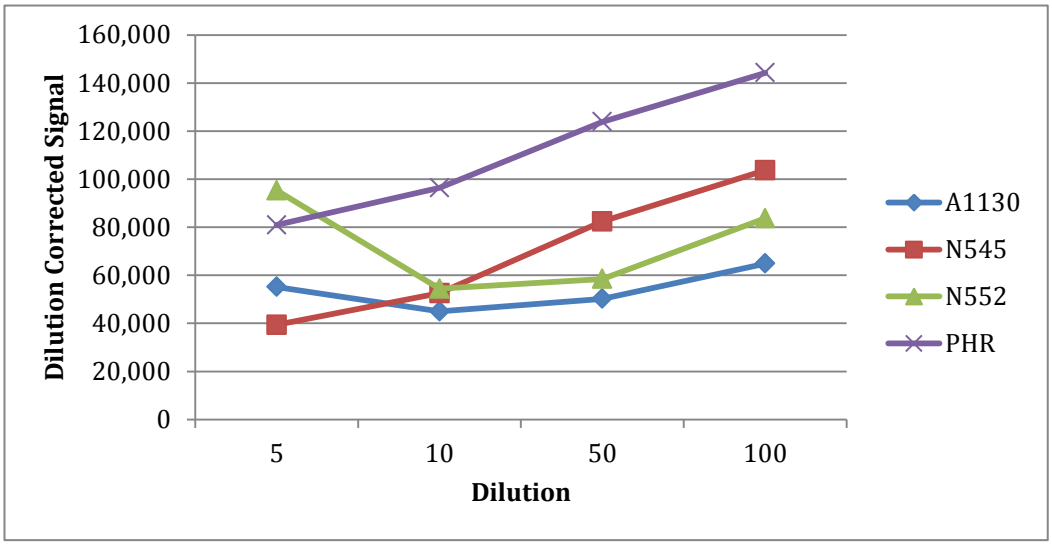
40: Linearity for IgG response to cdtA with 3.2 µg/ml coat & 0.4 µg/ml detection



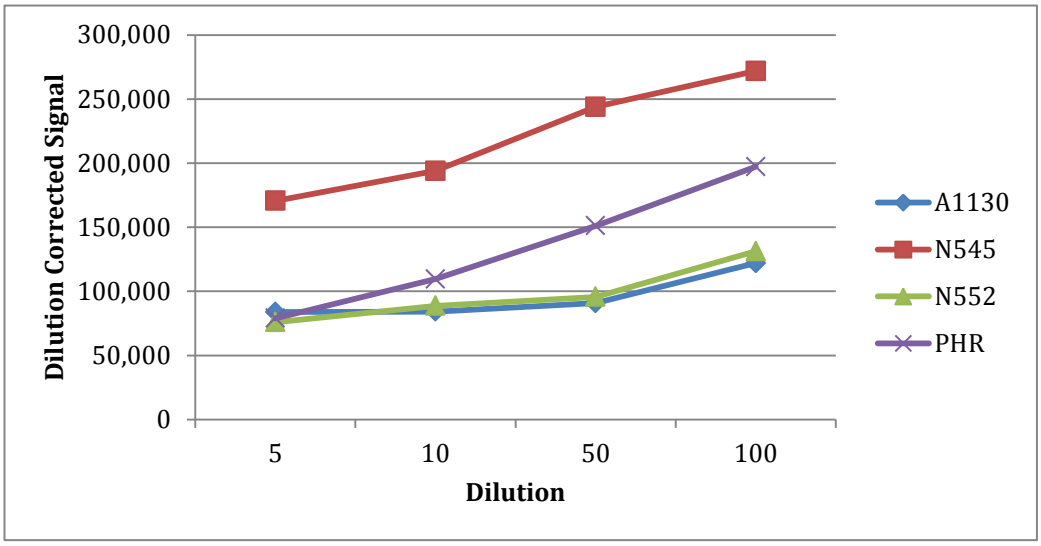
41: Dilution linearity plot for IgM response to cdtA



42: Dilution linearity plot for IgM response to cdtB-act

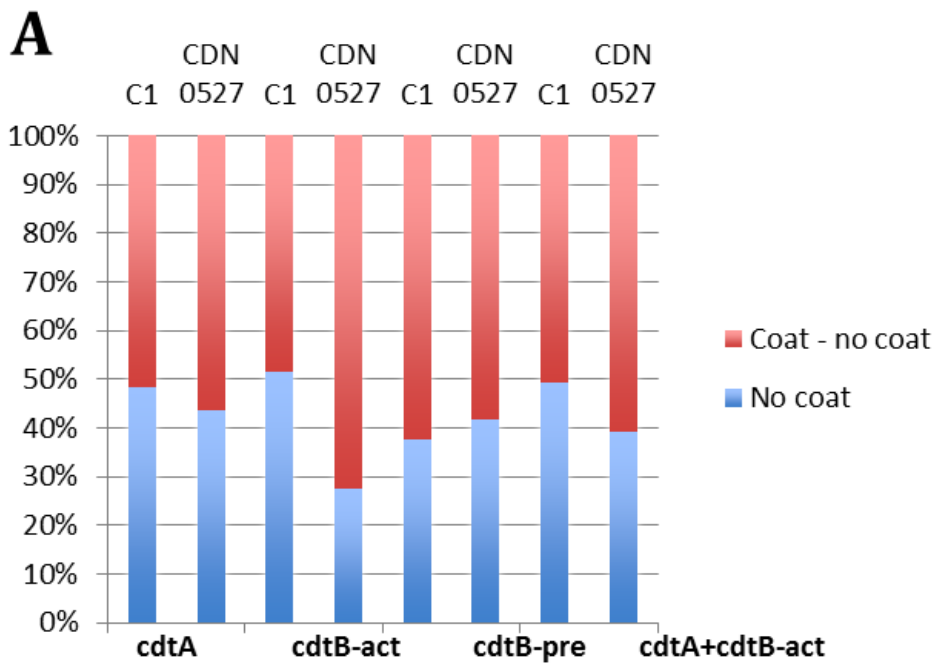


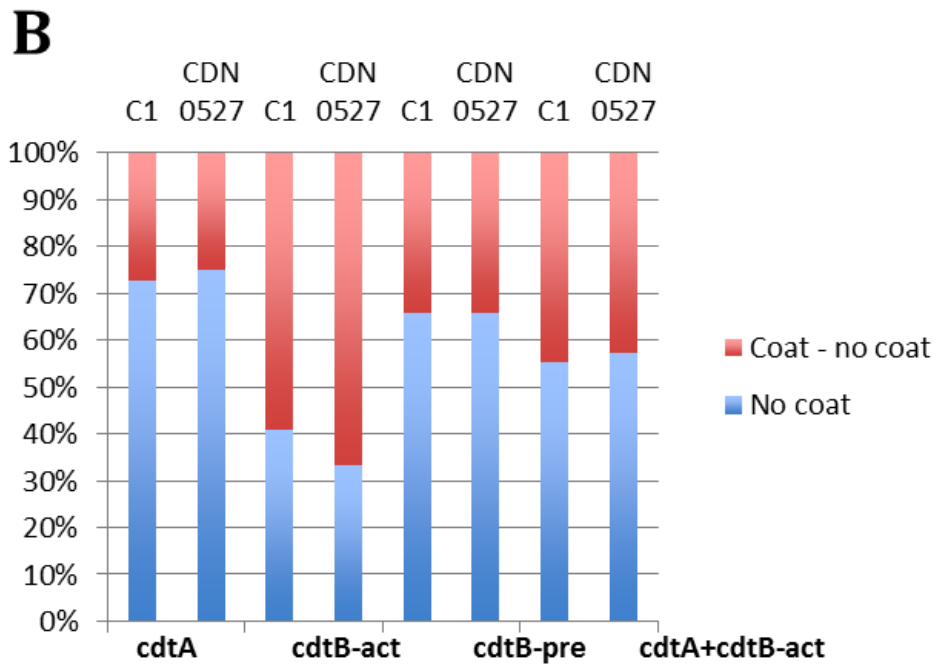
43: Dilution linearity plot for IgM response to cdtA+cdtB-act



44: Percentage of overall IgM response to all CDT analytes accounted for by background

(A) Blocker 1; (B) Casein Blocker





45: Final assay protocols

Toxin A & B

1. Take standard bind plate and to each well add 25 µl of 25 µg/ml tcdA/B, diluted in 1x PBS. Tap plate to ensure even coverage. Seal and incubate overnight at 4°C
2. Wash 3 x 150 µl with PBST
3. Add 150 µl PBST + 1% BSA + 1% Milk powder (Blocker 1) and seal. Incubate for 1 h at room temperature with shaking
4. Wash 3 x 150 µl with PBST
5. Add 25 µl human serum, diluted 1:40/1:2 (IgG/IgM) in PBST + 1% BSA + 1% Milk powder, and seal. Incubate for 2 h at room temperature with shaking
6. Wash 3 x 150 µl with PBST
7. Add 25 µl of polyclonal IgG/IgM antibody, diluted to 1 µg/ml in PBST + 1% BSA + 1% Milk powder, and seal. Incubate for 1 h at room temperature with shaking
8. Wash 3 x 150 µl with PBST
9. Add 150 µl of MSD Read Buffer (diluted to 2x working mixture with distilled water)
10. Read plate within 15 min

Binary toxin

- All components (IgG & IgM)
    - Plate = Standard bind
    - Coating buffer = PBS
    - Blocking buffer = Casein
- CDTb-act, CDTb-inact and CDTa+b-act (IgG/IgM)
    - Coating concentration = 0.1 µg/0.1 µg
    - Detection antibody concentration = 0.1 µg/ml/1.0 µg/ml
    - Sample dilution = 1:500/1:50
- CDTa (IgG/IgM)
    - Coating concentration = 0.04 µg
    - Detection antibody concentration = 0.1 µg/ml/0.8 µg/ml
    - Sample dilution = 1:500/1:50

- Take standard bind plate and coat with appropriate conc. of CDT component, diluted in PBS. Tap plate to ensure even coverage. Seal and incubate overnight at 4°C
- Wash 3 x 150 µl with PBST
- Add 25 µl casein and seal. Incubate for 1 h at room temperature with shaking
- Add 25 µl human serum, diluted 1:500/1:50 (IgG/IgM) in casein, and seal. Incubate for 2 h at room temperature with shaking
- Wash 3 x 150 µl with PBST
- Add 25 µl of polyclonal IgG/IgM antibody, diluted to appropriate concentration in casein, and seal. Incubate for 1 h at room temperature with shaking
- Wash 3 x 150 µl with PBST
- Add 150 µl of MSD Read Buffer (diluted to 2x working mixture with distilled water)
- Read plate within 15 min

46: Transferring MBL IVD ELISA kit onto MSD ECL platform

The transition process occurs via three key steps: -

1. Substituting the ELISA microtitre plate with one containing the carbon electrodes necessary for the redox reaction
2. Labelling the detection antibody with the electrochemiluminescent tag
3. Using MSD read buffer containing TPA

This method development workflow is slightly differently from the *C. diff* toxin assays as it involves translating an existing IVD ELISA kit (Sanquin) onto the MSD platform, as opposed to developing a novel assay as with the *C. diff* toxins. The issue I had here was taking the kit protocol that uses microtitre plates, specific wash buffers and dilution buffers etc. and using them as part of the MSD assay workflow.

Sulfo-tagging of unconjugated MBL1 antibody

The Sanquin kit utilises anti-HRP antibody. This would not work well on the MSD platform therefore I requested an unconjugated antibody from them. I then sulfo-tagged this unconjugated form, as with my IgG and IgM antibodies for the *C. diff* toxin assays. Using ZEBA desalting columns, I took 2.0 mg/ml of anti-MBL1 stock and diluted the resultant sulfo-tagged antibody 1:4 to obtain a working anti-MBL1 antibody solution of 0.5 mg/ml.

Optimisation 1: Determination of detection antibody concentration plus need for further work on all other conditions

For my first optimisation, I tried to keep everything as similar as possible, whilst making some slight tweaks that would allow it to be run via MSD. One notable change included attempting to reduce the amount of standard needed for each standard curve by having a lower top standard than their kit

recommended. This was done in the hope of reducing the financial burden of typing such a marker; using IVD ELISA kits for a large sample set can be very expensive. If I was able to use less standard then I could increase the number of samples being processed per kit. Further to this, standard bind MSD plates and two separate detection antibody concentrations (1.0 µg/ml and 2.0 µg/ml) were used. Recommended volumes were retained for all steps other than washing: this was reduced from 300 to 150 µl. The comparison protocol between Sanquin (black) and MSD (red) was as follows: -

- COATING: Dilute mannan stock 100-fold in 0.1 M bicarb/carb buffer (pH 9.6) and add 100 µl to each well
- COATING: Dilute mannan stock 100-fold in 0.1 M bicarb/carb buffer (pH 9.6) and add 100 µl to each well
- Incubate with lid on at room temp. overnight
- Incubate with seal on at 4°C overnight
- Stds = 350, 140, 56, 22.4 and 9 ng/µl (diluted using kit dilution buffer)
- Stds = 200, 100, 40, 16, 6.4 and 2.6 ng/µl (diluted using kit dilution buffer)
- Samples = 1:20 dilution (diluted using kit dilution buffer)
- Samples = 1:20 dilution (diluted using kit dilution buffer)
- STEP 1 = Wash x3 using 300 µl wash buffer
- STEP 1 = Wash x3 using 150 µl wash buffer
- STEP 2 = Add 100 µl of std curve, control and samples. Gently agitate and then incubate at room temp for 1 h
- STEP 2 = Add 100 µl of std curve, control and samples. Incubate at room temp for 1 h with shaking
- STEP 3 = Wash x3 using 300 µl
- STEP 3 = Wash x3 using 150 µl
- STEP 4 = Add 100 µl anti-human MBL-HRP antibody diluted 1:100 and incubate for 1 h
- STEP 4 = Add 100 µl sulfotagged MBL antibody diluted to working conc of 2 µg/ml or 1 µg/ml and incubate at room temp with shaking for 1 h

- STEP 5 = Wash x3 using 300 µl
- STEP 5 = Wash x3 using 150 µl
- STEP 6 = Incubate with 3,3′,5,5′-Tetramethylbenzidine (TMB)-containing substrate for 30 min in dark
- STEP 6 = Add 150 µl MSD read buffer (2x) and read plate
- STEP 7 = Add stop solution
- STEP 8 = Read plate at 450 nm

Initial analysis of the standard curves showed that the minimum detection level was not as low as that of the kit itself (Figures 46.1 & 46.2). It was evident that a blocking step may be beneficial, as with my other MSD assays. No significant difference was observed between calculated concentrations across the two detection antibody concentrations used (Table 46.1), nor minimum detection levels (Figures 46.1 & 46.2) and therefore only the 1.0 µg/ml concentration was taken forward for further optimisation.

Figure 46.1 - MBL1 standard curve using a detection antibody concentration of 1.0 µg/ml

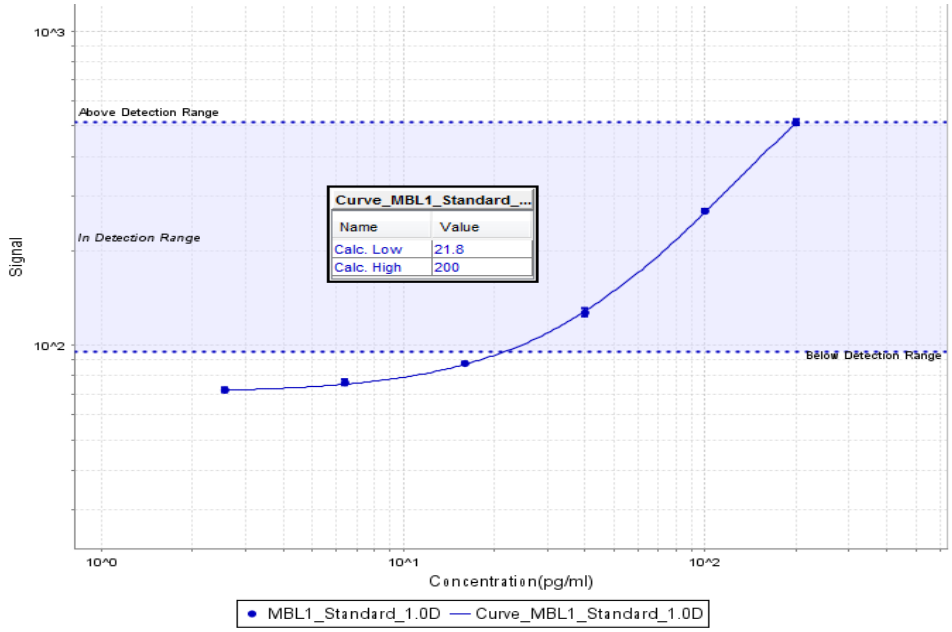


Figure 46.2 - MBL1 standard curve using a detection antibody concentration of 2.0 µg/ml

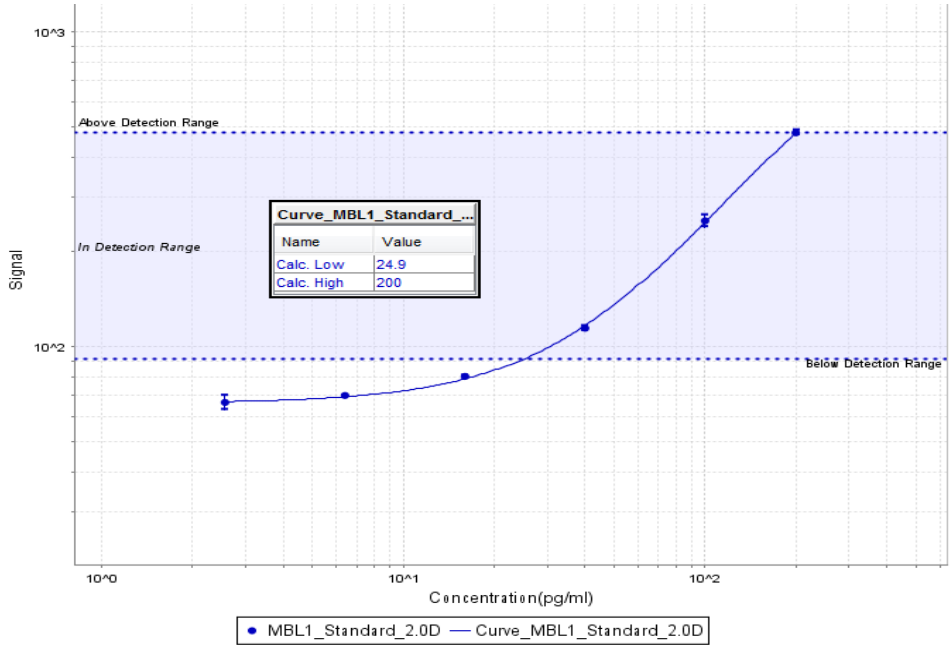


Table 46.1 – Comparison of calculated concentrations observed across two different anti-MBL1 antibody concentrations

Sample	Calculated concentration using 1.0 µg/ml D	Calculated concentration using 2.0 µg/ml D	CV
STD1 (200 ng/ml)	199.60	199.40	0.07
STD2 (100 ng/ml)	100.78	101.08	0.21
STD3 (40 ng/ml)	39.22	38.81	0.75
STD4 (16 ng/ml)	16.40	17.20	3.36
STD5 (6.4 ng/ml)	7.17	7.25	0.80
STD6 (2.56 ng/ml)	2.41	2.98	15.03
STD7 (0 ng/ml)	1.31	2.25	37.55
Kit control	1,160.42	1,220.07	3.54
Healthy control 1	652.36	741.66	9.06
Healthy control 2	1,778.05	1,772.25	0.23
Healthy control 3	5,153.50	4,918.73	3.30
Healthy control 4	1,691.45	1,690.33	0.05

CV: Coefficient of variation; D: Detection antibody; STD: Standard;

Optimisation 2: Determination of remaining optimal assay conditions

With most blocking buffers containg carbohydrates, lectin assays can therefore be difficult to optimise as they are known carbohydrate-binders. I therefore used Carbo-Free Blocking Solution (Vector Labs, Peterborough, UK). I also changed the washing step to utilise the standard PBST from my other MSD assays, as opposed to the recommended kit wash buffer, and tested high-bind plates alongside the standard plates. Furthermore, dilution linearity was investigated through inclusion of multiple sample dilutions ranging from 1:2.5 to 1:40. Zero coat control wells were also included.

The results demonstrated desirable dilution linearity on the high-bind plate between the 1:10 and 1:40 dilutions (Figure 46.3). Therefore, the IVD kit-recommended 1:20 dilution was deemed the best option. Samples did not dilute linearly on the standard bind plate (Figure 46.4) and this was excluded from further optimisation. Standard curve parameters (% CV, recovery & sensitivity) were also desirable using the high bind plate (Table 46.2), as was LLOD (0.5 ng/ml; Figure 46.5). Zero coat controls were low highlighting the utility of the carbo-free blocking buffer (data not shown). All samples tested at the 1 in 20 dilution were in the middle range of the curve, in terms of signal and concentration. However, as these are all healthy controls, the next step would be to revert to the kit recommended standard curve with a slightly higher top standard, including an extra standard point at the bottom of the curve, in order to ensure any clinical samples with potentially high titres will also be detected appropriately.

Figure 46.3 – Sample dilution linearity plot for MBL1 assay using high-bind plate

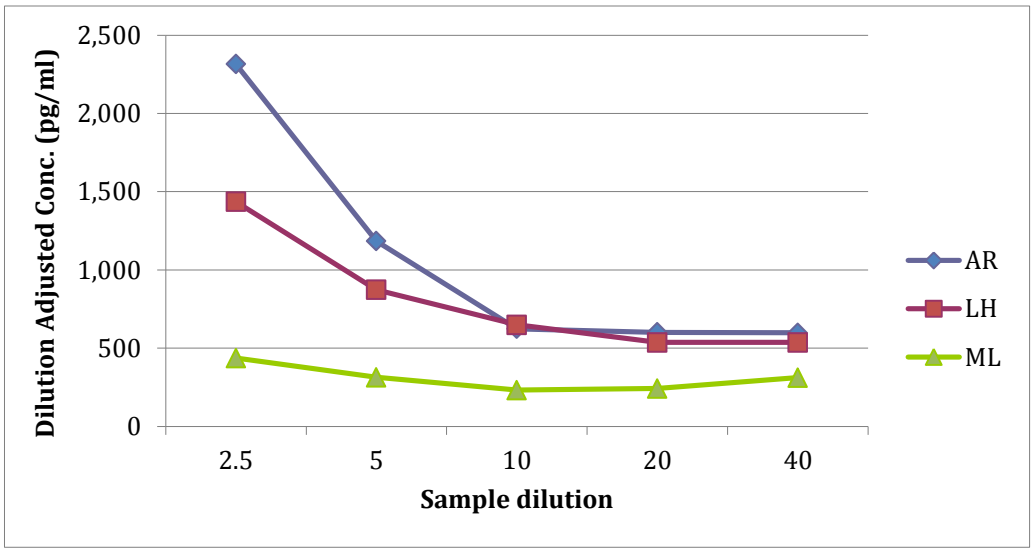


Figure 46.4 – Sample dilution linearity plot for MBL1 assay using standard bind plate

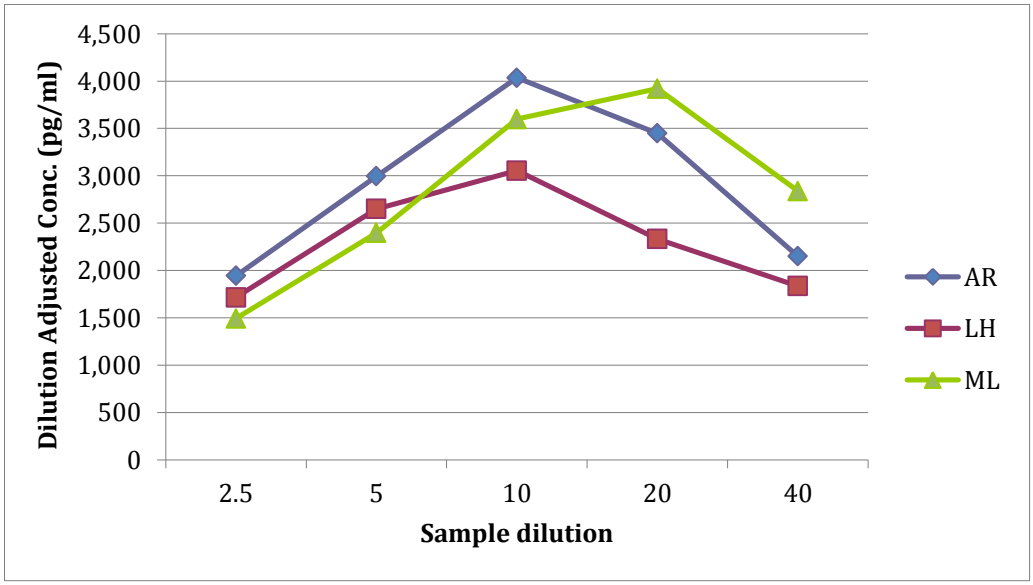
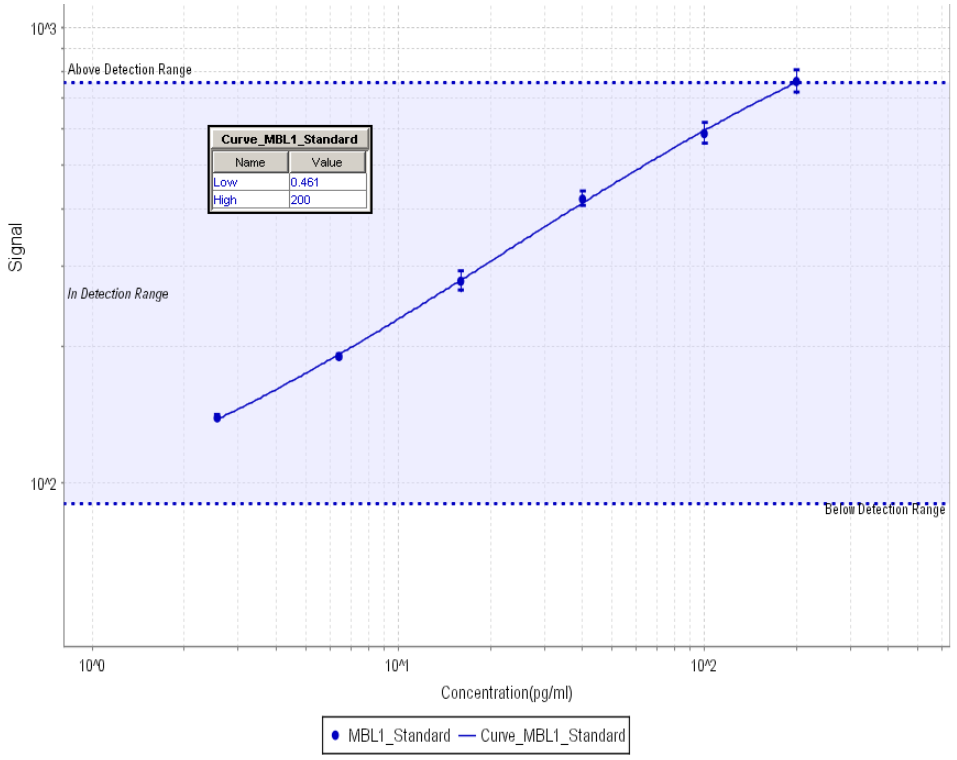


Table 46.2 - Standard curve statistics for MBL1 assay using high-bind plate

Sample	Concentration (ng/ml)	Mean signal	Signal CV	Mean % recovery	Mean calculated concentration	Calculated concentration: CV
STD1	200	763	5.7	102.3	204.6	17.8
STD2	100	586	5.1	96.4	96.4	13.3
STD3	40	421	3.5	105.0	42.0	8.4
STD4	16	278	4.8	98.8	15.8	11.4
STD5	6.4	190	1.1	96.9	6.2	2.9
STD6	2.56	139	1.0	101.6	2.6	3.1
STD7	0	65	2.2	NaN	0.0	141.2

CV: Coefficient of variation; NaN: Not a number; STD: Standard;

Figure 46.5 – MBL1 standard curve using high-bind plate and a detection antibody concentration of 1.0 µg/ml



Optimisation 3: Testing utility of recombinant MBL protein as an alternative standard curve

Alongside the heightened standard curve, the utility of recombinant MBL protein as a standard curve was also investigated. If successful, this would allow the curve to go higher than the kit recommended top standard, potentially allowing use of a lower sample dilution, which could improve the compressed sample range arising from the low signals described above. For storage purposes, 1% BSA was added to the recombinant MBL protein prior to use. To ensure the addition of BSA did not interfere with the assay, the recombinant protein prior to adding the 1% BSA was also tested.

The recombinant MBL protein standard curves with/without 1% BSA both failed and were therefore excluded from further work. Despite the LLOD being higher than previous optimisations (11.3 ng/ml; Figure 46.6), this is likely due to the high variation observed at Standard Point 7. With regards to CV and percentage recovery (LLOQ), the curve is accurate to the lowest standard (3.6 ng/ml; Table 46.3). All but two of the samples tested were above this level and the majority of observed CVs were <10% (Table 46.4).

Despite the low signal level, the assay is robust. A major problem when working in this signal range is that assay consistency can be affected when reagent batches are changed, particularly antibodies. The reagents for the assay in question are from a validated kit, therefore should be stable. Optimal assay conditions had now been identified.

Figure 46.6 – Standard curve for MBL1 using optimally identified assay conditions

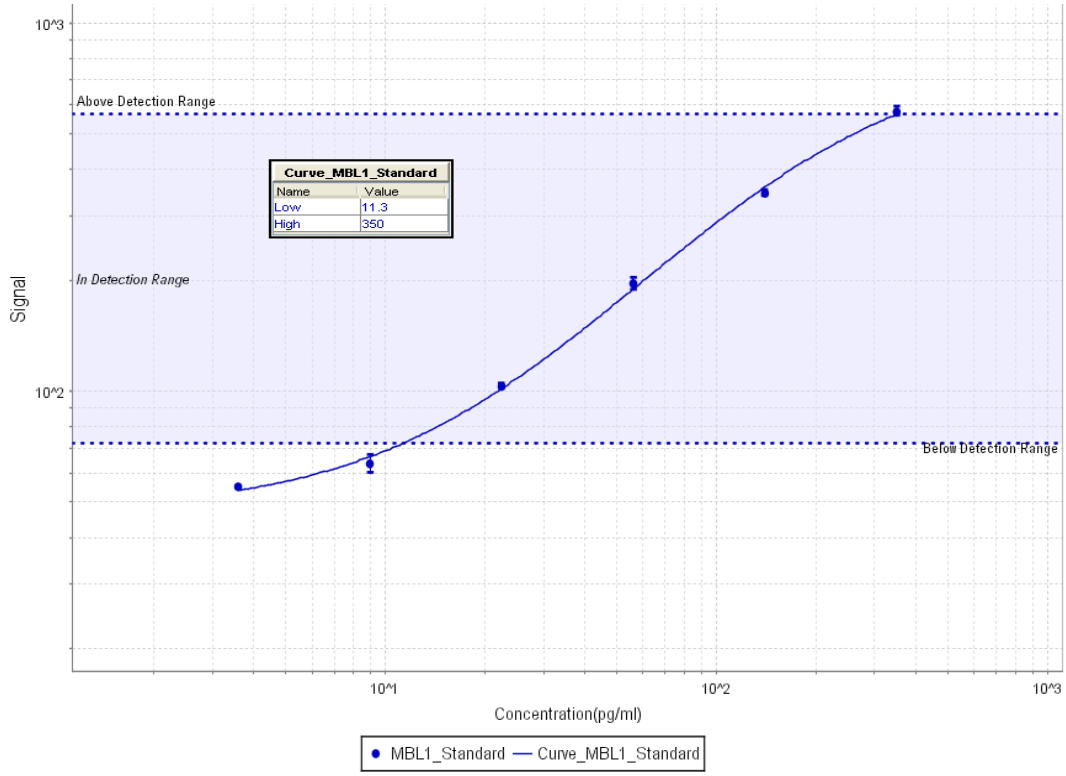


Table 46.3 - Standard curve statistics for MBL1 assay using optimally identified assay conditions

Sample	Concentration (ng/ml)	Mean signal	Signal CV	Mean calculated concentration (ng/ml)	Calculated concentration: CV	Mean % recovery
STD1	350	574	2.8	366.5	8.0	104.7
STD2	140	345	1.6	131.8	2.6	94.1
STD3	56	196	3.6	58.7	4.9	104.8
STD4	22.4	103	1.4	23.0	2.3	102.7
STD5	9	64	5.6	7.8	18.7	86.7
STD6	3.6	55	0.0	4.1	0.0	113.9
STD7	0	48	7.4	0.9	141.4	NaN

CV: Coefficient of variation; NaN: Not a number; STD: Standard;

Table 46.4 – Overview of sample values above or below LLOQ

Sample	Dilution	Mean signal	Signal CV	Mean calculated concentration (ng/ml)	Calculated concentration CV	Calculated concentration/ Dilution
Control	20	276	2.8	1,882.6	4.0	94.1
N536	20	225	6.9	1,418.8	9.5	70.9
N537	20	106	4.0	481.3	6.6	24.1
N539	20	218	15.6	1,362.5	21.3	68.1
N540	20	109	1.3	503.6	2.1	25.2
N541	20	285	2.5	1,976.7	3.6	98.8
N543	20	49	0.0	23.1	0.0	1.2
N545	20	147	0.5	784.4	0.7	39.2
N546	20	146	3.4	776.9	4.8	38.8
N547	20	64	NaN	160.0	NaN	8.0
N549	20	66	NaN	176.3	NaN	8.8
N550	20	195	NaN	1,165.4	NaN	58.3
N552	20	67	NaN	184.4	NaN	9.2
N553	20	53	NaN	64.2	NaN	3.2
N555	20	54	NaN	73.6	NaN	3.7
N556	20	168	NaN	949.9	NaN	47.5
N610	20	256	NaN	1,696.0	NaN	84.8

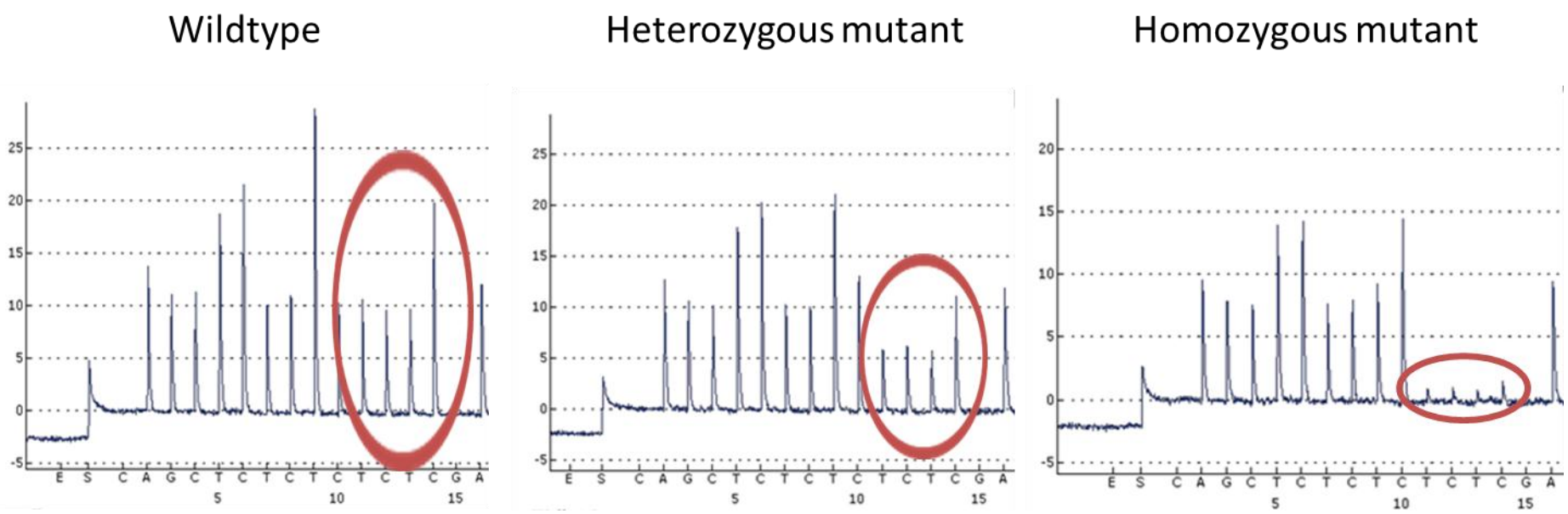
CV: Coefficient of variation; NaN: Not a number;

Final assay protocol

The kit control will be used across plates to determine the necessary correction factors, which will then applied to the appropriate plates. The optimised protocol is as follows: -

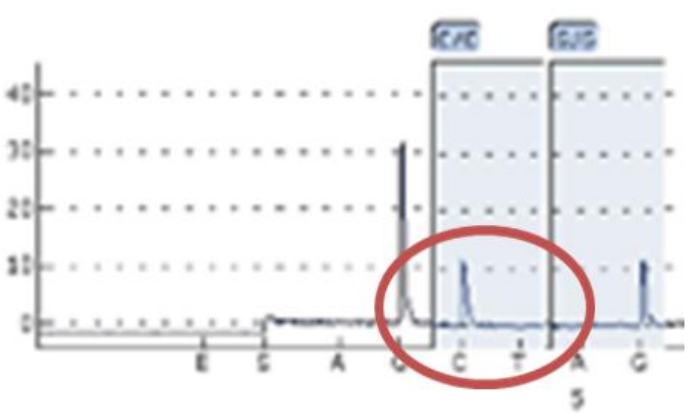
- COATING: Dilute mannan stock 100-fold in 0.1 M bicarb/carb buffer (pH 9.6) and add 100 µl to each well of high-bind plate
- Incubate with seal on at 4°C overnight
- Wash x3 using 150 µl PBST
- Add 100 µl carbo-free blocking buffer. Incubate at room temp for 1 h with shaking
- Wash x3 using 150 µl PBST
- Add 100 µl of standard curve, kit control and samples. Incubate at room temp for 1 h with shaking
    - Standards = 350, 140, 56, 22.4, 9 and 3.6 ng/µl (diluted using carbo-free blocking buffer)
    - Samples = 1:20 dilution (diluted using carbo-free blocking buffer)
- Wash x3 using 150 µl PBST
- Add 100 µl sulfotagged MBL antibody at 1 µg/ml (diluted using carbo-free blocking buffer) and incubate at room temp with shaking for 1 h
- Wash x3 using 150 µl PBST
- Add 150 µl MSD read buffer (diluted to 2x using distilled water)
- Read plate

47: Example pyrosequencing outputs for rs10554674

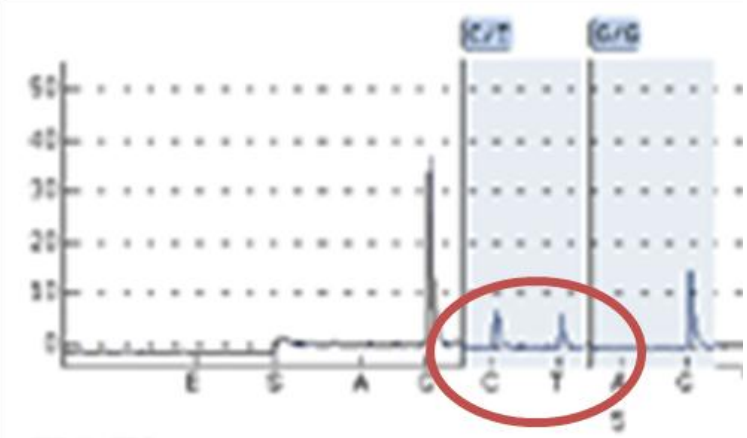


48: Example pyrosequencing outputs for rs1800451

Wildtype

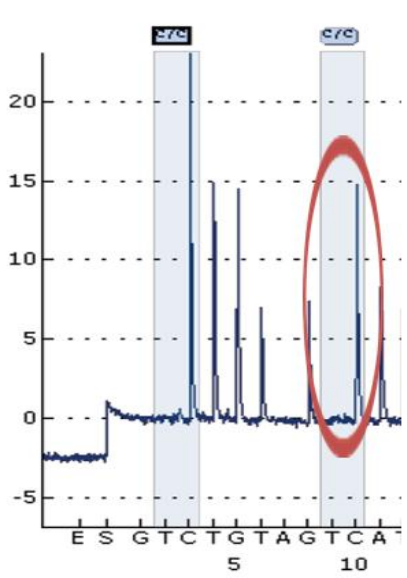


Heterozygous mutant

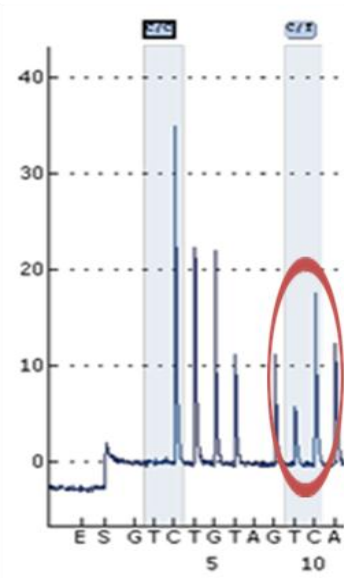


49: Example pyrosequencing outputs for rs1800450

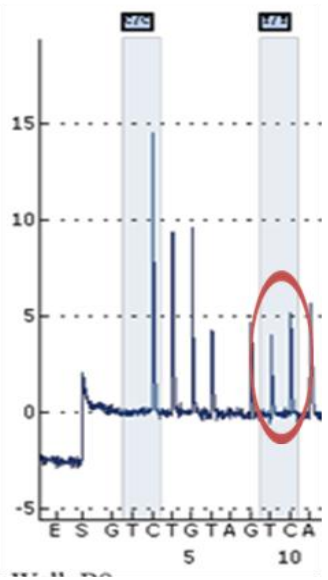
Wildtype



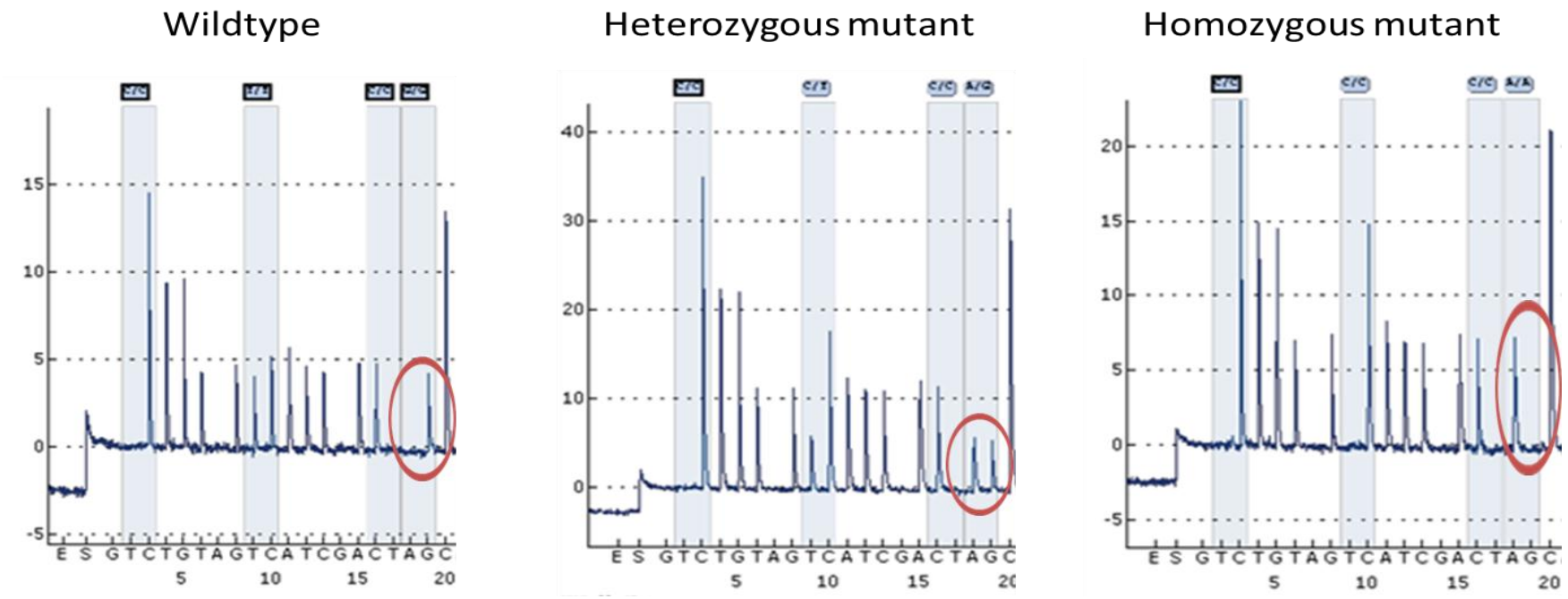
Heterozygous mutant



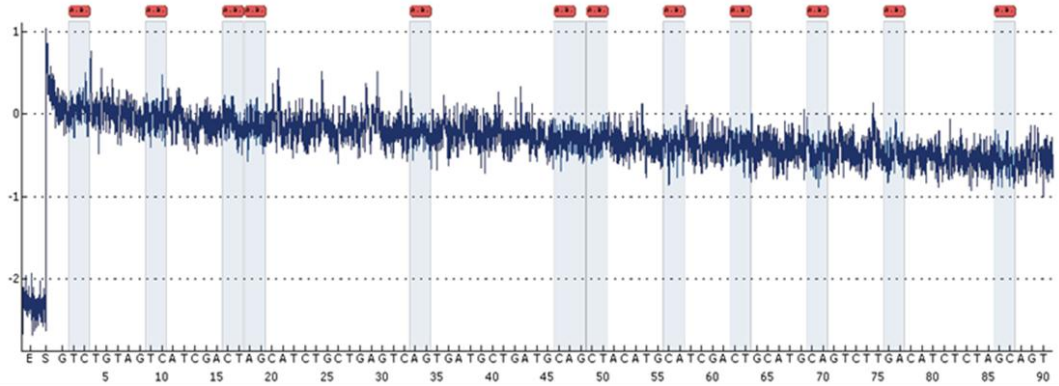
Homozygous mutant

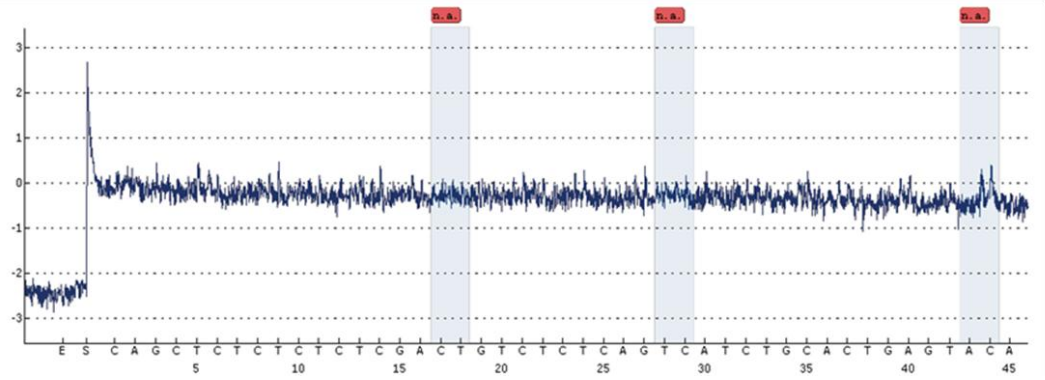


50: Example pyrosequencing outputs for rs5030737



51: Example pyrosequencing outputs for no calls/poor quality runs





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