Factors affecting the detection of *Clostridium difficile* in faecal samples

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds

School of Medicine

April 2019

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Section 6.2.1 of Chapter 4 contains work from a jointly authored publication; Davies K, Planche T and Wilcox M. The predictive value of quantitative nucleic acid amplification detection of *Clostridium difficile* toxin gene for faecal sample toxin status and patient outcome. PLoS One 13(12): e0205941 2018. The concept of the work was devised by Kerrie Davies, the analysis and initial draft of the manuscript was completed by Kerrie Davies with review by Dr Tim Planche and Prof Mark Wilcox.

Acknowledgements

There are so many people who I need to thank for their part in helping me to achieve this thesis.

The Department of Health study, for which I was scientific coordinator, was conducted prior to this PhD, but I have reused the data for further analysis within the thesis. I would therefore like to thank all of the sites that took part in the multi-centre study; Leeds Teaching Hospitals NHS trust, St Georges' University Hospitals NHS Foundation Trust, UCL Hospitals NHS Foundation Trust and Oxford University Hospitals NHS Foundation Trust, with special thanks to Dr Tim Planche for all of his support and guidance. I would also like to thank the Department of Health, who funded both this initial study and the PlaciD study.

The PlaciD study research was carried out by a team which has included Claire Berry (Clinical Scientist), Claire Brown (Research Nurse) and Faye Pinker (Research Nurse). My own contributions, fully and explicitly indicated in the thesis, for the PlaciD study were to devise the study, be study chief investigator, write the study protocol, apply for ethical approval, devise data collection sheets and databases, perform the majority of the laboratory testing of the samples and analyse the data. The other members of the team and their contributions have been as follows; The Research Nurses recruited all of the participants, collected their samples and their data; Claire Berry performed laboratory testing of the samples during my maternity leave (four months). Thanks go to all of the team. I would also like to thank all of the participants in the PlaciD study, who generously gave their time, and poo, to help make the study a success!

The gut models require a huge team effort to keep them running, therefore the research using this system has been carried out by a team which has included; Dr Tony Buckley (PI), Dr Caroline Chilton (PI), Dr Ines Moura (research assistant), Dr Hannah Harris (research assistant), Duncan Ewin (technician), Sharie Shearman (technician), Emma Clarke (technician), Kate Owens (technician), Bill Spittal (senior technician) and Flor Sporta (Summer student). My own contributions, fully and explicitly indicated in the thesis, have been to devise the experiments using gut model samples to monitor proliferation of *C. difficile* within the gut models using commercial assays, to test the samples, except where tested by Flor Sporta, as detailed within the thesis, and analyse the data. The other members of the team and their contributions have been as follows; individual gut model experiments devised and overseen by the PI in each case; all the technical aspects of keeping the gut models running was completed by the technical team (technicians and

research assistants), including taking the daily samples; Flor Sporta tested some of the gut model samples using the DS2 assays, as explicitly indicated in the thesis. I would like to thank each and every one of them for their assistance.

All PCR ribotyping was carried out by the *C. difficile* Ribotyping Network of England and Northern Ireland (CDRN), as explicitly stated within the thesis. I would like to thank Dr Warren Fawley and his team at CDRN for their contribution and support.

The Healthcare Associated Infections Research Group is a special place to work and I feel very privileged to be part of such a great team. Every single person deserves my thanks, but I'd especially like to thank Dr Jane Freeman, for being my ever steady friend who always steers me right; Dr Baljit Saundh, my dear friend who helps me to 'relax the chaos'; Georgina Davis, my great friend and dedicated cheering squad, who never lets me quit; and of course Prof Mark Wilcox, my PhD Supervisor and mentor, who has given me the opportunity to fly. A great debt of thanks also goes to Dr John Heritage; not only is he the reason I came to Leeds to study Microbiology as an undergraduate, but he was also my PhD co-supervisor, even after he had retired. I appreciate all of the wisdom and advice he has imparted over the many years I have known him.

Huge thanks go to my family; for standing by me, for keeping me encouraged and inspired. To my children Alf and Sid, for being patient when I couldn't play, and for making me laugh whenever they could; to my mum and dad for being so supportive and proud; to my extended Leeds family for supporting us all and providing 'donations' when needed. Special thanks obviously go to Graeme, my amazing husband; without him this thesis, my career and my self-belief would be naught.

This PhD thesis is dedicated to my dad; for inspiring me to be the scientist that he never got the chance to be.

Abstract

Clostridium difficile infection (CDI) laboratory diagnostic assays have variable performance, but reasons behind this variability are not well defined. In contrast to previous findings, the PCR ribotype of the organism only appears to be a factor in reduced sensitivity for toxin enzyme immunoassays (EIAs), not glutamate dehydrogenase (GDH) EIAs. Growth curve data demonstrated that GDH is produced during the exponential phase of growth, and the sensitivity of the GDH assay *in vivo* may be related to the amount of protein produced by the organism, as very high levels of GDH were detected during growth of *C. difficile* in an *in vitro* gut model. Indeed the levels of GDH, measured in both gut model and patient samples, correlated with organism bioload. In addition, the median faecal levels of GDH in recurrent CDI cases were significantly higher than in patients with a single infection episode. Interestingly, when patients had sequential faecal samples tested, 27% with an initial GDH-positive/toxin-negative result had a subsequent toxin positive sample, after a median of eight days. Further studies, with supplementary assays for gut inflammation, are required, to determine if these are 'missed' infections or insignificant sub-clinical levels of toxin. A laboratory test that could predict risk of recurrence would be an important tool to inform choice of appropriate *C. difficile* treatment and prevention options. Indeed GDH detection may offer such an opportunity; a cohort of patients has been identified who were consistently GDH positive, even after resolution of symptoms, who subsequently developed recurrent CDI following additional antimicrobial therapy. The cycle threshold (CT) value of PCR assays for the detection of toxin gene may also provide additional information, as low CT (<25) was significantly associated with toxin positivity, presence of PCR ribotype 027 and mortality. Low CT was also associated with recurrence but was not a significant finding.

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Abbreviations used in this thesis

AAD	Antibiotic associated diarrhoea
AUROC	Area under receiver operator curve
BHI	Brain heat infusion
CCEY	Cefoxitin cycloserine egg yolk agar (also known as Braizer's)
CCNA	Cell cytotoxin neutralisation assay
c-di-GMP	cyclic diguanylate
CDI	*Clostridium difficile* infection
Cfu/ml	Colony forming units per millilitre
CROP	Combined repetitive oligopeptide
CTC	Cytotoxigenic culture
CT	Cycle threshold
EIA	Enzyme immunoassay
FMT	Faecal microbiota transplant
GDH	Glutamate dehydrogenase
HCAI	Healthcare associated infection
NAAT	Nucleic acid amplification assay
NPV	Negative predictive value
OD	Optical density value
PCR	Polymerase chain reaction
PCDE	Potential *C. difficile* excretor
PBS	Phosphate buffered saline
PPV	Positive predictive value

RCT	Randomised controlled trial
ROC	Receiver operator curve
TVC	Total viable count
WGS	Whole genome sequencing

1.0 Introduction

1.1 Historical context

Clostridium difficile is widely recognised as the predominant cause of nosocomial diarrhoea in the western world (Crobach *et al,* 2009). This anaerobic spore-forming bacillus was first identified in 1935 as a commensal bacterium of children, although the authors did note that the organism was pathogenic when broth supernatants were injected into Guinea pigs (Hall and O'Toole, 1935). The organism was later shown to be the aetiological agent of pseudomembranous colitis and antibiotic-associated diarrhoea (AAD) (George *et al,* 1978; Bartlett *et al,* 1978), after the identification of a clostridial toxin that was neutralised by *Clostridium sordelii* antitoxin in the faeces of pseudomembranous colitis patients and patients with antibiotic-associated diarrhoea (Larson and Price, 1977; Rifkin *et al,* 1977). *C. difficile* infection only represents 10-20% of cases of antibiotic-associated diarrhoea (Kelly *et al,* 1994; Gerding, 1989); *Staphylococcus aureus, Clostridium perfringens* and *Klebsiella oxytoca* among others, have also been implicated in this condition (Sparks *et al,* 2001; Altemeier *et al,* 1963; Bartlett and Gerding, 2006; Högenauer, 2006). Importantly, pseudomembranous colitis may also be caused by *Salmonella* (Hovius and Rietra, 1982), which could cause misidentification as *Clostridium difficile* infection if imaging alone is used for diagnosis. In the majority of cases, however, no organism can be isolated from AAD patients, and it is postulated that diarrhoea in some of these patients is as a result of disrupted carbohydrate catabolism from depleted anaerobic faecal microbiota (Bartlett, 2002; Young and Schmidt, 2004). Also, some antibiotics, for example erythromycin and the clavulanic acid component of co-amoxiclav, can affect gut motility, and may therefore increase frequency and softness of motions in some patients (Barlett, 2002).

Recently, a new classification of C*lostridium difficile* has been proposed as *Clostridioides difficile* (Lawson *et al*, 2016). However, *Clostridium difficile* still appears to be the preferred option in the current literature and the bacterium will be referred to as such throughout this thesis.

1.2 Virulence factors

1.2.1 Toxins

The ability to produce toxins is one of the key factors that makes *C. difficile* such a successful pathogen. The organism produces two large glucosyltransferase protein toxins, toxin A and toxin B, both of which target the actin cytoskeleton of cells by inactivation of Rho proteins (Ras-homologous proteins), allowing the secretion of chloride ions into the gut lumen via cell tight junctions that have become opened (Just *et al*, 1995; Giry *et al*, 1995), resulting in diarrhoea. Toxin A is a 308,103 Da enterotoxin (Dove *et al*, 1990) with approximately 45% homology at the amino acid level with the 269,000 Da cytotoxin toxin B (Barroso *et al*, 1990; Aktories 1997). Although described as an enterotoxin, toxin A also has cytotoxic activity; however, toxin B is 100-fold more potent than toxin A (Tucker *et al*, 1990) as a cytotoxin. Both toxins are encoded on a 19.6Kb, non-mobile pathogenicity locus (PaLoc), alongside *tcdC* (regulator), *tcdD* (positive regulator, nomenclature later changed to *tcdR*) and *tcdE* (mediator of cell release) (Braun *et al*, 1996; Hundsberger *et al*, 1997; Voth and Ballard, 2005). Non-toxigenic strains of *C. difficile* do not contain the PaLoc within the chromosome (Rupnik *et al*, 1998).

The toxins bind to human epithelial cells via a combined repetitive oligopeptide (CROP) domain, and a hydrophobic protein sequence that allows insertion into host membranes (Dingle *et al*, 2008). Despite the high degree of homology, the toxins appear to have different effects in experimental models; in a mouse model toxin B causes damage to epithelia, and increases inflammation to a much greater extent than toxin A. Indeed, the incidence of mortality in this model is also higher with toxin B, compared with toxin A (Carter *et al*, 2015). In a hamster model, however, toxin A appears to be the more potent toxin (Carter *et al*, 2015). These differences may be due to the fact that the two toxins have different target receptors (Chaves-Olarte *et al*, 1997). In addition, toxin A may have a wider range of targets (GTPases) to which it can bind (Pruitt *et al*, 2012). Mutants lacking either *tcdA* (encoding toxin A) or *tcdB* (encoding toxin B) have been investigated to determine the role of each toxin in virulence (Lyras *et al*, 2009; Kuehne *et al*, 2014), with conflicting results; one study highlighted that only toxin B was required for virulence, since mutants expressing *tcdA* but not *tcdB* did not cause disease (Lyras et al, 2009), while the other study found that mutants with knocked out expression of either *tcdA* or *tcdB* could cause disease (Kuehne et al, 2014).

During the exponential phase of growth, the regulator *tcdC* is expressed in higher amounts, thereby inhibiting the expression of *tcdA* and *tcdB* (Matamouros *et al*, 2007); once in a stationary phase of growth, *tcdR* expression initiates expression of *tcdA* and *tcdB* (Mani and Dupuy, 2001). The product of expression of *tcdR* also promotes its own expression, in a positive feedback loop, thereby increasing the expression of toxins even further (Mani and Dupuy, 2001). Increased toxin production has been linked with changes to the negative regulator TcdC (Spigaglia and Mastrantonio, 2002; Murray *et al*, 2009). The *tcdC* gene of a type of *C. difficile* known as PCR ribotype 027, and certain other PCR ribotypes, has been shown to be truncated, producing an altered amino acid sequence (Spigaglia and Mastrantonio, 2002; Murray *et al*, 2009), potentially reducing the ability of the negative regulator to repress production of toxins A and B (Spigaglia and Mastrantonio, 2002). However, a strain with an intact *tcdC* gene has been shown to be a 'hyper-producer' of both functional toxins A and B, whilst conversely, a strain with a truncated *tcdC* produced low levels of functional toxins (Murray *et al*, 2009). There is also considerable sequence heterogeneity in *tcdB* between strains (Stabler *et al*, 2006), and purified toxin B from PCR ribotype 027 was found to be 4-fold more toxic in a mouse model, than toxin B from other PCR ribotypes (Lanis *et al*, 2013). This may imply that factors other than the *tcdC* gene have a role to play in the regulation of toxin production.

In addition to the two main toxins, a small number of *C. difficile* strains also produce another toxin (binary toxin), which has ADP-ribosyltransferase activity (Voth and Ballard, CMR 2005). This toxin is not encoded within the PaLoc, but within a separate Cdt locus; a 6.2kb region including two toxin genes, *cdtA* and *cdtB,* and a regulatory gene (*cdtR*) (Gerding *et al*, 2014). These two toxins, collectively known as binary toxin, are much smaller than toxin A or toxin B, with a relative molecular mass of only 53 kDa and 98.8 kDa for CdtA and CdtB respectively (Gerding *et al*, 2014). It is thought that binary toxin may increase cell adherence of *C. difficile* by causing fibronectin to be displayed on the surface of the target cell (Schwan *et al*, 2014). The clinical significance of binary toxin is still uncertain. In one cohort of clinical samples however, the presence of binary toxin genes was associated with samples with demonstrable toxin A or B, as measured by cell-cytotoxicity assay, compared with samples positive for the presence of a toxigenic strain, but not free toxin (Berry *et al*, 2017), suggesting a role in toxin-mediated disease. In addition, mortality was higher in the group positive for the binary toxin genes (RR 1.68). It should be noted that this study only detected the presence of *cdt* genes not free toxin however, as assays for functional binary toxin are not readily available.

The genes encoding binary toxin are carried by some of the strains of *C. difficile* termed 'hypervirulent', such as PCR ribotypes 027, 078 and 251 (Wehrhahn *et al*, 2018; Rupnik *et al*, 1998; Toxinotyping website database), and it is therefore postulated that binary toxin contributes to the more severe infections seen in these PCR ribotypes. Indeed, there have been reports of CDI caused by PCR ribotype 033, a non-toxigenic strain (in that it does not contain the PaLoc), but which does produce binary toxin (Eckert *et al*, 2013). Given that the mechanism of action of binary toxin is to increase adherence of *C. difficile* to the epithelial cells but without affecting cell leakage however (Schwan *et al*, 2014), it is unclear how toxin mediated disease occurred in these patients.

1.2.2 Spores

Clostridium difficile is an anaerobic spore-forming bacterium that forms sub-terminal spores on nutrient limitation (Sorg and Sonenshein, 2008). The spores provide protection for the organism when in an oxygen-rich environment, as the vegetative cells are extremely sensitive to the presence of oxygen (Sorg and Sonenshein, 2008). In addition, the spores are heat-stable and resistant to gastric acid, whereas the vegetative cells are not, thereby providing protection to the organism when passing through the upper GI tract (Sorg and Sonenshein, 2008). Chemicals that induce spores to outgrow into vegetative cells are known as germinants, and for *C. difficile* these include primary bile acids, such as taurocholate (Giel *et al*, ne 2010). These primary bile acids have been found to increase in antibiotic-disrupted gut microbiomes (Theriot *et al*, 2014), due to a decrease in the species of gut bacteria that metabolize primary bile salts into secondary bile salts. Indeed, one study found that bacteria within the phyla Firmicutes and Bacteroidetes were essential to this process, and microbiomes depleted in these organisms were more likely to support the germination of *C. difficile* spores (Theriot *et al*, 2014). For *Bacillus subtilis*, the process of germination is well documented; the germinant binds to specific receptors on the spore and leads to the rehydration of the core, which, in turn, causes the cortex of the spore (which surrounds the core) to undergo rehydration and expansion (Setlow, 2014). At this point, the spore has lost its dormancy and the outgrowth of the vegetative cell begins (Setlow, 2014). It could be assumed that the process of germination, albeit with different germinants from *B. subtilis*, is similar in *C. difficile* (De Hoon *et al*, 2010). It has been suggested that primary bile acids require glycine to act as a co-germinant for *C. difficile*

(Sorg and Sonenshein, 2008), as germination was increased when glycine was added to media containing taurocholate, compared with taurocholate alone.

Spores are able to survive in the environment for long periods, and are resistant to cleaning agents that do not contain hypochlorite (Fawley *et al*, 2007). Environmental contamination has been demonstrated in hospital settings where patients with CDI have been treated (Fawley *et al*, 2007; Eckstein *et al*, 2007) leading to a possible cross-infection risk to other patients. In addition, the fact that spores are alcohol-resistant means that alcohol hand cleansers are not adequate to decontaminate hands, and good hand-washing with soap and water is required to prevent further spread of spores (Wilson *et al*, 1985; Boyce and Pittet, 2002).

1.2.3 Other Virulence Factors

Given that the walls of cells in the intestines are covered in a mucus layer, *C. difficile* will need to bind to the mucus layer in order to achieve contact with the epithelial cell layer (Tasteyre *et al*, 2001). The flagella of the bacterium play a crucial role in this adherence, rather than providing motility to the organism (Tasteyre *et al*, 2001; Baban *et al*, 2013). Interestingly, *C. difficile* can adhere to the mucus layer of mice but not the mucus layer of pigs (Tasteyre *et al*, 2001), perhaps suggesting why mice can be used as an infection model for this organism. There are differences in the role of flagella in different strains of *C. difficile* (Baban *et al*, 2013), with some strains having a single flagellum, while others are peritrichously flagellated. In addition, flagella appear to play a role in expression of toxins A and B (Aubry *et al*, 2012), as mutants with the gene for flagella protein (*fliC*) removed had significantly increased expression of toxins A and B and the regulator protein TcdR, and had increased virulence in a hamster model. In contrast, mutants lacking other flagellar protein genes had decreased expression of toxins. A secondary cell signalling molecule, cyclic diguanylate (c-di-GMP), decreases expression of genes encoding flagella via the alternative sigma factor SigD, and therefore, c-di-GMP also affects expression of toxins (Purcell *et al*, 2012, McKee *et al*, 2013). Further cellular adherence during colonisation is provided by *C. difficile* surface layer proteins (SLP) (Calabi *et al*, 2002) which have been shown to bind to collagen, thrombospondin and vitronectin. Interestingly, however, they do not provide adherence to fibronectin (Calabi *et al*, 2002), which, as discussed above, is more highly expressed in cells affected by binary toxin, suggesting that alternative cell surface proteins are involved in adherence to cells after the release of binary toxin.

Increased intracellular levels of c-di-GMP also increase clumping of cells, potentially leading to biofilm formation (McKee *et al*, 2013), along with other virulence factors such as a putative quorum-sensing protein LuxS (Dapa *et al*, 2013). There may be a possible link between the formation of biofilm and sporulation, as a sporulation mutant, Spo0A, was unable to form biofilms (Dapa *et al*, 2013). In addition, these biofilms are more resistant to vancomycin than planktonic cells (Dapa *et al*, 2013).

One recent study highlights another possible virulence mechanism for *C. difficile*, as the organism appears resistant to the increased concentration of heme, found in the gut lumen when infected epithelial cells use a heme efflux pump (Knipple *et al*, 2018). Although capsule can be demonstrated on some strains of *C. difficile* by electron microscopy, *in vivo* the presence of capsule does not appear to be required for virulence, as many of the strains with capsules were toxin negative strains and were unable to produce infection in a hamster model (Davies and Boriello, 1990). In addition, capsule does not appear to be related to cell adhesion (Baldassarri *et al*, 1991). *C. difficile* also possesses several hydrolytic enzymes, although production of these enzymes varies between strains (Seddon *et al*, 1990). Virulence, as measured by the Syrian hamster model, appears to correlate with the presence of these hydrolytic enzymes (Seddon *et al*, 1990). It is therefore theorised that the enzymes are responsible for damaging tissues within the gut, aiding further adhesion of the bacterium (Seddon *et al*, 1990). A recent study, using whole-genome sequencing to identify differences in open reading frames (ORFs) within the accessory genome of different *C. difficile* strains, found several ORFs that were associated with higher virulence strains (Lewis *et al*, 2017). Some of the ORFs could be identified as cell-surface proteins, or proteins associated with transcription. However, there were several with unknown function, highlighting that there are possibly further virulence factors within the accessory genome that are still to be identified.

1.3 Clinical manifestations

The majority of patients with *C. difficile* infection (CDI) will have symptoms of diarrhoea, which can vary from mild to severe, with or without abdominal cramping/pain and colitis (including pseudomembranous colitis) (Burnham and Carroll, 2013). Conversely, it is recognised that some people can carry *C. difficile* in their gut without being symptomatic (asymptomatic carriage) (Shim *et al*, 1998).

In the majority of cases, CDI diarrhoea will begin within a few days of antibiotic use; however, onset can vary from concurrent with the antibiotic course to several weeks following completion of the course (Mogg *et al*, 1979). The watery diarrhoea can be extremely debilitating with >10 episodes per day, and often patients become afraid to move far from toilet facilities (Madeo and Boyack, 2010; Guillemin *et al,* 2014). Many patients also experience distress and embarrassment while symptomatic, and continued anxiety following infection due to the possible recurrence of symptoms (Madeo and Boyack, 2010; Guillemin *et al,* 2014).

In addition to diarrhoea, patients may also exhibit increased temperature, abdominal cramps and increased white blood cell count (Mogg *et al,* 1979). One study found fever in ~28% of cases, abdominal cramps in ~22% and increased white cell count in ~50% of cases (Bartlett *et al*, 1980). Leucocytes can also be detected in the faeces of CDI patients, although this test is rarely used clinically (Mogg *et al*, 1979). Increased leakage of albumin through the damaged gut can also lead to hypoalbuminaemia, detectable from patient serum (Olson *et al*, ICHE 1994).

Pseudomembranous colitis, the presence of distinctive plaques (volcano lesions) on the colonic surface, is associated with CDI, although it is not present in all cases, even in severe infection (Bartlett, 2002). Mogg *et al*. suggested several reasons why plaques may not be visualized on sigmoidoscopy or histology; obscuration by mucus and faecal material, failure to sample the plaque/mucosa junction itself, or the timing of the sigmoidoscopy (Mogg *et al*, 1979). In addition, the variation in severity of cases means that not all patients will develop pseudomembranous colitis (Bartlett and Gerding, 2008).

In severe disease, patients may develop paralytic ileus, where the bowel fails to move faecal contents through the bowel. If this occurs patients will not exhibit the classic diarrhoea seen in most CDI cases (Bartlett and Gerding, 2008). Paralytic ileus may develop into toxic megacolon, where the colon swells and dilates as faeces and gas builds up inside

the colon (Sunenshine and McDonald, 2006). This is a life-threatening condition, and patients may exhibit pain, fever, nausea/vomiting, tachycardia and lethargy (Mogg *et al*, 1979).

Disruption to the normal host gut microbiome provides a niche for the growth and proliferation of *C. difficile*. The phenomenon whereby the normal gut microbiota in healthy individuals prevents colonisation by pathogens was first named colonisation resistance by van De Waaij *et al.* in 1971, after observations that healthy mice were not colonised by repeated challenges of Enterobacteriacea, but once pre-treated with antibiotics, they became colonised (van de Waaij *et al*, 1971). Additionally, once the antibiotic challenge had been removed, colonisation resistance was gradually re-established. Further experiments highlighted that anaerobic bacteria, rather than aerobic bacteria, were fundamental to colonisation resistance (van de Waaij *et al*, 1971). Colonisation resistance provides protection against overgrowth of endogenous potentially pathogenic organisms (carried in small number by many individuals) and exogenous pathogens when ingested (Vollaard and Clasener, 1994). The presence of the right balance of microorganisms is further strengthened by the importance of other physiological factors such as an intact mucosal membrane, gastric acid pH and gut motility (van de Waaij *et al*, 1983).

1.4 Recurrence

C. difficile infection can recur in ~20% of patients, despite appropriate antimicrobial therapy (Cornely *et al*, 2012; Kelly and LaMont, 2008). In addition, patients who have suffered one recurrent episode are at increased risk of developing a second recurrence (40%), and then patients with a second recurrence are at even greater risk of developing a third recurrence (60%) (figure 1.1) (McFarland *et al*, 1994; McFarland *et al*, 2002). This repeated cycle of infection leaves many patients with heightened anxiety about additional infections long after resolution of symptoms (Madeo and Boyack, 2010; Guillemin *et al,* 2014) and with a higher risk of mortality compared with CDI patients without recurrence (36% *vs* 26%, p <0.001) (Olsen *et al*, 2015).

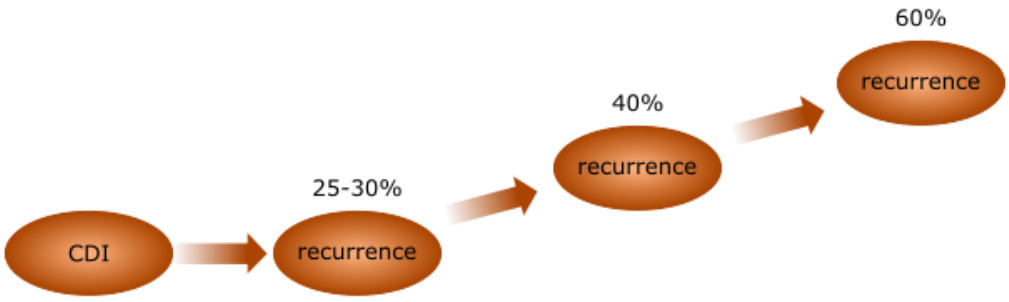


Figure 1.1 The increasing risk of recurrence of infection for patients with CDI

Recurrence is likely linked to a still depleted gut microbiome; indeed, antibiotics used to treat the initial primary case of CDI can also further deplete the natural gut microbiome (Kelly and LaMont, 2008). The majority of secondary infections usually occur within 4-8 weeks, but in one recent study 29% of recurrent cases were seen at up to 12 weeks after resolution of the initial infection (Wilcox *et al*, 2017). The depleted gut microbiome, with reduced colonisation resistance, leaves a niche that can be further exploited either by the strain responsible for the first infection or by a second strain (Wilcox *et al*, 1998). The recurrence rates in patients treated for their primary CDI with either metronidazole or vancomycin appear similar (20.2% *vs* 18.4% respectively) (Kelly and LaMont, 2008); however, patients treated with fidaxomicin have an ~50% reduced risk of recurrence compared with vancomycin, presumably due to the narrower spectrum of action of this

agent, and its more gut-sparing action (Cornely *et al*, 2012, Louie *et al*, 2011). Antibiotic treatments will be discussed in further detail later in section 1.7.

It has been postulated that in addition to lack of colonisation resistance, due to damage to the gut microbiome, the host immune response also has a part to play in the risk of recurrence. Patients with low levels of either toxin A immunoglobulin gamma (IgG) or toxin B IgG were significantly more likely to have recurrent CDI than patients with high levels of these immunoglobulins (Kyne *et al*, 2001a; Aronsson *et al*, 1985). A recently developed human monoclonal antibody against *C. difficile* toxin B (bezlotoxumab) significantly reduced the rate of recurrence, by ~38%, when used as an adjunctive therapy alongside normal CDI treatment antibiotics, compared with those patients who received antibiotics alone (Wilcox *et al*, 2017), highlighting the role of the immune system in preventing recurrent infections. Interestingly, subjects given a human monoclonal antibody against *C. difficile* toxin A (plus antibiotic) did not have a lower risk of recurrent CDI compared with those receiving antibiotics alone. This suggests, but does not prove, that toxin B has more importance in human infection than toxin A.

1.5 Asymptomatic carriage

The carriage of *C. difficile* in the absence of clinical symptoms (diarrhoea) is defined as asymptomatic carriage, although a consensus on the exact definition of this condition is lacking (Furuya-Kanamori *et al*, 2015; Crobach *et al*, 2018). One study indicated that asymptomatic carriage has a protective effect, with less risk of these individuals going on to develop CDI (Shim *et al*, 1998), and indeed IgG levels have been shown to be higher in asymptomatically colonised individuals (Kyne *et al*, 2001b), supporting this 'protective' effect. However, a study following asymptomatically colonised patients in the geriatric setting found that 16.3% of them went on to develop CDI (Nissle *et al*, 2016). In addition, of the eight cases of CDI in the study, seven (87.5%) of them had been asymptomatically colonised on admission to the facility (Nissle *et al*, 2016). This was a small study however, and the authors highlight that they did not manage to reach the sample size required to have adequate power within the study, mostly because of the difficulty in getting repeat specimens from the participants. In addition, the initial proportion of individuals that were positive for *C. difficile* on admission was high (16.4%), compared with other reported ranges of 0.6-15% in the elderly (Furuya-Kanamori *et al*, 2015), which means the results from this study may not be generalisable. Another, similarly small study, found that 37% of the asymptomatically colonised hospitalised individuals went on to develop CDI (Mcfarland *et al*, 1989). It is important however, in studies of this kind, that there is a clear differentiation between patients carrying toxigenic and non-toxigenic strains of *C. difficile*, as toxigenic strains appear to increase CDI risk, whereas colonisation by non-toxigenic strains is protective (Blixt *et al*, 2017; Gerding *et al*, 2018). The proportion of patients who developed CDI if they carried a toxigenic strain was three-times that of patients who carried a non-toxigenic strain in a recent cohort study (Blixt *et al*, 2017).

The proportion of asymptomatic carriers from different settings varies, for example carriage in healthy individuals ranges from 0-15%, while carriage in elderly patients in long-term care facilities ranges from 0-51% (Furuya-Kanamori *et al*, 2015). In the UK only 0.5% of healthy individuals in the community were found to carry *C. difficile*. Importantly, rates may be lower than reported, depending on how samples are screened; for example, PCR for toxigenic *C. difficile* will 'miss' any non-toxigenic strains (Furuya-Kanamori *et al*, 2015), although non-toxigenic strains will not go on to cause CDI. The duration that individuals remain colonised appears variable, with participants in one study remaining positive for *C. difficile* for a median of 8.5 days (Samore *et al*, 1994); however, these patients were only followed until discharge from hospital. Further, extended studies have

found 32% of individuals remained positive for *C. difficile* for 5-7 months (Kato *et al*, 2001), although these were healthy individuals, not previously hospitalised patients.

Treatment to eradicate colonisation is not recommended (SHEA/IDSA guidelines) (McDonald *et al*, 2018), as there has been varied success with attempts to use vancomycin and metronidazole to successfully eradicate *C. difficile* (Johnson *et al*, 1992). While vancomycin did appear to initially eradicate *C. difficile*, this effect was temporary, and carriage rates were actually higher in this group two months after treatment. There was no difference in faecal excretion of *C. difficile* between patients treated with metronidazole and those given a placebo. Given that antibiotic disruption of the gut microbiome is a predisposing factor for CDI, theoretically 'treating' colonisation, could actually drive development of CDI. In addition, faecal excretion of *C. difficile* is often transient (Johnson *et al*, 1992). Potentially, therefore, knowledge of colonisation status may be more useful in terms of antibiotic stewardship and infection prevention, as colonised individuals shed spores into the environment for up to six months (Riggs *et al*, 2007), and onto their skin, although at a lower rate than CDI patients (Guerrero *et al*, 2013). Whole genome sequencing of isolates from asymptomatic carriers and CDI cases has shown that transmission from carriers may be a rare event, but that carriers are a potential source of transmission (Eyre *et al*, 2013c). Indeed, the authors highlight that the rate of transmission is so low that in order to obtain sufficient power, ~3000 individuals would need to be included in a study to determine true transmission rates.

1.6 Risk factors

C. difficile infection is not an infection that occurs in otherwise healthy individuals. As discussed in section 1.3 of this introduction, perturbation of the gut microbiota provides a niche for the growth and proliferation of the organism. This perturbation may be caused by many factors, and as such, become risks for developing CDI (Barlett and Gerding, 2008). Risk factors for development of CDI include: advancing age, comorbidities, long hospital stay and exposure to antibiotics (Bartlett and Gerding, 2008).

1.6.1 Antibiotics

Almost all antibiotic classes have been associated with CDI, although third-generation cephalosporins and clindamycin are historically most often implicated in inducing CDI (Bartlett and Gerding, 2008). With the increasing use of alternative antibiotics however, several other agents/classes have also been implicated as risk factors for CDI, such as fluoroquinolones (Bartlett and Gerding, 2008). The propensity for a specific antibiotic to result in CDI could be related to the effect of that drug on the host gut microbiome (Pultz and Donskey, 2005), as there appears to be increased risk from those drugs that both disrupt the gut microbiome and have no activity against *C. difficile*. For example, *C. difficile* is generally resistant to cephalosporins, and *C. difficile* PCR-ribotype 027 is resistant to fluoroquinolones (Dingle *et al*, 2017). Indeed, fluoroquinolone resistance may have been a key driver of the increased prevalence of PCR-ribotype 027 prior to 2007 (Dingle *et al*, 2017.)

In a mouse model, antibiotics appear to form three distinct groups, each with decreasing propensity to induce CDI (Pultz and Donskey, 2005). As described above, this effect is related to both the activity of the drug against normal gut microbiome and against *C. difficile* itself, with those agents with the narrowest spectrum of action having the least propensity to induce infection in the mouse model. There are however, documented cases of CDI where the only antibiotic exposure was a single dose, given prophylactically before surgery (Privitera *et al*, 1991), and, indeed, surgery itself has been highlighted as a risk factor for CDI, presumably due to the antibiotic exposure (Abou Chakra *et al*, 2014). The bacterial phyla and families affected by different antibiotic classes have been investigated using 16S rRNA amplification and pyrosequencing (Vincent *et al*, 2013). In univariate analyses, the phylum Bacteroidetes and the families *Bacteriodaceae* and *Clostridiales Incertae Sedis XI* were significantly reduced in faecal samples from patients with CDI,

compared with controls. In addition, there was a bloom of *Enterococcaceae*. After multi-variate analysis, prior use of cephalosporins or fluoroquinolones by the patients and a decrease in Clostridiales Incertae Sedis XI within the patient's faecal samples were significantly associated with CDI. It should be noted however, that the faecal samples from patients classified as CDI cases, were identified by a mixture of diagnostic methods, including toxigenic culture, which only identified the presence of a toxigenic strain. It is possible therefore, that the some of the people in the case group were only colonised. These factors are still relevant for the acquisition of *C. difficile* itself however, regardless of whether it causes disease or not.

A systematic review and meta-analysis of risk factors for CDI in 1998 found that exposure to antibiotics was significantly associated with both CDI and carriage of *C. difficile* (Bignardi, 1998). In addition, antibiotics were ranked, by meta-analysis, with aztreonam having the highest odds ratio (OR) of 61.7, although the 95% confidence intervals (CI) were very wide at 8.6-444.5. Of interest, cefotaxime was second highest in the ranking with an OR of 36.2 (95% CI 19-68.9) and fluoroquinolones were about half way down the rankings with an OR of 8 (95% CI 4.5-14.3). As this meta-analysis was conducted in 1998 however, many of the studies demonstrating risk associated with fluoroquinolones had not been published, and so the risk associated with fluoroquinolones in this meta-analysis may be underestimated. Fluoroquinolones potentially cause relatively little disruption of the gut microbiota as they have little activity against anaerobes (BNF, 2019); however, this class of antibiotics have been shown to be a risk factor for the development of CDI. This may partially be related to the emergence of a fluoroquinolone-resistant strain of *C. difficile*, PCR ribotype 027 (Pepin *et al*, 2005). In one study in 2010, 55% of CDI cases had prior exposure to fluoroquinolones alone (Howell *et al*, 2010). In addition, a recent UK study has shown that country-wide antibiotic stewardship, restricting the use of fluoroquinolones, correlates with the decrease in cases across the UK since 2007 (Dingle *et al*, 2017), highlighting the impact of this antibiotic on driving C*. difficile* infection. Indeed, antimicrobial stewardship, not just of fluoroquinolones, offers the opportunity to modify the risk of CDI in patients.

1.6.2 Polypharmacy

If risk of CDI is related to disruption of the normal host gut microbiota, it follows that increased duration of therapy and multiple concomitant antibiotics would increase the CDI risk, as there would theoretically be even more disruption of the gut microbiota. Indeed, a

case/control study demonstrated that CDI cases were significantly more likely to have received multiple concomitant antibiotics (Gerding *et al*, 1986) than controls (80% *vs* 56% respectively, p <0.002). A cohort study in 2011 demonstrated that the risk of CDI increased from a hazard ration (HR) of 2.5 for two antibiotics up to 9.6 for those patients who received five antibiotics, when compared to patients who received one antibiotic (Stevens *et al*, 2011). In addition, in a recent multicentre case/control study the risk of CDI increased by 1.2 (95% CI 1.1-1.3) for each additional antibiotic the patient received (Davies *et al*, 2016c). In the same study, antibiotics were more likely to have been prescribed for treating an infection (acute use) than for prophylaxis. This may be because certain antibiotics are unlikely to be used for prophylaxis, or that prophylactic drugs are given for a shorter duration. Similarly, in an earlier study, there was a significant difference between cases and controls that received antibiotics to treat an infection (59% *vs* 31% respectively, p <0.0001) (Gerding *et al*, 1986). In addition, significantly more control individuals than cases had received short-courses of prophylactic antibiotics (38% *vs* 20% respectively, p <0.01).

Although the impact of duration of antibiotic therapy is not clear from the literature, due to the differing study methodologies used in published studies and the different prescribing habits/policies in study centres, one multivariate analysis did find a correlation between longer duration of therapy and increased risk of CDI (Pepin *et al*, 2005a). This was however, a retrospective analysis conducted at the height of a PCR ribotype 027 outbreak, so the results may not be generalisable. Another retrospective single centre study found a dose dependant effect, with an unadjusted HR for risk of CDI of 1.5 for four-seven days of antibiotic compared with <four days, increasing to a HR of 3.4 for 8-18 days and 9.8 for >18 days of antibiotic (Stevens *et al,* 2011). There was also an increase in risk of CDI with increasing daily doses of antibiotic (Stevens *et al*, 2011). The risk from antibiotics may not only be related to the effects of the agent on the gut microbiota. Of note, some antibiotics appear to stimulate increased toxin production over other antibiotics in an *in vitro* model of the human gut (Saxton e*t al*, 2009).

1.6.3 Exposure to healthcare facilities

CDI is most often diagnosed in patients with previous exposure to a healthcare facility, although there has been a rise in the number of cases in otherwise healthy individuals from the community setting (Chitnis *et al*, 2013). Multivariate analyses demonstrate that prior

hospitalisation is a risk factor for CDI, even after adjusting for other confounding factors such as age (Melzer *et al*, 2019; Davies *et al*, 2016c). Environmental contamination with spores of *C. difficile* has been shown to be higher in healthcare facilities than other environments (McFarland *et al*, 1989) and the spore acquisition rate in patients increases with increased length of stay in hospital facilities , from 13% during a two-week stay to 50% in stays >four weeks (Raveh *et al*, 2006). Indeed, the colonisation rate in hospitalised patients is higher than in the general population (10-40% *vs* 2-3%) (Bartlett, 1994; Sundram *et al*, 2009). It therefore follows that exposure to environments with higher bioload of spores would lead to a higher risk of developing CDI. Duration of hospital stay was significantly associated with CDI, but not carriage, in a qualitative review of 49 studies (Bignardi, 1998). Interestingly, the PCR ribotypes circulating in the hospital and community are largely similar, although certain PCR ribotypes are associated with either the community (002, 020 and 056) or hospital setting (027) (Fawley *et al*, 2016). In addition, although antibiotics are a risk factor for community associated CDI cases (Deshpande *et al*, 2013) there have been reports of these community-onset cases occurring without previous exposure to antibiotics (Bauer *et al*, 2008).

1.6.4 Increased Age

The number of cases of CDI in the elderly is disproportionately higher than for younger age groups, with 93% of deaths due to CDI in the USA in 2008 (McDonald *et al*, 2006; Miniño *et al*, 2011) and 84% in 2011 (Lessa *et al*, 2015) occurring in those greater than 65 years old. This may be due to a number of factors including waning immunity and the increased frequency of hospital visits likely to occur in this population (Di Bella *et al*, 2013). In addition, increasing Charleson comorbidity score, demonstrating frailty of patients due to the number of concomitant comorbidities, is significantly associated with risk of CDI in univariate analysis, but not on multivariate analysis after adjusting for age (Davies *et al*, 2016c). This may be due to the likelihood that aging patents are the most likely to have several comorbidities, and that several of these comorbidities, such as UTI, COPD and diabetes may increase the likelihood of the patients being prescribed antibiotic therapy (Abou Chakra *et al*, 2014). The influence of age on treatment outcomes in CDI can be mathematically modelled and has been shown to predict lower clinical cure (17%), greater recurrence (17%) and lower sustained clinical response (13%) for each increased decade of life (Louie *et al*, 2013).

1.6.5 Proton Pump Inhibitors

There is conflicting information regarding the risk for CDI that comes from taking proton pump inhibitors (PPIs) (Faleck *et al*, 2016; Dial *et al*, 2004; Vesteinsdottir *et al*, 2012), and one review of PPIs in 2014 highlights that there is insufficient evidence to either rule-in or rule-out PPIs as a risk factor for CDI (Biswal, 2014). The difference in risk reported between studies may be due to ascertainment bias; *i.e.* the choice of the groups in which to study this phenomenon (Novack *et al*, 2014). Indeed, a recent study comparing risk factors between a single-centre cohort and a multi-centre cohort found that PPIs were associated with an increased risk in the single-centre cohort (OR 1.8), but not in the multi-centre cohort (Davies *et al*, 2016c). Spores of *C. difficile* are resistant to gastric acid (Wilson *et al*, 1985), therefore, theoretically, increasing gastric pH by using PPIs should have little effect on acquisition of *C. difficile*. It is postulated that PPIs allow microbial colonisation of the upper GI tract (Bavishi and Dupont, 2011), however, whether this holds true for *C. difficile*, which usually proliferates in the colon, has yet to be proved. One *in vitro* study demonstrated increased expression of toxin A on exposure to alkaline environments and PPIs (Stewart and Hegarty, 2013). Again, however, it is unclear if PPIs would have this effect *in vivo*, as the pH of the colon is already more alkaline.

1.6.6 Risk factors for complicated CDI and recurrence

In addition to risk factors for a primary case of CDI, there are factors associated with increased risk for either complicated infection or recurrence. A systematic review of 24 studies found; increased age, continued use of antibiotics (following diagnosis with CDI), PPIs and the strain type of *C. difficile* were most frequently associated with recurrence (About Chakra *et al*, 2014). Unfortunately, as the authors did not perform a meta-analysis, due to the heterogeneity of the study methodologies, the level of significance of these factors cannot be assigned. Indeed, even for the factor >65 years old, the results are reported as a combination of HRs (range 1.75-1.92) and ORs (range 1.32-10.42), making interpretation of the data difficult. The same authors assessed 18 studies for risk factors for complicated CDI, and 30 studies for risk of mortality from CDI; increased age, raised white cell count, renal failure (as measured by raised serum creatinine) and the presence of comorbidities were frequently associated with complicated CDI cases, while mortality was associated with all of these plus hypoalbuminaemia and infection with PCR ribotype 027.

Current diagnostic guidelines define severe CDI using white cell counts >15cellx10^9/L, raised serum creatinine (>1.5mg/dL IDSA/SHEA, >133µM or ≥1.5 times baseline for ESCMID) and serum albumin <30g/dL (ESCMID only) (McDonald *et al*, 2018; Crobach *et al*, 2016).

1.6.7 Prediction of risk

There have been attempts to combine risk factors into prediction models for recurrent CDI with varied success. One recent study used data gathered from a derivation cohort of 7,538 patients to define the risk factors for the prediction model, before testing in a validation cohort of 15,077 patients (Reveles *et al*, 2018). Following logistic regression, risks for recurrence were identified as prior antibiotic therapy with 3rd- or 4th- generation cephalosporins, prior PPI use, prior use of anti-diarrhoea agents, CDI defined as non-severe and CDI with community onset. Each risk was assigned a score, which allowed the prediction model to classify risk as low, medium or high. In the validation set, those classified as low risk had a recurrence rate of 8.9%, those with medium risk had a recurrence rate of 20.2% and those with high risk had a recurrence rate of 35.0%. While the model appeared successful in the validation cohort, it should be noted that some of the factors identified as risks in this study have not been identified previously, such as anti-diarrhoeal agents, highlighting again the influence of the cohort that is studied and possible ascertainment bias. In addition, this study included cases that were coded as CDI within the hospital database. This would likely therefore, include cases diagnosed by NAAT for toxin gene; potentially diluting the pool of 'true' cases with some patients who simply had carriage of *C. difficile* (Polage *et al*, 2015).

In contrast, another group that used the same study design of a derivation (n = 9,386) and validation cohort (n = 1,865) were unable to produce a successful prediction model (Escobar *et al*, 2017). Although the sample size of the validation cohort was smaller than the first study, the derivation cohort was larger, suggesting that it was unlikely to be underpowered. Of note, the first study appears to have included fewer potential prediction factors than the second study, and combined several factors into a definition of 'severe CDI' where only at least one of the factors had to be positive, rather than using each of those variables as an independent predictor. It remains to be seen if the first model still performs as well when used on a dataset derived from different facilities or even from a different country.

1.6.8 Asymptomatic carriage

Risk factors for asymptomatic carriage are similar to risk factors for CDI and include previous hospitalisation, history of CDI, use of PPIs, and presence of anti-toxin B IgG (Loo *et al*, 2011; Kong *et al*, 2015). Interestingly however, asymptomatic carriers were less likely to have been prescribed an antibiotic in the previous eight weeks than CDI patients (OR 1.04 *vs* 5.25). Colonisation may therefore not depend on disruption to the gut microbiota, in the same way that CDI does (Furuya-Kanamori *et al*, 2015). Further research into risk factors for asymptomatic colonisation is required, especially in settings outside of the hospital.

1.7 Treatment

Guidelines are available for treatment options in *C. difficile* infection from both the European Society for Clinical Microbiology and Infectious Disease (ESCMID) and jointly from the Infectious Disease Society of America (IDSA) and the Society for Healthcare Epidemiology of America (SHEA) (Debast *et al*, 2014, McDonald *et al*, 2018). Whilst the IDSA/SHEA guidelines are up-to-date, the European guidelines are some six years old, and do not reflect some of the newer trials of recently developed antibiotics, or the latest information regarding older therapies.

Both sets of guidelines recommend that any predisposing antibiotic is stopped, although the evidence for this recommendation is unclear from the literature (Debast *et al*, 2014, McDonald *et al*, 2018). In addition, empirical treatment is recommended on suspicion of CDI, while awaiting laboratory test results, in the IDSA/SHEA guidelines (McDonald *et al*, 2018). It should be noted however, that diagnostic samples should be taken before staring any empirical therapy, as use of concomitant *C. difficile* targeting antibiotics can affect *C. difficile* recovery from faecal samples (Sunkesula *et al*, 2013).

1.7.1 Metronidazole

Metronidazole is a nitroimidazole antibiotic with activity against nucleic acid synthesis by disrupting the DNA of microbial cells (Freeman *et al*, 1997). The amount of drug excreted via the bowel is quite low, with the majority of excretion via bile and urine (Bartlett, 2010), which is not ideal for a treatment drug for CDI, however it is thought that metronidazole can cross into the bowel through the 'leaky' cells of the colon that have been affected by *C. difficile* toxin (Sullivan *et al*, 2001). Despite not being an ideal treatment option however, lack of alternative antibiotics with activity against CDI led to the promotion of metronidazole as a treatment of choice (Freeman *et al*, 1997).

Mounting evidence demonstrates the inferiority of metronidazole treatment compared with other CDI antibiotics. The time to resolution of diarrhoea for patients on metronidazole is significantly longer by ~1.5 days, than it is for those on vancomycin (Wilcox and Howe, 1995). In addition, there are reports of treatment failure on metronidazole; 38% in one study (Fernandez *et al*, 2004) and 22% in another (Musher *et al*, 2005). Of note, in the first study, patients with serum albumin level of <2.5g/L were more likely to fail metronidazole therapy (OR 11.7, 95% CI 4.0-31.6), suggesting that these were

patients with more severe infection. Data from a trial of another potential *C. difficile* agent, tolevamer, allowed the direct comparison of metronidazole and vancomycin, as both drugs were used as a comparator in the study. Clinical success, defined as resolution of diarrhoea and abdominal symptoms, was significantly higher in the vancomycin arm, compared with metronidazole (81.1 *vs* 72.7%, $p = 0.02$) (Johnson *et al*, 2014). In addition, when only severe cases were compared there was a higher proportion of patients that had clinical resolution in the vancomycin arm, compared with metronidazole, although this was not significant at the $p = 0.05$ level (78.5% *vs* 66.3% $p = 0.059$). The 2014 ESCMID guidelines still recommend oral metronidazole 500mg three times daily for treatment of non-severe CDI, while 2018 IDSA/SHEA guidelines only recommend using metronidazole when access to alternative treatments is restricted, and then only for non-severe cases (Debast *et al*, 2014, McDonald *et al*, 2018). In a recent network meta-analysis, metronidazole was only ranked 11 out of the 13 drugs reviewed (Beinortas *et al*, 2018), giving further weight to the argument for not using metronidazole. Others argue that there is still a role for metronidazole in the treatment of CDI, but that patients should be carefully selected, as younger patients (<65 years old) with mild disease were significantly more likely to have clinical resolution on metronidazole treatment than those patients who were older than 65 with mild disease (OR 1.63, 95%CI 1.29-2.06) (Appaneal *et al*, 2018).

1.7.2 Vancomycin

Vancomycin is a glycopeptide antibiotic with bactericidal activity against both aerobic and anaerobic Gram-positive bacteria. The drug binds to the cell wall, blocking polymerisation of glycopeptides (Hammes and Neuhaus, 1974). This action inhibits synthesis of the cell wall and damages the cytoplasmic membrane (Hammes and Neuhaus, 1974). Vancomycin is usually used intravenously to treat infections, however, oral administration is recommended for treatment of *C. difficile*, as there is very poor absorption via the gut, meaning that high levels of the drug can be achieved in the large intestine to treat the infection (Hammes and Neuhaus, 1974). Oral administration of vancomycin may select for vancomycin-resistant enterococci (Edlund *et al*, 1997), which meant at one point, metronidazole was used in preference to vancomycin for treatment of CDI (Pelaez *et al*, 2002). It should be noted however, that oral metronidazole treatment for CDI has also been associated with overgrowth of vancomycin resistant enterococci (Al-Nassir *et al*, 2008).

Current treatment guidelines state that vancomycin (or fidaxomicin in the US) should be used as the first treatment option for all initial episodes of CDI (2018 IDSA/SHEA) or for all initial cases of severe CDI (2014 ESCMID) at a dosage of 125mg four times daily (Debast *et al*, 2014, McDonald *et al*, 2018). This difference reflects the age of the ESCMID guidelines, as recent evidence does not support the use of metronidazole rather than vancomycin for mild infections, as described in the section above. Vancomycin is also the recommended treatment option for fulminant or severe CDI in the IDSA/SHEA guidelines, but at a dosage of 500mg four times daily, approximately four times the dose recommend by ESCMID, as the latter states that the current evidence for increased dosage of vancomycin is not sufficient, if the patient does not have ileus.

There are several dosing options for treating recurrent infections with vancomycin, including tapered and pulsed regimens (Debast *et al*, 2014, McDonald *et al*, 2018). Tapered vancomycin regimens decrease the total dose each day, gradually weaning the patient off the antibiotic (Debast *et al*, 2014, McDonald *et al*, 2018), and theoretically allowing the host microbiome to begin re-establishing, while still supressing the growth of any remaining *C. difficile*. One study found that vancomycin tapered regimens were significantly more successful when the final day contained three doses (81% cured), compared with one (61% cured, p =0.03), and when patients had only had ≤two recurrences of CDI (78% cured), compared with ≥three recurrences (62%, p = 0.13), although this was not a significant finding (Sirbu *et al*, 2017). Presumably, the higher success rate in patients with ≤two recurrences of CDI reflects the more damaged microbiome of those patients with ≥three recurrences, due to repeated treatments. Careful selection of patients suitable for tapered vancomycin treatment therefore needs to be considered. A randomised controlled trial comparing vancomycin taper with faecal microbiota transplantation (FMT), see section 1.9.3, was ended early, as futility analysis demonstrated lack of efficacy in both arms; vancomycin cure rate 58.3%, FMT cure rate 43.8% (Hota *et al*, 2017), and the authors cited patient selection as a factor in the failure of the study. A systematic review of different extended regimens of vancomycin for CDI found that taper-pulse regimens had a higher clinical success incidence than pulsed regimens alone (58-100% *vs* 26-81% respectively) (Murphy *et al*, 2018). It should be noted however, that there is a lack of good quality studies of these alternative dosing regimens, as the systematic review only included five studies.

Recent use of vancomycin as a longer-term prophylactic agent has shown promise, with a success rate of 95% over 200 patients-months of follow up (Zhang *et al*, 2019). This was however, a retrospective study, not a study designed to determine the clinical success of this regimen. In addition, although there were no reported side effects, there was no attempt to determine the level of vancomycin resistant enterococci carried by any of the treated individuals. Of note, repeated oral administration of vancomycin can lead to elevated serum levels (Edlund *et al*, 1997), despite poor uptake by the gut, so toxicity in long-term oral use of vancomycin should not be ruled out.

1.7.3 Fidaxomicin

Fidaxomicin is a macrocyclic antibiotic that inhibits bacterial RNA polymerase and therefore transcription (Lin *et al*, 2018). The drug has a narrower spectrum of action than vancomycin, and causes less damage to the host gut microbiota, specifically *Bacteroides* spp., and with variable effects on communities of bifidobacteria (Chilton *et al*, 2014b, Chilton *et al*, JAC 2015). In addition, recurrence of *in vitro* modelled CDI has been seen with vancomycin but not with fidaxomicin (Chilton *et al*, 2014b). Indeed, in a randomised controlled Phase III trial, in patients treated for a first recurrence of CDI, secondary recurrence within 28 days was reduced from 35.5% in the vancomycin arm to 19.7% in the fidaxomicin arm, a reduction of ~50% (p = 0.45) (Cornely *et al*, 2012,). The impact of fidaxomicin on secondary recurrence within 14 days was even more stark, with 27% of patients experiencing recurrence in the vancomycin arm compared with 8% in the fidaxomicin arm (p = 0.003). In a second Phase III clinical trial there was also a reduction in recurrence of ~50% in the fidaxomicin arm compared to patients treated with vancomycin (Louie *et al*, 2011). Fidaxomicin was only recommended for treatment of recurrent cases of CDI in the 2014 ESCMID guidelines, as the initial randomised controlled trials were only powered to determine non-inferiority of fidaxomicin compared with vancomycin (Cornely *et al*, 2012, Louie *et al*, 2011), and there was no evidence at this time regarding the use of fidaxomicin in severe infections (Debast *et al*, 2014). The 2018 IDSA/SHEA guidelines however, recommend fidaxomicin as an alternative treatment option to vancomycin, for first episodes of CDI (McDonald *et al*, 2018).

The high up-front-cost of fidaxomicin, compared with vancomycin, may influence choice of drug for initial episodes. However, economic analyses show that it is as cost-effective to use fidaxomicin for the initial case of CDI rather than vancomycin, due to the decreased downstream costs associated with the higher recurrence rate with vancomycin (Watt *et al*, 2016). Interestingly, sub-group analyses showed that fidaxomicin was cost-saving for cancer patients compared with vancomycin. Although this study was conducted in Germany and direct healthcare costs will differ across countries, there will be similarities in relative patterns of costs. For treating recurrent CDI cases, fidaxomicin is the second most cost-effective option, after FMT (Lapointe-Shaw *et al*, 2016).

A recent systematic review conducted a network meta-analysis, which allowed the authors not only to compare antibiotic treatments for CDI, but also to rank them (Beinortas *et al*, 2018). The comparison included 24 clinical trials (three unpublished at the time of the analysis) with 13 different antibiotic treatment options; vancomycin, fidaxomicin, metronidazole, tecioplanin, fucidic acid, bacitracin, ridinilazole, LFF571, cadazolid, nitazoxanide, surotomycin, tolevamer, rifaximin. They were unable to include any of the studies on faecal microbiota transplant (FMT), probiotics or immunotherapies, as they did not meet the inclusion criteria. For sustained clinical cure, teicoplanin (OR 0.37, 95% CI 0.14-0.94) and fidaxomicin (0.67, 0.55-0.82) performed significantly better than vancomycin, and vancomycin performed significantly better than metronidazole (0.73, 0.56-0.95). The evidence for the use of teicoplanin is limited, however, with only two small trials of ~ 50 patients each, both of which date from before 1996. Importantly however, vancomycin was superior to all agents for primary symptomatic cure, but vancomycin and metronidazole were only ranked ninth and 11th for prevention of recurrence. Patients on fidaxomicin had significantly fewer episodes of recurrence than patients on vancomycin or metronidazole; thereby having greater sustained clinical cure. This analysis gives further weight to the argument of using fidaxomicin as a first-line treatment for CDI. Indeed, as discussed above, it may also be the most cost-effective option, due to the decreased risk of recurrence and therefore decreased patient time spent in hospital (Watt *et al*, 2016; Burton *et al*, 2017).

In addition to its action in sparing the gut microbiota, fidaxomicin appears to persist on the spores of *C. difficile* and inhibits the outgrowth of the vegetative cell from the spore, then by turn, toxin production by the cell *in vitro* (Chilton *et al*, 2016). The fact that active fidaxomicin can be detected for extended periods after dosing has been completed was

first seen in the *in vitro* human gut model, where fidaxomicin was detectable in the model for 21 days after installation had finished, compared with four days for vancomycin (Chilton *et al*, 2014b; Chilton *et al*, 2015). In addition, detection of toxin from sequential patient faecal samples in an *in vivo* study, demonstrated that while both fidaxomicin and vancomycin reduced toxin (A and B) detection at the mid-point (3-5 days) of therapy, only samples from fidaxomicin-treated patients maintained low toxin A levels up to the end of follow up (19-38 days after treatment initiation) (Thabit *et al*, 2016). Interestingly, this drop in the level of toxin A was even seen in patients treated with fidaxomicin who developed recurrence. There was also a significant association between high toxin levels (both A and B) and presence of vegetative cells (p = 0.003 for toxin A and 0.007 toxin B respectively) and spores (p <0.001 for both). This evidence for reduced numbers of spores in faecal samples, and the direct effect of fidaxomicin on spore outgrowth discussed above, means that it is possible, therefore, that fidaxomicin could reduce onward transmission, as well as treating the infection. This hypothesis is supported by a small study *in vivo* which demonstrated reduced environmental contamination in the rooms of patients treated with fidaxomicin compared with those treated with vancomycin; 36.8% of environmental samples showed contamination *vs* 57.6% respectively, p = 0.02. (Biswas *et al*, 2015). A larger, multicentre study substantiates the results of the first, small study but has yet to be published (Davies *et al*, 2019a).

Similar to the different dosing regimens for vancomycin, extended fidaxomicin dosing has been investigated, as theoretically this would be less detrimental to the gut microbiota. Pulsed-tapered and tapered-pulsed dosing allowed recovery of bifidobacteria within an *in vitro* model, whilst still clearing *C. difficile* infection, and with no signs of recurrence during the length of the experiment (Chilton *et al*, 2015). These data have been replicated in a randomised controlled trial comparing pulsed fidaxomicin over a longer time period than standard therapy (200mg twice daily for five days, followed by 200mg once daily on alternate days from day 7 to 25) with standard vancomycin therapy of 125mg four times a day for ten days (Guery *et al*, 2018). Sustained clinical cure, defined as initial resolution of symptoms and no recurrence, was assessed at three time points; days 40 (30 days post end of vancomycin treatment), 55 (30 days post end of fidaxomicin treatment) and 90. There was no difference in initial resolution of systems, however the proportion of patients on extended-pulsed fidaxomicin with sustained clinical cure at day 30 (after treatment) was significantly higher than for those patients on standard vancomycin (70% *vs* 59% respectively, p = 0.03). The difference in sustained clinical cure remained significant at day

90, with a 14% difference between fidaxomicin- and vancomycin-treated patients (p = 0.007). Importantly, the authors also looked at the bacterial diversity in the faecal samples from the two different patient groups and showed that bacterial diversity increased in the fidaxomicin group over the study period to a greater extent than those in the vancomycin arm. Additional data are required to compare extended-pulsed vancomycin regimens with the extended fidaxomicin regimen studied here, especially the impact of vancomycin on the microbiota over that time. Without a randomised controlled trial comparing the two extended-pulsed regimens it is difficult to know the best choice for patients and, indeed, decisions should be taken on a patient-by-patient basis.

1.8 Novel antimicrobial treatments

1.8.1 Tigecycline

The antibiotic tigecycline, a broad-spectrum agent, has been suggested as a possible treatment for CDI, as it has shown activity against *C. difficile in vitro*, with very low MICs of 0.06mg/L (Baines *et al*, 2006). As a broad-spectrum agent tigecycline does affect the gut microbiome, particularly affecting the bifidobacteria and bacteroides both *in vitro* and *in vivo*, although to a lesser extent for bacteroides *in vivo* (Baines *et al*, 2006, Nord *et al*, 2006). However, the drug did not induce proliferation of the organism or production of toxins in the *in vitro* model (Baines *et al*, 2006). Of note, in both experiments there was an increase in some enterobacteria in patients who received tigecyline (Nord *et al*, 2006), most notably *Klebsiella* spp. Enterobacterial bloom has been demonstrated with an *in vitro* model, including gene exchange from carbapenemase-resistant *Enterobacteriaceae* (CPEs) (Rooney *et al*, 2017a; Rooney *et al*, 2017b). There is the possibility therefore, that using tigecycline could leave patients at increased risk of carbapenemase-resistant Enterobacteriaceae colonisation. It should be emphasised, however, that tigecycline has no regulatory approval for use as CDI treatment option.

1.8.2 Cadazolid

Cadazolid, a quinoxolidinone antibiotic was another agent that showed promise in *in vitro* studies against *C. difficile*. Because the drug inhibits bacterial protein synthesis, it decreases production of both toxins and spores of *C. difficile* (Locher *et al*, 2014), and appeared bactericidal *in vitro*. In addition, Phase I studies demonstrated that active cadazolid was found in the faeces of patients at a peak of 5675 times the levels of the MIC_{90} 0.25mg/L, and daily levels of > 1651 times higher than the MIC_{90} (Gehin *et al*, 2015). Cadazolid reduced the total viable count of *C. difficile* and the titre of cytotoxin within an *in vitro* gut model to below the limit of detection, when used at doses comparable to those found within the human gut, and appeared to have minimal impact on the gut microbiota (Chilton *et al*, 2014a). In addition, there was no recurrence of infection observed within the model. Cadazolid had similar efficacy to vancomycin for the treatment of *C. difficile* in a Phase II study (Louie *et al*, 2015) but with greater sustained clinical cure due to a reduction in recurrence of 18.2-25.0% for the different dose regimens tested compared with 50% recurrence in the vancomycin arm. A recent report however, from two Phase III randomised clinical trials of cadazolid compared with vancomycin, only demonstrated non-

inferiority to vancomycin in one of the two studies (Gerding *et al*, 2019) and the drug is no longer being developed for use in CDI. Importantly however, cadazolid was found not to be inferior to vancomycin in either of the trials when the investigators determined if clinical cure had occurred, instead of a per protocol analysis. The authors highlighted that the lack of a standardised definition of clinical cure makes such per protocol analyses difficult.

1.8.3 Ridinilazole

Antibiotics that target *C. difficile* but with a narrow spectrum of action can potentially provide greater protection to the gut microbiota. One such agent, ridinilazole is currently in Phase III trials (Clinicaltrial.gov, 2019). Ridinilazole has a low MIC_{90} of 0.25µg/ml against *C. difficile*; lower than fidaxomicin MIC_{90} 0.5µg/ml (Goldstein *et al*, 2013), but MICs were raised for isolates with known multiple resistance to other agents, although this was not statistically significant (Freeman *et al*, 2015). Exposure to sub-MIC levels of ridinilazole reduced production of toxin A and B by 91 and 100% respectively when measured using a commercial EIA with a limit of detection of 0.31ng/mL (Bassères *et al*, 2016). In addition, sub-MIC levels of the antibiotic appear to inhibit the formation of the cell septum during cell division, thereby affecting the ability of the organism to proliferate (Bassères et al, 2016).

The narrow spectrum of action of this antibiotic is demonstrated when used within the human gut model, as *Clostridium* spp. were the only commensal bacteria that declined after the installation of the drug to the model (Baines *et al*, 2015). Unlike the cadazolid gut model study described above however, the total count of *C. difficile* did not fall below the limit of detection, although counts did fall by ~1.5 $\log_{10}$ cfu/mL over the seven days of installation, with a further fall of 2 $\log_{10}$ cfu/mL by the end of the experiment. In addition, the level of toxin, as measured by cell-cytotoxicity assay, dropped below the limit of detection ~ 7 days after the end of the instillation of the drug. Ridinilazole was found to be safe and well tolerated in a Phase I study, with a mean concentration of 547 times the MIC_{90} detected within faecal samples of patients on day 5 of receiving 250mg twice daily (Vickers *et al*, 2015).

In a Phase II randomised controlled trial comparing ridinilazole with vancomycin that was powered for non-inferiority, ridinilazole actually demonstrated superiority to vancomycin, with 66.7% of patients having sustained clinical cure at 30 days, compared with 42.2% in

the vancomycin arm (Vickers *et al*, 2017), estimated difference 21.1% (90% CI 3.1-39.1, p = 0.0004). In several sub-group analyses, sustained clinical cure was higher with ridinilazole, however due to the low sample size these were not significant. For PCR ribotype 027 sub-group analyses, sustained clinical cure was higher in the vancomycin group, but again this was not significant. In addition, there was a high proportion of participants in this study who were <65 years old, which may not truly reflect the population most likely to require this treatment option. The Phase III study has only just begun recruiting, so results from this study will not be available for at least another two years.

1.8.4 Surotomycin

Another antibiotic that was found not to be inferior to vancomycin in a Phase II RCT was surotomycin, a daptomycin-derived antibiotic (Lee *et al*, 2016). The rate of initial clinical cure was similar between the two different doses of surotomycin (125mg twice a day and 250mg twice a day) compared with vancomycin standard therapy with >86% success in all three groups. As with other newly developed drugs with a narrower spectrum of action than vancomycin, the recurrence rate of 17.2% for 250mg surotomycin twice daily was significantly lower than the recurrence rate of 35.6% in the vancomycin arm (p = 0.035). Sustained clinical cure was not significantly different between any of the comparisons, however the sustained clinical cure in the 250mg surotomycin arm was 70.1% compared with 56.1% in the vancomycin arm. Interestingly, although the recurrence rate was higher in patients from any of the arms receiving concomitant antibiotics, the recurrence rate in these patients in the 250mg surotomycin arm was lower than for vancomycin patients who received concomitant antibiotics (37.5 *vs* 58.8%). The Phase II data demonstrating non-inferiority for initial clinical cure was supported by both *in vitro* model data and animal model data, where surotomycin was effective at killing *C. difficile*, and was comparable to vancomycin (Chilton *et al*, 2014c, Mascio *et al*, 2012). It should be noted however, that surotomycin did not prevent recurrence in the *in vitro* model and led to overgrowth of *Enterobacteriacae* (Chilton *et al*, 2014c).

The Phase II results did not appear to translate into efficacy in Phase III RCTs however (Boix *et al*, 2016; Daley *et al*, 2017). In one Phase III trial the proportion of patients with clinical cure when treated with 250mg twice daily of surotomycin (79%) was lower than for patients treated with standard vancomycin therapy (84%), and surotomycin therefore failed to show non-inferiority (Boix *et al*, 2016). Importantly, the reduction in recurrence

seen in the Phase II studies was not seen in this Phase III study, as sustained clinical cure was similar between the two arms of the study with 60.6% of surotomycin patients remaining cured of their infection compared with 61.4% of patients treated with vancomycin. Interestingly sustained clinical cure was higher for patients treated with surotomycin *vs* those treated with vancomycin if the infection was caused by PCR ribotype 027; however this was not statistically significant. In contrast, in the second Phase III study, surotomycin was found not to be inferior to vancomycin for initial clinical cure; the authors suggest this difference may be due to some differences in the patient populations in these studies, with those in the second study being younger (median age 57.1 *vs* 61.3% years) and with milder disease (32.6 *vs* 25.6%) (Daley *et al*, 2017). However, again, the decrease in cases of recurrence demonstrated in the Phase II study was not seen (Daley *et al*, 2017), as sustained clinical cure was not significantly different between the two arms (63.3% *vs* 59.0% respectively). There was a higher impact on sustained clinical cure for PCR ribotype 027 infections with a significantly higher number of patients remaining free from recurrence when treated with surotomycin (64.4%) compared with those treated with vancomycin (37.8%), similar to the previous study (Boix *et al*, 2016; Daley *et al*, 2017). The authors suggest that the conflicting results between the Phase II and Phase III studies may be due to the high rate of recurrence seen in the patients treated with vancomycin in the Phase II study, as the lower rates of recurrence in the vancomycin arm of the surotomycin Phase III studies were similar to those seen in Phase III studies of other antibiotics used to treat *C. difficile* (Daley *et al,* 2017). In addition, the definition of CDI in the Phase II and III studies differed, with diagnosis in Phase II requiring a positive stool test for toxin A and/or B, while diagnosis in the Phase III studies was based on a positive stool test by either a toxin detection assay or by NAAT for toxin genes. Indeed >50% of cases in the Phase III studies were diagnosed by NAAT (Boix *et al*, 2016; Daley *et al*, 2017). This highlights the difficulty in designing randomised controlled trials, as the impact of the baseline characteristics of the patients enrolled, how CDI is diagnosed, and the definitions of cure and recurrence, can influence the results of the study, as demonstrated by both the surotomycin and cadazolid studies. It is very unlikely that either of these two antibiotics will be taken further in development as a treatment option for CDI.

1.8.5 Other novel antibiotics

Several other antibiotics are being developed or are being used for the treatment of CDI, including ramoplanin, fusidic acid, nitazoxanide, LFF571 and rifaximin, with varying rates of success (Petrosillio *et al*, 2018). Current evidence on the efficacy of these agents is insufficient however to show superiority over fidaxomicin and ridinilazole (Beinortas *et al*, 2018). Rifaximin, although bactericidal for *C. difficile*, is not recommended for use in the treatment of CDI due to the high levels of resistance found in *C. difficile* isolates (NG *et al*, 2019). However, rifaximin used as a follow-on treatment for either metronidazole or vancomycin standard therapy has been shown to reduce recurrence by ~50% from 29.5% in the placebo group to 15.9% in those that received rifaximin, although this was not a statistically significant finding (Major *et al*, 2018).

1.9 Alternative treatment options

Alternative methods, other than traditional antimicrobial therapy for curing CDI, have been investigated. As CDI is a toxin-mediated disease, it makes sense that binding the toxin within the gut lumen may reduce the impact the toxin can have on the gut tissue. One agent developed for this purpose is tolevamer, a polymer that neutralises both toxin A and toxin B (Braunlin *et al*, 2004). Tolevamer not only resolved infection in 80% of the treated mice in a CDI mouse model, but also none of the animals had a relapse of infection when treatment was stopped (Kurtz *et al*, 2001). This is in contrast to those mice that had been treated with metronidazole where 80% had relapse of infection after withdrawal of the metronidazole treatment. When used to treat a simulated CDI infection in an *in vitro* gut model however, tolevamer was not able to reduce cytotoxin levels within the model (Baines *et al*, 2009). Indeed, tolevamer failed to show efficacy in Phase III studies (Johnson *et al*, 2014). In addition, in a recent meta-analysis tolevamer was significantly inferior when compared to all of the agents in the comparison, with the exception of LFF571 and bacitracin (Beinortas *et al*, 2018).

1.9.1 Immunoglobulin

Pooled human immunoglobulin was suggested as an adjunctive therapy in 1997, but current guidelines do not recommend its use, due to a lack of evidence regarding efficacy (Salcedo *et al*, 1997; Debast *et al*, 2014, McDonald *et al*, 2018). Indeed, a systematic review in 2016 only found a total of 17 published studies examining passive immunotherapy in humans (Diraviyam *et al*, 2016). While the studies were generally favourable, there was a lack of rigorous, controlled studies to enable the true effect of this treatment option to be determined (Diraviyam *et al*, 2016).

1.9.2 Bezlotoxumab

A more focussed immunotherapy has been developed, rather than using pooled immunoglobulins as described above. Two humanised monoclonal antibodies were developed, one anti-toxin A, actoxumab, and one anti-toxin B, bezlotoxumab; when mixed together denoted as actoxumab-bezlotoxumab. Bezlotoxumab binds within the CROP domain of toxin B, thereby preventing toxin B from binding to cells of the gut epithelium (Orth *et al*, 2014). In a mouse model of acute infection, actoxumab-bezlotoxumab

significantly reduced fluid accumulation and protected the gut epithelium, with no evidence of inflammation or damage to the epithelial cells (Yang *et al*, 2015). In addition, when mice, pre-treated with actoxumab-bezlotoxumab and that survived an initial primary infection with *C. difficile,* were re-challenged with an antibiotic, they were protected against further infection, unlike mice that had not been pre-treated with actoxumab-bezlotoxumab (Yang *et al*, 2015). Warn *et al* have used rodent models to demonstrate a period after primary infection during which the risk for recurrent infection is highest (Warn *et al*, 2016). This 'at-risk window' is due to possible spore outgrowth when the antibiotic that had been used to treat the primary infection has been stopped and the concentration has fallen, combined with a lack of recovery of the host microbiota. In rodent models, actoxumab-bezlotoxumab reduces the risk of recurrence during this 'at-risk window' and thereby provides time for the microbiota to recover, leading to fewer recurrent infections (Warn *et al*, 2016).

In a pooled analysis of two randomised controlled trials for standard therapy combined with actoxumab-bezlotoxumab, actoxumab alone or bezlotoxumab alone, bezlotoxumab significantly reduced recurrence by ~38% compared with standard therapy plus placebo, with no impact on initial clinical cure (Wilcox *et al*, 2017). Interestingly, although there was reduced recurrence in the actoxumab-bezlotoxumab arm compared with placebo, there was no additional benefit over the arm devoted to bezlotoxumab alone. This supports the hypothesis that toxin B is more important for clinical infection than toxin A, as discussed in the virulence section of this introduction. However, sub-group analyses demonstrated a greater effect from actoxumab-bezlotoxumab in participants that had an infection caused by PCR ribotype 027, compared with bezlotoxumab alone, although this was not significant and numbers in each group were small. *In vitro* data suggests that changes in some of the epitopes on the toxin B protein in the CROP region in some strains of *C. difficile*, including PCR ribotype 027, correlated with decreased bezlotoxumab potency (Hernandez *et al*, 2015). While further studies in humans, with much larger numbers are required to understand fully any decreased effect against certain PCR ribotypes of *C. difficile*, bezlotoxumab provides a way to reduce not only recurrent infections, but also the number of days a patient spends in hospital (Basu *et al*, 2018). Reduced hospital readmissions and costs associated with recurrent CDI, such as additional antibiotics, nursing and other healthcare costs, make bezlotoxumab a cost-effective option, with 0.12 quality-adjusted life-years (QALYs) gained compared with placebo (Prabhu *et al*, 2017), and $19,824 saved per QALY gained.

1.9.3 Faecal Microbiota Transplantation

Faecal microbiota transplantation (FMT) is recommended for patients that have had multiple recurrences of CDI, and who have failed antimicrobial therapy (Debast *et al*, 2014, McDonald *et al*, 2018). Augmentation of a patient's gut microbiome by using an infusion of donor faeces was first reported in 1958 by Eiseman *et al*, and the idea gained increased interest after the publication of a randomised controlled trial between FMT and vancomycin with or without bowel lavage for treating recurrent CDI (van Nood *et al*, 2013). Patients who had had at least one recurrence of CDI were eligible for inclusion, although the majority of cases 35/43 had >one recurrence. For those randomised to receive FMT, 13/16 (81.3%) were cured (with no recurrence within ten weeks) after one infusion, while a further two patients were cured after a second infusion from a different donor; giving overall cure in 93.8% of patients with one or two infusions. In comparison, only 31% of the patients in the vancomycin arm and 23% of patients in the vancomycin with bowel lavage arm remained free from CDI after ten weeks. The trial showed superiority of FMT over both vancomycin regimens early (p <0.0001), and the study was stopped, as it would have been unethical to continue with patients in the failing vancomycin arms. It should be noted that the proportion of patients who failed to have resolution of symptoms after treatment with vancomycin was higher than anticipated, which could have been due to the selection of patients with multiple recurrences (van Nood *et al,* 2013). Interestingly, the vancomycin regimen was 500mg four times a day, rather than 125mg, and it could be argued that this higher dose of vancomycin would be even more detrimental to the gut microbiome, and thereby not allow re-establishment of colonisation resistance, leading to further recurrences. Another randomised controlled trial of 64 patients however, also found a significant difference between the numbers of patients with no recurrence at eight weeks when treated with FMT *vs* 125mg four times per day of vancomycin (Hvas *et al*, 2019). FMT was also found to be superior to fidaxomicin (200mg twice a day) with 71% of patients treated with FMT remaining symptom free at eight weeks compared with 33% of patients treated with fidaxomicin (p = 0.009) (Hvas *et al,* 2019).

Many of the randomised controlled trials on FMT are small, and therefore more liable to be influenced by confounders, such as the population studied. Meta-analysis can be used to determine if the pooled results are generalisable to a larger population. A recent meta-analysis of FMT randomised controlled trials included eight studies in the analysis,

with a total of 537 patients (Hui *et al*, 2019). Pooled relative risk for recurrence with FMT was 0.38 (95% CI 0.16-0.87, p = 0.02) with an overall recurrence rate of 11% (Hui *et al*, 2019), demonstrating the effectiveness of this treatment option.

Guidelines for the use of FMT have been issued jointly by the British Society of Gastroenterology (BSG) and the Healthcare Infection Society (HIS) (Mullish *et al*, 2018) to try to provide some standardisation to the use of this currently unregulated treatment option. Concerns over safety are partially addressed, with recommendations over the selection of potential donors (Mullish *et al*, 2018). The need for FMT should be judged on an individual patient basis however, balancing patient cure with possible unknown long term consequences. Donor selection is not only based on the results of screening for infectious diseases, which needs to be extensive, but also concerns the general health of the donor; the link between gut microbiome composition and health is still being investigated but there are increasing reports of the role that the microbiome plays in other health conditions (Skonieczna-Żydecka *et al*, 2018; Zheng *et al*, 2019). One recommendation in the guidelines, for example, is to use donors with a body mass index (BMI) of ≥18 and ≤30kg/m^2, as there have been reports in mice of donor faeces from obese mice causing thin mice to become obese (Ridaura *et al*, 2013). In addition, there have been case reports of patients dying from aspiration pneumonia when FMT was delivered via nasogastric (NG) or nasoduodenal (ND) tube (Bang *et al*, 2017; Baxter *et al*, 2015; van Beurden *et al*, 2017). The current recommendation is to use NG, ND, nasojejunal tube or enema for infusion of FMT (Mullish *et al*, 2018), but that NG should be used with caution in patients with risk of regurgitation. An alternative to liquid FMT is pre-prepared gastric-resistant faecal capsules, which can be swallowed (Youngster *et al*, 2016).

1.9.4 Microbiome therapeutics

Attempts have been made to reduce the risks associated with FMT by producing preparations of organisms that may perform the same function of FMT, namely resolution of the gut microbiome. RBX2660 is a suspension of live microbes that has been used with some success when used in a similar way to FMT, with an overall success of 87.1% in preventing a further episode of recurrence within eight weeks (Orenstein *et al*, 2016). It should be noted that the primary end-point of this study was product related adverse events (AEs), and 28/31 patients experienced at least one AE. These were mostly gastrointestinal in nature, and the majority resolved within seven days; there were no

product related serious AEs. An alternative agent being developed, SER109 only uses spores from *Firmicutes*, rather than a suspension of microbes (Khanna *et al*, 2016). Theoretically, this reduces the possibility of transferring an unknown infectious agent to the recipient. However, although Phase Ib trial results appeared successful with 96.7% of patients remaining recurrence free, the Phase II trial results of this agent have so far not proved successful (Khanna *et al*, 2016; press release from Seres Therapeutics 2019). A post-trial analysis by the company suggests that the decreased efficacy of SER-109 could have been due to suboptimal dosing, as a lower dose was used than the Phase Ib study. In addition, CDI was defined using PCR-based diagnostics; further testing of samples demonstrated that a significant number of these patients did not have *C. difficile* toxin in their samples (press release from Seres Therapeutics, 2019). If patients who were only carriers of *C. difficile* rather than having *C. difficile* infection were included on the study, this would have impacted the outcome measure for the trial. Further studies of SER-109 are therefore continuing (press release from Seres Therapeutics, 2019).

In addition, the use of non-toxigenic *C difficile* to enhance colonisation resistance and, therefore, reduce recurrence, has been investigated via RCT (Gerding *et al*, 2015). The oral formulation of spores was found to be well tolerated and patients in the treatment arm reported fewer events of diarrhoea and abdominal pain than patients in the placebo arm. Four different doses of spores were evaluated, and a dose dependant effect was found, with faecal colonisation seen in 71% of patients treated with the highest dose, compared with 63% of patients treated with the lowest dose. In addition, reduction of recurrence was dose dependant, with the lowest recurrence rate in the patient group that received the highest dose of spores (5% vs 30% placebo, OR 0.28, P=0.006). Although not demonstrated *in vivo*, the PaLoc has been transferred to non-toxigenic strains within the laboratory (Brouwer *et al*, 2016), suggesting that there may be a potential risk if using non-toxigenic *C. difficile* as a treatment option.

1.9.5 Microbiome protective therapeutics

In addition to the therapeutics discussed above, that have been designed to enhance the microbiome, there are investigational drugs that are designed to reduce the negative impact of antibiotics on the gut microbiota, and so reduce the risk of CDI. SYN-004 (ribaxamase) is a beta-lactamase that is designed to be taken orally at the same time as a beta-lactam antibiotic (Connelly *et al*, 2017). The beta-lactamase then degrades the

antibiotic that is excreted within the gastrointestinal tract of the patient, thereby protecting the gut microbiome from damage by the antibiotic. Results from Phase IIa and IIb studies indicate that SYN-004 is effective at reducing the level of ceftriaxone in the gut of patients to below the level of detection (Kokai-Kun *et al*, 2017a), and reduced the risk of subsequent CDI by 2.4% (95% CI -0.6-5.9, p=0.045) (Kokai-Kun *et al*, 2017b). Another agent in development is DAV-132. This is an oral adsorbent agent, which binds to antibiotics excreted via the gastrointestinal tract, thereby lowering the antibiotic concentration and protecting the microbiota (de Gunzberg *et al*, 2015). DAV-132 is currently being investigated in an RCT in Europe (clinicaltrial.gov, 2019).

1.10 Infection control and prevention

The financial burden on the healthcare economy from this pathogen is considerable. Each case has been estimated to cost the NHS approximately £7,000 due to the increased use of isolation facilities, specialised barrier nursing and extended hospital stay (Wiegand *et al*, JHI 2012), with estimates of $5.4-6.3 billion per year in the US (Zhang *et al*, BMC Infec Dis 2016). In the UK, there is increased financial pressure on NHS Trusts as a result of financial penalties for failing to meet infection reduction targets set by the Department of Health (Department of Health, 2016). Therefore, although the driver of Department of Health targets is reduction of morbidity and mortality, the reduction of the burden of *C. difficile* infection also has important economic implications, as well as those for the care and well-being of patients, and there are several measures that can be taken to reduce *C. difficile* burden.

1.10.1 Transmission

Transmission of *C. difficile* is via the faecal-oral route, following ingestion of spores, presumably primarily from contaminated hands (Durovic *et al*, 2018). A recent review of 24 transmission studies found 53.3% of studies reported that, in the hospital setting, direct contact with a previous case was the source of infection; however 40% reported that contact with the hospital environment was the source for cases of CDI (Durovic *et al*, 2018). In addition, the most commonly cited environmental source was the patients' room (25% of studies), followed by wards (13%), bathrooms (13%) and toilets (13%). Indeed, the risk of acquiring *C. difficile* after being nursed in a room that previously housed a patient positive for *C. difficile* was 1.73 (95% CI 1.15-2.55), with a mean time to acquisition of 3.2 days, compared with 18.9 days for those patients nursed in areas that did not previously house *C. difficile*-positive patients (MacFarland *et al*, 1989). A multivariate analysis of factors affecting acquisition of *C. difficile* found admission to a room that previously housed a patient positive for *C. difficile* was independently associated with *C. difficile* acquisition (hazard ratio 2.35) (Shaughnessy *et al*, 2011). The risk appears to be highest within the first 48 hours of exposure to that environment with a relative risk of 2.23 (95% CI 1.24-4.01) (MacFarland *et al*, 1989). There have been several recent transmission studies, using a combination of typing techniques and spatial links to demonstrate likely transmission from a previous CDI case (Walker *et al*, 2012; Martin *et al*, 2018; Mawer *et al*, 2017; Eyre *et al*, 2017; Eyre *et al*, 2013a). In all of these studies a direct or indirect link between previous

CDI cases or *C. difficile* positive patients could only be demonstrated for ~20% of cases. This highlights that there may be alternative sources of transmission, in addition to previous CDI cases. Infection control and prevention methods however try to reduce the likelihood of transmission via these pathways.

Hand hygiene is a key way of reducing the risk of *C. difficile* transmission and/or acquisition. In one study, when the hands of nursing staff were checked for contamination after contact with patients positive for *C. difficile* carriage, 59% of them became contaminated (MacFarland *et al*, 1989), including their fingernails, fingertips, palms and the underside of rings. One important difference for hand hygiene with *C. difficile* however, is that alcohol skin gels are ineffective against the spores of the bacterium. Indeed, as discussed in section 1.2.2, the spores are resistant to alcohol (Wilson *et al*, 1985). Hands can however, be effectively cleaned by physical washing, with standard soap and water or antibacterial soaps (Boyce and Pittet, 2002). However current ESCMID guidelines only recommend switching from alcohol-based gels to soap and water in a *C. difficile* outbreak setting, due to the effectiveness of alcohol-based gels for preventing other hospital-acquired infections (Tschudin-Sutter *et al*, 2018). The guidelines also recommend using personal protective equipment (PPE) including gowns and gloves in both outbreak and endemic settings, although the use of PPE is a stronger recommendation for the outbreak situation than the endemic setting (Tschudin-Sutter *et al*, 2018). Isolation of patients is recommended in both endemic and outbreak situations (Tschudin-Sutter *et al*, 2018) although there is no recommendation on if this should be in individual rooms, or via cohorting of symptomatic patients together, presumably due to the differing availability of individual side rooms in different institutions.

Individual side rooms may make decontamination of the environment easier, as all high-touch areas should be decontaminated, using an appropriate cleaning agent, on a daily basis, and after vacation of the room by the patient (Tschudin-Sutter *et al*, 2018). The spores of *C. difficile* have been shown to remain in the hospital environment for up to 12 weeks, with only a ≤ 3 $\log_{10}$ reduction from the starting inoculum during that time (Otter and French, 2009), and are more likely found in room of patients with symptomatic CDI, as opposed to asymptomatic carriage (MacFarland *et al*, 1989; Riggs *et al*, 2007). In one study, where floors were intentionally contaminated with a *C. difficile* spore preparation of 10^6cfu, spores could be recovered up to five months later (Kim *et al*, 1981). When the starting inoculation was 10^4cfu however, spores could only be recovered at a maximum of four

weeks later; demonstrating that a higher starting inoculum enhances the chance that some spores will remain in the environment long-term. In addition to contamination via contaminated hands, the environment can also be seeded by spores via aerosolisation (Best *et al*, 2010), most commonly when there is activity near a symptomatic patient. Aerosolised spores can also be recovered from the air above toilets following flushing, thereby further contaminating the environment (Best *et al*, 2012). Hypochlorite-based agents are more effective than those containing quaternary ammonium compounds at removing *C. difficile* spores from the environment (Fawley *et al,* 2007), and accelerated hydrogen peroxide-based agents are comparable with bleach containing 5g/L free chlorine, at inactivating spores in ≤10 minutes (Perez *et al*, 2005).

Cleaning practices must be maintained at an appropriate level; one outbreak of *C. difficile* has been associated with failure to adequately clean the re-usable mop-heads that were used to clean the ward (Sooklal *et al*, 2014). Several of the cleaning agents described above can be hazardous to cleaning staff, if not used appropriately (Perez *et al*, 2005), and require all high-touch areas to be physically cleaned adequately. Alternative, no-touch systems for room decontamination have now been developed. These consist of systems using either ultra-violet-C (UVC) light, or hydrogen peroxide vapour (HPV). UVC systems, where the light damages the bonds in DNA, require direct line-of-sight with the surface to be decontaminated and reduce the number of spores on surfaces by 2-4$\log_{10}$ (Havill *et al*, 2012; Nerandzic *et al*, 2010). HPV systems, in contrast, which damage membrane lipids, DNA and protein by free radicals, reduce the number of spores on surfaces by ~6$\log_{10}$ (Havill *et al*, 2012). As highlighted by a review in 2015, however, currently most studies of these systems have been tested on seeded environmental surfaces (Barbut, 2015) and further evaluation of their clinical impact is required.

1.10.2 Surveillance

Surveillance of CDI cases is recommended in both the endemic and outbreak setting (Tschudin-Sutter *et al*, 2018), however the evidence level for this is low. Nevertheless, it is logical that up-to-date knowledge of the current number and location of cases, along with the strain associated with those cases can help to direct available infection prevention and control resources better. Surveillance requires accurate diagnosis however; methods that can also detect colonised patients as well as actual cases of CDI could falsely inflate reported CDI rates (Davies *et al*, 2017; Longtin *et al*, 2013). In Europe, the European Centre

for Disease Prevention and Control (ECDC) has initiated a standardised programme of surveillance of *C. difficile* (ECDC, 2019.) to overcome the issue that several European countries do not have national CDI surveillance programmes of their own. The results of the pilot project, reported in 2016, demonstrate that two of those countries without their own national systems had high levels of PCR ribotype 027 (van Dorp *et al*, 2016), perhaps due to a lack of knowledge about circulating outbreak strains. Additional to the issue of accurate diagnostics, surveillance systems can only record those cases that were clinically suspected and therefore tested; thereby missing potential cases. Results from a study of 482 hospitals across 20 countries in Europe in 2012-2013 estimated that there were a total of ~40,000 missed cases per annum in those 482 hospitals (Davies *et al*, 2014). Indeed, the proportion of cases attributed to PCR ribotype 027 in a country was inversely correlated with the testing rate in that country (Davies *et al*, 2014), again suggesting that lack of knowledge about circulating strains prevents appropriate outbreak control methods being put in to place. An earlier study of 97 hospitals in 34 countries in Europe demonstrated a correlation between the number of tests performed/10,000 patient bed days (pbds) and the number of cases/10,000pbds (Bauer *et al*, 2008), supporting the adage 'the more you look, the more you find'. However, this was not replicated in the later study (Davies *et al*, LID 2014) for either reported, or actual measured cases of CDI. This may be due to the increased sample size of the later study, thereby possibly including centres with greater heterogeneity of testing policies (Davies *et al*, 2014). These differences however, highlight the need for standardised case definitions, diagnostic methods, clinical parameters and appropriate denominators for rate data, to make surveillance successful.

1.10.3 Antibiotic stewardship

C. difficile infection is most often precipitated by antimicrobial exposure, as discussed earlier in the introduction. Antimicrobial stewardship may therefore reduce the burden of CDI; reducing the number of potentially susceptible patients would reduce the number of CDI cases. The decrease in cases since 2007 seen in two areas within the UK, has been correlated with the decrease in fluoroquinolone and cephalosporin prescribing over the same period within those regions, but not with overall levels of antimicrobial prescribing (Dingle *et al*, 2017). Importantly, PCR ribotype 027 is resistant to fluoroquinolones, in contrast to many other types of *C. difficile* (Freeman *et al*, 2018), and in one of the two regions the proportion of cases due to PCR ribotype 027 dropped from ~67% to ~3% during

the period of decreased fluoroquinolone use. Currently, antibiotic stewardship is largely driven by the policies of local centres, or by national guidelines (Debast *et al*, 2014; McDonald *et al*, 2018). In the developing era of personalised medicine however, there may be potential for diagnostic assays that can predict those patients who would be most susceptible to *C. difficile* infection and, therefore, those in whom antimicrobial stewardship would be most advised. These tests may focus on markers of dysbiosis of the gut microbiota, such as the levels of bacterial metabolites found in the urine (Obrenovich *et al*, 2017), or on carriage of specific organisms, such as *C. difficile*.

1.11 Typing methods

Investigation of *C. difficile* outbreaks is reliant on being able to distinguish between different strains of the bacterium, for which several methods have been developed. Gurtler developed a method using the DNA sequence of the region encoding the intergenic spacer region between the 16S and 23S ribosomal subunits of *C. difficile* (Gurtler, 1993). This highly variable region of DNA can be cleaved into sections using restriction endonucleases, with the resultant unique pattern of DNA fragments attributed to a specific PCR ribotype (Stubbs *et al*, 1999). Improvements made to the sequence of the primers used, improved the reproducibility of the method (Bidet *et al*, 1999), and this method was then used by the *Clostridium difficile* Ribotyping Network of England and Northern Ireland (CDRN). The CDRN was developed as a response to the growing number of CDI cases in England, with an aim to drive down transmission (Wilcox, *et al*, 2012). Recent advances in technology have allowed a shift from visualisation of the fragments using gel electrophoresis, to measurement of fragment lengths on a sequencer (Fawley *et al*, 2015), enabling greater standardisation of the method. The current library of strains held at the CDRN is >900 (personal communication).

PCR ribotyping, although used world-wide, is not the only typing system for *C. difficile,* for example PCR ribotype 027 is also known as pulsed field gel electrophoresis (PFGE) NAP1, restriction enzyme assay (REA) B1 or toxinotype III (McDonald *et al*, 2005; Rupnik *et al*, 1998). Due to the large sections of DNA, cleaved by enzymes from the whole genome of the organism in PFGE, separation of the fragments takes longer than standard electrophoresis, and requires alternating and rotating currents to be applied to the gel (Schwartz and Cantor, 1984; Wren and Tabaqchali, 1987). PFGE was first used to define strains of *C. difficile* in 1996 (Kato *et al*, 1996), demonstrating that some patients had relapse of their original infecting strain, while others acquired a new infection with a different strain (reinfection). PFGE has modest discrimination for strains of *C. difficile*, and some strains are non-typeable using this method, due to DNA degradation (Kato *et al*, 1996; Alonso *et al*, 2005). Improvements to the method however, have reduced DNA degradation, and therefore, increased the number of isolates for which a PFGE type can be derived (Corkill *et al*, 2000; Alonso *et al*, 2005).

C. difficile can also be typed based on the toxin genes within the genome, when compared to a reference strain, VP1 10463 (Rupnik *et al*, 1998). Restriction fragment length polymorphism (RFLP)-PCR amplifies the section of DNA containing the toxin genes *tcdA* and

tcdB, which is then cut with DNAse enzymes and checked for polymorphisms (Rupnik *et al*, 1998). Ten overlapping PCR amplification products enable the entire PaLoc to be analysed (Rupnik *et al*, 1998), with 31 toxinotypes currently known (Toxinotype website database, 2019).

Several other typing methods have been used for *C. difficile*, including serogrouping (Delmee *et al*, 1985), restriction endonuclease analysis (REA) (Devlin *et al*, 1987), multilocus sequence type (MLST) (Marsh *et al*, 2010) and multilocus variable-number tandem repeat analysis (MLVA) (Fawley *et al*, 2008; Fawley and Wilcox, 2011). Each method has a different level of discrimination; MLVA does achieve increased discrimination over PCR ribotyping, and can be used for enhanced analysis of transmission where the same PCR ribotype has been identified (Fawley *et al*, 2008; Fawley and Wilcox, 2011). MLST is less discriminatory than MLVA but can provide information on the genetic lineage of the isolate (Marsh *et al*, 2010). Advances in technology have made whole genome sequencing (WGS) of *C. difficile* available to many laboratories. Although termed whole genome sequencing, the sequence of the entire genome is not determined, for example the region used for PCR ribotyping is not included in WGS (Eyre *et al*, 2013b), making it impossible to derive a PCR ribotype from WGS currently. One advantage of methods such as PCR ribotyping, MLVA and WGS over other typing methods, is that they can be used to track transmission as they can be adjusted to examine the relatedness of isolates, not just place isolates into a defined category, as, for example, PFGE does (Eyre *et al*, 2013b).

1.12 Epidemiology

C. difficile infection became of public interest in the UK and worldwide after several high-profile incidents in healthcare institutions (Kostkova *et al* 2013). These outbreaks were often associated with the PCR ribotype 027 (McDonald *et al*, NEJM 2005; Pepin *et al*, 2004; Loo *et al*, 2005). Typing of *C. difficile* isolates is therefore, a useful tool for examining the spread of infection and understanding outbreaks, and new typing methods, such as WGS, provide new information on transmission pathways for CDI. Indeed, although CDI was traditionally thought to spread from case to case, the source of infection in the majority of cases remains unclear, as a recent study reported that only 20-25% of inpatient CDI cases could be linked with another CDI case when using WGS (Walker *et al*, 2012). Of note, in-patient asymptomatic carriage rates of *C. difficile* have been shown to be higher (up to 21%) than those seen in the community (1.6%) (Rea *et al*, 2012), and may contribute to transmission within the hospital setting (Furuya-Kanamori, 2015).

1.12.1 Changing patterns of epidemiology

Typing isolates of *C. difficile* also demonstrates how patterns of *C. difficile* types have changed over time and how they vary between locations; *e.g.* hospital *vs* community, or by country. Before the world-wide outbreaks of PCR ribotype 027, the predominant strain in the UK was PCR ribotype 001, with little variation seen in circulating hospital strains (Freeman *et al*, 2010). Increased infection control and prevention measures in the UK, including mandatory reporting of all CDI cases from hospitals, led to a decline of outbreaks, including those caused by PCR ribotype 027 (Wilcox *et al*, 2012). After the decline of PCR ribotype 027 in the UK, the heterogeneity of circulating strains within the hospital environment increased and, indeed, appears similar to the diversity of strains within the community (Fawley *et al*, 2016).

CDI case incidence has continued to increase across Europe in the last decade, with a mean of 4.1/10,000patient bed days (pbds) across 34 countries in 2008 and a mean of 7.0/10,000pbds across 20 countries in 2012/2013 (Bauer *et al*, 2011; Davies *et al*, 2014); albeit with marked variation between countries. In the same period, the testing rate also increased from a mean of 52.1tests/10,000pbds to 65.8/10,000pbds, and when undiagnosed cases are added to those actually reported, the case rate rises by 2.4-2.9 fold, highlighting ascertainment bias (Davies *et al*, 2014). Targeted testing of patients may also impact on reported CDI incidence; those tested for CDI are significantly older in Italy than in

four other countries studied, suggesting that testing in Italy is targeted to those thought to be most 'at risk' (Davies *et al*, 2016b; Davies *et al*, 2017). Comparisons of case incidences between centres and countries are therefore compounded by factors such as clinical suspicion, testing policies and testing methodologies (Davies *et al*, 2014; Longtin *et al*, 2013; Davies *et al*, 2016b; Davies *et al*, 2017).

A recent systematic review has attempted to determine the global burden of *C. difficile* (Balsells *et al*, 2019), and estimates a mean of 3.54 hospital-associated cases/10,000pbds and 2.24/1,000 admissions across the 41 countries included in the studies in the meta-analysis. The incidence was substantially lower in the community with a mean of 0.55/1000 admissions. It should be noted however, that this may not be the most appropriate measure for community infections, as it will only capture those that were sick enough to require hospital admission. Interestingly, the authors note that the CDI incidence was highest in North America; however, although they mention that diagnostic methods may affect incidence data, they fail to mention that laboratories in the US largely use stand-alone PCR assays for CDI diagnosis, which have been shown to overcall the number of true cases of CDI (Davies *et al*, 2016b; Polage *et al*, JAMA 2015). This may therefore partly explain the high incidence levels reported in the USA.

The diversity of PCR ribotypes across Europe has increased from a total of 65 different PCR ribotypes found in the 2008 study to the 125 found in the 2012/2013 study (Bauer *et al*, 2011; Davies *et al*, 2016a). Distribution of ribotypes varies markedly across Europe, with the greatest proportion of PCR ribotype 027 in Eastern Europe (figure 1.2), a shift from the high prevalence seen in the UK in 2008 (Davies *et al*, 2016a, Davies *et al*, 2019c, Bauer *et al*, 2011). The prevalence of PCR ribotype 027 and 176 (a closely related strain), is inversely related to the overall diversity of PCR ribotypes within a country; *i.e.* outbreaks of one strain dominate in these countries, while those countries with fewer outbreaks have increased diversity of circulating strains (Davies *et al*, 2016a). Within-country clustering is seen, using WGS, for PCR ribotypes 356, 018, 176, 001/072 and 027, while other PCR ribotypes, such as 078, 015, 002, 014 and 020, appear to have a European-wide distribution (Eyre *et al*, 2018). This is suggestive of two different patterns of spread for these PCR ribotypes, and that those with European-wide distribution may have a common source (Eyre *et al*, 2018). Some PCR ribotypes are found in animal faecal samples, such as 078 and 014 (Knetch *et al*, 2014; Knight *et al*, 2016), it is therefore postulated that the food

chain may be a potential source for those PCR ribotypes distributed across Europe (Eyre *et al*, 2018).

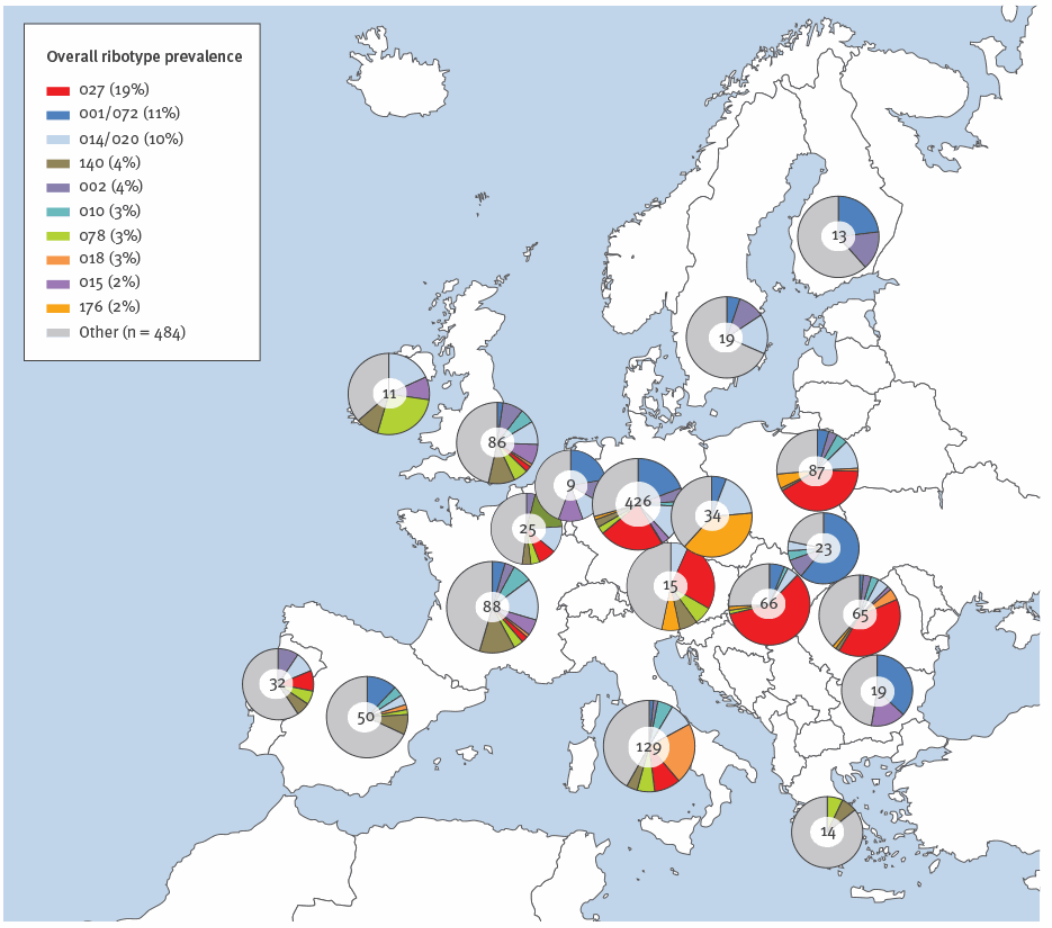


Figure 1.2. Reproduced from Eurosurveillance (with permission). Geographical distribution of *Clostridium difficile* PCR ribotypes, by participating European country[a], in the EUCLID study, 2012–13 and 2013[b] (n = 1,196). Pie charts show the proportion of the most common ribotypes by country and the number in the centre of the charts is the number of typed isolates in the country. [a] Austria, Belgium, Bulgaria, Czech Republic, Finland, France, Germany, Greece, Hungary, Ireland, Italy, the Netherlands, Poland, Portugal, Romania, Slovakia, Spain, Sweden and United Kingdom. None of the faecal samples submitted from Slovenia during the two sampling days were found to be positive for *C. difficile* or its toxins. [b] The countries submitted inpatient diarrhoeal samples on two sampling days (one day in winter, in December 2012 or January 2013, and one day in summer, in July or August 2013)

1.12.2 PCR ribotype 027

Further to epidemiological information, typing can also highlight different clinical outcomes between strains. Increased severity of infection and greater mortality has been associated with PCR ribotype 027 (Loo *et al*, 2005; Pepin *et al*, 2005b). In a study of 1,144 CDI cases the presence of PCR ribotype 027 was associated with severe CDI (OR 1.73, 0.037), and mortality (OR 2.02, $p = 0.009$) compared with infection caused by an alternative PCR ribotype, after multivariate analysis (Rao *et al*, 2015). One difficultly however, when comparing studies describing the relationship of PCR ribotype 027 and severity of infection, is the variation in the definition of severity. In the ESCMID guidelines, severity is defined as severe colitis and/or the presence of one of several other markers, including raised white cell count above $15x10^9$/L, serum albumin <30g/L, rise in serum creatinine >1.5X baseline values or >133µM (Debast *et al*, 2014). Severity can also be described based on ICU admission, serious comorbidities or immunodeficiency in patients aged >65 years (Debast *et al*, 2014). IDSA/SHEA guidelines describe severe infection in similar terms to ESCMID, but presence of hypotension, shock, ileus or megacolon is defined as fulminant infection (McDonald *et al*, 2018). One study of 236 cases of CDI found no association between presence of PCR ribotype 027 and severity; however, severity was based on death within 30 days (Cloud *et al*, 2009). In this case then PCR ribotype 027 was not associated with mortality, rather than not being associated with severity as the study title suggests; however this study may have been underpowered for mortality as an outcome measure. A later study also found no association between PCR ribotype and severity, after multivariate analysis to control for other confounders, such as age and raised white blood cell count, although presence of PCR ribotype 027 was associated with severity in univariate analysis (OR 2.33, $p = 0.035$) (Walk *et al*, 2012). Severity in this study was defined as admission to ICU, requiring interventional surgery for CDI symptoms, or death within 30 days of diagnosis, and the authors acknowledge that they may have missed some cases of severe infection using these criteria (Walk *et al,* 2012).

One reason for the increased severity of infection with this PCR ribotype may be increased production of toxin by this strain (Warny *et al*, 2005), although this has been disputed by both batch and continuous culture system experiments (Merrigan *et al*, 2010; Freeman *et al*, 2007). Experiments can also be compounded by the choice of comparator non-027 strain, as production of toxin in batch culture appears to be strain dependant (Merrigan *et al*, 2010). In addition, continuous culture systems, rather than batch culture show that PCR ribotype 027 produces toxin for longer than does PCR ribotype 001, but not at a higher

level (Freeman *et al*, 2007). The high levels seen in the batch culture model may therefore be due to an accumulation of toxin and potentially do not give a true representation of toxin production by this strain. An alternative method that overcomes the limitations of batch culture experiments uses a continuous culture system to model the interplay between *C. difficile,* normal bowel microbiota and antimicrobials introduced into the system (Freeman *et al*, 2003). This human gut model has been validated against the colonic microbiota of sudden death victims (Macfarlane *et al*, 1998), and has been shown to be clinically reflective of *in vivo* CDI (Freeman *et al*, 2002). Indeed, the model has successfully predicted clinical failure of treatment options *in vitro* that went on to fail Phase II clinical trials, such as tolevamer (Baines *et al*, 2009; Johnson *et al*, 2014).

In addition to robust toxin production, increased sporulation and an increased binding capacity of toxin B, over other PCR ribotypes, has also been observed with PCR ribotype 027 (Merrigan *et al*, 2010; Stabler *et al*, 2008). Patients with PCR ribotype 027 may also have higher levels of C-reactive protein (CRP) and faecal calprotectin, a measure of gut inflammation, over those with other PCR ribotypes (Goldenberg and French, 2011; Peretz *et al*, 2016). The emergence of fluoroquinolone resistance in PCR ribotype 027 has been suggested as a reason for the proliferation of this strain, as historical strains (collected prior to 2001) were fully susceptible to gatifloxacin and moxifloxacin (McDonald *et al*, 2005). In contrast 100% of tested strains of PCR ribotype 027 collected after 2001 were resistant to gatifloxacin, and 42% were resistant to moxifloxacin (McDonald *et al*, 2005). Indeed, more recent strains of PCR ribotype 027, collected from across Europe, demonstrate multi-drug resistance, specifically to moxifloxacin, rifampicin, imipenem and metronidazole (Freeman *et al*, 2018). The emergence of fluoroquinolone resistance has been shown to have been acquired from two different lineages; FQR1 and FQR2, with spread throughout Europe largely being due to FQR2 (He *et al*, 2013).

1.13 Laboratory diagnosis

From a clinical point of view, it is important to understand the status of the disease of the patient, to ensure they receive the correct treatment and that the appropriate infection-control precautions are used (Bignardi *et al*, 2013). This requires accurate diagnostics. Laboratory diagnostics of CDI have been a contentious subject for some time with no agreement on the optimal method for detection as numerous comparison studies have been published showing conflicting variations in the performance of diagnostic assays (Fang *et al*, 2017; Planche and Wilcox, 2015; Planche *et al*, 2013). Indeed, at several microbiology and infectious disease conferences in recent years, debates have been held on the virtues of differing diagnostic algorithms (Fang *et al*, 2017). It is important to note that the prevalence of a disease in the study population affects the positive and negative predictive value of a detection assay. The higher the prevalence, the better the assay will perform (Planche *et al*, 2008). It is a weakness of many studies that they use a selected population for study with a prevalence level much higher than that seen in routine clinical practice, so falsely inflating the predictive value of the assays in question. The decision as to which assay to use is compounded by the issue of what to detect; the organism, its toxins or its DNA (Planche *et al*, 2013).

1.13.1 Assays for detection of toxin

Detection of toxin B directly from a diluted faecal sample using a cell cytotoxicity assay is often quoted as the gold standard method for laboratory diagnosis (Planche *et al*, 2013), however this method is not without its limitations. For example, whilst some positive results can be detected within six hours, negative results cannot be confirmed until 48 hours after inoculation of the tissue culture. Additionally, there is no consensus on methodology, including but not limited to which cell line and faecal dilution to use, which may account for the different sensitivities reported for this assay. Indeed, human foreskin fibroblast (HFS) cells have been shown to be more sensitive for detection of toxin B than Chinese hamster ovary (CHO), human lung fibroblasts or Hep-2 cells (Tichota-Lee *et al*, 1987).

Rapid enzyme immunoassays (EIAs) were developed to detect *C. difficile* toxin A directly from a faecal sample and to reduce the time to diagnosis to less than two hours (Lyerly *et al*, 1983). Subsequently these assays were modified to detect both toxins A and B after the discovery of toxin A negative/toxin B positive disease-causing strains (Rupnik M *et al*, 1998;

Borriello *et al*, 1992). Comparison studies of commercially available toxin detection EIAs show huge variation in the performance of these assays with some sensitivities <50% in a low-prevalence setting (Planche *et al*, 2008; Eastwood *et al*, 2009). Alternate views on the need for repeat testing using EIAs has led to further confusion about appropriate CDI diagnostics (Mohan *et al*, 2006; Manabe *et al*, 1995). Department of Health (UK) guidance, based upon one large study in 2009, recommended that diagnosis of CDI should not be based on the sole use of a toxin EIA (Eastwood *et al*, 2009; Wilcox and Eastwood, 2009; Department of Health, 2009) but did not offer an alternative diagnostic strategy.

1.13.2 Assays for detection of C. difficile

Due to the long turn-around time and the technical difficulty of using cell lines to detect toxin, alternative methods have been developed for CDI diagnosis. As people can carry non-toxigenic strains of *C. difficile* in their gut (Shim *et al*, 1998), culture of *C. difficile* from a faecal sample is not diagnostic for CDI. Cytotoxigenic culture aims to solve this problem by identifying patients with pathogenic *C. difficile* isolates in their faeces. Ensuring that this test is only performed on diarrhoeal faecal samples (usually Bristol stool chart types 5-7) should ensure that people with asymptomatic carriage, those in whom *C. difficile* is detected but who have no symptoms (Shim *et al*, 1998), are not falsely diagnosed with CDI. Following standard *C. difficile* culture, the isolates are tested for the ability to produce toxin (Bouza *et al*, 2001); however, this does not indicate the presence of toxin in the original faecal sample, only the ability of the isolate to produce toxin in laboratory conditions. Importantly, the detection of free-toxin, not the detection of an isolate with toxin producing ability has been shown to be associated with mortality (Planche *et al*, 2013). Unfortunately, because of the need for two incubation steps in this method, the turn-around time can be even longer than that for the cell cytotoxicity assay.

Commercial assays with alternative targets for detection of *C. difficile* have been developed, such as a test for glutamate dehydrogenase (GDH), an enzyme associated with the cell surfaces of many bacteria (Anderson *et al*, 1993). The variability seen in the performance of toxin EIAs does not seem to be present with *C. difficile*-specific GDH EIAs (Shetty *et al*, 2001), perhaps suggesting that there is less target heterogeneity *i.e.* GDH has less antigen variability than *C. difficile* toxins. One disadvantage of GDH assays, however, is that they cannot distinguish between toxigenic and non-toxigenic strains, meaning that they have to be used in conjunction with another, toxin-detecting assay to diagnose CDI.

Detection of toxin gene DNA using nucleic acid amplification tests (NAAT) offer an alternative, rapid and potentially high-throughput method for screening faecal samples for toxigenic *C. difficile*. It has been suggested that a PCR assay for toxin genes (Cepheid GeneXpert® *C. diff*, US) is more sensitive for certain PCR ribotypes of *C. difficile* than GDH EIAs (Tenover *et al*, 2010), even though the genes for GDH have been shown to be ubiquitous in *C. difficile* (Carman *et al*, 2012). It should be noted, however, that the number of samples examined in this study was small in total and hence the numbers of each PCR ribotype even smaller (Tenover *et al*, 2010). In addition, NAATs, in similarity with cytotoxigenic culture, cannot detect free toxin within a faecal sample, only the presence of a potentially toxigenic strain; indeed, it may also be detecting dead bacteria rather than living cells or cells in which expression of the gene encoding toxin cannot be expressed. In order to determine if the gene is being expressed, a reverse transcriptase assay would be required, to convert mRNA to DNA, but there is no such current *C. difficile* molecular assay available.

The potential for standalone NAATs to overdiagnose CDI has therefore, been highlighted (Polage *et al*, 2015), as patients with samples that are NAAT-positive/toxin-positive were significantly associated with higher bacterial load, greater antibiotic exposure, inflammation of the gut, presence of diarrhoea (all p <0.001), and longer duration of diarrhoea (p = 0.03), compared with patients that had NAAT-positive/toxin-negative samples (Polage *et al*, 2015). In addition, CDI-attributable mortality was significantly higher in NAAT-positive/toxin-positive patients compared with NAAT-positive/toxin-negative patients (8.4% *vs* 0.6%, p = 0.001). This was confirmed by a small study in 2018 where 30-day mortality was significantly higher in the toxin-positive group compared with the NAAT-positive group (31% *vs* 18%, p = 0.03) (Avni *et al*, 2018). In contrast however, a more recent study demonstrated that there was not a significant difference between CDI-related complications or 30-day mortality between NAAT-positive and toxin-positive patients; although toxin-positivity was significantly associated with recurrence (adjusted OR 1.89, 161-2.23) (Guh *et al*, 2019). It should be noted however that the first study was a prospective study designed and powered to compare NAAT and toxin assay results, while the last study was a retrospective data analysis.

Ensuring accurate diagnosis is important not just for the treatment of patients and for infection prevention, but because often CDI rates are used as a performance measure within healthcare facilities. A study in the US reporting the number of deaths per annum

related to CDI had to add a sensitivity analysis to account for the different diagnostic methods used by the hospitals included within the study; such is the effect of diagnostic methodology (Lessa *et al*, 2015). Indeed, within the US, a diagnostic assay factor is applied to all centrally reported CDI incidence data to allow for meaningful comparison between different healthcare institutions (Lessa *et al*, 2015) The phenomenon of diagnostic methods impacting on reported CDI rates has also been observed within Canada and Europe (Boagty *et al*, 2017; Davies *et al*, 2016b; Davies *et al*, 2017).

To try to reduce the potential for increased detection of false positives when using standalone NAATs, some centres have tried to employ diagnostic stewardship (Goret *et al*, 2018; Yen *et al*, 2018). One recent study demonstrated that moving to standalone NAATs increased compliance with faecal sample rejection policies (Goret *et al*, 2018). There have also been attempts to enhance current faecal sample rejection policies by asking for Infection Prevention review of potential cases before a sample is even sent to the laboratory. A study at one US centre demonstrated that diagnostic stewardship and education reduced the rate of requests for NAAT testing by 2-fold, and therefore, also reduced the reported CDI incidence (Yen *et al*, 2018). Whilst improved testing criteria may seem like a suitable solution, it often relies on clinical suspicion, which has been shown to 'miss' 23% of CDI cases in a large, multi-centre, European study of 482 hospitals (Davies *et al*, 2014). In addition, missed cases were significantly younger than those that were diagnosed at the hospital, suggesting that clinical suspicion is higher in older patients; while increasing age is a known risk factor for CDI (McDonald *et al*, 2006; Minino *et al*, 2011, Lessa *et al*, 2015), it does not mean that younger patients cannot have the infection. Another multi-centre European study demonstrated that patients tested in Italy were significantly older than in the other four countries that were studied (Davies *et al*, 2016), suggesting targeted testing of patents in Italy, and younger cases will be missed. Diagnostic stewardship may therefore exacerbate the situation, by missing potential cases that do not fit the traditional risk factors for CDI, leaving greater opportunity for lack of effective infection prevention precautions and the possibility of complications in undiagnosed patients.

1.13.3 Algorithmic approach

Combining different types of tests into diagnostic algorithms for CDI has been suggested as an alternative to poorly performing stand-alone assays (Crobach *et al*, 2016, McDonald *et*

al, 2018). Assays detecting GDH are often used as the first assay in many of these two- or three-stage algorithms (Crobach *et al*, 2016, McDonald *et al*, 2018). Following a large multi-centre UK study, an optimal algorithm was determined: GDH (or NAAT) followed by toxin detection (by EIA or cell cytotoxicity) (Planche *et al*, 2013). This led to updated guidance from the Department of Health in England in 2012 which was later followed by both Wales and Scotland (Department of Health, 2012). In addition, the ESCMID diagnostic guidelines were updated to reflect these recommendations (Crobach *et al*, 2016), while the latest US guidelines from IDSA/SHEA partially recommended the algorithm, but with allowance for using standalone NAAT with appropriate diagnostic stewardship (McDonald *et al*, 2018). The algorithm divides patients into three groups; those with CDI, those without CDI and those with *C. difficile* but no CDI (as they have no demonstrable toxin in their stool), also termed potential *C. difficile* excretors (PCDEs) (Planche *et al*, 2013). Additionally, this study also confirms the findings described above, that the presence of toxin in a patient's faecal sample correlates with mortality and severity of infection, in contrast to the presence of toxin genes (DNA) or toxigenic strains (Planche *et al*, 2013; Longtin *et al*, 2013).

1.13.4 Novel technologies

The most recent advances in CDI diagnostic methods have been ultrasensitive *C. difficile* toxin assays (Pollock, 2016; Song *et al*, 2015; Banz *et al*, 2018). Although not all of these assays are commercially available yet (one was launched at the European Congress for Clinical Microbiology and Infectious Disease 2019), they offer the potential to detect *C. difficile* toxin levels below the limit of detection of CCNA (Banz *et al*, 2018). These assays also offer the potential to give greater clarity to samples where current diagnostic methods give discrepant results. A recent study using this methodology demonstrated that there were significant differences between median toxin concentrations in faecal samples from CDI cases and asymptomatic carriers, but only when cases/carriers were defined by standard toxin detection and not when defined by NAAT positivity (NAAT cases had significant diarrhoea, NAAT carriers had no diarrhoea and no prior antibiotics) (Pollock *et al*, 2019). Further studies are required using this technology to determine the clinical significance of low-level toxin-positive results.

1.13.5 Additional laboratory assays that could be used to aid CDI diagnosis

Laboratory tests that can highlight the extent of gut inflammation may offer some further ways of stratifying those patients with the most severe disease. Both lactoferrin, and calprotectin, released by polymorphonuclear leucocytes in the gastrointestinal tract, have been investigated for this purpose (Langhorst and Boone, 2012). A case/control study found higher levels of both inflammatory markers in patients with CDI, compared with controls (Barbut *et al*, 2017). In addition, the levels in cases were higher in those patients with demonstrable free toxin in their faecal samples (Barbut *et al*, 2017), highlighting the effect of toxin on the gut mucosa. Calprotectin has also been found to be a marker for severe infection, with an AUROC of 0.821 for severe infection (Kim *et al*, 2017). Another study however, found that raised faecal lactoferrin levels significantly correlated with severity of CDI, while faecal calprotectin levels were raised, but not significantly (Swale *et al*, 2014). Further research into the use of adjunctive tests for CDI diagnosis is required but lactoferrin has been suggested as an adjunct to a test algorithm for CDI (Wren *et al*, 2009).

1.13.6 Clinical context

Although many studies have highlighted the variability in performance of CDI laboratory diagnostic assays, there is a paucity of data on the reasons for these differences. There are also few data on the clinical course of CDI and the optimal time for each of the different diagnostic assays to be used. For instance, depending on the stage of disease a test may 'perform' badly or well as a marker of CDI. For example, if a patient is in recovery phase when tested, is toxin still detectable? What does a GDH-positive/toxin-negative or GDH-positive/culture-negative result mean; *i.e.* the presence of disease or the presence of a non-viable organism with detectable GDH? These potential confounding issues have not been considered when patient samples are tested in evaluations of CDI tests, even though they may have a bearing on performance. Only in large, multicentre studies can these possible effects be minimised and true differences between assays of the same type be highlighted. Indeed, within one large multicentre study variability in assay performance was seen across four different hospital sites (Planche *et al*, 2013). These geographical differences would not be seen in a single-centre study and could possibly skew assay performance data.

1.14 Aims

This study aims to determine if sample, patient and/or bacterium factors could affect the performance of CDI diagnostic assays and to determine the optimal time for each of these assays to be used during the clinical course of CDI, or highlight alternative uses for current diagnostic assays.

1.14.1 Primary objectives

To determine if the PCR ribotype of *C. difficile* strains affects the performance of *C. difficile* diagnostic assays;

to study the proliferation of *C. difficile* within a human gut model in order to elucidate how the different detection targets increase and decrease over the course of the infection;

to study the clinical course of *C. difficile* infection within patients to determine if the results seen *in vitro* can be extrapolated to patients;

to determine the optimal times to use each of the diagnostic assays during the clinical course of *C. difficile* infection.

2. Materials and methods

This section gives details of methods that were commonly used throughout the study. Specific methods, especially where they deviate from the methods listed below, are detailed within each chapter. In addition, methods only used within one chapter are described within the relevant chapter. Please note that wherever faecal samples are tested, they are mixed using a vortex generator (Fisons Scientific Equipment, UK) for 30 seconds to ensure a homogenised sample, before removal of part of that sample for testing.

2.1 Reference methods

2.1.1 Cell-cytotoxicity neutralisation assay

Monolayers of Vero cells (European collection of animal cell cultures), UK) were grown in 96-well flat bottomed microtitre trays (VWR, UK) in 160µl of Dulbecco medium (Invitrogen, UK). Cell monolayers were prepared by the research technicians within the Healthcare Associated Infections (HCAI) research group, and then passed to the researcher. To test the faecal samples for the presence of *C. difficile* toxin(s) they were diluted 1:5 in phosphate-buffered saline, pH 7.5, and mixed with a vortex (Fisons Scientific Equipment, Loughborough, UK) for 30 seconds before being spun in a centrifuge at 16000*g* for 10 minutes. Twenty microliters of diluted sample were added to one well of the microtitre tray, mixed and then 20µl was removed and added to the next well. This pattern was repeated for the two wells on the row below on the microtitre tray, which had been protected by the prior addition of 20µl of *C. sordelli* antitoxin (Prolab Diagnostics, UK). The microtitre trays were incubated for 48 hours in an incubator with 10% CO_2 (Panasonic MCO-5AC-PE, Japan) at 37°C. The cells were examined for rounding after 24 and 48 hours using x40 magnification (Leica DMIL, UK); >50% rounding in only the unprotected cells indicated the presence of *C. difficile* toxin. Where samples appeared contaminated after incubation in the cell-monolayer, the original diluted sample was pushed through a 0.45µm syringe filter (Nalgene CA membrane filter, VWR, UK) using a two mL syringe (Fisher Scientific, UK), and the resultant filtrate was retested using the procedure outlined above. If the cell-cytotoxicity result was still unclear after a second re-test it was recorded as such.

2.1.2 Isolation of C. difficile from faecal samples

One millilitre (or equivalent volume) of faecal samples was added to one millilitre 50:50 v/v ethanol and water to kill vegetative organisms before inoculation onto Brazier's agar (Oxoid, UK) supplemented with 250mg/L cycloserine and 8mg/L cefoxitin (Oxoid, UK) and 2% lysed horse blood (Oxoid, UK). Plates were incubated anaerobically for 48 hours at 37°C in an A95 anaerobic workstation (Don Whitley, UK) then examined for growth typical of *C. difficile* (grey-brown colonies with an irregular edge and horse manure odour). Suspect isolates were checked for green/yellow fluorescence under longwave (365 nm) UV light and latex agglutination for somatic antigen (Microgen Bioproducts Ltd, UK). Confirmation of ID of the organism was based on the criteria in table 2.1.

	C. difficile	*C. bifermentans* *C. sordellii* *C. glycolicum*	*C. innocuum*
Fluorescence under UV at 365 nm	+	-	+
Latex agglutination	+	+	-

Table 2.1 Criteria used to determine the identity of isolates demonstrating typical colony morphology for *C. difficile* on CCEY

2.1.3 Cytotoxigenic culture assay

Isolates confirmed as *C. difficile* were sub-cultured into four ml pre-reduced brain heart infusion (BHI) broth (Oxoid, UK). After anaerobic incubation at 37°C for 48h, one ml of broth supernatant was removed and spun at 16,000*g* in a centrifuge for 10 minutes. Twenty microliters of this BHI broth supernatant was then tested for the presence of toxin using the cell-cytotoxicity assay as described above.

2.1.4 Total viable counts and spore counts

Total viable counts were determined by a variation of the method of Miles and Misra (Miles *et al*, 1938, Levett 1991); samples were diluted 10-fold in pre-reduced peptone water to 10^{-6} with 20µL inoculated onto pre-reduced Brazier's agar supplemented as described in section 2.1.2., in triplicate for each dilution. The agar plates were incubated anaerobically for 48 hours in an A95 anaerobic workstation (Don Whitley, UK) before colonies were counted.

Spore counts were determined after alcohol shock; one ml of the batch culture broth was added to one ml ethanol (50% w/v) and left at room temperature for 1 hour. Ten-fold dilutions were prepared from the alcohol shock fluid and plated out using the Miles and Misra technique as described above.

2.1.5 Storage of isolates at -20°C

For each sample positive for C*. difficile, a C. difficile* colony isolated on Brazier's agar, supplemented as described in section 2.1.2., was harvested using a sterile loop and used to inoculate a Columbia blood agar plate (Oxoid, UK), streaking out for single colonies. Plates were incubated at 37^{o}C in an A95 anaerobic workstation (Don Whitley, UK) for seven days to encourage germination and out-growth of spores. All growth (if pure) was harvested from the plate using a sterile cotton tip swab and inoculated into a cryogenic microtube (Sarstedt Inc, UK) containing one ml nutrient broth with 10% glycerol. Microtubes were stored in Nalgene cryoboxes (Sarstedt Inc, UK) in a -80^{o}C freezer (New Brunswick, Austria).

2.1.6 PCR ribotyping

All the *C. difficile* isolates identified from the study were typed by the *C. difficile* Ribotyping Network of England and Northern Ireland (CDRN) laboratory using PCR ribotyping as described by the CDRN protocol (Stubbs *et al*, 1999). Briefly, DNA was extracted from isolate suspensions in water using the DX kit on the Qiagen Xtractor (Qiagen Ltd, UK) automated DNA extraction platform. DNA was amplified using primers targeted to the 16s-23s rRNA intergenic spacer region designed by Bidet *et al* (1999) on the Verti thermocycler (Life Technologies, UK). Amplified products were either visualised on agarose gels following electrophoresis on the Midi I 128mmx110mm (Thermofisher Scientific Ltd) (used for isolates from the Department of Health study) or fragment lengths were measured on the

3130XL Gene Analyser (Thermofisher Scientific Ltd, UK) sequencer (used for isolates from the PlaciD study). Band patterns from both methods were compared to library reference strains of *C. difficile* using bioNumerics (Applied maths, Belgium) to determine PCR ribotype.

2.2 Commercially available methods

2.2.1 C. diff chek-60™ (Techlab Ltd, USA); a commercial enzyme immunoassay for the detection of C. difficile glutamate dehydrogenase (GDH).

One hundred microliters of faecal samples were diluted in 400µL of kit sample diluent; to ensure there was enough sample volume (including dead volume) these were double the recommended volumes. Samples were mixed using a vortex mixer then spun in a centrifuge at 10,000g for 10 minutes. Sample tubes were loaded onto the DS2 instrument (Dynex Technologies, USA) along with all of the kit consumables; wash buffer (phosphate-buffered saline containing detergent and 0.2% thimerosal), conjugate (a highly specific mouse monoclonal antibody to GDH conjugated to horseradish peroxidase), substrate (tetramethylbenzidine and peroxide), stop solution (0.6 N sulphuric acid), and the required number of pre-coated wells from the microplate kit. The wells were pre-coated with an immobilised polyclonal antibody to glutamate dehydrogenase. The DS2 was pre-programmed to carry out the following steps; 50µL of conjugate and 100µL of diluted sample were added to individual wells, plates were shaken to mix the contents thoroughly whilst they are incubated at 37°C for 20 minutes (this is shorter than the 50 minutes stated in the kit instructions, as the shaking reduces the incubation time; this is recommended by the manufacture), each well was washed with 350µL wash buffer for a total of three times before the addition of 100µL of substrate. After a further 10 minutes of shaking incubation at room temperature, 50µL of stop solution was added to each well. The resultant colour change was read using a dual wavelength (450/620nm) spectrophotometer built into the DS2. On each plate run, a positive and negative control well was added. The test procedure for the controls was as described, with the exception that 100µL of either positive or negative control (included in the kit) were added to the control wells instead of diluted sample. For the results of a microplate run to be accepted, the positive and negative control OD values had to fall within quality control limits; positive control >0.500 and negative control <0.080. If the QC failed, the testing for that sample batch was repeated on the same day. As the kit is designed for diagnosis from patient specimens, there are set

cut-off values stated in the kit insert; an OD ≥0.080 indicates a positive result, with <0.080 indicting a negative result.

2.2.2 Tox AB II™ (Techlab Ltd, USA); a commercial enzyme immunoassay for the detection of C. difficile toxins A and B

The sample buffer for the *TOX AB II™* assay and the *C. diff Chek-60*™ assay are the same, as they are made by the same manufacturer (Techlab Ltd, USA). The initial sample preparation steps are therefore the same and only need to be carried out once, in order to perform both assays. Additionally, the same reagents and test procedure can be used for both assays, with the exception of the microplate wells, the positive control and the conjugate, which are specific to each assay. The microplate wells in the *TOX AB II*™ assay are coated with a goat polyclonal antibody against both toxins (A and B) which has been affinity purified and immobilised onto the wells. The conjugate contains a mixture of a monoclonal mouse toxin A antibody and a polyclonal goat toxin B antibody, both conjugated to horseradish peroxidase. The cut-off values and QC values for the *TOX AB II™* kit are the same as for the GDH kit, described above.

2.2.3 Premier Toxins A & B™ (Meridian Bioscience, Inc. Europe*, UK); a commercial enzyme immunoassay for the detection of C. difficile toxins A and B*

Two hundred microliters of faecal sample were added to 800µL of kit sample diluent; these were double the recommended volumes, to ensure there was enough residual volume, included dead volume, to run the assay on the DS2. These volumes were recommended by the manufacturer (verbal communication). Samples were mixed using a vortex mixer (Fisons Scientific Equipment, UK) then spun in a centrifuge at 2,750g for 5 minutes. Sample tubes were loaded onto the DS2 instrument along with all of the kit consumables; wash buffer (phosphate-buffered saline containing detergent and 0.2% thimerosal), conjugate (polyclonal goat anti-toxin A and anti-toxin B antibodies conjugated to horseradish peroxidase), substrate (tetramethylbenzidine and peroxide), stop solution (1M phosphoric acid), and the required number of pre-coated wells from the microplate kit. The wells were pre-coated with an immobilised mouse monoclonal anti-toxin A antibody and a polyclonal goat anti-toxin B antibody. The DS2 was pre-programmed to carry out the following steps;

50µL of conjugate and 100µL of diluted sample were added to individual wells, plates were shaken to mix the contents thoroughly whilst they were incubated at 37°C for 20 minutes (as with the Techlab assays, this is shorter than the 50 minutes stated in the insert, as the shaking reduces the incubation time, but is also recommended by the manufacturer of this assay). Each well was washed with 350µL wash buffer for a total of three times before the addition of 100µL of substrate. After a further 10 minutes of shaking incubation at room temperature, 50µL of stop solution was added to each well. The resultant colour change was read using a dual wavelength (450/630nm) spectrophotometer built into the DS2. On each plate run, a positive and negative control well was added. The test procedure for the controls was as described, with the exception that 100µL of either positive or negative control (included in the kit) were added to the control wells instead of diluted sample. For the results of a microplate run to be accepted, the positive and negative control OD values had to fall within quality control limits; positive control >0.100 and negative control <0.100. If the QC failed, the testing for that sample batch was repeated on the same day. As the kit is designed for diagnosis from patient specimens, there are set cut-off values stated in the kit insert; an OD ≥0.100 indicates a positive result, with <0.100 indicting a negative result.

2.2.4 GeneXpert®, C.diff (Cepheid UK Ltd., UK); a commercial multi-plex PCR assay for the detection of the tcdB gene, the truncated form of the tcdC gene, and the binary toxin gene from faecal samples

A Copan dual swab (Copan Diagnostics, USA) was used to remove a small amount of faecal material from the sample; this was the only validated swab for use with this assay at the time. The swab was dipped into the faecal sample until half of the swab tip was covered by faecal material (as per manufacturer's instructions); only one swab of the pair within the dual swab was used (as per manufacturer's verbal instructions). The swab was snapped off into the kit sample buffer before the buffer was mixed using a vortex (Fisons Scientific Equipment, UK) for 30 seconds. A plastic pipette was used to transfer the entire volume of the sample buffer into the sample well within the GeneXpert® cartridge. All other test reagents are already pre-prepared within the cartridge. The cartridge lid was snapped shut and the cartridge placed into the GeneXpert® XVI machine (for samples in the Department of Health study) or the GeneXpert® IV machine (for all other analyses) (both Cepheid, USA). The assay has a run time of 47 minutes, and inbuilt internal controls to check both the extraction and amplification stages. Pre-specified algorithms within the machine software

determine if the test is valid (if the internal control has been detected within the correct cycle threshold [CT] value parameters). The algorithm also produces results for detection of *tcdB*, the truncated form of *tcdC*, and the binary toxin gene, based upon both the CT value and the end-point threshold value. Results were recorded as positive or negative, as determined by the algorithm, for each of these genes; the software algorithm can also produce a 'presumptive 027' result, if all three of the targets are detected within the correct parameters. The actual CT values and end point values were also recorded. If a sample result was invalid, the sample was tested once more. The result of the second test was recorded in the study database (even if it was invalid again).

2.2.5 BD Max™ C.diff (Becton Dickinson and Company, Sparks, MD, USA); a commercial multiplex assay for the detection of the tcdB gene from faecal samples

A plastic 10µL loop (Starstedt Inc, UK) was used to remove a 10µL portion of a faecal material from the sample before adding to the sample buffer tube supplied with the kit. A septum cap (included in the kit) was added to the tube before the contents were mixed using a vortex (Fisons Scientific Equipment, UK). One test strip from the kit was used per sample; strips contained lysis tube, extraction tube (magnetic DNA affinity beads, Achromopeptidase and sample processing control in a freeze dried pellet), *C. difficile* master mix (*tcdB* specific primers and probe, sample processing control specific probe in a freeze dried pellet), wash buffer, elution buffer, neutralisation buffer, a waste reservoir and pipette tips. The sample tube and test strip were added to the BD MAX™ system. The assay has a run time of 56 minutes, and has inbuilt controls to check both the extraction and amplification stages. The software within the BD Max™ system contains a pre-specified algorithm to determine in a test is valid, and what the result of a test is; positive (*tcdB* detected), negative (no *tcdB* detected), unresolved (no amplification of internal control, possibly due to sample inhibition), indeterminate (no results due to system failure) or incomplete (run did not complete). If a sample result was unresolved, indeterminate or incomplete, the sample was tested once more. The result of the second test was recorded in the study database (even if it was unresolved again).

2.3 Gut model

The gut model is a temperature-controlled, triple chemostat system linked by weir cascade, representing the pH and nutrient availability of the proximal to distal colon (figure 2.1), using bespoke glassware (Soham Scientific, UK). The system was sparged with oxygen-free nitrogen from a nitrogen generator (Parker Balston, UK) to keep the system anaerobic. The pH of each vessel was monitored using MLO pH meters (Brighton Systems Ltd, UK) and either acid or alkali was added automatically to ensure that each vessel was kept at the correct pH; vessel one pH 5.5 ($^{+/-}$0.2), vessel two pH 6.2 ($^{+/-}$0.2), vessel three pH 6.8 ($^{+/-}$0.2). A 10% w/v pooled faecal slurry was prepared using saline and donated healthy stools (collected under ethical approval from University of Leeds HSLTLM/12/061 and MREC15-070) that were mixed using a 400 paddle blender stomacher (Seward Ltd, UK), before 300mL of the pooled faecal slurry was added to the system to introduce gut microbiota to the vessels. A defined growth medium, as previously described (Freeman *et al*, JAC 2003), was added to the system at a continuous flow rate of 0.015h^{-1} via a masterflex digital (HV-77921-60) peristaltic pump (Cole Palmer Ltd, UK), to mimic bowel transit time. Each vessel was kept at 37°C as it is jacketed by a water filled layer, circulating through a Grant P5 heated waterbath (Grant Instruments Ltd, UK).

The levels of faecal bacteria were monitored by culturing gut model fluid on selective agars (Freeman *et al*, 2003); once populations of bacteria had stabilised the model was classed as reaching 'steady state'. *C. difficile* spores (~10^7 cfu/mL) were added twice, each dose one week apart, and again the bacterial populations in the models were left to stabilise. Clindamycin was instilled into the gut model (33.9 mg/L four times per day, for 7 days) to induce simulated CDI. *C. difficile* total viable counts, spore counts and toxin levels were enumerated daily. A CDI treatment antibiotic was then added to the system, *C. difficile* total viable counts, spore counts and toxin levels were monitored for further defined periods; the specifics of each gut model experiment are given in Chapter 2. The gut models were set-up, maintained and sampled by the technicians and research assistants within the HCAI research group, under the guidance of the PI for that set of models; Kerrie Davies tested gut model fluid from each model using the commercial assays. Permission was granted for the use of excess gut model fluid to be used by Kerrie Davies for this PhD project from the funder of each set of gut models.

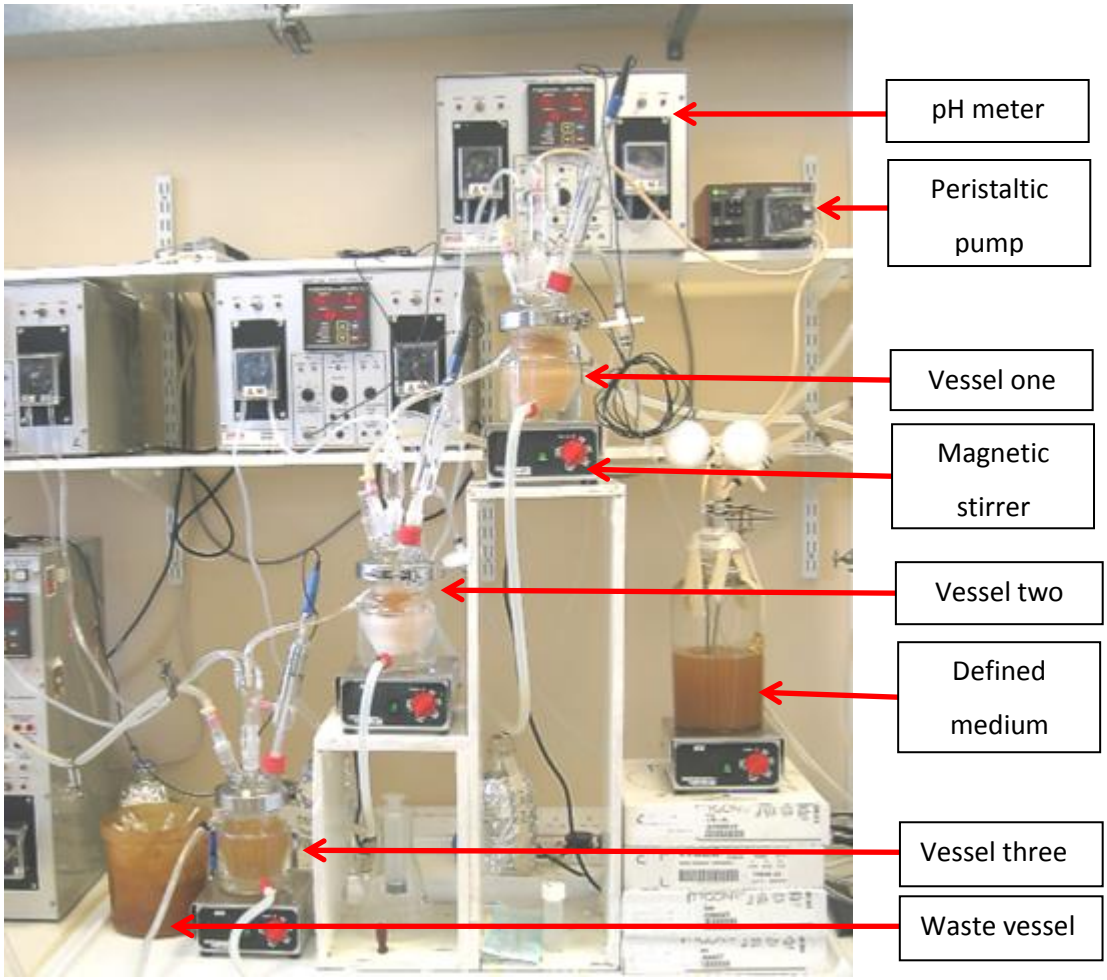


Figure 2.1. Image of the triple-stage chemostat gut model

3. Organism factors affecting detection by commercial assays

3.1 Introduction

Rapid enzyme immunoassays (EIAs) were developed to detect *C. difficile* toxins A and B directly from a faecal sample and to reduce the time to diagnosis to less than two hours (Lyerly *et al*, 1983). Comparison studies of commercially available toxin detection EIAs show huge variation in the performance of these assays with some sensitivities <50% in a low-prevalence setting (Eastwood *et al*, 2009). In addition, commercial assays with alternative targets for detection of *C. difficile* have been developed, such as a test for glutamate dehydrogenase (GDH), an enzyme associated with the cell surfaces of many bacteria (Anderson *et al*, 1993). The variability seen in the performance of toxin EIAs does not seem to be present with *C. difficile*-specific GDH EIAs (Shetty *et al*, 2011), perhaps suggesting that there is less target heterogeneity *i.e.* GDH has less antigen variability than *C. difficile* toxins. One disadvantage of GDH assays, however, is that they cannot distinguish between toxigenic and non-toxigenic strains, meaning that they have to be used in conjunction with another, toxin-detecting assay to diagnose CDI.

Detection of toxin gene DNA using nucleic acid amplification tests (NAAT) offer an alternative, rapid and potentially high-throughput method for screening faecal samples for toxigenic *C. difficile*. It has been suggested that a PCR assay for toxin genes (Cepheid geneXpert® *C. diff*, US) is more sensitive for certain PCR ribotypes of *C. difficile* than GDH EIAs (Tenover *et al*, 2010), even though the genes for GDH have been shown to be ubiquitous in *C. difficile* (Carman *et al*, 2012). It should be noted, however, that the number of samples examined in this study was small in total and the number of each PCR ribotype even smaller (Tenover *et al*, 2010).

Here a previously collected data set, comparing commercial *C. difficile* detection assays, was interrogated to determine if the PCR ribotype of the organism affected detection by these assays. In addition, the production of GDH by the organism was explored using growth curves. Key findings in this chapter are;

- Performance of commercial toxin detection assays varied for different PCR ribotypes, specifically non-027 ribotypes
- Performance of commercial GDH detection assays did not vary for different PCR ribotypes

- GDH appeared to be produced during the exponential phase of growth of the organism

3.2 Methods

3.2.1 Effect of PCR ribotype on EIA performance (GDH and toxins)

3.2.1.1 Analysis of existing data set for evidence of decreased sensitivity for detection of certain PCR ribotypes by commercial enzyme immunoassays from faecal samples

Hypothesis

The commercial enzyme immunoassays for both GDH and toxin detection have decreased sensitivity for certain ribotypes of *C. difficile*.

Methods

Diarrhoeal faecal samples, collected as part of a large, multicentre evaluation of CDI laboratory diagnostics were tested with two reference methods; cell cytotoxicity and cytotoxigenic culture, and by three enzyme immunoassays (EIAs); one for detection of glutamate dehydrogenase (*C. diff CHEK-60*™, Techlab, USA) and two for the detection of toxins A and B (Toxin AB II™, Techlab, USA and Premier Toxins A & B™, Meridian, USA) (Planche *et al*, 2013). Assays and reference methods were performed on the same day by the same evaluator at three sites (one in Leeds and two in London) or two evaluators in one site (Oxford). In total, one evaluator worked at each of the two London sites, two at Leeds, and three at Oxford. Kerrie Davies was one of the evaluators at the Leeds site and scientific coordinator for the entire study. All analysis from these data within this thesis was completed by Kerrie Davies. Ethical approval for the extended use of the study data for this thesis was granted by the NHS REC, approval number 12/EE/0495. The initial study ethical approval number was 10/H0715/34.

Commercial enzyme immunoassays

All of the commercial EIAs were performed on an automated platform (DS2, Dynex Magellan Biosciences, USA) to reduce operator error, following manufacturer's protocols given in the kit insert, with minor changes listed within materials and methods section 2.2. The optical density (OD) of each test was recorded as well as the result defined by the assay (using manufacturer's set cut-offs).

Reference methods

The cell-cytotoxicity neutralisation assay, culture and the cytotoxigenic culture assay were performed as described in materials and methods section 2.1.

PCR ribotyping

The *C. difficile* isolates identified from the study were typed using PCR ribotyping by the *C. difficile* Ribotyping Network of England and Northern Ireland (CDRN) as previously described (Stubbs *et al*, 1999) and as expounded in the methods section 2.1.6.

Analysis

Median optical densities (OD) for different PCR ribotypes of *C. difficile* were compared using Mann-Whitney tests to determine if certain PCR ribotypes produced higher optical density readings than others. The detection rates of the EIAs for different PCR ribotypes were compared using Chi-squared (χ^2) tests, thereby highlighting if particular PCR ribotypes were more likely to be missed (give a negative result) by an assay than other PCR ribotypes. Only the results of toxigenic strains of *C. difficile* were analysed for the two-toxin detection EIAs, whereas all strains were included for the GDH assay analysis as this assay can detect both toxigenic and non-toxigenic strains of *C. difficile*.

3.2.1.2 Investigation of batch cultures for evidence of decreased sensitivity for detection of certain PCR ribotypes by commercial enzyme immunoassays

Hypothesis

The commercial enzyme immunoassays for both GDH and toxin detection have decreased sensitivity for detecting certain ribotypes because different ribotypes produce different amounts of the assay target molecule.

Methods

Different strains of *C. difficile* were inoculated into 200µL BHI to give monocultures of each strain. Broths were incubated anaerobically (A95 workstation, Don Whitley, UK) for 48 hours, to obtain peak levels of GDH and toxin production. After incubation, the broths were serially diluted 10-fold in sterile phosphate-buffered saline (PBS). Each dilution was tested with the commercial assays (as per manufactures' instruction and described in materials and methods section 2.2) substituting the patient specimen with the dilution of broth to be tested. Total viable counts for each broth were determined by serial plating of 20µL

volumes onto CCEY (materials and methods section 2.1.4) and incubating anaerobically for 48 hours before counting colonies. All assays were performed in triplicate.

Isolates tested included representatives of PCR ribotypes 001, 002, 005, 014, 015, 020, 027, 106 (CDRN library strains). These isolates were selected as they were either the most common strains in previous studies (001, 027, 015 and 014) (Davies *et al*, 2014), the most commonly 'missed' strains during the Department of Health study (005, 0202 and 014) (Planche *et al*, 2013), or have been highlighted as strains where there are discrepancies between methods (002, 027 and 106) (Tenover *et al*, 2010).

3.2.1.3 Dilutions after 24 hours growth

Hypothesis

The commercial enzyme immunoassays for both GDH and toxin detection have decreased sensitivity for detecting certain ribotypes because different ribotypes produce different amounts of the assay target molecule at 24 hours

Methods

The previous experiment was repeated for three strains (002, 014, 027), but with only an initial incubation period of 24 hours and using 2-fold dilutions of the final broths instead of 10-fold dilutions.

3.2.1.4 Investigation of the growth curve of *C. difficile* to determine where in the growth cycle GDH detection peaks

Hypothesis

That GDH is produced as the *C. difficile* cells proliferate, allowing detection of GDH early in the growth cycle of the bacterium.

Methods

Growth curve 1

A 0.5 Macfarland suspension of *C. difficile* growth harvested from overnight culture of *C. difficile* on CCEY was made in PBS. The strain used was a clinical strain from a case of CDI in Maine Medical centre, Portland, USA in 2005, kept in research group archive; it is PCR-ribotype 027 and is susceptible to clindamycin (MIC of 0.5mg/L). This strain is used

throughout this thesis for growth curves and gut model experiments. A 200µl volume of the 0.5 McFarland suspension was added to 200mL of pre-reduced BHI broth before anaerobic incubation on a rotary shaker (Compact orbital shaker, Cole Palmer, UK).

The broth was sampled for total viable counts/spore counts/GDH EIA OD/Toxin EIA OD and toxin titre (cell-cytotoxicity neutralisation assay) (materials and methods sections 2.1 and 2.2) at the following time points; 0, 3, 6, 24, 27, 30, 48, 51, 54, 72, 75 and 78 hours post inoculation.

For the GDH and toxin EIAs**,** 100µL of the batch culture broth was added to 400µL EIA buffer (to simulate a patient sample). Samples were then tested as per manufacturer's instructions for patient samples on the DS2 instrument (materials and methods section 2.2). Where samples tested using the enzyme immunoassays reached the maximum OD value, they were diluted (1/10 dilution series) and tested again. The OD value was then calculated from the dilution. See sections 4.2.1.3 and 4.3.1.3 in Chapter 2 for an explanation of the maximum OD threshold of the enzyme immunoassays.

Cytotoxin titres were determined using the cell-cytotoxicity neutralisation assay (CCNA) using the method described previously (materials and methods section 2.1.1) with the exception that 20µL of the batch culture fluid was diluted across the toxin tray by doubling dilutions up to a maximum of six times.

Growth curve 2.

The second growth curve was set up as per the first growth curve with the following exceptions;

1) The initial inoculum was 200µL of a spore preparation of known concentration, to give a final spore concentration of $1.6x10^4$cfu/mL in the 200mL BHI. There will be an initial lag phase before growth begins but this may give some interesting information about whether GDH can be detected at that time or not, as in the gut model there was some 'turnover' of the organism before germination was seen to occur. This was in the gut model, however, and conditions will therefore be different from those seen in batch culture.

2) The batch culture fluid was tested for total viable counts/spore counts/GDH EIA OD/Toxin EIA OD and cytotoxin titre (cell-cytotoxicity) at the following time points; 0, 2, 4, 6, 8, 24, 26, 28, 30, 32, 48, 50, 52, 54, 56, 72, 74, 76, 78 and 80 hours post inoculation.

3.2.2 Effect of the presence of spores and vegetative cells on the diagnosis of CDI when using PCR to detect C. difficile toxin genes

3.2.2.1 Can the commercial *C. difficile* toxin gene PCR assay detect DNA from *C. difficile* spores as well as DNA from vegetative cells?

Hypothesis

That the commercial *C. difficile* toxin gene PCR assay Xpert® C.diff cannot detect DNA from *C. difficile* spores.

Methods

Cleaning spore preparations to remove residual DNA

Five microliters of commercial DNAse I suspension (Sigma Aldrich, UK) and 5µl of DNAse buffer (Sigma Aldrich, UK) were added to 50µL of a spore preparation before it was incubated at room temperature for 15 minutes, as per manufacturer's instructions. After 15 minutes, 5µl of STOP solution (Sigma Aldrich, UK) were added and the spore preparation was incubated at 70°C for a further 10 minutes. The spore preparation was allowed to cool before 50µL was tested with the Cepheid GeneXpert® C. diff PCR assay.

As the PCR assay gave a positive result for the spore preparation after it had been treated appropriately with DNAse, the spore preparation was then diluted using 10-fold serial dilutions and each dilution was treated with DNAse as before. Each dilution was then tested using the Cepheid GeneXpert® C. diff PCR assay.

In addition, to determine if spores are lysed by sonication (the method of DNA extraction used by the Cepheid GeneXpert® C. diff PCR assay), the spore preparation that had been diluted to 1/1000 was sonicated for 10 minutes in a sonicating waterbath (45 kHz, USC100T, VWR Scientific, UK). This preparation was not treated with DNAse before it was tested with the Cepheid GeneXpert® C. diff PCR assay.

3.3 Results

3.3.1 Effect of PCR ribotype on EIA performance (GDH and toxins)

3.3.1.1 Analysis of existing data set for evidence of decreased sensitivity for detection of certain ribotypes by commercial enzyme immunoassays from faecal samples

There were 1295 samples from which *C. difficile* was isolated; 1291 (99.6%) of which had an OD available for *C. DIFF Tox AB II*™, and *C. DIFF CHEK-60*™assays, whilst there were only 961 (74.2%) with an OD result for the Premier™ Tox A/B assay. The three most common PCR ribotypes isolated were 014 (n = 128), 015 (133) & 027 (104). The median OD values for both toxin EIAs were significantly higher for PCR ribotype 027 than for other PCR ribotypes (table 3.1). In addition, the median OD value for PCR ribotype 015 was higher than for other PCR ribotypes when using the *C. diff Tox AB II™* assay, but not for the other toxin EIA (table 3.1). The higher OD values for PCR ribotype 027 may account for the increased sensitivity of the test for this strain, as both toxin EIAs were significantly less likely to 'miss' PCR ribotype 027 than any other PCR ribotype (p = <0.0001). There were significant differences between the OD values for the two toxin assays for PCR ribotypes 027 and 015, with the median ODs being significantly higher with the *C. DIFF Tox AB II*™ assay than the Premier™ Tox A/B assay (027, p = 0.007; 015, p = 0.03) (figure 3.1). It is interesting to note, however, that although there were differences in the median ODs for these PCR ribotypes between the two assays, they failed to detect *C. difficile* toxins in similar numbers of samples containing these isolates. The Premier™ Tox A/B missed 16% of samples containing PCR ribotype 027 and 37% of samples containing PCR ribotype 015 compared with 15% (027) and 38% (015) using the *C. DIFF Tox AB II*™ assay.

The two toxin EIAs most commonly failed to detect toxin in samples containing the same three PCR ribotypes of *C. difficile*, 005, 014 and 020, although they were not statistically more likely to 'miss' these PCR ribotypes than other PCR ribotypes (table 3.2.). Cytotoxigenic culture confirmed that 95% of these isolates were capable of producing toxin, however direct cell-cytotoxicity was only positive in 30% of the isolates tested.

The variation of median OD values across PCR ribotypes seen with toxin EIAs was not present for the *C. DIFF CHEK-60*™ (GDH) assay (table 3.1). Additionally, this assay was not statistically more likely to miss any one PCR ribotype over others. In fact, even the three most commonly missed PCR ribotypes were in very low numbers; 002 (9.9% missed), 015 (6.8% missed) and 027 (6.7% missed).

Assay	Median OD for *C. difficile* PCR ribotype (RBT)								
	RBT 014	Non-014 RBT	Mann Whitney (*p* value)	RBT 015	Non-015 RBT	Mann Whitney (*p* value)	RBT 027	Non-027 RBT	Mann Whitney (*p* value)
Premier™ Tox A/B	0.03	0.02	0.4	0.04	0.02	0.1	1.93	0.02	<0.0001
C. DIFF TOX AB II™	0.11	0.05	0.12	0.38	0.04	<0.0001	3	0.03	<0.0001
C. DIFF CHEK-60™	3	3	0.07	3	3	0.5	3	3	0.06

Table 3.1 The median OD of the three most common PCR ribotypes in the DoH study for each EIA, with comparisons to the median OD for PCR ribotypes other than the one selected

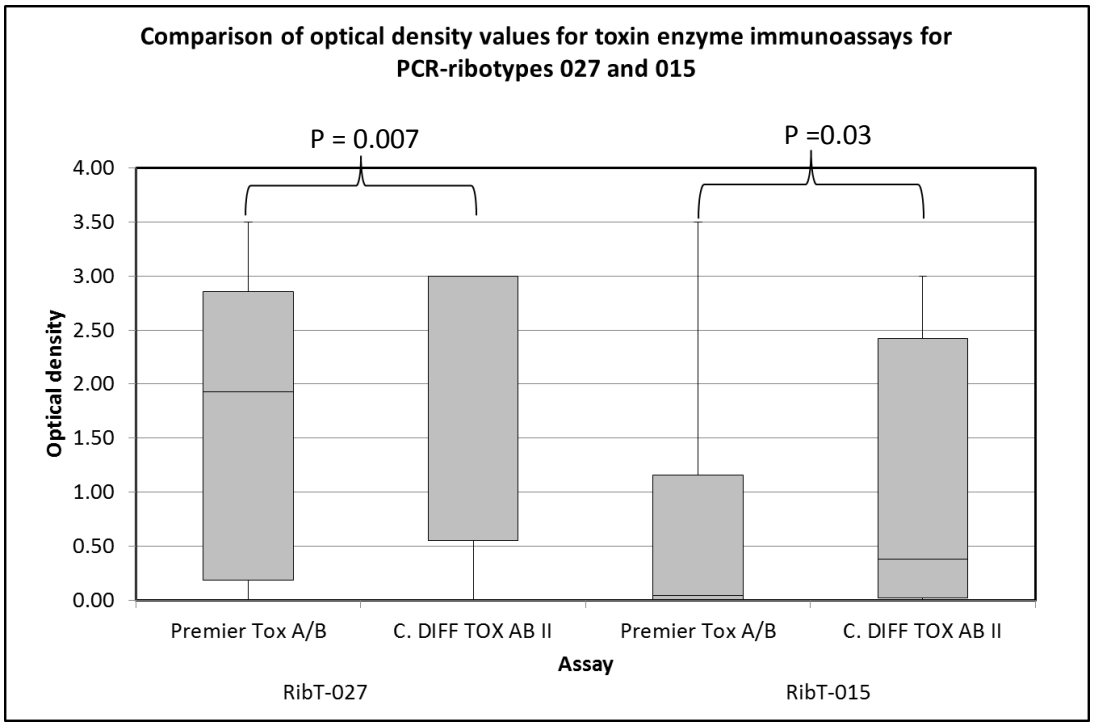


Figure 3.1 Comparison between the OD values of the two toxin EIAs for PCR ribotype 027 and 015

	Percentage of PCR ribotype 005 missed (%)	Significance of missing PCR ribotype 005 over other PCR ribotypes (χ^2 *p* value)	Percentage of PCR ribotype 014 missed (%)	Significance of missing PCR ribotype 014 over other PCR ribotypes (χ^2 *p* value)	Percentage of PCR ribotype 020 missed (%)	Significance of missing PCR ribotype 020 over other PCR ribotypes (χ^2 *p* value)
Premier™ Tox A/B	64.7	0.107	58.3	0.346	55.9	0.806
C. DIFF Tox AB II™	54.4	0.073	46.1	0.571	46.8	0.667

Table 3.2. The three PCR ribotypes most commonly 'missed' by the two toxin EIAs and the significance of the 'missed' result

3.3.1.2 Investigation of batch cultures for evidence of decreased sensitivity for detection of certain PCR ribotypes by commercial enzyme immunoassays

There is little variation in the quantity of GDH detected by the EIA for the different PCR ribotypes, as shown by both the OD values and the titre at which the test first becomes negative (figure 3.2). PCR ribotype 002 does, however, appear to have less GDH detected than the other PCR ribotypes; the dilution at which the test first becomes negative is 10-fold lower than any other PCR ribotype (figure 3.2) and the median OD values are substantially lower than any other PCR ribotype (Figure 3.3). PCR ribotype 014 also appears to have lower median OD values than other PCR ribotypes, although they are not as low as 002.

The same pattern of results is observed with the toxin EIA OD values, where PCR ribotypes 002 and 014 have the lowest median OD values and 002 becomes negative at a dilution 100-folder lower than the other PCR ribotypes (figures 3.2 and 3.4). The dilutions at which 014 becomes negative were inconsistent; there was a 100-fold difference between the three replicates. It should be noted that the cell-cytotoxin titre was also lower for PCR ribotype 002 than other PCR ribotypes.

The drop-off between dilutions is very stark, which may be a product of the 10-fold dilutions used. The experiment was therefore repeated for PCR ribotypes 002, 014 and 027 in the next experiment using 2-fold dilutions to try to close the gap on the steep drop-off.

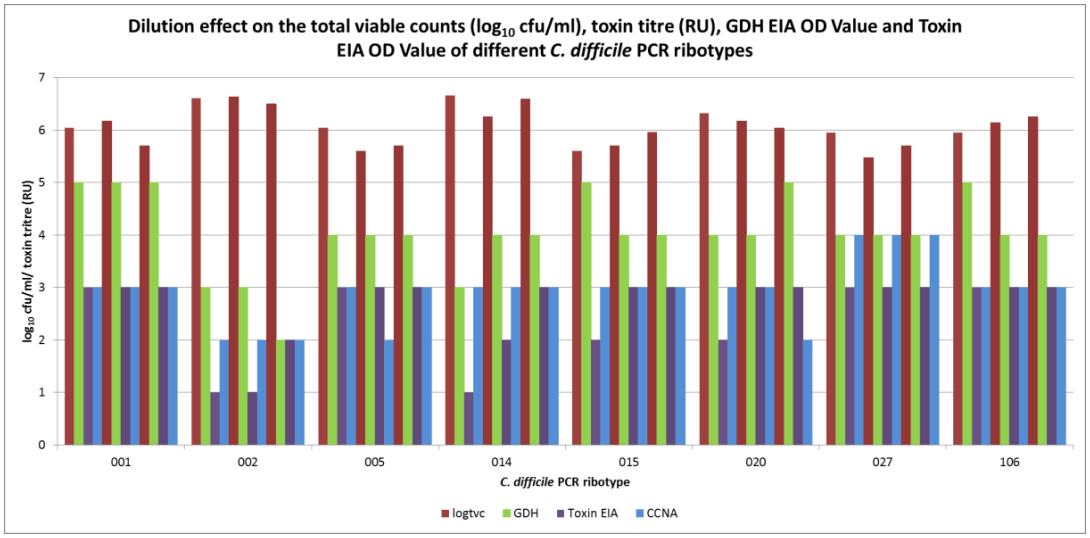


Figure 3.2. Graph showing the first titre for each *C. difficile* PCR ribotype that gave a negative result on the GDH and toxin EIAs, along with the Log_{10} total viable count and cytotoxin titre for the starting broth

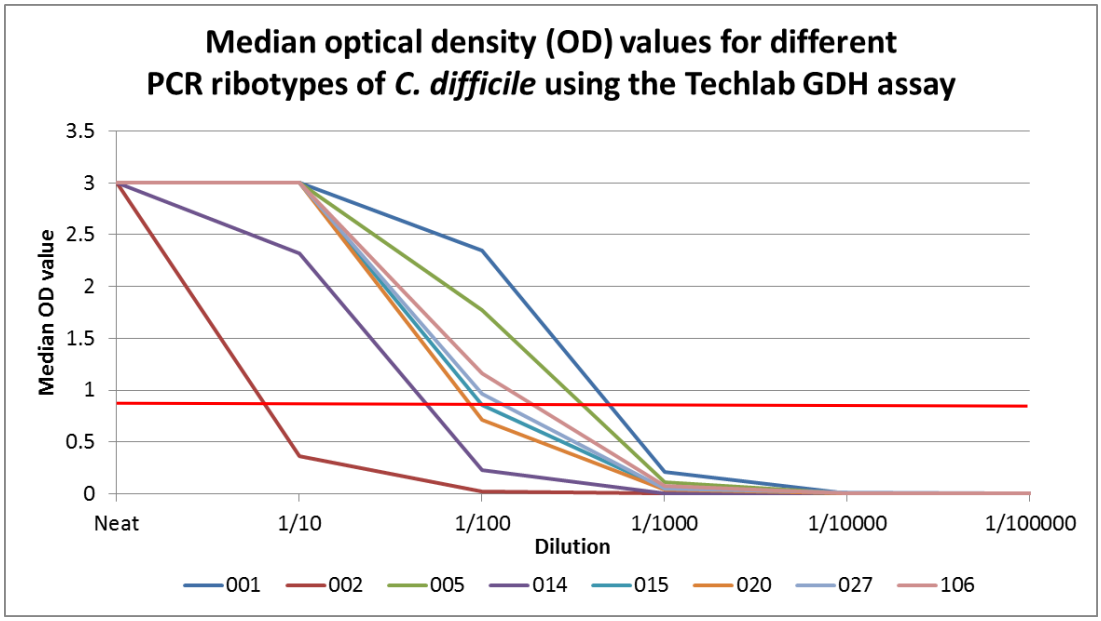


Figure 3.3. The decreasing median OD values of the broths for each of the different *C. difficile* PCR ribotypes, when tested using the GDH EIA (*C. DIFF CHEK-60*™). Red line indicates the diagnostic cut-off value for a positive sample (as defined by the kit insert)

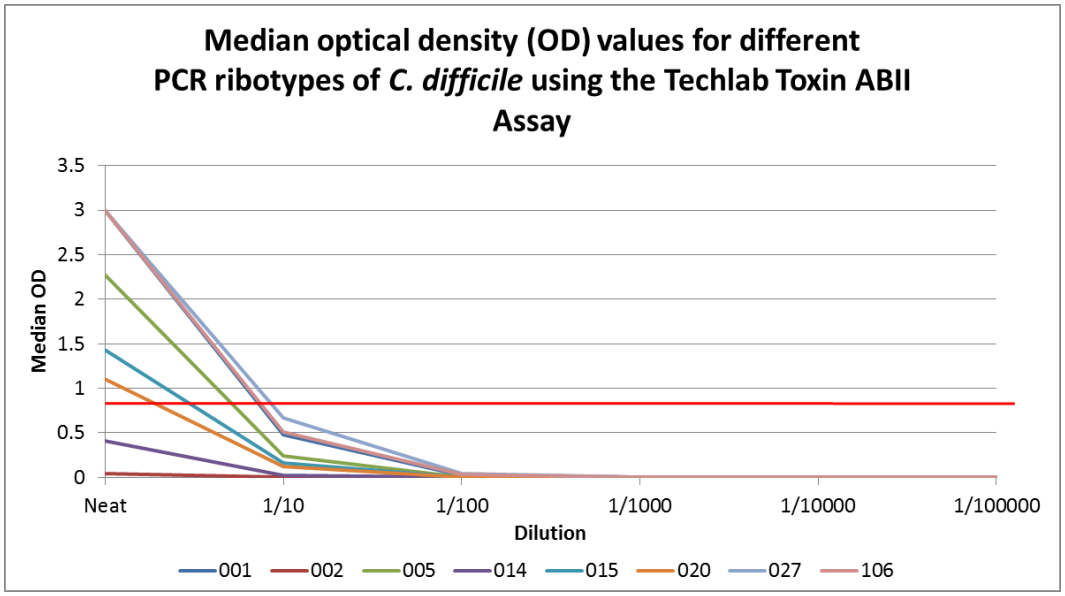


Figure 3.4. The decreasing median OD values of the broths for each of the different *C. difficile* PCR ribotypes, when tested using the toxin EIA (*C. DIFF Tox AB II*™). Red line indicates the diagnostic cut-off value for a positive sample (as defined by the kit insert)

3.3.1.3 Dilutions after 24 hours incubation.

There was less variation between the PCR ribotypes for the level of GDH and toxin detected by the enzyme immunoassays than seen in the previous experiment (figure 3.5). Previously, the amount of GDH detected for ribotypes 002 and 014 was much lower than that detected for ribotype 027. In this experiment, however, there is little difference (figure 3.6). The same pattern is true for the level of toxin detected; previously there were higher levels for isolates of ribotype 027 (figure 3.7). Interestingly, although the level of toxin detected by the EIA appears to be similar between the three ribotypes, there is a large difference between the toxin titres when measured using the CCNA, with isolates of ribotype 027 having a titre of 12 compared with 7 or 9 for isolates of ribotypes 002 and 014 respectively. The logTVCs were lower (by about one $\log_{10}$) than the previous experiment, they were however, reasonably consistent across the ribotypes with a standard deviation of $\log_{10}$ TVC = 0.236. Interestingly, there were no spores detected for isolates of ribotype 002. It should be noted that the cultures were only grown for 24 hours this time, compared with 48 hours in the previous experiment.

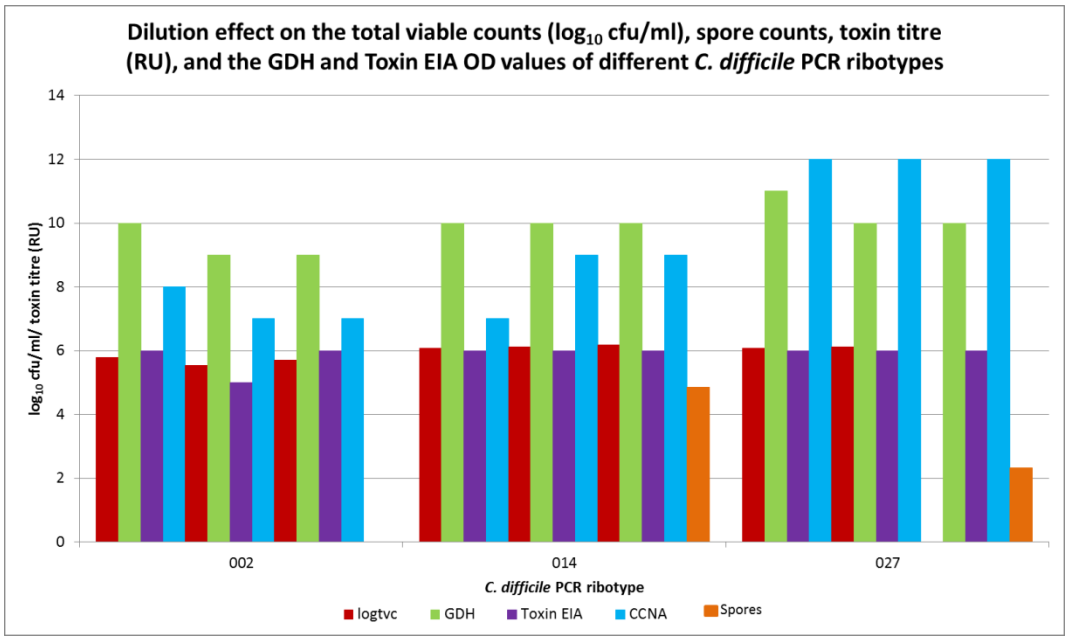


Figure 3.5 Graph showing the first titre for each *C. difficile* PCR ribotype that gave a negative result on the GDH and toxin EIAs, along with the log10 total viable count and cytotoxin titre for the starting broth

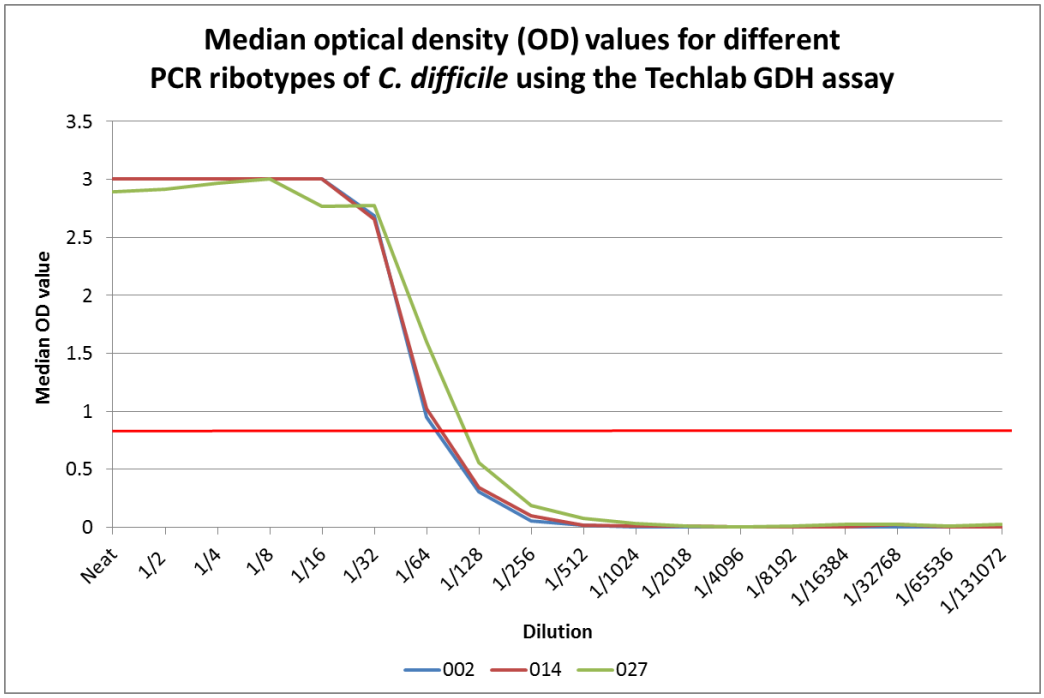


Figure 3.6. Graph showing the decreasing median OD values on the GDH EIA assay (*C. DIFF CHEK-60*™) for each of the different *C. difficile* PCR ribotypes. Red line indicates the cut-off value for a positive sample

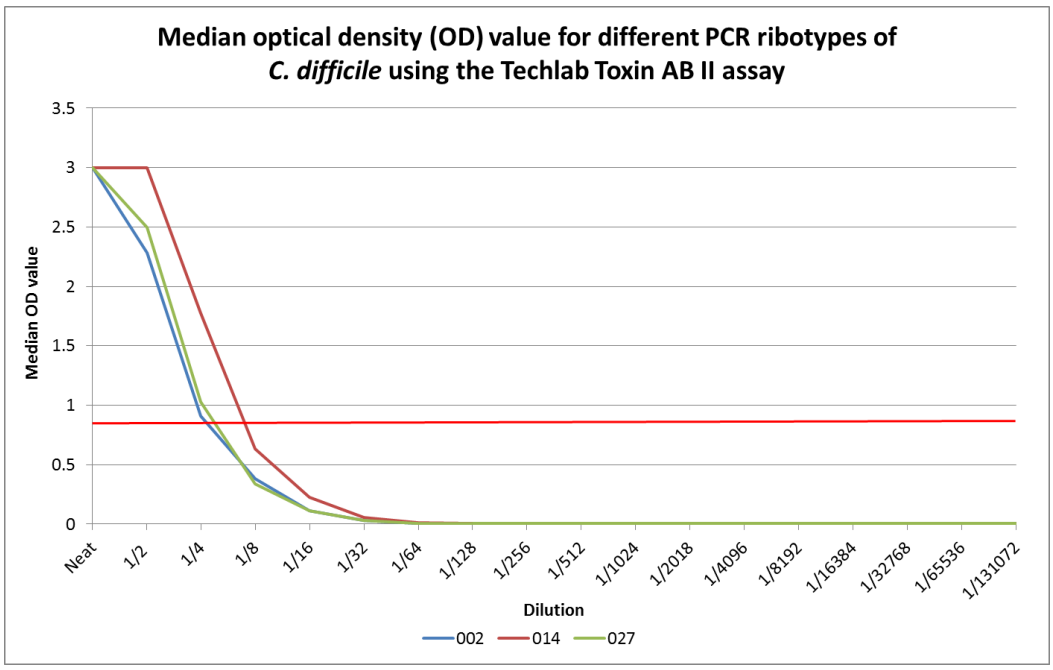


Figure 3.7. Graph showing the decreasing median OD values on the Toxin AB II EIA assay (*C. DIFF Tox AB II*™) for each of the different *C. difficile* PCR ribotypes. Red line indicates the cut-off value for a positive sample

3.3.1.4 Investigation of the growth curve of *C. difficile* to determine where in the growth cycle GDH detection peaks

First growth curve

The growth curve (figure 3.8) shows the initial logarithmic phase of growth within the first 24 hours. After this point, growth plateaus as the organism enters stationary phase. The GDH curve mirrors that of the total viable counts, with the biggest increase in GDH detection between 6 and 24 hours, although it does appear to lag slightly behind the growth curve (as measured by TVCs).

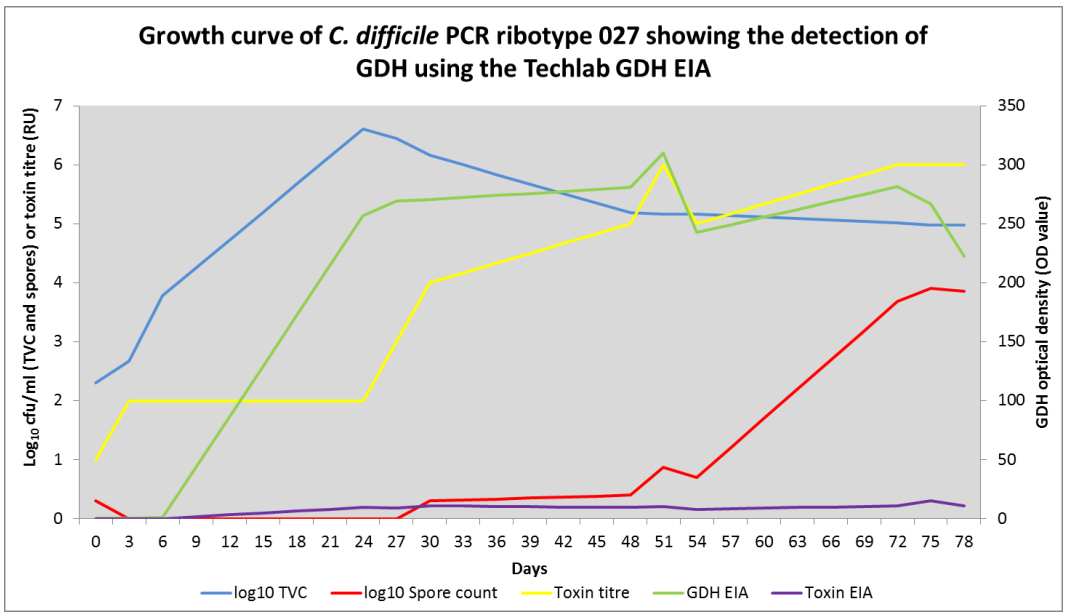


Figure 3.8. Growth curve showing growth of *C. difficile* as measured by total viable counts, spore counts, GDH production and toxin titre; starting inoculum was viable cells

Second growth curve

The growth curve of PCR ribotype 027 with a starting inoculum of spores shows a lag period before the exponential growth phase (figure 3.9). The vessel was not sampled between 8 and 24 hours so the line between these points is extrapolated. GDH was detected before germination was detected using TVCs.

It should be noted that the amount of toxin produced in this experiment, as measured by both toxin titres (CCNA) and toxin EIA, is almost twice as high as the previous growth curve. Nevertheless, the log_{10} viable counts are about the same for both experiments.

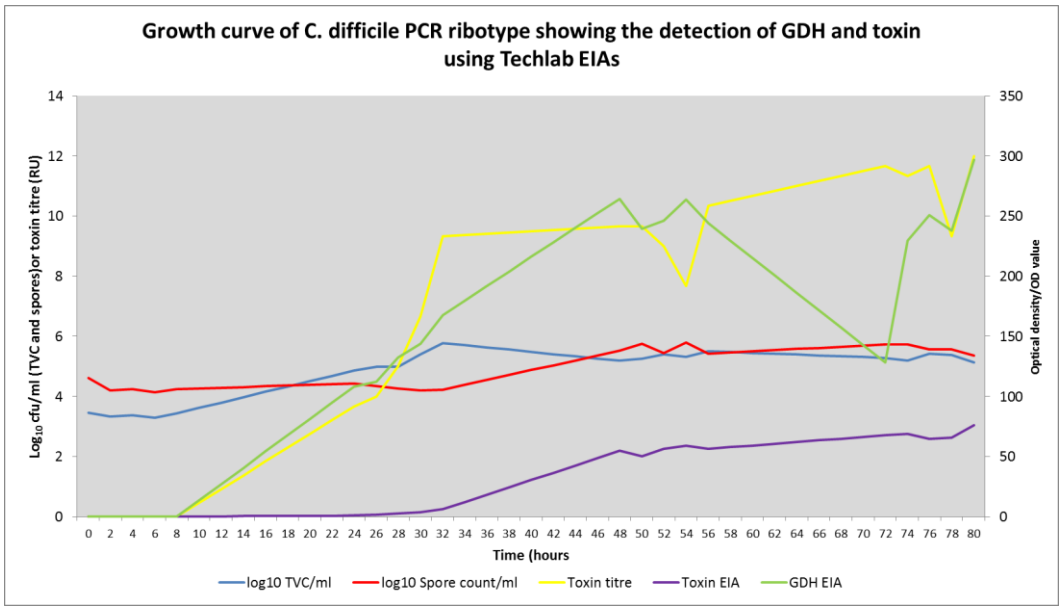


Figure 3.9 Extrapolated growth curve showing growth of *C. difficile* as measured by total viable counts, spore counts, GDH production and toxin titre; starting inoculum was spore preparation

3.3.2 Effect of spores and vegetative cells on the diagnosis of CDI by PCR for C. difficile toxin genes

3.3.2.1 Can the commercial *C. difficile* toxin gene PCR assay detect DNA from *C. difficile* spores as well as DNA from vegetative cells?

Cleaning spore preparations to remove residual DNA

The Cepheid geneXpert® C. diff PCR assay gave a positive result for the spore preparation after treatment with DNAse. The CT values for all three targets in the assay were comparable between the two samples (table 3.3).

	CT value at which the assay became positive for each target		
	tcdB	*cdt*	*tcdC*
DNAse	19.9	19.1	20.7
no DNAse	18.8	18.1	19.4

Table 3.3 The CT values at which the Cepheid assay became positive for different dilutions of a *C. difficile* spore preparation after treatment with DNAse. The label *tcdB* indicates detection of toxin B gene, *tcdC* indicates detection of the deletion in the *tcdC* gene associated with PCR ribotype 027, *cdt* indicates detection of binary toxin gene.

To allow for the possibility that there was too much starting material for the DNAse to work appropriately, the spore preparation was diluted and then each diluted sample was treated with DNAse, as per the kit instructions. Diluting the spore preparation before treatment with DNAse does not, however, appear to have affected the detection of DNA within the sample, as all four dilutions were still positive by the assay (figure 3.10), with roughly a four CT increase between dilutions (table 3.4 and figure 3.10).

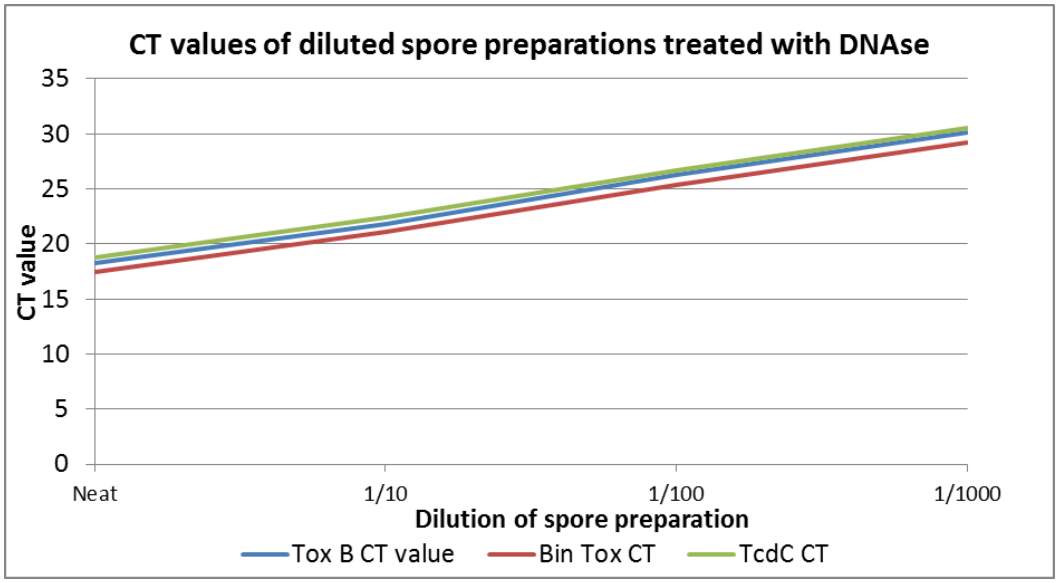


Figure 3.10 Graph showing the CT values at which the Cepheid assay became positive for different dilutions of a *C. difficile* spore preparation after treatment with DNAse

	CT value at which the assay became positive for each target		
Dilution	*tcdB*	*cdt*	*tcdC*
Neat	18.3	17.4	18.8
1/10	21.8	21.1	22.4
1/100	26.3	25.4	26.7
1/1000	30.1	29.2	30.5

Table 3.4. The CT values at which the Cepheid assay became positive for different dilutions of a *C. difficile* spore preparation after treatment with DNAse. The label *tcdB* indicates detection of toxin B gene, *tcdC* indicates detection of the deletion in the *tcdC* gene associated with PCR ribotype 027, *cdt* indicates detection of binary toxin gene.

After the spore preparation was sonicated, the CT value increased for all three targets, suggesting that the amount of target was lower in these samples (table 3.5).

	CT value at which the assay became positive for each target		
Dilution	*tcdB*	*cdt*	*tcdC*
1/1000 DNAse with no sonication	30.1	29.2	30.5
1/1000 DNAse with sonication	34.1	33.4	34.6

Table 3.5. The CT values at which the Cepheid assay became positive for different dilutions of a *C. difficile* spore preparation after treatment with DNAse, with and without sonication. The label *tcdB* indicates detection of toxin B gene, *tcdC* indicates detection of the deletion in the *tcdC* gene associated with PCR ribotype 027, *cdt* indicates detection of binary toxin gene.

3.4 Discussion

The issue of sub-optimal performance of CDI laboratory diagnostics is complex. In contrast to a previous study (Tenover *et al*, 2010), the toxin and GDH EIAs were not found to be less sensitive for specific PCR ribotypes. Indeed, they were not statistically more likely to 'miss' any one PCR ribotype over another, indicating that there are additional factors affecting the performance of EIAs other than which PCR ribotype is in the sample. The two toxin EIAs were, however, statistically less likely to give a negative result for a sample containing PCR ribotype 027 and both assays had statistically higher median OD values (table 3.1.) for samples containing this PCR ribotype than PCR ribotypes other than 027 (figure 3.1.). The reported increase in toxin yield for this strain (Warney *et al*, Lancet 2005; Freeman *et al*, 2007) may partially explain this result, as it is perhaps of note that the assays were developed in the USA against a background of high incidence of PCR ribotype 027. It is possible, therefore, that the assay cut-off values were optimised to detect toxin in samples containing this PCR ribotype. In addition, lack of sensitivity for strains other than 027 could also be related to the specificity (avidity/affinity) of the antibodies used in the assays. Whilst both assays use polyclonal antibodies for toxin B, the antibody for toxin A is monoclonal, which potentially may reduce the detection of the numerous toxinotypes (n = 31) so far discovered (Toxinotype website database, 2019)

The variability in performance of the two toxin EIAs previously reported is supported by this study (Eastwood *et al*, 2009; Planche *et al,* 2008, Planche *et al* 2013) as they had significantly different median ODs for PCR ribotypes 027 and 015, two of the three most common PCR ribotypes found in this study (table 3.2). Although these assays are not truly quantitative, theoretically, increasing ODs correlate with increasing levels of antigen (in this case toxin) detected. It would seem, therefore, that one of the assays, the Premier™ Tox A/B, is not able to detect as much toxin as the other assay, as shown by the lower OD values for these PCR ribotypes. This hypothesis does, however, rely on the assumption that the scale of ODs between the two assays is comparable, which is yet to be demonstrated. The fact that 30% of the samples containing a toxigenic isolate of *C. difficile* that were negative by the toxin EIAs were positive by the cell-cytotoxicity assay supports the assumption that the EIAs are 'missing' true toxin positive samples. Cell cytotoxicity has been shown to be more sensitive than both the toxin detection EIAs (Eastwood *et al*, 2009), and so could be detecting low levels of toxin in the other 30% of samples. An alternative explanation for the 'missed' detection of positives by the toxin EIAs is that these patients could be colonised by toxigenic strains of *C. difficile*, but that these are not currently producing toxin; designated potential *C. difficile* excretors (PCDE) in the UK Department of Health guidelines (Planche *et al*, 2013; Department of Health, 2012).

The contrast between previous results (Tenover *et al*, 2010) and those seen in this study could be explained by the increased number of samples in this study. There were four times as many as those studied previously; giving this study greater power to elucidate differences between assays and PCR ribotypes (Tenover *et al*, 2010). The previous study was not able to show differences between the two toxin EIAs as, due to low numbers of samples, the results of samples tested on either assay were pooled. Indeed, no one sample was tested using both assays in that study (Tenover *et al*, 2010).

In contrast to the toxin EIAs, the performance of the GDH EIA does not seem to be adversely affected by PCR ribotype, as there were no significant differences between OD values for any one PCR ribotype over another. This result conflicts with the work from a previous study that showed that the GDH EIA was less sensitive for PCR ribotypes other than 027 (Tenover *et al*, 2010), although as already discussed that was on a much smaller sample size than the current study. To elucidate fully what a GDH positive/toxin negative (PCDE) result means, it is necessary to understand the course of *C. difficile* infection, which

can be achieved by collecting sequential samples either from patients or an *in vitro* CDI model.

To investigate this phenomenon further the amount of both GDH and toxin, as detected by the commercial EIAs was investigated for different PCR ribotypes of *C. difficile* using batch culture. Although there was little difference between the median GDH values for most PCR ribotypes there was both a lower median GDH and toxin OD value for PCR ribotype 002 (figures 3.3 and 3.4). In addition, the cytotoxin titre was lower for PCR ribotype 002, which suggests that there was a smaller amount of toxin produced by this PCR ribotype, rather than that the toxin assay has less affinity for this PCR ribotype. Differences between PCR ribotype 002 and other PCR ribotypes have been demonstrated previously, although in those studies PCR ribotype 002 had increased sporulation (Cheng *et al*, 2011) and comparable toxin titre to those of hypervirulent strains (Baines *et al*, 2015). The total viable counts for all the strains were fairly consistent (standard deviation of $\log_{10}$ TVC = 0.359), and, most notably, the TVCs for the strains with the lowest GDH and toxin ODs were the highest of all the strains. This suggests that the lower OD values are not because there was less growth of these strains, but that they produced less toxin and less GDH than other strains. When only incubated for 24 hours, these differences were not as clear, perhaps as peak levels are only reached after 48 hours (as shown in the growth curve experiments).

GDH appears to be produced by *C. difficile* during the exponential growth phase of the organism (figures 3.8 and 3.9), which has not previously been demonstrated in the literature. Once the organism begins to slow vegetative growth and produce spores, the level of GDH begins to decline (figure 3.8). From one experiment it would appear that GDH might be detected before germination is detected using TVCs, however, this could be an artefact from the way the graph has been constructed (figure 3.9). The amount of toxin produced in the growth curve experiment that was started with a spore preparation, was almost twice as high, as measured by both toxin titres (CCNA) and toxin EIA, as the previous growth curve. It is possible that this could be due to the way the growth was started; the first was started from viable growth rather than from spores. The reason for this is unclear; although perhaps if the growth starts from spores it induces greater toxin production in the cells? It should be noted that the $\log_{10}$ viable counts are about the same for both experiments, suggesting that the increased toxin production was not due to the amount of growth but some other factor. Batch culture is not the ideal method to investigate the growth of *C. difficile*, as nutrient and pH limitations do not truly reflect the growth

conditions of the organism *in vivo*. In addition, the metabolites produced are neither washed out of the system nor denatured, and so their concentrations could therefore be artificially increased. The *in vitro* gut model system provides the ability to model the growth of *C. difficile* in a more clinically reflective manner (Freeman *et al*, 2003), and experiments in chapter 2 will use this methodology.

Finally, as it proved difficult to get a totally 'clean' spore prep, with no residual DNA from broken down vegetative cells, it is hard to be certain that the Cepheid GeneXpert® C. diff PCR assay cannot detect DNA from spores as well as from vegetative cells. The addition of DNAse to the samples did not affect the CT values for neat spore preparations or diluted preparations (table 3.3, figure 3.10). For diluted samples the CT values produced a straight line (figure 3.10), as would be expected for a 10-fold dilution series, with ~3.3 CT increase per 10-fold dilution (Thermofisher, 2019) (table 3.4), so, again, there was no impact of the DNAse. After the spore preparation was sonicated, the CT value increased for all three targets of the PCR assay, suggesting that the amount of starting material had gone down, perhaps as DNA was destroyed by shear forces (table 3.5). If sonication (the method used by the Cepheid assay to release DNA from faecal samples) released DNA from spores that could then be detected by the assay, the CT values should have decreased; they did not change. An alternative method would be to determine if the CT values for the PCR assay decrease (become positive earlier) as spores germinate (Chilton *et al*, PLOS One 2016); it would be possible to follow spore germination using phase contrast microscopy. If the PCR assay is not detecting the spores themselves, the CT value of the assay should decrease (become positive earlier) as spores germinate. There was not scope within this study to pursue this further but this is an important point to consider, as detection of spores by the PCR assay would further cloud the interpretation of this assay when used for the diagnosis of CDI.

4. Detection of GDH in an *in vitro* gut model of CDI using a commercial assay

4.1 Introduction

Continuous culture systems, rather than batch culture, show that PCR ribotype 027 produces toxin for longer than does PCR ribotype 001 but not at a higher level (Freeman *et al*, 2007). The high levels seen in the batch culture model may therefore be due to an accumulation of toxin and potentially do not give a true representation of toxin production by this strain. To overcome the limitations of batch culture experiments, a validated continuous culture system was used as a human gut model in this project. This model allows investigation of the interplay between *C. difficile,* normal bowel microbiota and antimicrobials (Freeman *et al*, 2003). From a clinical point of view, it is important to understand the status of the disease of the patient, to ensure they receive the correct treatment and that the appropriate infection control precautions are used (Bigardi *et al*, I 2013). This requires accurate diagnostics. Although many studies have highlighted the variability in performance of CDI laboratory diagnostic assays (see section 1.13 of introduction), there is a paucity of data on the reasons for these differences. There are also few data on the clinical course of CDI and the optimal time for each of the different diagnostic assays to be used. For instance, depending on the stage of disease a test may perform 'badly' or 'well' as an assay for CDI. For example, if a patient is in the recovery phase when tested, is toxin still detectable? What does a GDH positive/toxin negative or GDH positive/culture negative result mean; *i.e.* the presence of disease or the presence of a non-viable organism with detectable GDH?

Here, the *in vitro* gut model was used to examine the rise and fall in GDH production, compared with total viable and spore counts of *C. difficile*, against a background of colonic microbiota. The key findings from this chapter are;

- GDH was produced during the exponential phase of growth and the amount detected increased and decreased in phase with total viable counts of the organism
- GDH could be detected from the system when total viable counts had either begun to be detected, or, had gone below the limit of detection
- The amount of GDH produced was partly due to the bioload of *C. difficile* within the system

4.2 Methods

4.2.1 Methods for measuring C. difficile glutamate dehydrogenase in an in vitro gut model

4.2.1.1 Measuring *C. difficile* glutamate dehydrogenase in an *in vitro* gut model by both enzyme immune assay and polymerase chain reaction assay.

Hypothesis

The amount of GDH produced by *C. difficile* over the course of a simulated *C. difficile* infection will vary in line with the proliferation of *C. difficile* as measured by culture.

Methods

Gut model

C. difficile GDH was measured from fluid collected daily from an *in vitro* gut model of CDI, set up as previously described (Freeman *et al*, 2003) and as described in methods section 2.3. Briefly, the model consists of a three-stage continuous weir cascade culture system, pre-loaded with a human faecal emulsion. The faecal bacteria were allowed to proliferate and population levels were stabilised over a period of about two weeks. *C. difficile* spores (~10^7 cfu/mL) were added twice, each dose one week apart, and again the models were left to equilibrate. Clindamycin was instilled into the gut model (33.9 mg/L QDS for seven days) to induce simulated CDI. *C. difficile* total viable count, spore count and toxin level in each vessel were enumerated daily. A CDI treatment antibiotic was then added to the system, *C. difficile* total viable count, spore count and toxin level was monitored daily for a further three weeks. Two models were run in parallel; denoted 'A' and 'B'. The gut models were set up, maintained and sampled by the technicians within the HCAI Research group, under the guidance of the PI for that set of models. Kerrie Davies tested daily samples using the commercial assays and performed the analysis discussed in this thesis. Permission was granted for the use of excess gut model fluid to be used for this PhD project from the funder of the set of gut models.

Glutamate dehydrogenase enzyme immunoassay

A commercial automated GDH EIA, *C. DIFF CHEK-60*™, was used to measure the level of GDH within the model daily. Once samples had been collected from the model, they were frozen at -20°C immediately; they were then defrosted once before testing with the

enzyme immunoassays with the remainder stored at 4°C until all testing had been completed. The samples from this set of gut models were tested after four months in storage, due to maternity leave. The assay was performed according to manufacturer's modified instructions for use on the automated DS2 platform (Dynex Magellan Biosciences, USA) (see materials and methods section 2.2). One hundred microliters of gut model samples were diluted in 400µL of kit sample diluent; to ensure there was enough sample volume (including dead volume) this was double the recommended volumes, and was recommended by the manufacturer, as described in the materials and methods section.

Glutamate dehydrogenase gene (gluD) PCR assay

A polymerase chain reaction (PCR) assay for the GDH gene *gluD* was also performed on the gut model fluid samples. DNA was extracted from samples that were diluted 1/10 in STAR buffer (Roche, Germany) containing 1/10 chloroform (v/v), on the QiaXtractor using the DX kit (Qiagen Ltd, UK) after the addition of an internal control (*Yersinia ruckerii*). Primers and probes for *gluD* and yersi (internal control) (table 4.1.) (Berry *et al*, 2017; Davies *et al*, 2015) were added to Brilliant QPCR multiplex master mix (Agilent, UK) along with template DNA. Amplification was performed on a Stratagene MX3000P (Agilent, UK) using the following thermocycling conditions; strand separation at 95°C for 10 minutes, followed by 45 cycles comprising strand separation at 95°C for 30 seconds, primer annealing at 60°C for 30 seconds and replication at 72°C for 30 seconds, with no final extension step. Samples without a positive internal control result were re-extracted and PCR repeated using the new template DNA.

Oligo Name	Sequence	5' modification	3' modification
Yersi F1	GGAGGAAGGGTTAAGTGTTA		
Yersi R1	GAGTTAGCCGGTGCTTCTT		
Yersi P1	GCGAGTAACGTCAATGTTCAGTGC	Cy5	BHQ2
GluD F3	GTCTTGGATGGTTGATGAGTAC		
GluD R2	TTCCTAATTTAGCAGCAGCTTC		
GluD P1	AAGCCAGTTGAATTTGGTGG	FAM	BHQ1

Table 4.1 Sequence of primers and probes used in the *gluD* PCR assay (Berry *et al*, 2017; Davies *et al*, 2015)

4.2.1.2 Measuring *C. difficile* glutamate dehydrogenase in *in vitro* gut models A and B using a commercial enzyme immunoassay

Hypothesis

The amount of GDH produced by *C. difficile* over the course of a simulated CDI infection will vary in line with the proliferation of *C. difficile* as measured by culture.

Methods

Gut model

C. difficile GDH was measured from fluid collected daily from an *in vitro* gut model of CDI, set up as previously described (Freeman *et al*, 2003). Briefly, the models were used to compare standard and extended vancomycin treatment for CDI. The faecal bacteria were allowed to proliferate and population levels to stabilise over a period of about two weeks. *C. difficile* spores (~10^7 cfu/mL) were added and again the model is left to equilibrate. Clindamycin was instilled into the gut model (33.9 mg/L QDS) to induce simulated CDI. *C. difficile* total viable count, spore count and toxin level were enumerated daily. A CDI treatment antibiotic was then added to the system, *C. difficile* total viable count, spore count and toxin levels were monitored for a further three weeks. Two models were run in parallel; denoted 'A' and 'B'. The gut models were set up, maintained and sampled by the technicians within the HCAI Research group, under the guidance of the PI for that set of models. Kerrie Davies tested daily samples using the commercial assays and performed the

analysis discussed in this thesis. Permission was granted for the use of excess gut model fluid to be used for this PhD project from the funder of the set of gut models.

GDH enzyme immunoassay

A commercial automated GDH EIA, *C. DIFF CHEK-60*™, was used to measure the level of GDH within the model daily. Once samples had been collected from the model they were frozen at -20ºC immediately; they were then defrosted once before testing with the enzyme immunoassays with the remainder stored at 4°C until all testing had been completed. The samples from this set of gut models were tested after two years in storage, due to the maternity leave and other work commitments of Kerrie Davies. The assay was performed as described above. Assay positivity (as defined by the kit) and optical density (OD) values were compared with the pattern of *C. difficile* proliferation and toxin production.

4.2.1.3 Investigating the quantitative potential of the commercial glutamate dehydrogenase enzyme immunoassay

Hypothesis

The commercial glutamate dehydrogenase enzyme immunoassay is quantitative and can be diluted to give a result over the current maximum threshold optical density value.

Methods

A colony of *C. difficile* was inoculated into 20mL Brain Heart Infusion (BHI) broth and incubated overnight at 37°C in an A95 anaerobic workstation (Don Whitley, UK). After incubation, quadruplicate 10-fold dilutions of BHI were made in phosphate-buffered saline (PBS). Each dilution was added to the Chek-60 kit diluent (100µL in 400uL diluent) before the GDH assay was performed using the DS2 platform as described above. The OD value for each dilution was recorded and $\log_{10}$ transformed.

Twenty microliters of each dilution were spread plated, in triplicate, onto cefoxitin, cycloserine, egg yolk agar (CCEY) and incubated at 37°C in an A95 anaerobic workstation (Don Whitley, UK) for 24 hours. The number of colonies was counted on the dilution that gave the most easily readable colony count. The number of colonies was multiplied by the dilution factor to determine the colony count per mL and $\log_{10}$ transformed.

Log_{10} cfu/mL and log_{10} OD values were plotted for the OD values that fell below the maximum threshold of the assay ($\geq$3.0) and the equation of the line derived. The equation was resolved to give the 'true' log_{10} OD value for those OD values that were above the maximum threshold. The inverse log_{10} OD values were then calculated to find the 'true' OD value.

To confirm the results of this experiment, it was repeated in triplicate. The intra-assay variation of the OD values of the diluted samples was also compared.

4.2.1.4 Measuring *C. difficile* glutamate dehydrogenase and toxins A & B in *in vitro* gut models O and M using commercial enzyme immunoassays

Hypothesis

The amount of glutamate dehydrogenase varies over the time of a simulated course of CDI in the *in vitro* gut model, with periods where GDH is the only detectable marker of the presence of *C. difficile*.

Methods

Gut model

C. difficile GDH and Toxins A and B were measured from fluid collected daily from an *in vitro* gut model of CDI, set up as previously described (Freeman *et al*, 2003). Briefly, the model consists of a three-stage continuous weir cascade culture system, pre-loaded with a human faecal emulsion. The faecal bacteria were allowed to proliferate and population levels to stabilise over a period of about two weeks. *C. difficile* spores (~10^7 cfu/mL) from a PCR ribotype 027 strain were added on two occasions, one week apart, and again the model was left to equilibrate. As this pair of models (denoted 'O' and 'M') were being used to compare two antibiotics for their potential to induce CDI, one had omadacycline (O) added (430mg/L) and the other moxifloxacin (M) (43mg/L) once daily for seven days. *C. difficile* total viable count, spore count and toxin level were enumerated daily. *C. difficile* total viable count, spore count and toxin level were monitored for a further three weeks. The gut models were set up, maintained and sampled by the technicians within the HCAI Research group, under the guidance of the PI for that set of models. Kerrie Davies oversaw the testing of the daily samples using the commercial assays and performed the analysis discussed in this thesis. Permission was granted for the use of excess gut model fluid to be used for this PhD project from the funder of the set of gut models.

GDH and C. difficile toxin enzyme immunoassays

A commercial automated GDH EIA, *C. DIFF CHEK-60*™, was used to measure the level of GDH and the *TOX AB II*™ assay was used to measure the level of toxins within the model daily. Once samples had been collected from the model they were frozen at -20ºC immediately; they were then defrosted once before testing with the enzyme immunoassays with the remainder stored at 4°C until all testing had been completed. The samples from this gut model were tested after one year in storage, due to the maternity leave and other work commitments of Kerrie Davies. The GDH assay was performed as described above. The procedure for the toxin assay mirrors that of the GDH assay and the same reagents can be used for both assays, except for the microplate wells, the positive control and the conjugate, which are specific to each assay (see materials and methods section 2.2). Assay positivity (as defined by the kits) and optical density (OD) values were compared with the pattern of *C. difficile* proliferation and toxin production. In addition, where samples produced a maximum OD value of ≥3.0, they were serially diluted and tested again. The resultant OD value was multiplied by the dilution factor to produce a final OD value. The enzyme immunoassay testing on the DS2 was performed by a summer intern within the HCAI Research Group, Miss Flor Saporta, under the supervision of Kerrie Davies.

4.2.1.5 Measuring *C. difficile* glutamate dehydrogenase and toxins A & B in *in vitro* gut model X using commercial enzyme immunoassays

Hypothesis

The amount of glutamate dehydrogenase varies over the time of a simulated course of CDI in the *in vitro* gut model, including for an episode of CDI recurrence, with periods where GDH is the only detectable marker of the presence of *C. difficile*.

Methods

Gut model

C. difficile GDH and Toxins A and B were measured from fluid collected daily from an *in vitro* gut model of CDI, set up as previously described (Freeman *et al*, 2003). Briefly, the model for this experiment was used to examine CDI recurrence. For this model the faecal slurry had been pre-prepared and then snap frozen using liquid nitrogen before being stored at -80°C. The slurry was defrosted at room temperature before inoculation into the model. The faecal bacteria were allowed to proliferate and population levels to stabilise

over a period of about four weeks. *C. difficile* spores (~10^7 cfu/mL) of strain 210 were added on two occasions, one week apart, and again the models were left to equilibrate. Clindamycin was instilled into the gut model (33.9 mg/L QDS for seven days) to induce simulated CDI, followed by treatment with clinically reflective doses of vancomycin (125mg/L QDS for seven days) once a peak toxin level had been reached (as measured by cell-cytotoxicity assay). *C. difficile* total viable count, spore count and toxin level were enumerated daily for a further four weeks. One model was run in for this experiment; denoted 'X'. The gut model was set up, maintained and sampled by the technicians within the HCAI Research group, under the guidance of the PI for this set of models. Kerrie Davies oversaw the testing of the daily samples using the commercial assays and performed the analysis discussed in this thesis. Permission was granted for the use of excess gut model fluid to be used for this PhD project from the funder of the set of gut models.

GDH and C. difficile toxin enzyme immunoassays

A commercial automated GDH EIA, *C. DIFF CHEK-60*™, was used to measure the level of GDH and the *TOX AB II*™ assay was used to measure the level of toxins within the model daily. Once samples had been collected from the model they were frozen at -20ºC immediately; they were then defrosted once before testing with the enzyme immunoassays with the remainder of the samples stored at 4°C until all testing had been completed. The samples from this set of gut models were tested after four months in storage, due to the maternity leave and other work commitments of Kerrie Davies. The assays were performed as described above. Assay positivity (as defined by the kits) and optical density (OD) values were compared with the pattern of *C. difficile* proliferation and toxin production. In addition, where samples produced a maximum OD value of $\geq$3.0, they were serially diluted and tested again. The resultant OD value was multiplied by the dilution factor to produce a final OD value. The enzyme immunoassay testing on the DS2 was performed by a summer intern within the HCAI Research Group, Miss Flor Saporta, under the supervision of Kerrie Davies.

4.2.1.6 Measuring *C. difficile* glutamate dehydrogenase and toxins A & B in *in vitro* gut models E, F and G using commercial enzyme immunoassays

Hypothesis

The amount of glutamate dehydrogenase varies over the time of a simulated course of CDI in the *in vitro* gut model, with periods where GDH is the only detectable marker of the

presence of *C. difficile*, and that the amount of GDH within the model increases with each CDI episode

Methods

Gut model

C. difficile GDH and Toxins A and B were measured from fluid collected daily from an *in vitro* gut model of CDI, set up as previously described (Freeman *et al*, 2003). Briefly, the models for this experiment were used to compare CDI recurrence in a faecal microbiota transplant (FMT) model and two models treated with a manufactured 'microbiota'. For these models, the faecal slurry used was the same snap frozen, pre-prepared slurry used during Model X above. The slurry was defrosted at room temperature before inoculation into the model. The faecal bacteria were allowed to proliferate and population levels stabilise over a period of about four weeks. After the stabilisation period, *C. difficile* spores (~10^7 cfu/mL) of strain 210 were added on two occasions, one week apart, and again the models were left to equilibrate. Clindamycin was instilled into the gut model (33.9 mg/L QDS for seven days) to induce simulated CDI, followed by treatment with clinically reflective doses of vancomycin (125mg/L QDS for seven days) once the peak toxin level had been reached (as measured by cell-cytotoxicity assay). Following the vancomycin treatment, a simulated faecal microbiota transplant (FMT) (pooled faecal slurry) was added to one of the models, and the proprietary 'microbiota' was added to the other two models; one receiving a single dose, and one receiving three doses. *C. difficile* total viable count, spore count and toxin level were enumerated daily for a further four weeks. Three models were run in parallel; denoted 'E' (three doses of proprietary agent), 'F' (one dose of proprietary agent) and 'G' (FMT with pooled faecal slurry). The gut models were set up, maintained and sampled by the technicians within the HCAI group, under the guidance of the PI for that set of models. Kerrie Davies oversaw the testing of the daily samples using the commercial assays and performed the analysis discussed in this thesis. Permission was granted for the use of excess gut model fluid to be used for this PhD project from the funder of the set of gut models.

GDH and C. difficile toxin enzyme immunoassays

A commercial automated GDH EIA, *C. DIFF CHEK-60*™, was used to measure the level of GDH and the *TOX AB II*™ assay was used to measure the level of toxins within the model daily. Once samples had been collected from the model they were frozen at -20ºC immediately; they were then defrosted once before testing with the enzyme

immunoassays with the remainder stored at 4°C until all testing had been completed. The samples from this set of gut models were tested in one batch after completion of the model experiments, after a maximum of two months in storage, with the majority tested within two weeks of being placed in storage. The assays were performed as described above. Assay positivity (as defined by the kits) and optical density (OD) values were compared with the pattern of *C. difficile* proliferation and toxin production. In addition, where samples produced a maximum OD value of $\geq$3.0, they were serially diluted and tested again. The resultant OD value was multiplied by the dilution factor to produce a final OD value. The enzyme immunoassay testing on the DS2 platform was performed by a summer intern within the HCAI Research Group, Miss Flor Saporta, under the supervision of Kerrie Davies.

4.3 Results

4.3.1 Results of measuring glutamate dehydrogenase in an in vitro gut model

4.3.1.1 Measuring *C. difficile* glutamate dehydrogenase in an *in vitro* gut model by both enzyme immunoassay and polymerase chain reaction assay.

In both models (A and B) the GDH protein was detected by the EIA in Vessels Two and Three of the triple stage system (figures 4.1a-c and 4.2a-c). There was a rise in GDH during the period where the model was left to achieve steady state after the addition of spores. In Model A this occurred about nine days after the introduction of spores, whilst in Model B it was after only two days for Vessel Two, and three days for Vessel Three. The GDH increased to the maximum measured by the assay within six days in Model B and remained at this level for four days in Vessel Two and five days for Vessel Three, before falling back to zero over the next eight days. At the same time, there was a small amount of toxin production detected in Vessel Three only; this is low level (1 Relative Unit) and lasted for two days.

Once germination had been induced, the GDH level increased in phase with the total viable count of the organism and the production of toxin. It is interesting to note, however, that although the initial increase of GDH matched that of both the total viable count and toxin production; it remained elevated within the system for longer than both of these after the introduction of the treatment antibiotic. Once the GDH began to be washed out of the system, it did so at the same rate as the toxin, but lagged behind the toxin wash-out by at least two days. The overall pattern of GDH rise and fall was mirrored in Vessels Two and Three, although Vessel Two was about two days ahead of Vessel Three for both models. There was no germination or GDH detected in Vessel One of either system, apart from one raised GDH level on one day in Model B.

Although *gluD* was detected in the model by the PCR assay, the results were inconclusive (figures 4.3 a-b). DNA extraction from the model may not be optimal using this method as the CT values for the PCR assay do not fit the pattern of peaks and troughs shown by the total viable count, spore and GDH enzyme level. It is outside the scope of this PhD project to determine the optimal method for extracting DNA from the complex fluid extracted from the gut model. This method was therefore not used in any further gut models within this project.

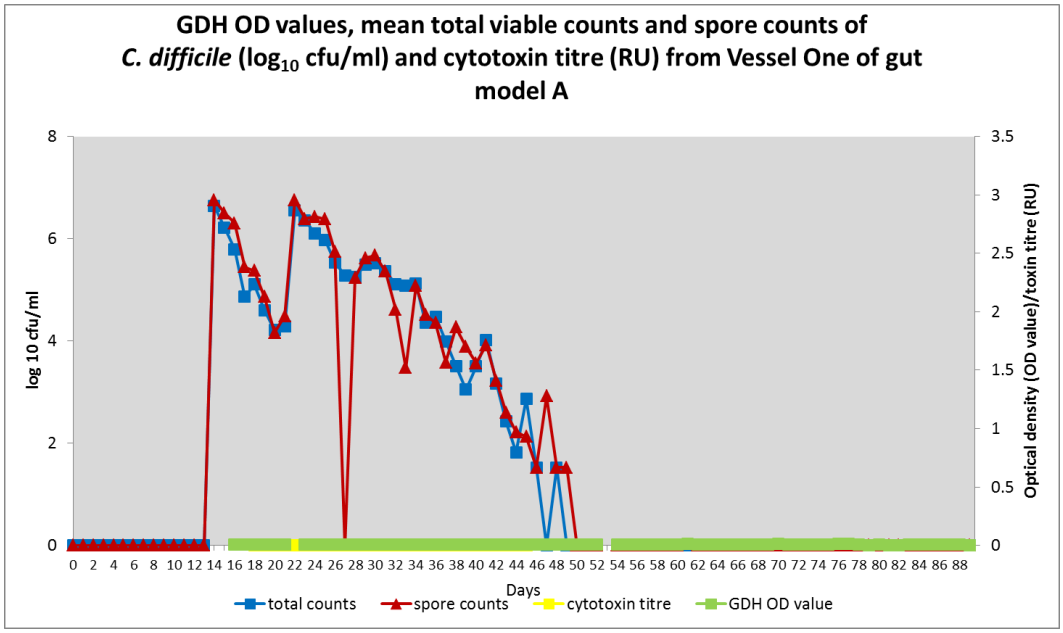


Figure 4.1.a Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model A

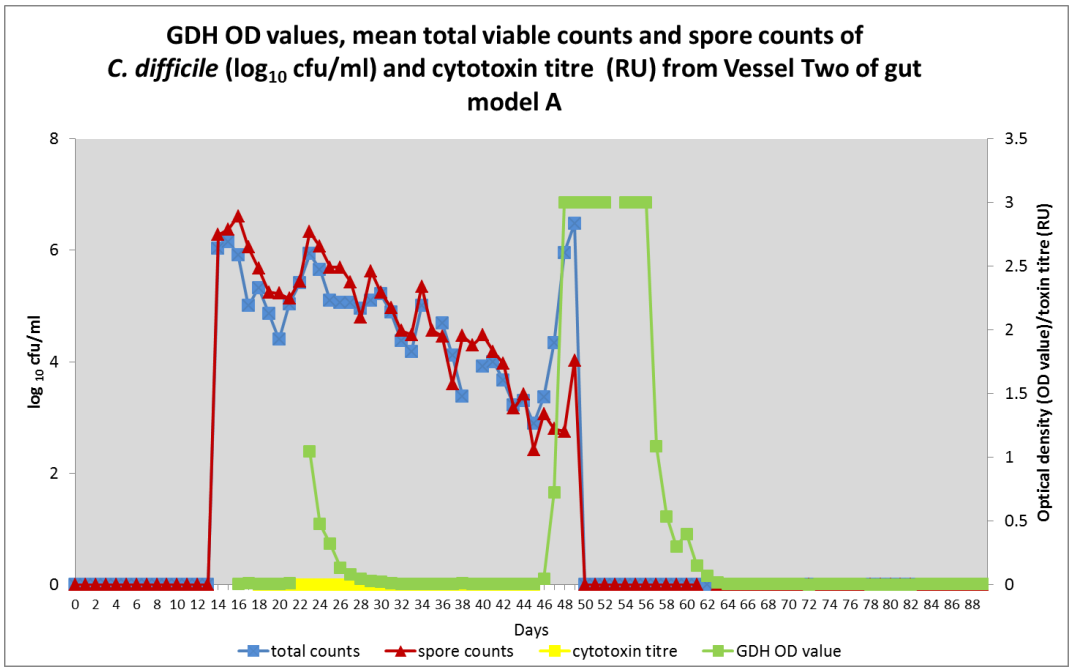


Figure 4.1.b Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model A

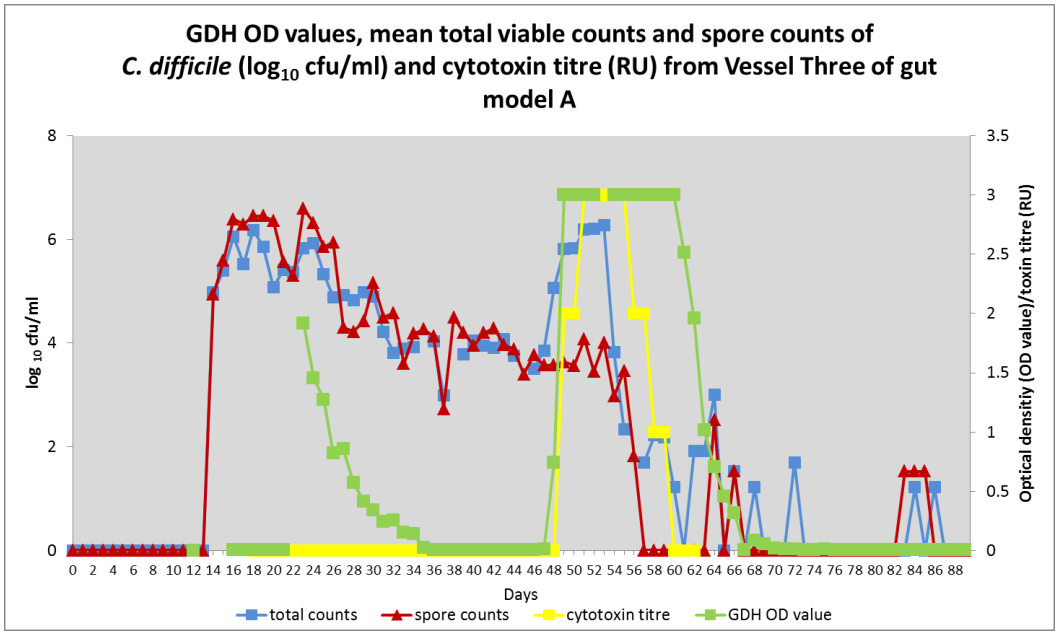


Figure 4.1.c. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model A

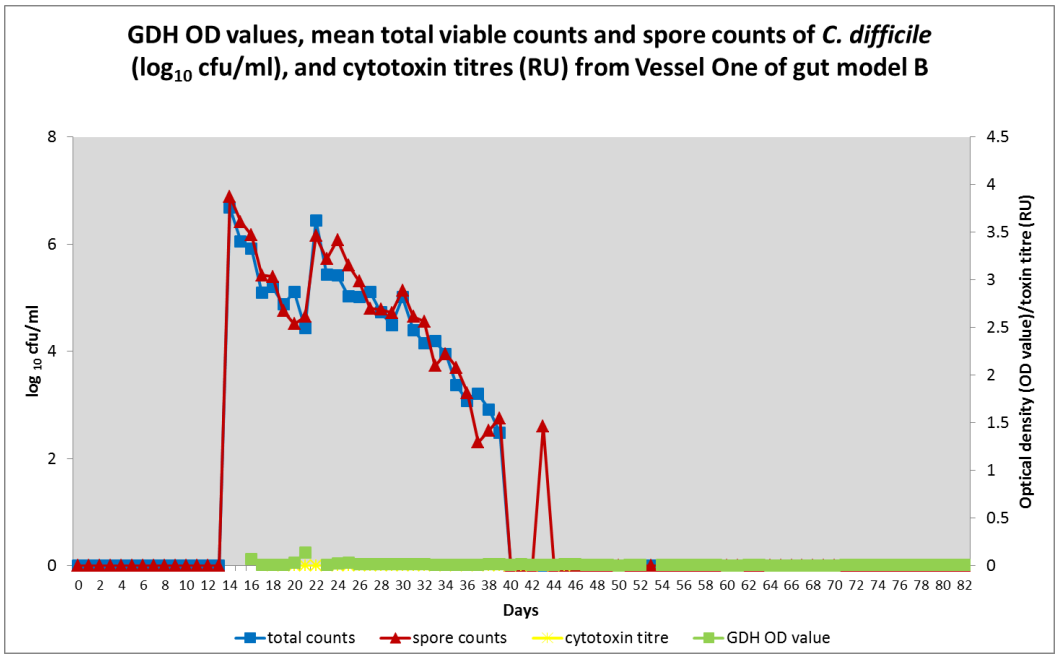


Figure 4.2.a. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model B

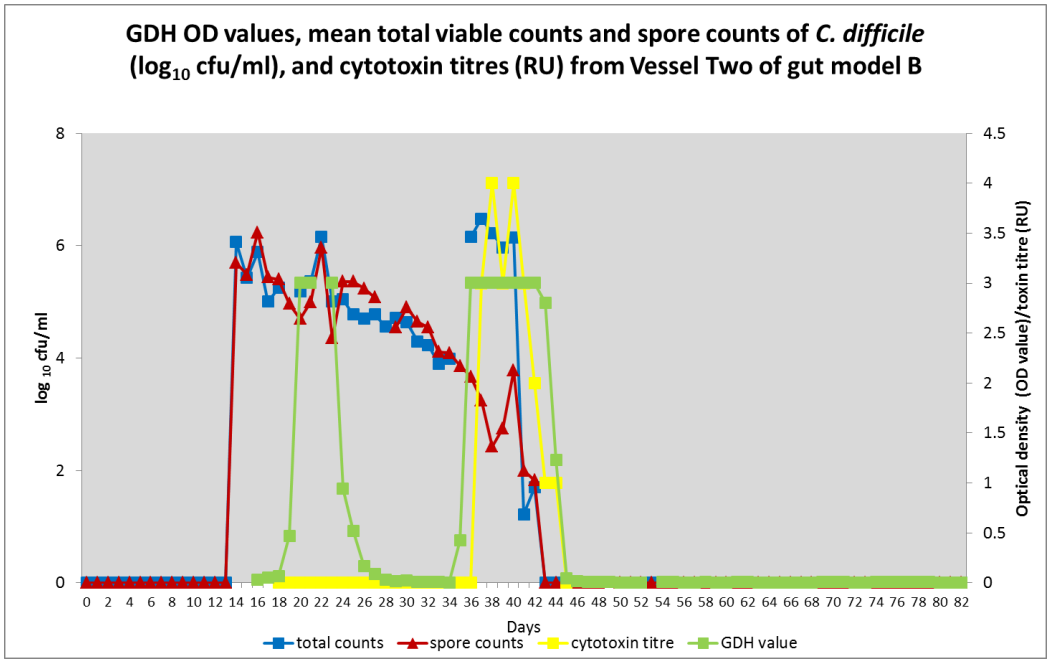


Figure 4.2.b Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model B

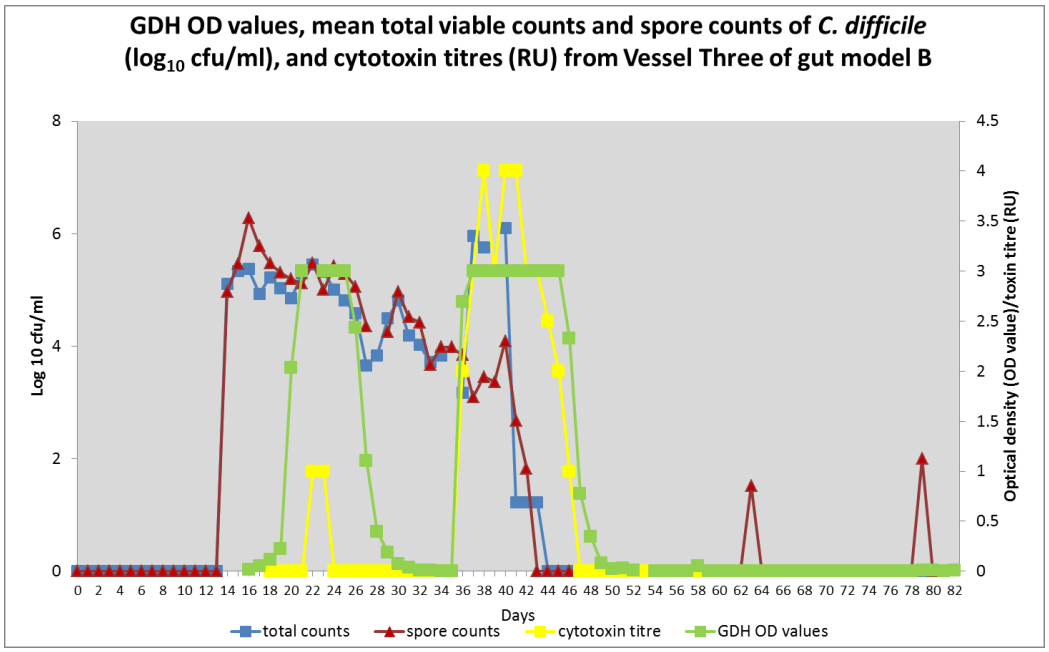


Figure 4.2.c Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model B

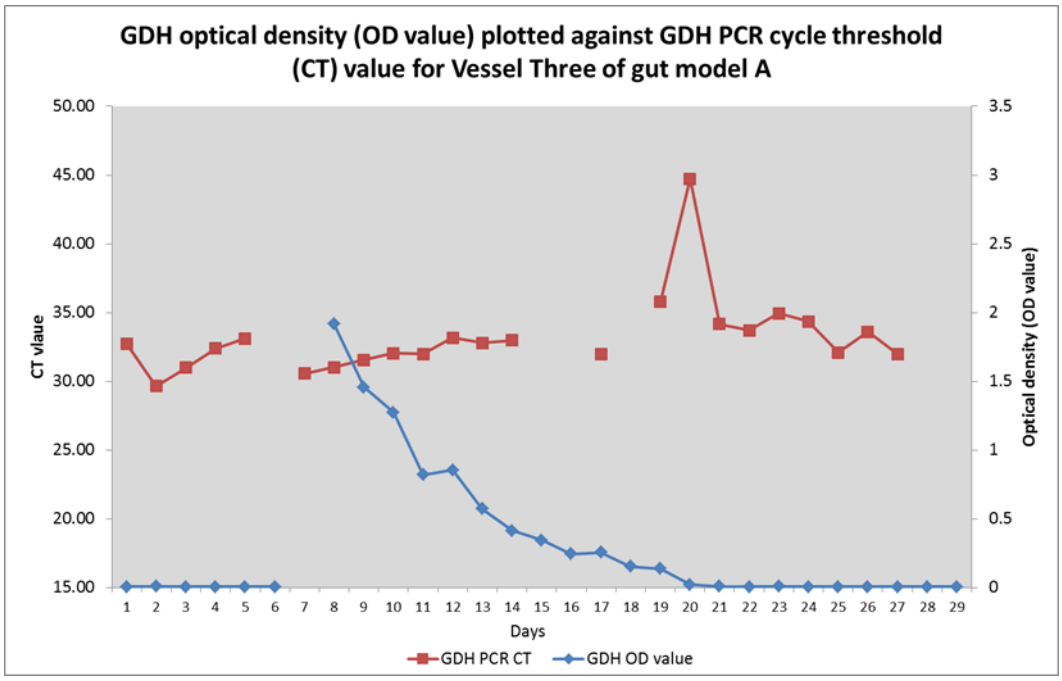


Figure 4.3.a. GDH EIA OD values and GDH PCR CT values from faecal slurry from gut Model A

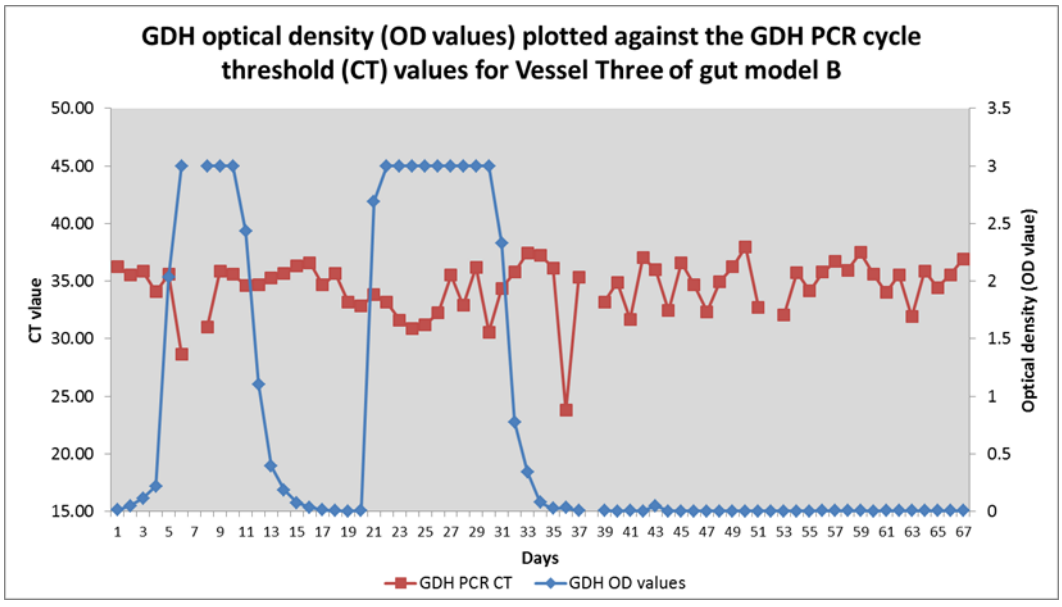


Figure 4.3.b. GDH EIA OD values and GDH PCR CT values from faecal slurry from gut Model B

4.3.1.2 Measuring *C. difficile* glutamate dehydrogenase in *in vitro* gut models A and B by enzyme immunoassay

Model A

In Vessel One there was a low level of GDH detected between Days 43-46, although this did not coincide with any increase in total viable count or germination (figure 4.4a). Germination occurred at Day 55, with total viable count and spore count diverging, with a concurrent increase in GDH from Days 54 to 61. The total viable count fell to an undetectable level by Day 58, however GDH was still detectable for a further three days. There was no toxin detected in Vessel One by the cell-cytotoxicity neutralisation assay.

In Vessel Two there was an increase in GDH OD value, as measured by the enzyme immunoassay, on Day 37, which coincided with germination (figure 4.4b). The OD value rose to a peak five days later, again coinciding with a peak in the total viable count. GDH remained detectable by the enzyme immunoassay until Day 67, while the total viable count began to fall on Day 59 and was below the limit of detection by Day 62. There were, therefore, five days where GDH was the only marker of *C. difficile* that could be detected in the model. Toxin was detected on Day 42, coinciding with the peak in the total viable count and peak GDH OD value. The highest peak of toxin was on Day 55.

Germination, and the concurrent increase in GDH, occurred on Day 37 in Vessel Three; the same day as Vessel Two (figures 4.4b and 4.4c). Both GDH OD value and total viable count rose to a peak by Day 42, with the GDH OD value remaining at a peak level until Day 63. The fluid from Vessel Three was still positive for GDH, using the enzyme immunoassay, at the end of the experiment on Day 67. In contrast, total viable count began to fall on Day 59 and was below the limit of detection by Day 62. Toxin was detected earlier in Vessel Three than Vessel Two, at Day 37, although the peak toxin concentration coincided in both vessels, at Day 56.

In both Vessel Two and Vessel Three, the GDH OD value reached the maximum threshold OD within a few days, producing a plateau on the graph. The assay in its current form was unable to show if the GDH level continued to rise past this level or if it did, indeed, plateau. The graphs also appear to show that the GDH OD value did not begin to decline at the same time as the total viable count; however this could be an artefact of the assay threshold, masking the true picture of GDH rise and fall.

Model B

In Vessel One, there is a small amount of GDH detected at Day 20, in the absence of any increase in the total viable count (figure 4.5a). Germination started at Day 51, with an increase in the total viable count and a divergence from the spore count. A coincident rise in the GDH level was detected from Days 51-56 and Days 57-71, indicated by two small peaks on the graph. The total viable count began to decline at Day 56 and was below the level of detection by Day 58. From the total viable count values, there appeared to be only one episode of germination, however, there were two peaks of GDH detection within this period; the reason for the decline and subsequent rise again to give these two peaks is unclear. Unlike the right-hand-side model there was a small amount of toxin detected in Vessel One between Days 52-54; during the proliferation of the organism after germination at Day 51.

Once again, there was a small amount of GDH detected from Vessel Two (Days 18-20), in the absence of any apparent *C. difficile* proliferation; as there was no concurrent rise in the total viable count (figure 4.5b). Germination started at Day 44; earlier than seen in Vessel One (Day 51). There was a lag of four days before the level of GDH began to rise, peaking at Days 50-60, before falling to an undetectable level by Day 64. The peak total viable count was seen at Day 50, with the count falling from Day 58 to below the level of detection within two days. *C. difficile* toxin was detected from Days 50-56; following the peak of *C. difficile* proliferation. There were, therefore, four days when GDH is the only marker of *C. difficile* that could be detected in the model.

A very similar pattern was seen in Vessel Three, with the same small amount of GDH detected early after introduction of spores within the model, but without signs of germination (figure 4.5c). Again, increasing GDH and toxin levels were detected during germination and proliferation. As with Vessel Two, there was a period of ~four days when GDH was the only marker of *C. difficile* that could be detected in Vessel Three of the model, following the decline of the total viable count below the level of detection. Again, in both Vessel Two and Vessel Three, the GDH OD value reached the maximum threshold OD within a few days, producing a plateau on the graph. As discussed above, this could be an artefact of the assay threshold, masking the true picture of GDH rise and fall.

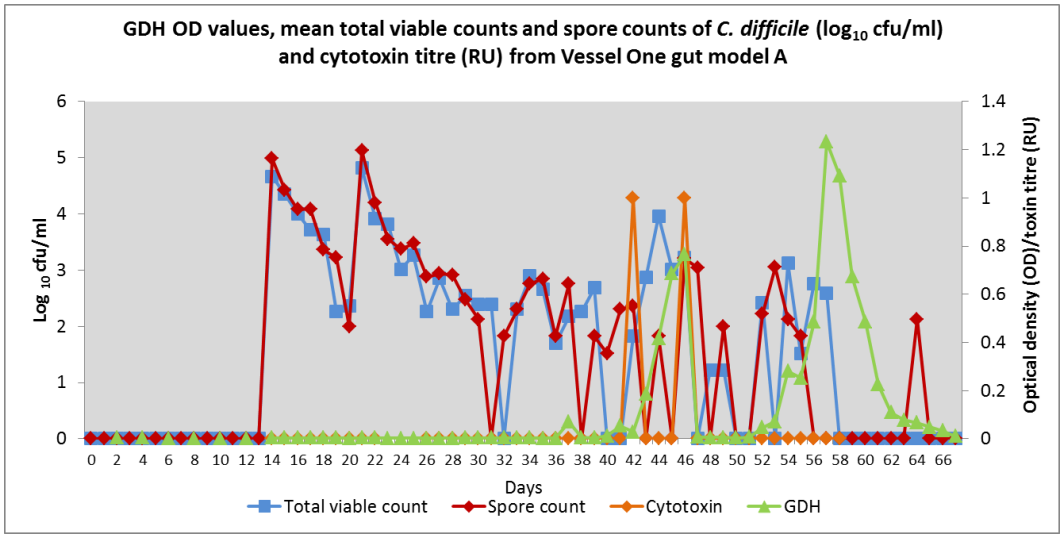


Figure 4.4.a. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model A

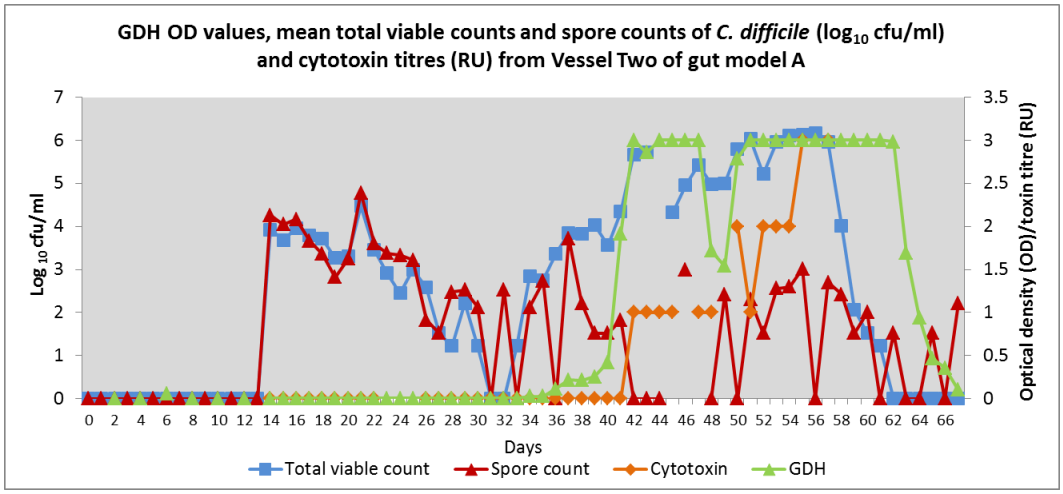


Figure 4.4.b. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model A

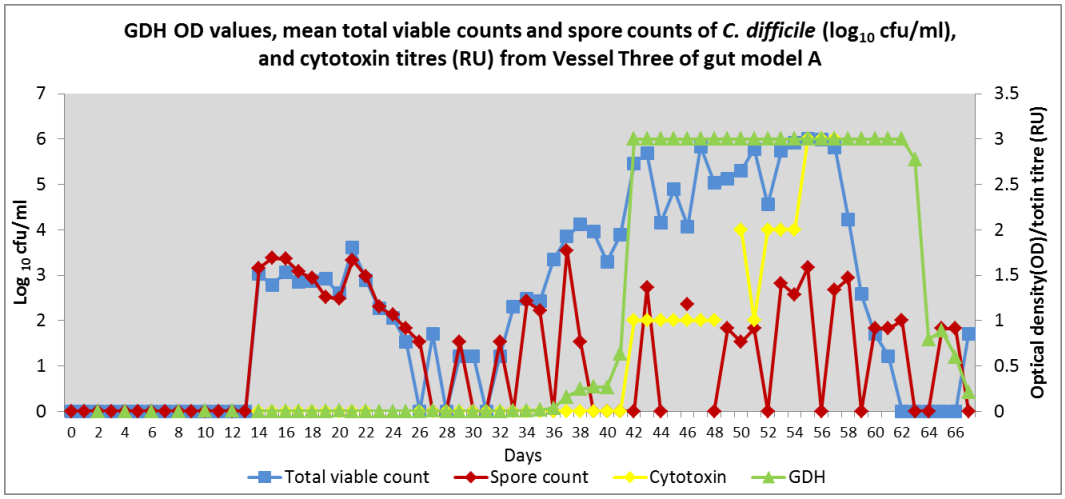


Figure 4.4.c. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model A

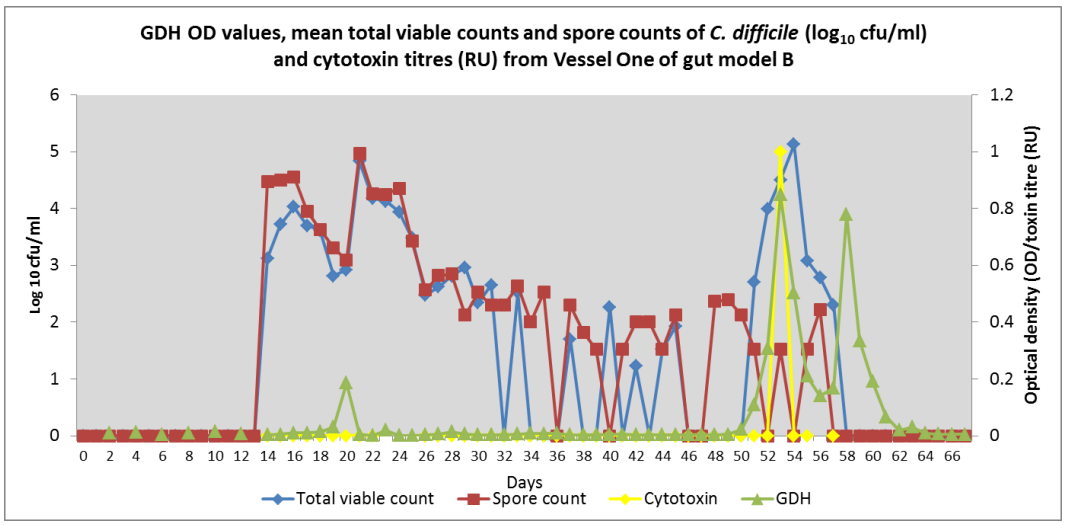


Figure 4.5.a. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model B

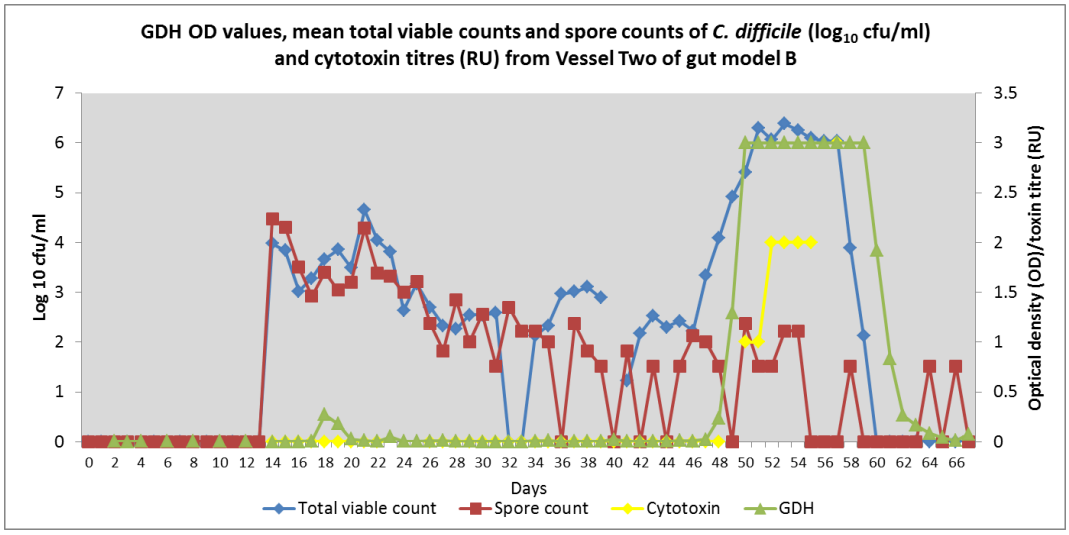


Figure 4.5.b. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model B

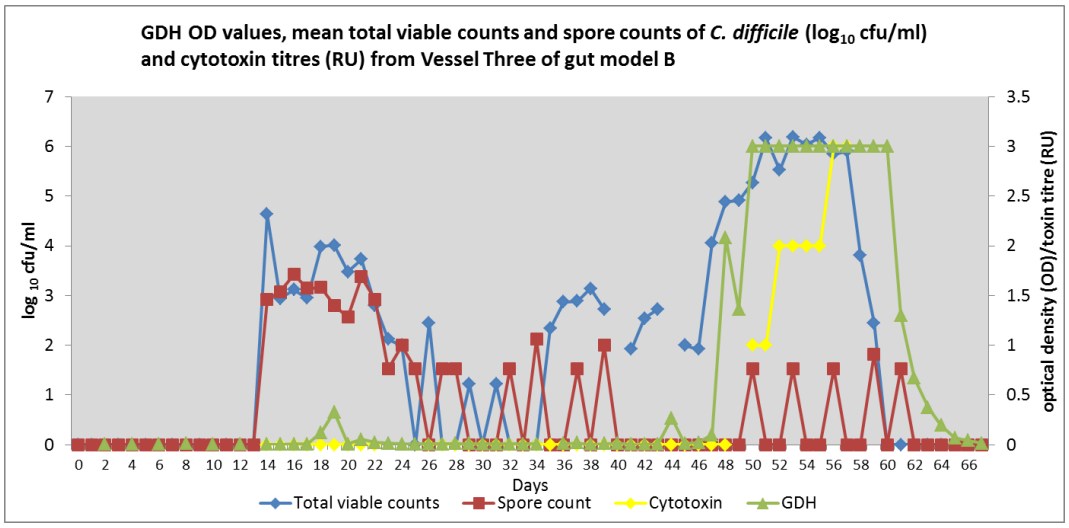


Figure 4.5.c. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model B

4.3.1.3 Investigating the quantitative potential of the commercial glutamate dehydrogenase enzyme immunoassay

For experiment one, the OD of each dilution was measured on the DS2 and the mean cfu/mL for each dilution was calculated, after counting colonies on the 1/1000 dilution plates (table 4.2). The mean colony count for each dilution was then $\log_{10}$ transformed and

plotted against the $\log_{10}$ OD value for each dilution with the exception of dilutions that gave an OD value of $\geq$3.0, as this was the maximum threshold of the assay and would skew the results (figure 4.6a). The equation for the line was calculated and extrapolated to calculate the $\log_{10}$ OD value for the neat and 1/10 dilutions (table 4.2). The inverse $\log_{10}$ OD value was then calculated for each dilution to give the actual OD value (had the threshold limit not been reached). To check the accuracy of the method, the equation of the line was also used to reverse calculate the cfu/ml and compare to the actual cfu/ml.

The calculated OD values and calculated cfu/ml was very close to the actual OD values and cfu/ml for the bottom three dilutions (1/100-1/10,000) (table 4.2.). However, the calculated cfu/ml for the neat and 1/10 dilutions underestimates the true bioload within the sample; therefore, any calculated OD values using this method would also underestimate the true OD value. These results therefore show that the GDH EIA is quantitative as long as the OD is below the threshold of $\geq$3.0. Therefore, samples could be diluted before they are tested on the GDH assay on the DS2 then multiplied by the dilution factor to find the actual OD. To confirm these results, the experiment was repeated. The second experiment confirmed the result of the first (table 4.3.), with the GDH assay demonstrating that it is quantitative, as long as the OD value is below that of the threshold ($\geq$3.0). The intra-assay variation between diluted samples is low, showing good reproducibility within the assay (table 4.4).

The OD value in the neat sample was calculated by multiplying the OD value in the diluted sample by the dilution factor (table 4.3). When compared with the calculated OD value (using the equation of the line (figure 4.6b), the two OD values for the neat solution were within the same order of magnitude. Whilst not an exact representative of the 'true' OD value, the order of magnitude of GDH can be calculated, and the pattern of rise and fall of GDH within the model can be followed. In future experiments, where the OD is the maximum value of $\geq$3.0, the sample will be diluted 1/100 and 1/1000, and the resultant OD will be multiplied by the dilution factor to give the 'true' OD value in the neat sample.

Dilution factor of the sample	Mean cfu/mL	Log_{10} cfu/mL	Mean OD value	Log_{10} of OD value	Calculated log_{10} OD value	Calculated OD value	Calculated log_{10} cfu/mL	Calculated cfu/mL
Neat	410000	5.61	3.001	0.477	2.630	426.646	3.81	6423.95
10^{-1}	41000	4.61	3.001	0.477	1.437	27.376	3.81	6423.95
10^{-2}	4100	3.61	1.641	0.215	0.245	1.757	3.59	3872.58
10^{-3}	410	2.61	0.109	-0.963	-0.948	0.113	2.60	398.65
10^{-4}	41	1.61	0.004	-2.398	-2.141	0.007	1.40	24.95
Neat	375000	5.57	3.001	0.477	2.584	383.572	3.81	6423.95
10^{-1}	37500	4.57	3.001	0.477	1.391	24.612	3.81	6423.95
10^{-2}	3750	3.57	1.741	0.241	0.198	1.579	3.61	4069.49
10^{-3}	375	2.57	0.195	-0.710	-0.994	0.101	2.81	649.21
10^{-4}	37.5	1.57	0.006	-2.222	-2.187	0.007	1.54	35.06
Neat	425000	5.63	3.001	0.477	2.649	445.327	3.81	6423.95
10^{-1}	42500	4.63	3.001	0.477	1.456	28.575	3.81	6423.95
10^{-2}	4250	3.63	0.93	-0.032	0.263	1.833	3.38	2405.59
10^{-3}	425	2.63	0.165	-0.783	-0.929	0.118	2.75	564.36
10^{-4}	42.5	1.63	0.005	-2.301	-2.122	0.008	1.48	30.09
Neat	277500	5.44	3.001	0.477	2.428	267.843	3.81	6423.95
10^{-1}	27750	4.44	3.001	0.477	1.235	17.186	3.81	6423.95

10^{-2}	2775	3.44	0.93	-0.032	0.042	1.103	3.38	2405.59
10^{-3}	277.5	2.44	0.165	-0.783	-1.150	0.071	2.75	564.36
10^{-4}	27.75	1.44	0.005	-2.301	-2.343	0.005	1.48	30.09

Table 4.2. The measured and calculated OD values for each dilution of *C. difficile* culture. The cfu/ml and OD were $\log_{10}$ transformed before plotting on a graph (Figure 4.6a). Because the top two dilution skewed the data (as the OD values were the maximum threshold of the kit [$\geq$3.0]) they were removed from the graph. Using the equation of the graph the 'real' OD of the top two dilutions could be calculated. The inverse $\log_{10}$ OD values then need to be calculated in order to find the 'true' OD.

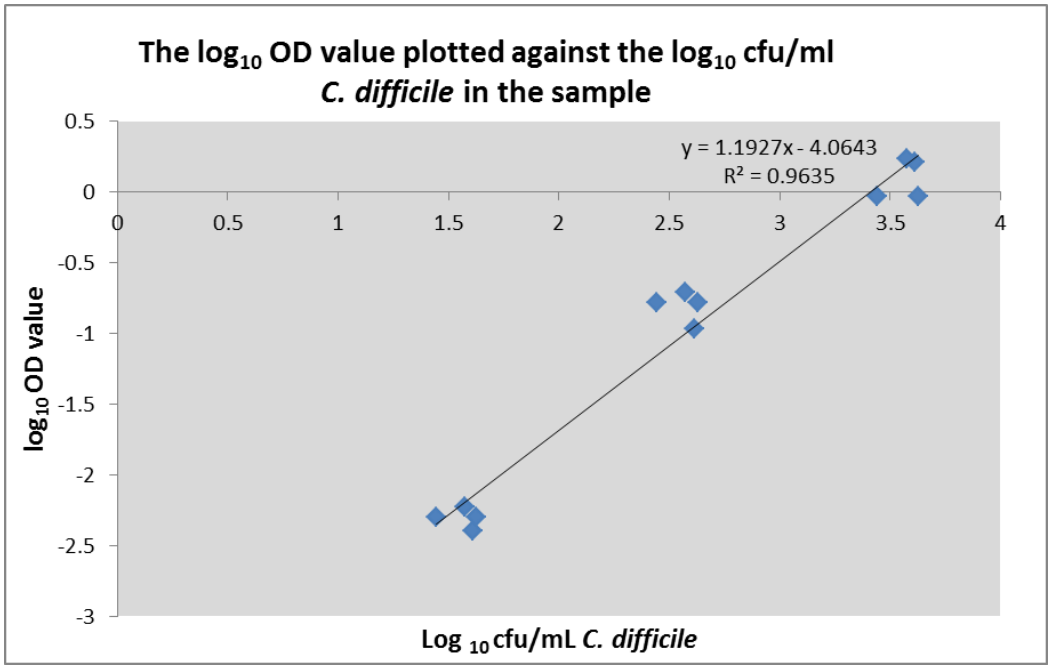


Figure 4.6.a. Graph showing the $\log_{10}$ OD values for the different dilutions of *C. difficile* (plotted as $\log_{10}$ cfu/ml) for experiment one. The neat and 1/10 dilution OD results were not plotted, as they gave the maximum threshold OD value, and skewed the results. The equation of the line was calculated and used to calculate the $\log_{10}$ OD value of the neat and 1/10 dilutions (see table 4.2).

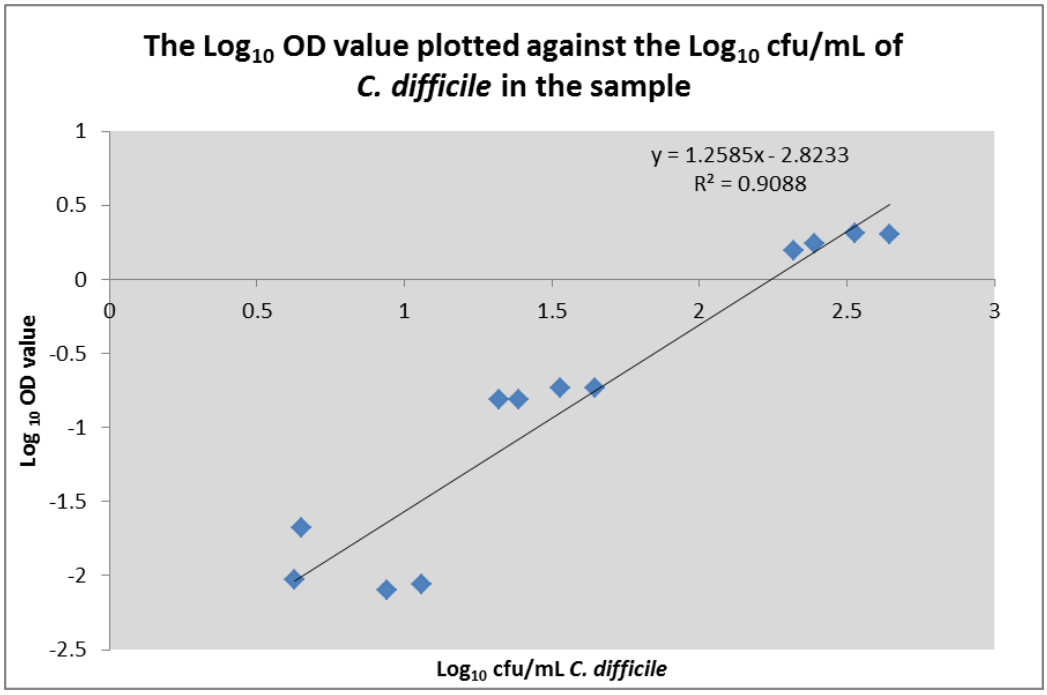


Figure 4.6.b. Graph showing the log_{10} OD values for the different dilutions of *C. difficile* (plotted as log_{10} cfu/ml for experiment two. The neat and 1/10 dilution OD results were not plotted, as they gave the maximum threshold OD value, and skewed the results. The equation of the line was calculated and used to calculate the log_{10} OD value of the neat and 1/10 dilutions (see table 4.3).

Dilution factor of the sample	Mean cfu/ml	Log_{10} mean cfu/ml	Mean OD value	Log_{10} mean OD	Calculated log_{10} OD value	Calculated OD value in the relevant diluted sample (inverse log_{10})	Calculated OD in the neat sample
Neat	24500	4.39	3.001	0.477	2.700	501.725	n/a
10^{-1}	2450	3.39	3.001	0.477	1.442	27.667	n/a
10^{-2}	245	2.39	1.754	0.244	0.183	1.526	175.370
10^{-3}	24.5	1.39	0.156	-0.808	-1.075	0.084	155.670
10^{-4}	4.5	0.65	0.021	-1.678	-2.001	0.010	210.000
Neat	33750	4.53	3.001	0.477	2.876	750.814	n/a
10^{-1}	3375	3.53	3.001	0.477	1.617	41.403	n/a
10^{-2}	337.5	2.53	2.042	0.310	0.359	2.283	241.670
10^{-3}	33.75	1.53	0.186	-0.731	-0.900	0.126	185.670
10^{-4}	11.5	1.06	0.009	-2.062	-1.488	0.032	86.670
Neat	44250	4.65	3.001	0.477	3.024	1055.800	n/a
10^{-1}	4425	3.65	3.001	0.477	1.765	58.221	n/a
10^{-2}	442.5	2.65	2.023	0.306	0.507	3.211	202.300
10^{-3}	44.25	1.65	0.184	-0.736	-0.752	0.177	183.670

10^{-4}	8.75	0.94	0.008	-2.097	-1.638	0.023	80.000
Neat	21000	4.32	3.001	0.477	2.616	413.250	n/a
10^{-1}	2100	3.32	3.001	0.477	1.358	22.788	n/a
10^{-2}	210	2.32	1.563	0.194	0.099	1.257	156.300
10^{-3}	21	1.32	0.154	-0.812	-1.159	0.069	154.340
10^{-4}	4.25	0.63	0.009	-2.030	-2.032	0.009	93.340

Table 4.3. The measured and calculated OD values of the dilution series of *C. difficile*, based on the graph in Figure 4.6b. Calculated $\log_{10}$ OD is based on resolving the equation of the line, the inverse log was calculated to produce the calculated OD. The calculated OD values for the neat solution if the actual OD of the dilution is multiplied by the dilution factor is very close to that for the higher dilutions (1/100-1/1000). In future experiments, where the OD is the maximum value of $\geq$3.0, the sample will be diluted 1/100 and 1/1000, and the resultant OD will be multiplied by the dilution factor to give the actual OD in the neat sample.

Dilution factor of the sample	Standard deviation Run 1	Standard deviation Run 2	Standard deviation Run 3	Standard deviation Run 4
Neat	0	0	0	0
1/10	0	0	0	0
1/100	0.0448	0.0493	0.0056	0.0615
1/1000	0.0084	0.0032	0.0032	0.0045
1/10000	0.0025	0.0025	0.001	0.0006

Table 4.4. The intra-assay variation in OD values for each set of sample dilutions. All OD values for the neat and 1/10 dilutions were ≥3.0 (the maximum threshold), hence a standard deviation of 0.

4.3.1.5 Measuring *C. difficile* glutamate dehydrogenase and toxins A & B in *in vitro* gut models O and M using commercial enzyme immunoassays

Model O

The antimicrobial agent added to this model did not induce CDI, and therefore there was no increase in total viable count or GDH level and no germination (figures 4.7a-c). There was also no increase in GDH after the instillation of spores in Vessel One, although there was a very slight rise in GDH after the first and second doses of spores in Vessel Two, and for one sample in Vessel Three following the second dose of spores.

Model M

In Vessel One, both the total viable count and GDH level began to rise concurrently as germination occurred at Day 29, with toxin being produced during the proliferation phase (as detected by cell-cytotoxicity assay) (Figures 4.8a-c). The total viable count peaked on the following day but the level of GDH continued to rise until Day 32. Previously this has not been observed, due to the maximum threshold of the GDH OD value. The total viable count then started to fall, along with the level of GDH, after a short lag period, before both rose again to a second peak. Interestingly, the second peak of the total viable count was higher than the first, whilst the second peak of GDH was lower than the first. The total viable count, spore count, toxin and GDH levels began to decline at a similar time, but there are two days where GDH could still be detected after both total viable and spore counts had gone below the limit of detection.

In Vessel Two the GDH level started to rise after the installation of the first dose of *C. difficile* spores, increasing after the second dose. It continued to increase after germination and proliferation of the organism in the model, as it began to produce toxin. Both the total viable count and level of GDH peaked at the same time and then began to fall in phase with each other, and indeed toxin production. There was a small second peak in both total viable count and level of GDH, again in phase with each other. Once again GDH could be detected for a further two days after total viable and spore counts had gone below the limit of detection. A very similar pattern of in-phase rise and fall of total viable count and GDH level were seen in Vessel Three, including the two days at the end of the experiment where GDH was the only marker of *C. difficile* that could be detected from the gut model fluid. The occurrence of the peak total viable count and peak GDH OD value were very similar for both Vessel Two and Three.

Although toxin production could clearly be seen within Vessels One and Two when using the cell-cytotoxicity assay, there was no concurrent rise in the OD value of the toxin enzyme immunoassay. In Vessel Three there was a very small rise OD value for the toxin assay in one sample, around the time of peak toxin production. The reasons for the low OD values with the toxin enzyme immunoassay are unclear; it is possible that something within the complex gut model fluid is inhibiting the assay.

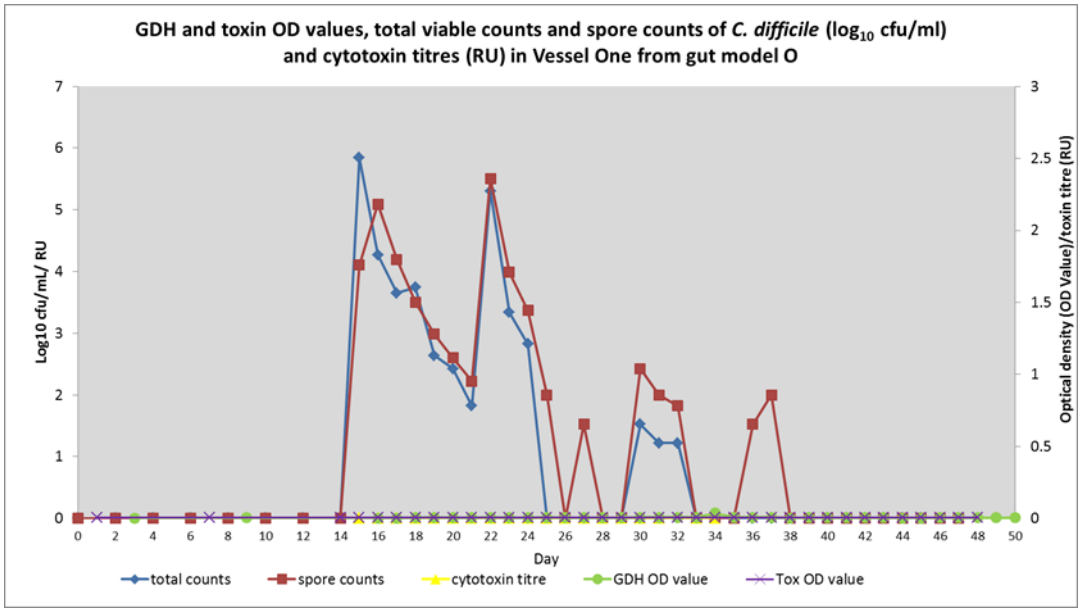


Figure 4.7.a. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model O

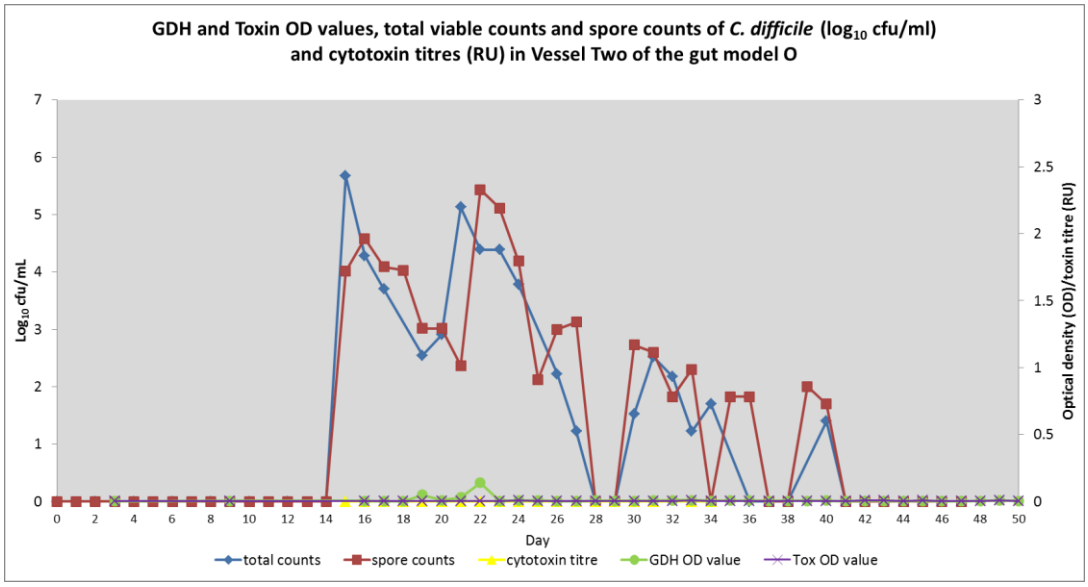


Figure 4.7.b. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model O

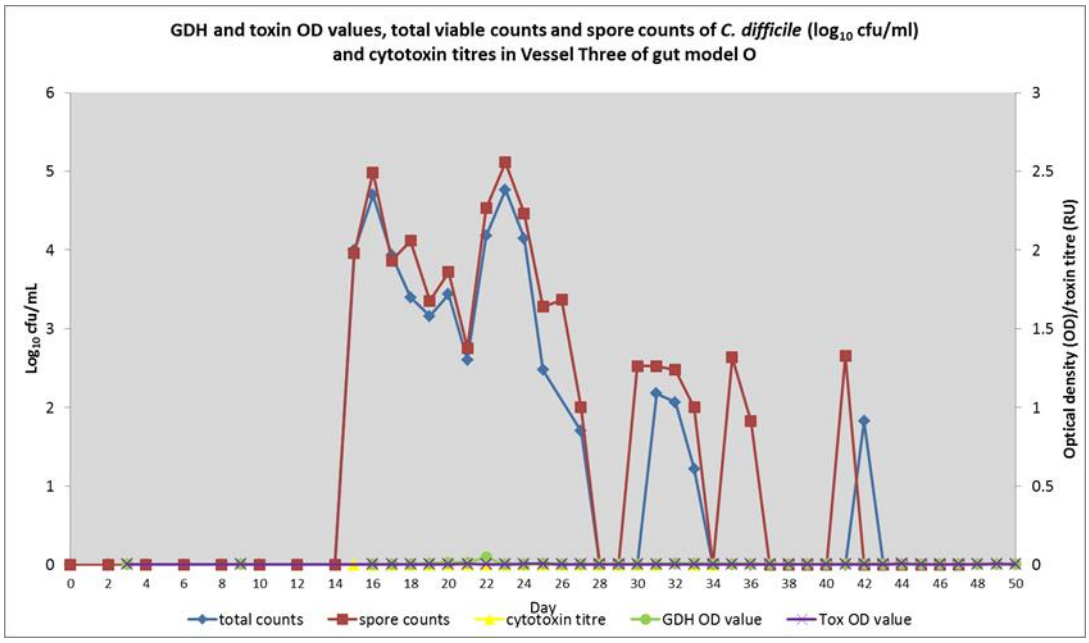


Figure 4.7.c. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model O

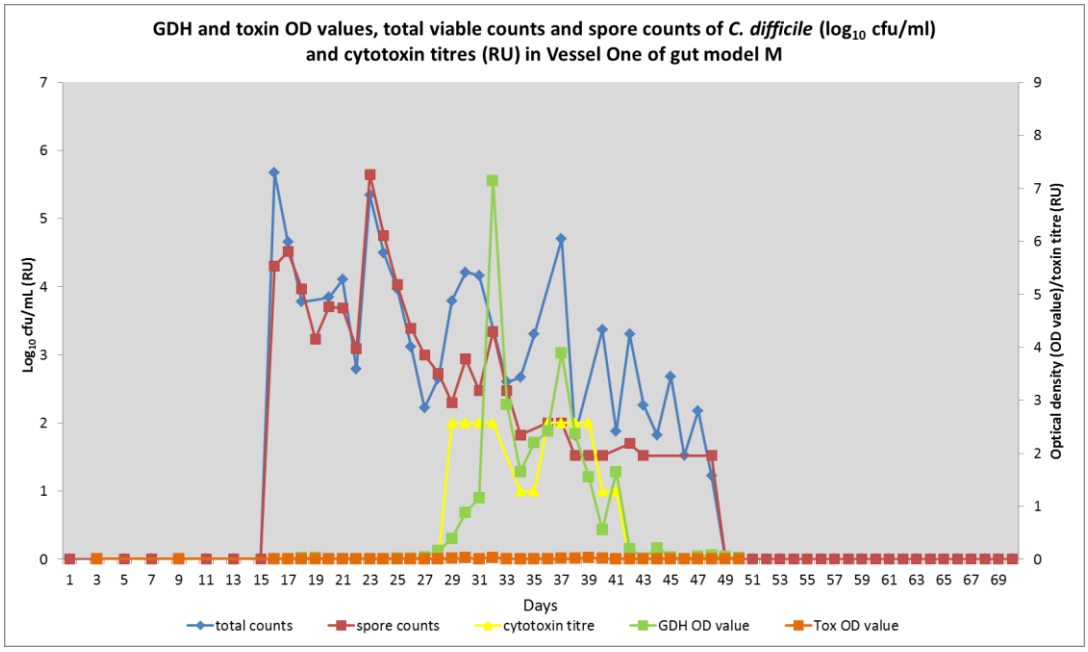


Figure 4.8.a. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model M

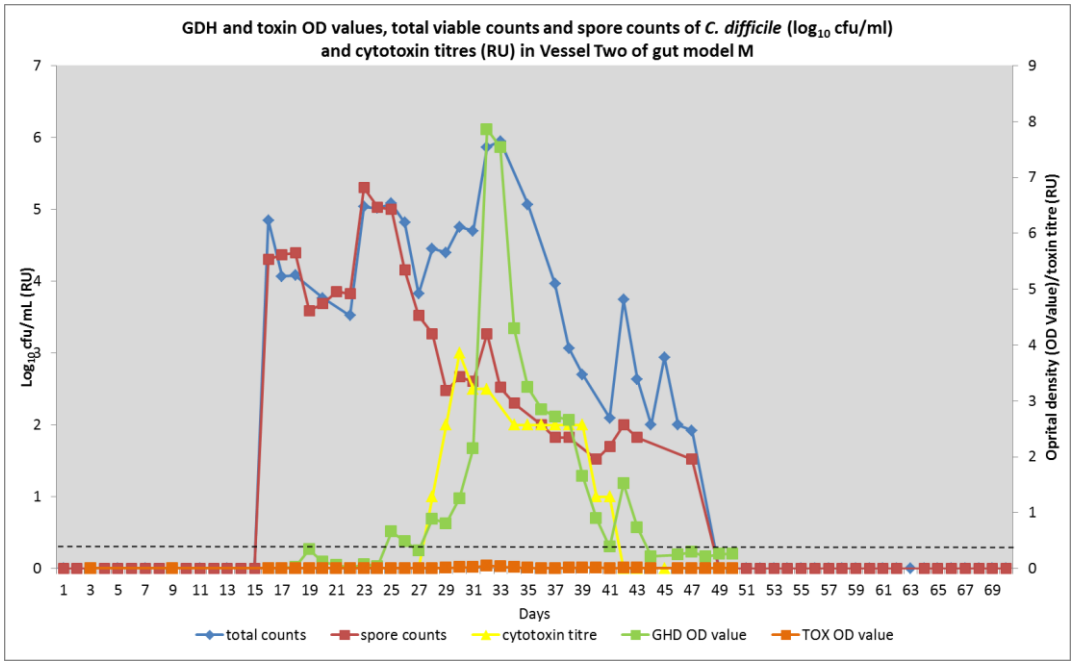


Figure 4.8.b. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model M

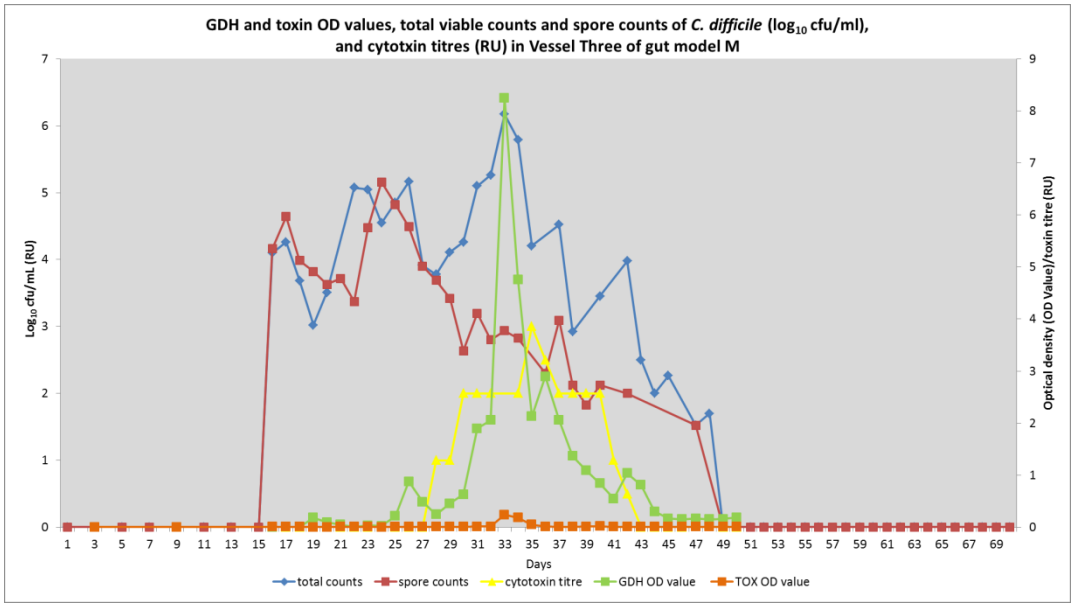


Figure 4.8.c. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model M

4.3.1.6 Measuring *C. difficile* glutamate dehydrogenase and toxins A & B in *in vitro* gut model X using commercial enzyme immunoassays

Model X

In Vessel One, there was an increase in GDH after the instillation of the first dose of spores, as seen with previous models (figures 4.9a-c). The level of GDH then starts to increase, in-line with the increase in the total viable count that signals the start of germination, with both reaching peak levels at the same time. As shown previously in the other models, the GDH level declined in line with the total viable count, however GDH could still be detected for a period of ~two days after total viable count was below the limit of detection. Recurrence occurred approximately 25 days after the end of the previous episode, with an increase in total viable and spore counts. There was no increase in the OD value representing GDH, however, and no toxin was produced.

The peak in GDH level after instillation of the spores was markedly higher in Vessel Two, than that seen in Vessel One, or indeed, any other model to date (~OD = 10, compared with 0.165 in Vessel One). GDH then declined back to zero. Once germination began, the total viable count and level of GDH increased in-phase and reached peak levels at the same time. It is interesting to note, however, that the peak GDH level was higher than seen in previous

models (OD~16 compared with ~eight in the previous model) despite the total viable counts being similar in both models, with a peak of ~six log_{10}cfu/mL. The peak in toxin production coincided with the peak in growth and declined as the total viable count and GDH level declined. As with previous models, GDH could be detected from the gut model fluid after the total viable count had gone below the limit of detection, however, this time this was for a period of ~seven days compared with ~two days in previous models. Again, recurrence occurred approximately 25 days after the end of the previous episode, with an increase in total viable and spore counts. Unlike Vessel One, however, there was an increase in GDH and toxin levels in Vessel Two during this recurrence episode. There was a strange 'dip' in total viable and spore counts and the level of GDH on one day, with no concurrent drop in toxin level. For GDH, this drop was back to zero, which seems unlikely, given that normally GDH can be detected for many days after the total viable count.

Whilst there was a large peak in GDH after instillation of spores in Vessel Three, it was not as high as is seen in Vessel Two (OD~5 vs ~10 in Vessel Two). Germination occurred on the same day in Vessel Three as Vessel Two (Day 59), reaching a similar maximum OD value as Vessel Two (peak OD in Vessel Three ~17 vs ~16 in Vessel Two). Again, the peak total viable count and GDH level coincided with the peak toxin level, and all started to decline at the same time. As with Vessel Two, GDH could still be detected for a further seven days after the total viable count was below the level of detection. In accord with Vessels One and Two, recurrence occurred approximately 25 days after the end of the first episode. Whilst the pattern of increasing total viable count and GDH OD value seen in Vessel Two was repeated In Vessel Three, the sample taken on the final day has a markedly high GDH OD value of ~25.

There was no detection of *C. difficile* toxin by the enzyme immunoassay in any of the vessels, despite detection of toxin by the cell-cytotoxicity assay in both Vessels Two and Three, similar to the previous models. As with the previous gut model experiment, the reason for this is not clear.

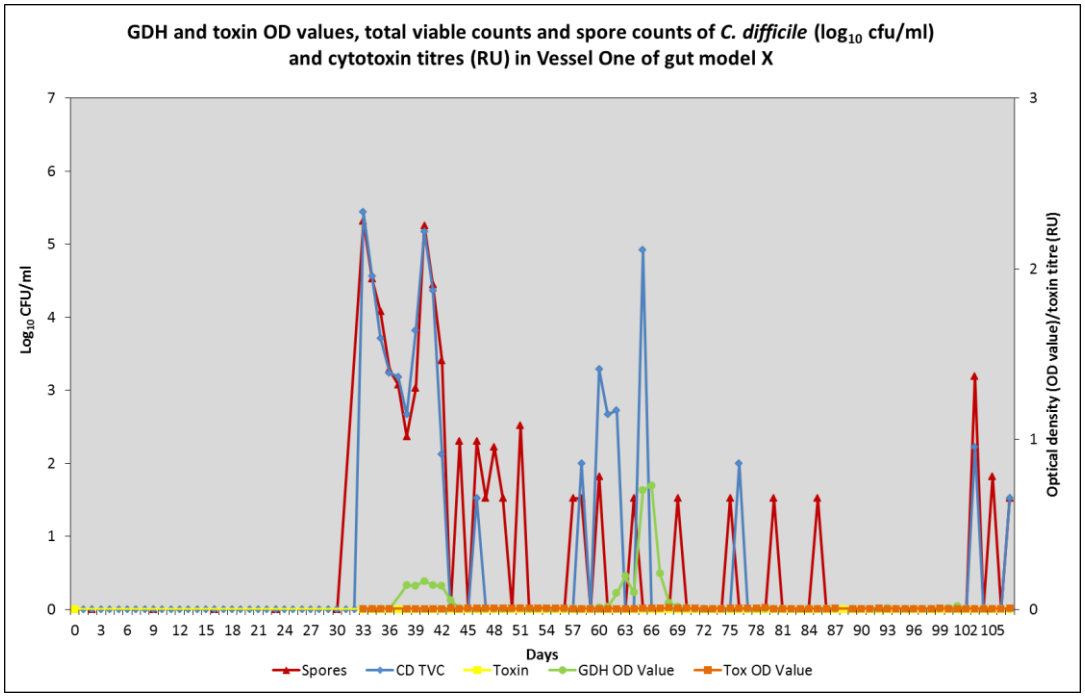


Figure 4.9.a. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model X

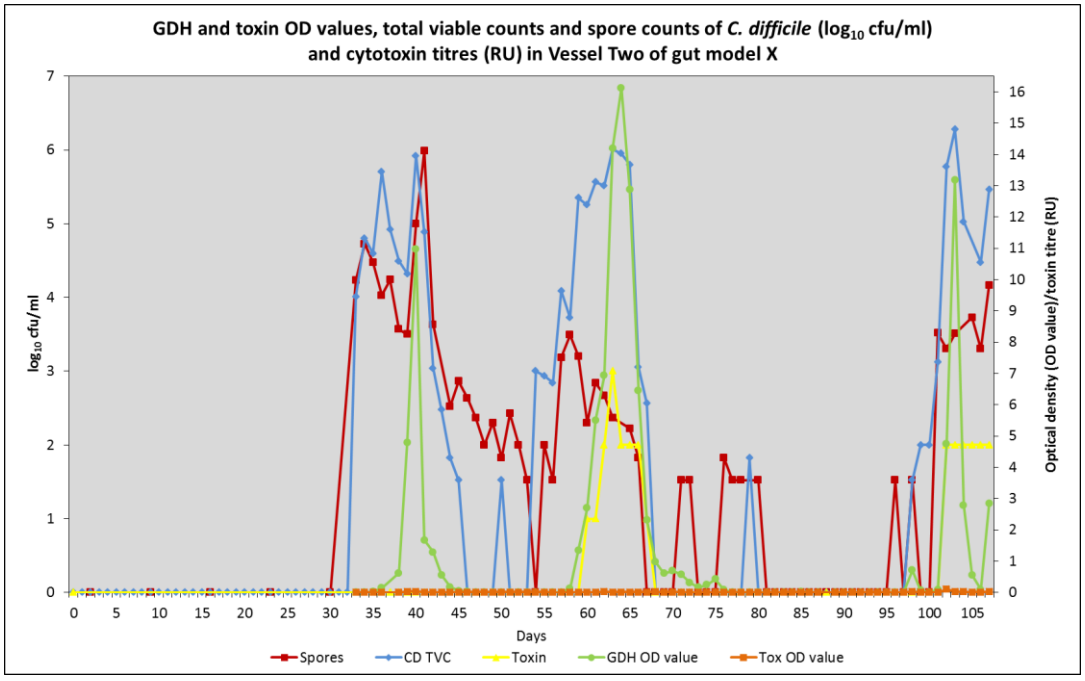


Figure 4.9.b. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model X

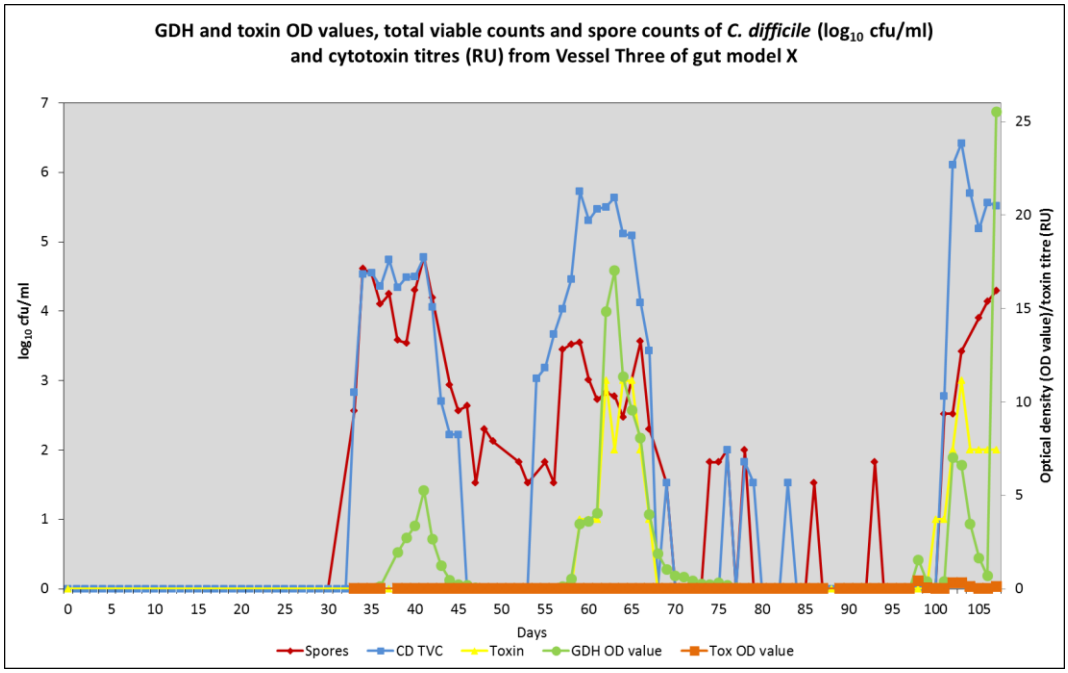


Figure 4.9.c. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model X

4.3.1.7 Measuring *C. difficile* glutamate dehydrogenase and toxins A & B in *in vitro* gut models E. F and G using commercial enzyme immunoassays

Model E

Unlike previous models there was no peak in GDH level seen in any of the three vessels after the instillation of spores (figures 4.10a-c). Germination occurred at Day 53 in all three vessels, with a rise in the total viable count. There appeared to be a lag of three days before the GDH level subsequently starts to rise, although the peak total viable count and GDH level do coincide, along with peak toxin level in Vessels Two and Three. The highest OD level of GDH was seen in Vessel Two (~34) followed by Vessel Three (~24) and then Vessel One (~1.5). It is interesting to note however that there was not a significant difference in the total viable count between the vessels however. The GDH level started to decline after the total viable count was already falling, and in all three vessels GDH could still be detected from the model fluid for ~10 days after the total viable count had fallen below the limit of detection. Whilst this detection of GDH after total viable count has declined had been seen in previous models, this was the longest period for which this lag has occurred. It is important to note that not only could the GDH be detected, *i.e.* it had an

OD raised from baseline, but the OD was also above the cut-off the assay that indicates a 'positive' result (had this been a clinical sample).

There was an episode of recurrence within this model, with the total viable count rising again at Day 100. There was a lag of ~two day in all three vessels before the level of GDH begin to rise, although the peak OD value was much lower than that seen for the first episode.

There was no toxin detected in any of the vessels within this model by either the cell-cytotoxicity assay or the enzyme immunoassay.

Model F

Unlike Model E, but in agreement with earlier models, there was a peak in the level of GDH after the instillation of spores; this peak was higher than previously seen however and GDH could be detected from the gut model fluid in every sample, from all three vessels, from this point on until the end of the simulated CDI episode (figures 4.11a-c). It should be noted however that although the OD was raised in these samples for four days the OD was below that which wold indicate a 'positive' result if the test were used clinically.

Germination occurred around Day 44, with increasing total viable count and GDH level. The peak value for GDH in this episode was the highest seen in any model and coincided with a very high level of toxin (as measured by the cell-cytotoxicity assay). The highest GDH level was seen in Vessel Two (OD = ~250), followed by Vessel Three (~200) and then Vessel One (~10). The total viable count was also slightly higher than previously seen with 6 $\log_{10}$cfu/mL compared with 5 $\log_{10}$cfu/mL seen previously. Unlike Model E, the GDH level fell very quickly, and went below the limit of detection just before the total viable count and toxin also reached an undetectable level.

Recurrence occurred at Day 81; as with Model E, there was an increase in the total viable count followed by an increase in GDH OD value ~three days later. The peak GDH level coincided with the peak of toxin production; toxin levels were very high, with 5RU, compared with 3RU seen in most previous models. The total viable count was also higher than usually seen. Although the toxin level was higher in the recurrent episode the GDH level was lower than the first simulated CDI episode in this model. For example, the peak GDH OD in Episode One in Vessel Two was ~250, but it was ~100 in the recurrent episode. The same pattern of GDH production seen during the first episode was also seen during

recurrence; the highest level was in Vessel Two (~100) followed by Vessel Three (~30) and then Vessel One (0.3). This contrasts with the toxin level and total viable count, however, which were similar across Vessels Two and Three.

Interestingly, unlike previous models, the toxin enzyme immunoassay did detect toxin, and the OD values for the assay followed the rise and fall of toxin production as detected by the cell-cytotoxicity assay.

Model G

There was no rise in the GDH level in any of the vessels after the instillation of spores in Model G (figures 4.12a-c); this was in contrast to most of the models but was also observed in Model E (figures 4.10a-c). Germination was not seen in Vessel One, however there was a small peak in GDH in Vessel One around the time of peak proliferation in the other vessels (figures 4.12a-c). As previously observed, the GDH level rose during germination and peaked with the total viable count and peak toxin production in Vessels Two and Three. The level of GDH was high, although not as high as for Model F, and was higher for Vessel Two (OD= ~ 84) than for Vessel Three (~74). Once again, the GDH level dropped quickly and the OD value went below the limit of detection about two days before the total viable count. In this model, toxin could still be detected from Vessel Two and Three after the total viable count and GDH level were below the limit of detection.

Two days after the toxin was washed out of the system there was a small rise in GDH OD in both Vessels Two and Three, which lasted for two days before dropping again. There was a subsequent rise in total viable and spore counts after two days, but the total viable count did not diverge from the spore count and no toxin was detected; indicating that although the organism is growing in the model, this was not a recurrent episode.

Once again, the toxin enzyme immunoassay detected toxin, and the OD values followed the rise and fall of toxin as detected by the cell-cytotoxicity assay. The level of toxin in this model was similar to those seen in earlier models (3RU), but not as high as seen in Model F (figures 4.11a-c).

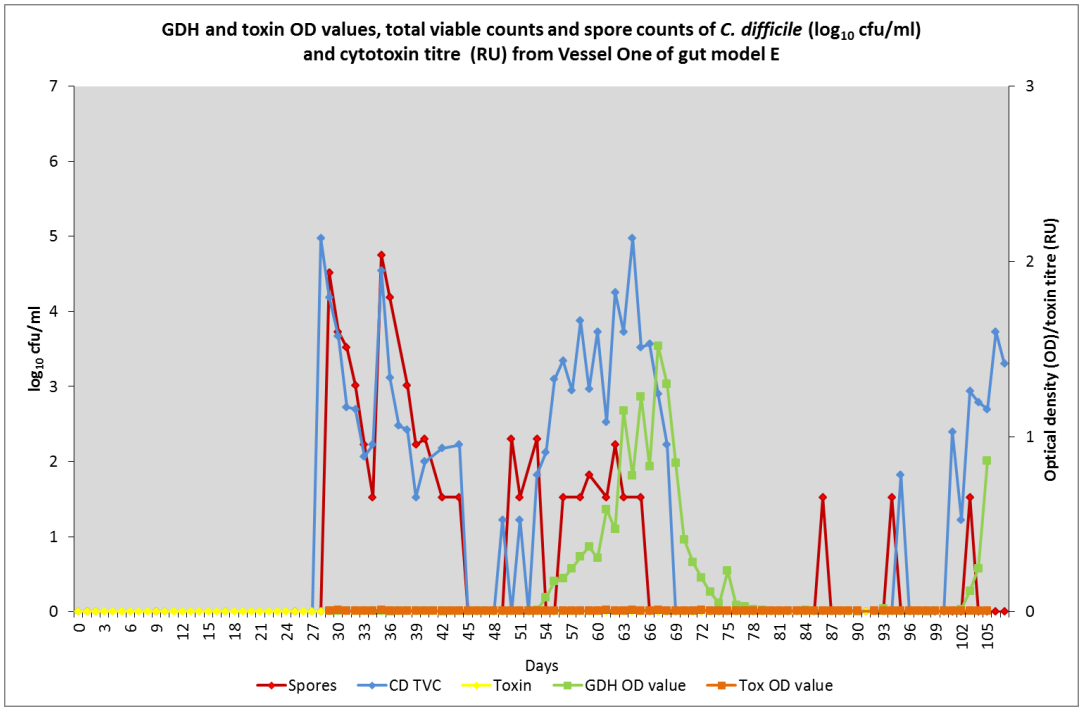


Figure 4.10.a. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model E

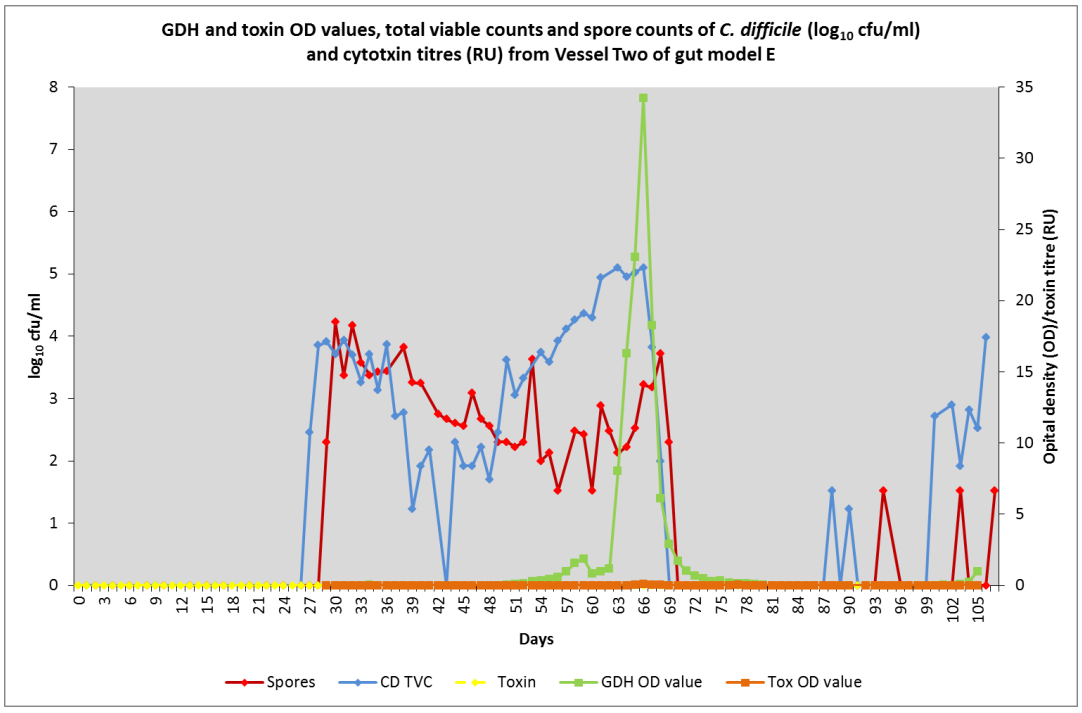


Figure 4.10.b. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model E

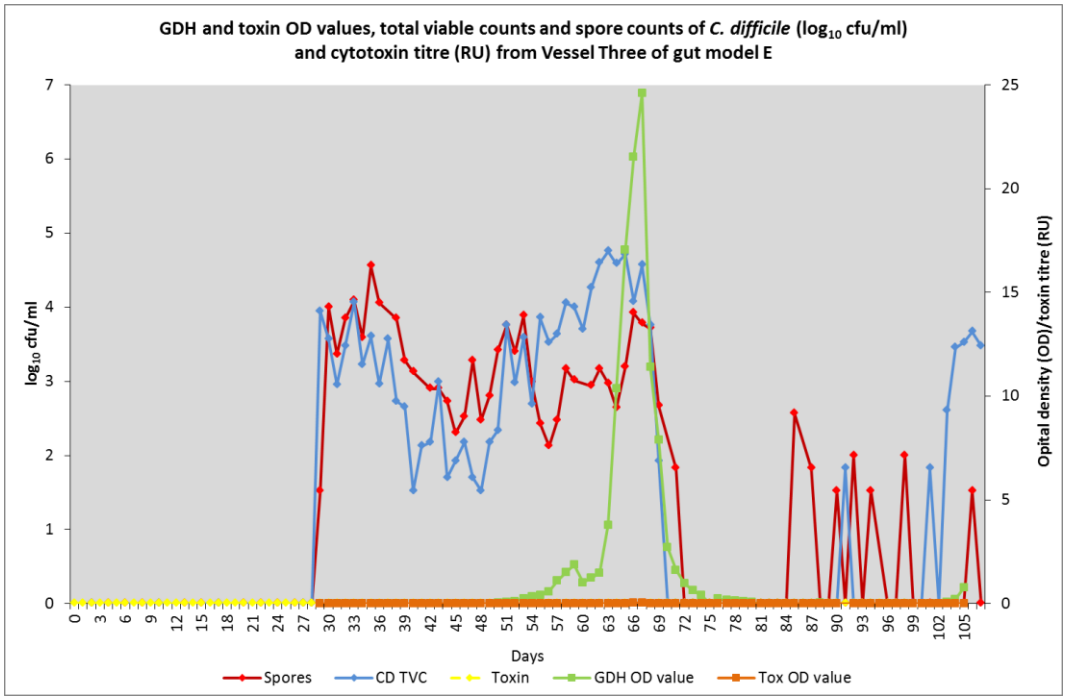


Figure 4.10.c. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model E

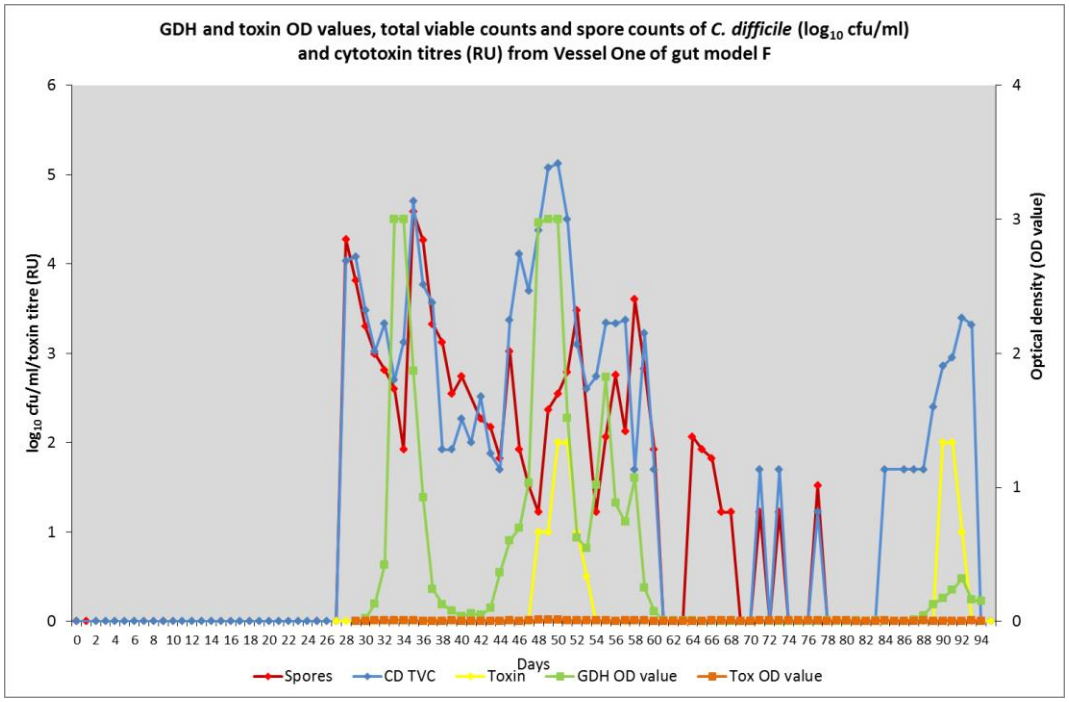


Figure 4.11.a. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model F

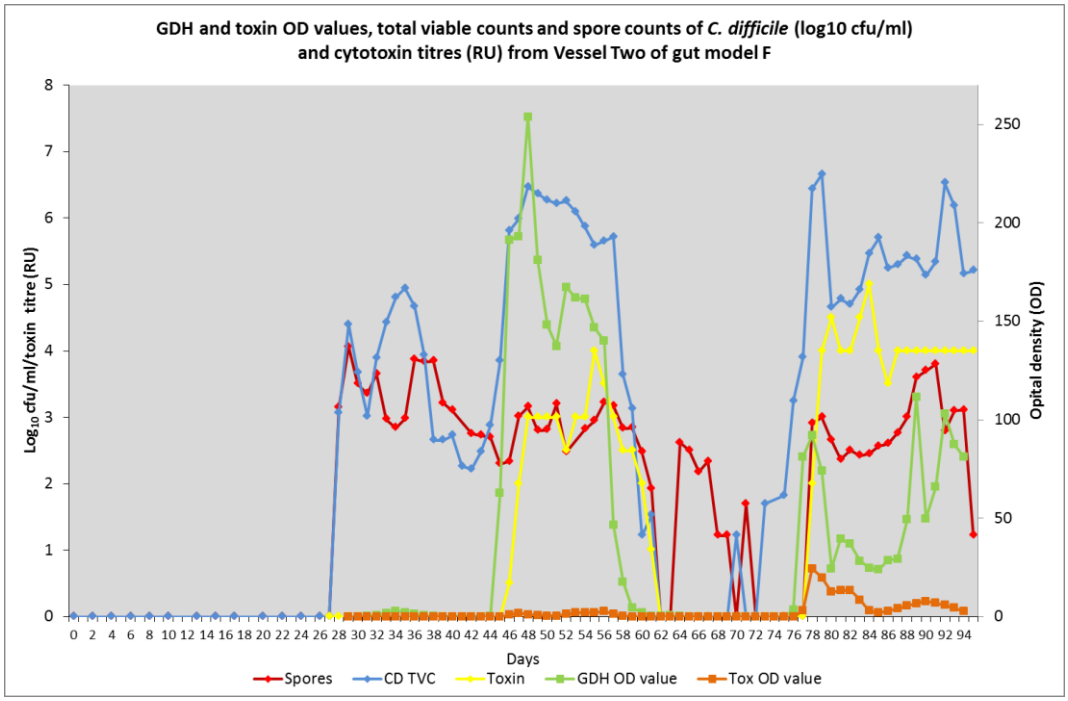


Figure 4.11.b. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model F

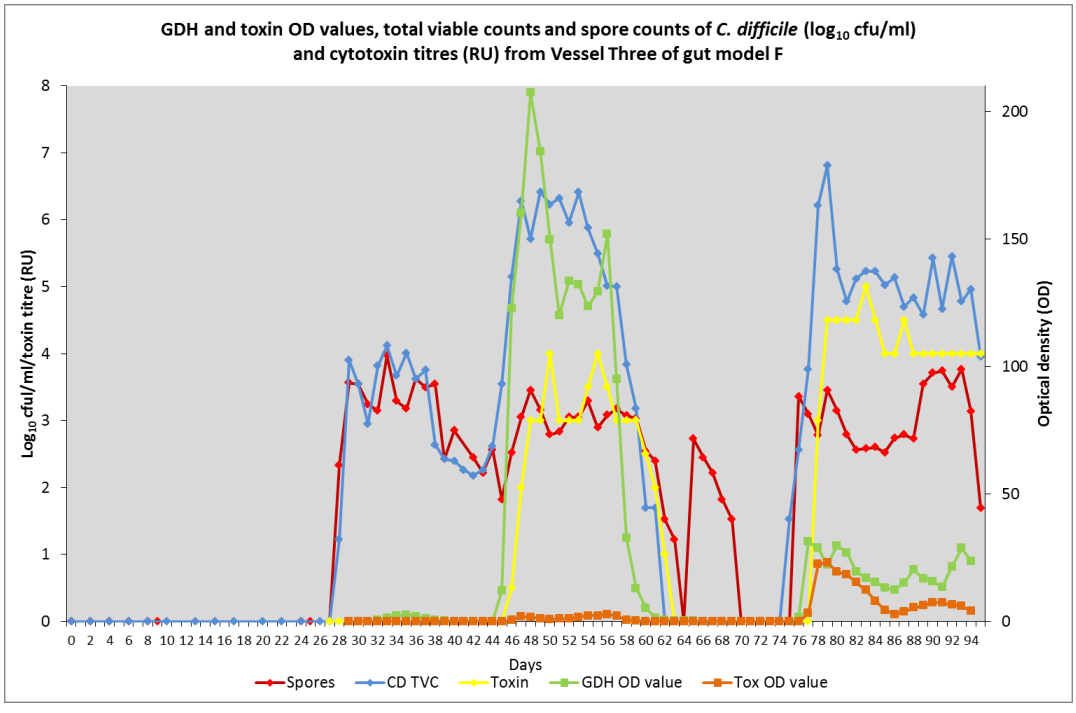


Figure 4.11.c. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model F

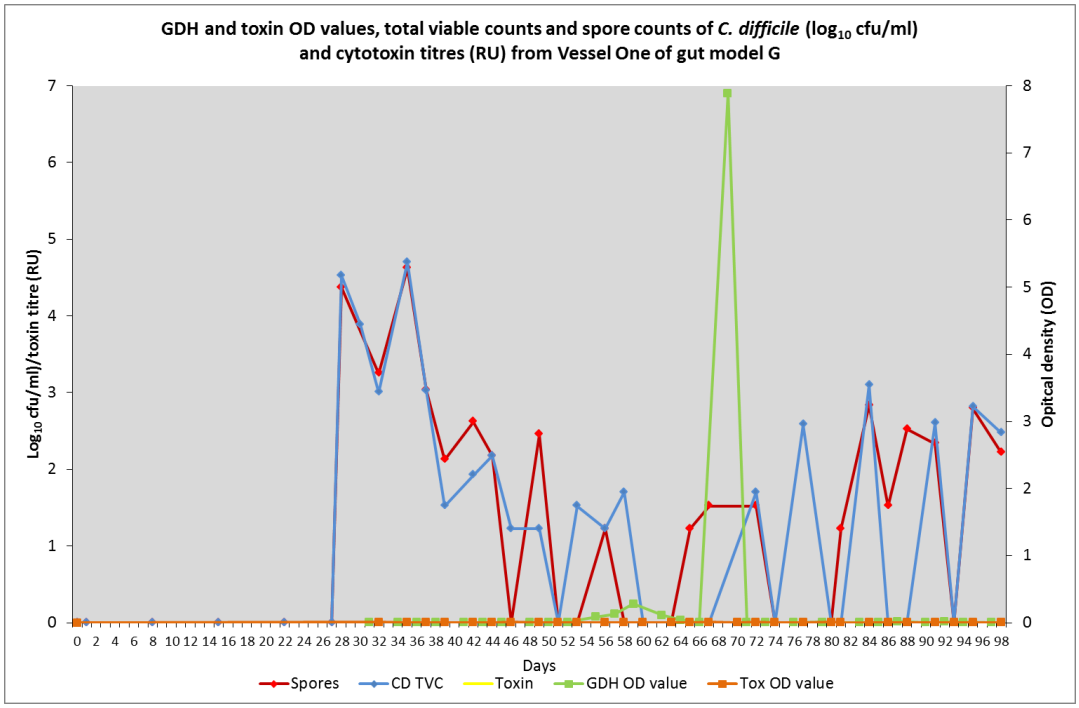


Figure 4.12.a. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel One of gut Model G

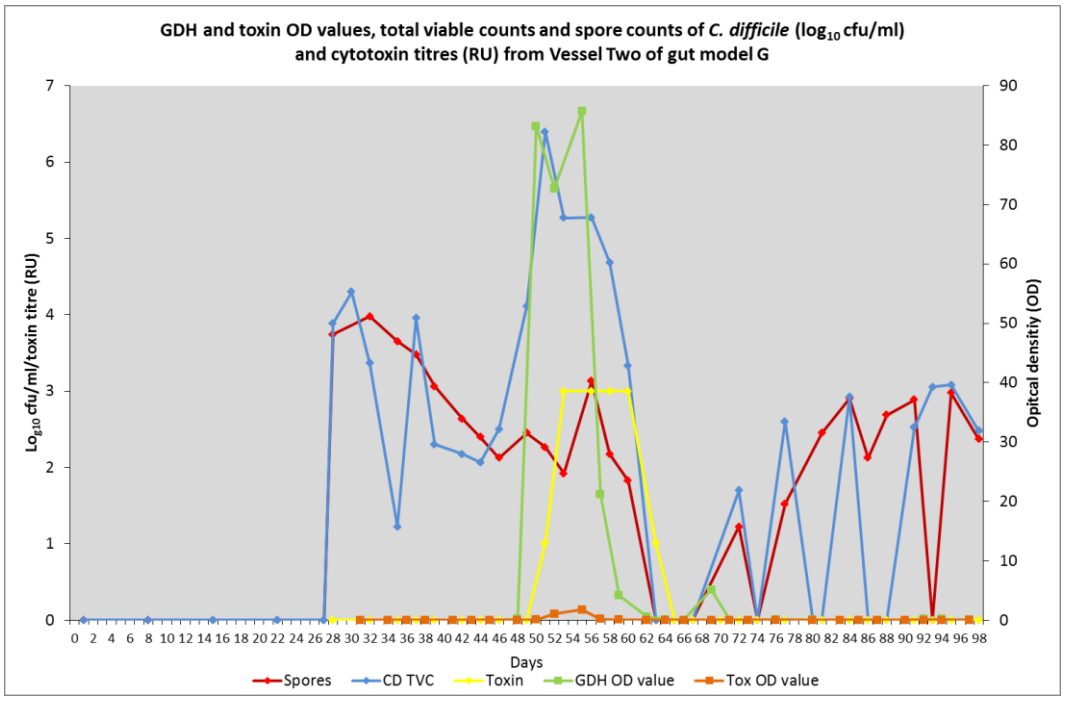


Figure 4.12.b. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Two of gut Model G

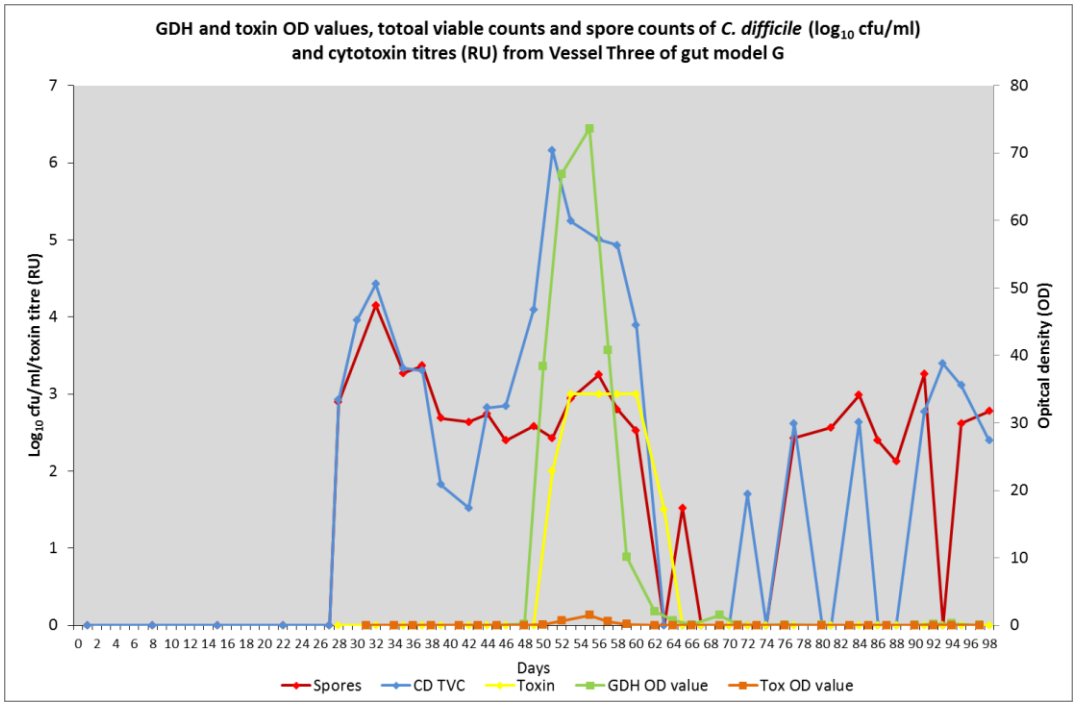


Figure 4.12.c. Spore counts, total viable counts, toxin production and GDH measured by EIA in Vessel Three of gut Model G

4.4 Discussion

The enzyme immunoassay was able to detect GDH from the gut model fluid and showed rise and fall in line with the proliferation of the organism in the model. The *gluD* PCR assay however, produced consistently high CT values, and unfortunately does not appear to be optimised for use in the gut model and so could not help to explain the pattern of rise and fall of GDH. It would be expected that as total viable count increases, the level of *gluD* within the system would also increase but that was not seen with the current *gluD* assay. Further optimisation of this assay is required before it can provide useful information on the proliferation of *C. difficile* within the model (figures 4.3 a-b). Additionally, further work is needed to clarify whether the PCR assay is detecting DNA in spores as well as in vegetative cells and/or free *gluD* in the system (figures 4.3 a-b). In addition, detection of mRNA may also provide insight into the proliferation of *C. difficile*. It is unfortunately outside of the scope of this project to develop the *gluD* assay further, and so this assay was not used for any further gut model experiments.

The bacterial populations within the human gut model have been shown to be very similar to those seen at autopsy in the colon of patients who suffer sudden death, making this

model extremely useful for examining enteric pathogens (Macfarlane *et al*, 1998). The human gut model has been used to observe the effects of antibiotics on the growth and toxin production of *C. difficile* (Freeman *et al*, 2003; Baines *et al*, 2006; Freeman *et al*, 2007; Baines *et al*, 2009; Saxton *et al* 2009; Chilton *et al*, 2014b; Baines *et al*, 2015); the total viable count of *C. difficile* and toxin level rise and consequently fall in phase with each other during simulated CDI. This pattern was repeated for the gut model experiments described here, where the values for the total viable count and toxin production remained in phase (figures 4.1, 4.2, 4.4, 4.5, 4.7-4.12). In contrast, for the first two sets of models, whilst the level of GDH did increase in phase with the total viable count and toxin level, it remained at peak levels in the model for longer. Once the level of GDH began falling, however, it was cleared from the model within the same number of days as the cells and toxin of *C difficile.* This may indicate that GDH is a very stable protein that is not broken down within the system, or possibly that it is sequestered in the model, perhaps in biofilm, and therefore takes longer to be cleared from the system. Alternatively, stores of GDH may be released by dying *C. difficile* cells, 'flooding' the model with GDH and increasing the period during which it can be detected. The gut model is clinically reflective (Freeman *et al*, 2003), suggesting that there could be periods where patient faecal samples could be positive for GDH when there are no viable organisms proliferating within their gut.

Monitoring the level of GDH level in these models was impaired by the maximum threshold OD value for the GDH enzyme immunoassay. Using the assay in its current format does not show the 'true' peak levels of GDH within the model and could therefore be masking any subsequent rise and fall of GDH during this 'plateau' period. Dilution series demonstrated that below the maximum threshold OD of the assay, the results were quantitative, and diluted samples could be multiplied by the dilution factor to overcome the maximum threshold (tables 4.2-4.4, figures 4.6 a-b).

Subsequent gut model experiments showed that the GDH level did indeed continue to increase over the previous maximum threshold OD value, during organism proliferation (figures 4.7-4.12). The peak GDH OD varied between models, and between vessels. The highest GDH level seen in any experiment was those within gut Model F, which had a higher peak total viable count than any other gut model ($7\log_{10}$cfu/mL vs $5\text{-}6\log_{10}$cfu/mL, figures 4.11a-c). Interestingly, this model also had a higher peak toxin level (6 RU vs 3-5 RU). This suggests, that the level of GDH is related to organism bioload within the model, with a higher bioload resulting in a higher level of GDH in the gut model fluid. When

comparing within models, the highest level of GDH was seen in Vessel Two, followed by Vessel Three, and then Vessel One. Given that the total viable counts did not vary significantly between Vessels Two and Three for these models, the GDH level does not appear to be solely related to organism bioload.

When comparing the GDH levels seen in the initial simulated CDI episode and those when there is a recurrent episode (Models E and F), the levels in the recurrent episodes are lower. In Model F, for example, the peak GDH OD in the first episode in Vessel Two was ~250, but was ~100 in the recurrent episode. The peak total viable count and toxin level were actually higher than the first episode in this model, again suggesting that the level of GDH is not solely related to the bioload within the model. In addition, there was a lag from the time the total viable counts began to increase and the GDH subsequently began to increase. This lag period was not as clear for the first episodes in any model compared with the recurrent episodes in Models E and F (figures 4.10a-c and 4.11a-c).

There was often a rise in the level of GDH detected in the models when the spores were added to the system. This rise in GDH was not accompanied by a divergence between the total viable count and the spore count. This suggests that while there may be 'turn over' of the organism within the model, there was not exponential growth and proliferation. This rise in the level of GDH could, therefore, be a result of the organism reaching equilibrium within the model. If so, does this also occur when a human first encounters *C. difficile* spores? Is there a similar period of equilibrium, and could GDH be detected from their faeces at this point? It is possible that the spike in GDH is only seen once after the introduction of *C. difficile* spores to the model, or that if the period of steady state was extended, serial increases and decreases in GDH may be seen, as there is continual 'turnover' of the organism. Unfortunately, the opportunity to sample a gut model with an extended steady state did not present itself within the timescale of this project.

As well as detecting GDH at the beginning of the model, in the absence of proliferation, GDH was also been detected in the absence of any organisms in the model at all; after the total viable count had gone below the limit of detection. In four of the sets of models GDH was the only marker of *C. difficile* that could be detected in the gut model fluid for a median of 7.5 days (range of 2-10 days). However, in two of the models (F and G) the GDH levels fell very quickly and were below the limit of detection before all viable cells are washed out from the model (figures 4.11a-c and 4.12a-c). Interestingly, Model F had the highest level of GDH of any of the models, suggesting that the decline and wash out time of

GDH is not related to the load within the system. Further studies are necessary to clarify release and degradation of GDH in both simple and complex systems. It is possible that GDH is being sequestered in biofilm; such sequestration may account for the delayed clearance of GDH from the system compared with toxin titres and total viable counts. If this is the case, there may therefore have been less biofilm formation in Model F, creating higher loads of GDH in the planktonic system and making it easier to wash the GDH out of the system, after production had ceased.

The toxin enzyme immunoassay did not function very well in detecting toxin within the gut model fluid, in contrast to the cell-cytotoxicity assay. The enzyme immunoassay did detect toxin in the last two models to be tested. As samples had been taken from the models and then frozen before testing, it is possible that freezing the fluid influenced the integrity of the toxin. It is known that toxin can be degraded by repeated freezer/thaw episodes (Freeman and Wilcox, 2003); however, all samples were only thawed once before testing to attempt to alleviate this fact. It is possible therefore that the storage time in the freezer had an effect; those samples with the shortest length of storage were those models tested last, and in which toxin was detected by the enzyme immunoassay.

5. Detection of GDH and toxin from longitudinal patient samples using commercial assays

5.1 Introduction

Many studies have highlighted the variability in performance of CDI laboratory diagnostic assays; however, there is a paucity of data on the reasons for these differences as discussed in section 1.13 of the introduction. In addition, there are also few data on the clinical course of CDI and the optimal time for each of the different diagnostic assays to be used. For instance, depending on the stage of disease a test may 'perform' badly or well as a marker of CDI. The data from gut model experiments of CDI showed marked differences in when the diagnostics targets of *C. difficile* or CDI can be detected (Chapter two). It is not clear however if the performance of the diagnostics assays in these *in vitro* experiments can be translated into their performance during clinical CDI. For example, what does a GDH positive/toxin negative or GDH positive/culture negative result mean for a patient? Does this indicate the presence of disease or the presence of a non-viable organism in the presence of detectable levels of GDH? These potential confounding issues have not been considered when patient samples are tested in laboratory evaluations of CDI tests, even though they may have a bearing on performance.

Here a sequential faecal sampling protocol was used to examine the detection of *C. difficile* targets at different points in the patient's clinical course of infection, and during any potential recurrence. The key findings in this chapter are;

- GDH could be detected from sequential faecal samples collected earlier than faecal samples that were positive for *C. difficile* toxin
- Of the participants recruited as GDH-positive/toxin-negative, 28% went on to have a toxin positive faecal sample, a median of eight days later
- GDH could be detected from sequential samples after other markers of *C. difficile* were no longer detected
- GDH EIA appeared more sensitive than PCR for toxin genes

5.2 Methods

5.2.1 Is the sensitivity of C. difficile diagnostic assays affected by the day on which a patient is tested?

5.2.1.1 Analysis of PlaciD dataset to determine the difference between cases and controls when tested for *C. difficile*, its toxins and its DNA

Hypothesis

There are significant differences between cases of CDI and controls when using different *C. difficile* detection assays over time

Methods

CDI cases (diagnosed toxin positive by the routine microbiology laboratory GDH/CCNA), potential *C. difficile* excretors (PCDE's, diagnosed GDH positive/toxin negative by the routine microbiology laboratory) and controls (≥50 year olds from the same hospital but who had no diarrhoeal symptoms) were recruited and followed during their hospital admission; faecal samples were collected daily where possible with routine clinical data. Samples were tested using culture for *C. difficile*, cell-cytotoxicity assay (CCNA), cytotoxigenic culture (CTC), enzyme immunoassays (EIAs) for glutamate dehydrogenase (GDH) and toxins (Chek-60 and ToxABII, Techlab, USA) and PCR for toxin genes (BD Max™, BD). Kerrie Davies was the study chief investigator for the PlaciD study and devised the study, wrote the study protocol and gained ethical approval, designed the data collection and study databases and analysed all the data. Patients were recruited by research nurses, who also collected patient samples and data. All samples were tested by Kerrie Davies, except for a four month period of maternity leave, when samples were tested by Claire Berry, Clinical Scientist within the Healthcare Associated Infections research group.

Commercial enzyme immunoassays

All of the commercial EIAs were performed on an automated platform (DS2, Dynex Magellan Biosciences, USA) to reduce operator error, following manufacturer's protocols given in the kit insert, with minor changes listed within materials and methods chapter section 2.2, and as discussed with the manufacturer. The optical density (OD) of each test was recorded as well as the result defined by the assay (using manufacturer set cut-offs).

Reference methods

The cell-cytotoxicity neutralisation assay, culture and cytotoxigenic culture assay were performed as described in materials and methods chapter, section 2.1.

Commercial molecular test

The commercial molecular test was performed on a BD Max™ instrument following the protocol in the manufacturer's kit insert, as described in material and methods chapter section 2.2.5.

PCR ribotyping

The *C. difficile* isolates identified from the study were typed using PCR ribotyping by the *C. difficile* Ribotyping Network of England and Northern Ireland (CDRN) as previously described (Stubbs *et al*, 1999) and as expounded in materials and methods chapter section 2.1.6.

Analyses:

Positive results for each *C. difficile* assay, white cell counts, serum albumin and serum creatinine values were compared between cases, PCDE's and controls. Patient demographics, antibiotic use and proton pump inhibitor (PPI) use were compared between cases, PCDE's and controls. For cases and PCDE's, day 0 was defined as the day of the routine sample that allowed entry into the study. For controls, day 0 was defined as the day of entry into the study.

Means are displayed for continuous variables, medians where data is not normalised. Continuous variables were compared by *t* test for independent samples or Mann-Whitney (non-parametric data); categorical variables were compared by χ^2 or Fisher's exact.

Sensitivity, specificity, positive and negative predictive values were calculated for each assay compared with the case definition (diagnostic sample result), CCNA, culture and CTC.

5.3 Results

5.3.1 Patient recruitment

There were 342 patients recruited to the study; 167 GDH positive (both toxin negative and positive) and 175 controls. However, faecal samples were received from only 147 GDH positive patients (both toxin negative and positive) and 81 controls, totalling 1173 sampling days. Of the 147 GDH positive patients, 65 had no detectable toxin in their initial routine diagnostic specimen; they were therefore designated PCDE's. No controls became CDI positive during their stay.

5.3.2 Patient demographics

Controls recruited to the study were significantly older than those recruited as GDH positive (80 Vs 71 years respectively, p <0.0001) (table 5.1). This was still true once the participants without samples in the study had been removed, with median ages for those with at least one sample included in the study of 80 years and 70 years for controls and GDH positive participants respectively (p <0.0001). CDI cases were significantly older than PCDEs (median age 75 *vs* 62 years respectively, P <0.0001). There were no significant differences in the gender of participants either recruited or with at least one sample in the study. However, there was a significantly higher proportion of females in the PCDEs than in the CDI cases (60% *vs* 40.2% respectively, *P* = 0.03).

A significantly higher proportion of participants recruited as GDH positive had at least one antibiotic in the previous eight weeks when compared to participants recruited as controls (90.7% *vs* 47.7%, P <0.0001). This was also true for all participants with at least one sample in the study (controls = 50.6% vs GDH positive 91.9%, p <0.0001). CDI cases were significantly more likely to have had an antibiotic in the previous eight weeks than PCDEs, although the proportions in both groups were high (96.3% Vs 84.6% respectively, p = 0.03). There was no significant difference between the proportion of recruited controls that had a previous drug (other than an antibiotic) in the previous eight weeks compared with recruited GDH positive patients; there was also no significance difference when only those with at least one sample in the study were compared, although the proportion in GDH positive patients was higher in both cases. PCDEs had a higher proportion of patients that had been taking a drug other than an antibiotic in the previous eight weeks compared to CDI cases, but this was not significant (72.3% Vs 64.6%, p = 0.19).

Most patients did not want to have a rectal swab taken (90.9%), and the proportion of those refusing was not significantly different between those recruited as controls or those recruited as GDH positive. Interestingly of those patients that had at least one sample in the study, a significantly higher proportion of GDH positive patients refused the rectal swab than control patients (91.2% Vs 79.0%, p = 0.03). Although there was a higher rate of refusal in the CDI cases than PCDEs this was not significant (96.3% Vs 84.6%, p = 0.19).

	Recruited to the study			Had at least one sample in the study			Had at least one sample in the study and initial diagnostic specimen was GDH positive		
	Control (n = 175)	GDH positive (n = 167)	Significance (*p*)	Control (n = 81)	GDH positive (n = 147)	Significance (*p*)	PCDE (n = 65)	CDI case (n = 82)	Significance (*p*)
Age									
median	80	71	<0.0001	80	70	<0.0001	62	75	<0.0001
mean	79	69	<0.0001	78	68	<0.0001	62	74	0.016
min	41	18		55	18		18	27	
max	100	101		100	101		101	101	
Gender									
Female	92	80	0.41	44	72	0.53	39	33	0.03
Male	77	82		37	75		26	49	
unknown	6	5							
Had a rectal swab taken									
No	150	151	0.24	64	133	0.03	56	77	
Yes	25	16		17	14		9	5	0.19
Had an antibiotic in the previous 8 weeks									
Yes	80	147	<0.0001	41	134	<0.0001	55	79	0.03
No	89	15		40	13		10	3	
unknown	6	5							
Had a drug (other than an antibiotic in the previous 8 weeks)									

Yes	104	112	0.08	48	100	0.15	47	53	0.19
No	64	44		32	42		14	28	
unknown	7	9		1	5		4	1	

Table 5.1. Patient demographics of all cases and controls, regardless of if they had samples in the study and if they had at least one sample included in the study. Those patients that had at least one GDH positive sample are divided into potential *C. difficile* excretors (PCDEs) and CDI cases based on the presence or absence of *C. difficile* toxins in the original routine sample.

For all recruited participants, those in the control arm were significantly more likely to have been admitted following a fall than those recruited into the GDH positive arm (p <0.0001) (table 5.2). In addition, participants in the control arm were significantly more likely than GDH positive participants to be admitted with a fractured neck of femur, fracture of the knee, fracture of the shaft of the femur and septic arthritis (table 5.2). Participants recruited into the GDH positive arm were significantly more likely than control participants to have been admitted with diarrhoea, diarrhoea and vomiting, and abdominal pain (table 5.3).

For all participants that had at least one sample included in the study those in the control arm were significantly more likely to have been admitted following a fall than those recruited into the GDH positive arm (p <0.0001) (table 5.2). In addition, participants in the control arm were significantly more likely than GDH positive participants to be admitted with a fractured neck of femur, fracture of the knee, fracture of the shaft of the femur and septic arthritis (table 5.2). Participants recruited into the GDH positive arm were significantly more likely than control participants to have been admitted with diarrhoea, diarrhoea and vomiting, abdominal pain, with pyrexia, and for being 'unwell' (table 5.3). GDH positive participants with CDI were significantly more likely to be admitted for being 'unwell' than PCDE participants.

Control participants recruited into the study were significantly more likely than GDH positive participants to have a past medical history of cancer, dementia and hypertension (table 5.4). However, participants recruited as GDH positive were significantly more likely than control participants to have a past medical history of alcoholism, asthma, pancreatitis and renal failure (table 5.5). Once only participants that had at least one sample in the study were compared, only a past medical history of dementia remained significantly more likely in control participants than GDH participants (table 5.4). Alcoholism and asthma remained as the only conditions listed in past medical histories that were significantly associated with GDH participants compared with control participants (table 5.5). CDI participants were significantly more likely to have had angina, heart failure and epilepsy than PCDE's (table 5.5).

	Recruited to the study				Had at least one sample in the study				Had at least one sample in the study and initial diagnostic specimen was GDH positive			
	Control n (%)	GDH positive n (%)	Total n	Significance (*p*)	Control n (%)	GDH positive n (%)	Total n	Significance (*p*)	PCDE n (%)	CDI n (%)	Total n	Significance (*p*)
Fall	87 (49.7)	13 (7.7)	100	<0.0001	36 (44.4)	11 (7.5)	47	<0.0001	3 (4.6)	8 (9.7)	11	0.20*
Fractured neck of femur	58 (33.0)	2 (1.2)	60	<0.0001	17 (21.9)	2 (1.4)	19	<0.0001	0 (0.0)	2 (2.4)	2	0.31*
Planned surgery	9 (5.1)	19 (11.4)	28	0.06	7 (8.6)	18 (22.2)	25	0.54	11 (16.9)	7 (8.5)	18	0.20
Fracture of the Knee	7 (4.0)	0 (0.0)	7	0.009*	5 (6.1)	0 (0)	5	0.005*	0 (0)	0 (0.0)	0	N/A
Fracture of the shaft of the femur	7 (4.0)	0 (0.0)	7	0.009*	5 (6.1)	0 (0)	5	0.005*	0 (0.0)	0 (0.0)	0	N/A
Not known	7 (4.0)	0 (0.0)	7	0.009*	1 (1.2)	0 (0.0)	1	0.36*	0 (0.0)	0 (0.0)	0	N/A
Septic arthritis	6 (3.4)	0 (0.0)	6	0.017*	4 (4.9)	0 (0)	4	0.015*	0 (0.0)	0 (0.0)	0	N/A
Total	175	167			81	147			65	82		

Table 5.2. The top five reasons for admission (by n size) for control patients that were recruited to the study, regardless of if they had samples in the study and if they had at least one sample included in the study. Those patients that had at least one GDH positive sample are divided into potential *C. difficile* excretors (PCDEs) and CDI cases based on the presence or absence of *C. difficile* toxins in the original routine sample. Full table of admission for all participants are shown in appendix table A3.1. Each participant could have more than one reason for admission recorded. *indicates where Fishers exact tests was used instead of χ^2

	Recruited to the study				Had at least one sample in the study				Had at least one sample in the study and initial diagnostic specimen was GDH positive			
	Control n (%)	GDH positive n (%)	Total n	Significance (*p*)	Control n (%)	GDH positive n (%)	Total n	Significance (*p*)	PCDE n (%)	CDI n (%)	Total n	Significance (*p*)
Diarrhoea[abc]	1 (0.6)	21 (12.6)	22	<0.0001	1 (1.2)	19 (12.9)	20	0.006	6 (9.2)	13 (15.9)	19	0.34
Planned surgery[abc]	9 (5.1)	19 (11.4)	28	0.06	7 (8.6)	18 (12.2)	25	0.54	11 (16.9)	7 (8.5)	18	0.20
Diarrhoea and vomiting[abc]	0 (0.0)	14 (8.4)	14	0.0003	0 (0)	14 (9.5)	14	0.002*	7 (10.8)	7 (8.5)	14	0.43
Fall[ac]	87 (49.7)	13 (7.8)	100	<0.0001	36 (44.4)	11 (7.5)	47	<0.0001	3 (4.6)	8 (9.8)	11	0.20*
Abdominal pain[abc]	1 (0.6)	10 (5.0)	11	0.011*	1 (1.2)	9 (6.1)	10	0.07*	4 (6.1)	5 (6.1)	9	0.62*
Shortness of breath[b]	N/A	N/A			3 (0.4)	7 (4.8)	10	0.49*	5 (7.6)	2 (2.4)	7	0.13*
Pyrexia[c]	N/A	N/A			0 (0)	7 (4.8)	7	0.04*	2 (3.0)	5 (6.1)	7	0.32*
Unwell[c]	N/A	N/A			0 (0)	6 (4.1)	6	0.07*	0 (0.0)	6 (7.3)	6	0.03*
Total	175	167			81	147			65	82		

Table 5.3. The top five reasons for admission (by n size) for [a]GDH positive patients that were recruited to the study , [b]PCDEs or [c]CDI patients, regardless of if they had samples in the study and if they had at least one sample included in the study. Those patients that had at least one GDH positive sample are divided into potential *C. difficile* excretors (PCDEs) and CDI cases based on the presence or absence of *C. difficile* toxins in the original routine sample. Full table of admission for all participants are shown in appendix table A3.1. Each participant could have more than one reason for admission recorded. *indicates where Fishers exact tests was used instead of χ^2

	Recruited to the study				Had at least one sample in the study				Had at least one sample in the study and initial diagnostic specimen was GDH positive			
	Control n (%)	GDH positive n (%)	Total n	Significance (*p*)	Control n (%)	GDH positive n (%)	Total n	Significance (*p*)	PCDE n (%)	CDI n (%)	Total n	Significance (*p*)
Atrial fibrillation[ab]	15 (8.6)	13 (7.8)	28	1.0	10 (12.3)	13 (8.8)	23	0.54	3 (4.6)	10 (12.2)	13	0.19
Cancer[ab]	28 (16.0)	41 (24.6)	69	0.07	14 (17.3)	39 (27.5)	53	0.16	22 (33.8)	17 (20.7)	38	0.11
CABG[ab]	10 (5.7)	5 (3.0)	15	0.33	5 (6.2)	3 (2.0)	8	0.10*	1 (1.5)	2 (2.4)	3	0.67*
CKD[ab]	10 (5.7)	3 (1.8)	13	0.11	5 (6.2)	3 (2.0)	8	0.10*	0 (0.0)	3 (3.7)	3	0.33*
COPD[ab]	12 (6.9)	10 (6.0)	22	0.92	4 (4.9)	10 (6.8)	14	0.40*	6 (9.2)	4 (4.9)	10	0.24*
Dementia[ab]	15 (8.6)	3 (1.8)	18	0.01	6 (7.4)	3 (2.0)	9	0.05*	0 (0.0)	3 (3.7)	3	0.33*
Diabetes[ab]	33 (18.9)	30 (18.0)	63	0.92	19 (23.5)	26 (17.7)	45	0.38	9 (13.8)	19 (23.2)	28	0.22
Heart disease[ab]	14 (8.0)	8 (4.8)	22	0.32	7 (8.6)	6 (4.8)	13	0.19*	1 (1.5)	6 (7.3)	7	0.10*
Hypertention[ab]	55 (31.4)	34 (20.4)	89	0.02	22 (27.2)	30 (20.4)	52	0.32	13 (20.0)	18 (22.0)	31	0.92
Hypothyroid[ab]	10 (5.7)	5 (3.0)	15	0.33	3 (3.7)	4 (2.7)	7	0.48*	1 (1.5)	3 (3.7)	4	0.63*
MI[ab]	15 (8.6)	8 (4.3)	23	0.24	8 (9.9)	8 (5.4)	16	0.32	5 (7.7)	3 (3.7)	8	0.24*
Ostearthritis[ab]	15 (8.6)	13 (7.8)	28	1.0	6 (7.4)	12 (8.2)	18	1.0	5 (7.7)	7 (8.5)	12	0.82
Osteoporosis[ab]	11 (6.2)	4 (2.4)	15	0.13	4 (4.9)	4 (2.7)	8	0.30*	0 (0.0)	4 (4.9)	4	0.22*

Rheumatoid[ab]	9 (5.1)	8 (4.8)	17	0.92	6 (7.4)	8 (5.4)	14	0.37*	4 (6.2)	4 (4.9)	8	0.51*
Stroke[ab]	17 (9.7)	15 (9.0)	32	1.0	6 (7.4)	11 (7.5)	17	0.80	3 (4.6)	8 (9.8)	11	0.54*
Angina[b]	N/A	N/A	N/A		3 (3.7)	3 (2.0)	6	0.36*	4 (6.2)	2 (2.4)	6	0.07*
Epilepsy[b]	N/A	N/A	N/A		4 (5.0)	7 (4.8)	11	0.59*	5 (7.7)	2 (2.4)	7	0.03*
na[b]	N/A	N/A	N/A		3 (3.7)	2 (1.4)	5	0.24*	0 (0.0)	2 (2.4)	2	0.48*
PVD[b]	N/A	N/A	N/A		3 (3.7)	3 (2.0)	6	0.36*	2 (3.1)	1 (1.2)	3	0.22*
Total	175	167			81	147			65	82		

Table 5.4. The top ten conditions listed within past medical history (by n size) for [a]control patients that were recruited to the study regardless of if they had samples in the study and [b]if they had at least one sample included in the study. Those patients that had at least one GDH positive sample are divided into potential *C. difficile* excretors (PCDEs) and CDI cases based on the presence or absence of *C. difficile* toxins in the original routine sample. Full table of past medical history for all participants are shown in appendix table A3.2. Each participant could have more than one reason for admission recorded. *indicates where Fishers exact tests was used instead of χ^2

	Recruited to the study				Had at least one sample in the study				Had at least one sample in the study and initial diagnostic specimen was GDH positive			
	Control n (%)	GDH positive n (%)	Total n	Significance (p)	Control n (%)	GDH positive n (%)	Total n	Significance (p)	PCDE n (%)	CDI n (%)	Total n	Significance (p)
Alcoholic[abc]	4 (2.3)	14 (8.4)	18	0.02	1 (1.2)	12 (8.2)	13	0.02*	5 (7.7)	6 (7.3)	11	0.59*
Asthma[abcd]	5 (2.9)	20 (12.0)	25	0.002	2 (2.5)	18 (12.2)	20	0.02	9 (13.3)	9 (11.0)	18	0.78
Atrial fibrillation[abd]	15 (8.6)	13 (7.8)	28	1.0	10 (12.3)	13 (8.8)	23	0.54	3 (4.6)	10 (12.2)	13	0.19
Cancer[abcd]	28 (16.0)	41 (24.6)	69	0.07	14 (17.3)	39 (27.5)	53	0.16	22 (33.8)	17 (20.7)	38	0.11
COPD[abc]	12 (6.9)	10 (6.0)	22	0.92	4 (4.9)	10 (6.8)	14	0.40*	6 (9.2)	4 (4.9)	10	0.24*
Diabetes[abcd]	33 (18.9)	30 (18.0)	63	0.92	19 (23.5)	26 (17.7)	45	0.38	9 (13.8)	19 (23.2)	28	0.22
Epilepsy[abc]	5 (2.9)	7 (4.2)	12	0.71	4 (4.9)	7 (4.8)	11	0.59*	5 (7.7)	2 (2.4)	7	0.14*
Heart disease[a]	14 (8.0)	8 (4.8)	22	0.32	7 (8.6)	7 (4.8)	14	0.19*	1 (1.5)	6 (7.3)	7	0.10*
Hypertention[abcd]	55 (31.4)	34 (20.4)	89	0.02	22 (27.2)	30 (20.4)	52	0.32	13 (20.0)	18 (22.0)	31	0.92
MI[abc]	15 (8.6)	8 (4.3)	23	0.24	8 (9.9)	8 (5.4)	14	0.32	5 (7.7)	3 (3.7)	8	0.24
Ostearthritis[abc]	15 (8.6)	13 (7.8)	28	1.0	6 (7.4)	12 (8.2)	18	1.0	5 (7.7)	7 (8.5)	12	0.92
Pancreatitis[a]	1 (0.6)	7 (4.2)	8	0.02*	0 (0.0)	5 (3.4)	5	0.11*	3 (4.6)	2 (2.4)	5	0.39*
Renal failure[ab]	2 (1.1)	8 (4.8)	10	0.04*	1 (1.2)	8 (5.4)	16	0.11*	4 (6.2)	4 (4.9)	8	0.51*

Rheumatiod[ab]	9 (5.1)	8 (4.8)	17	0.92	6 (7.4)	8 (5.4)	9	0.37*	4 (6.2)	4 (4.9)	8	0.51*
Stroke[ab]	17 (9.7)	15 (9.0)	32	1.0	6 (7.4)	11 (7.5)	17	0.81	3 (4.6)	8 (9.8)	11	0.20*
Heart failure[b]	N/A	N/A			1 (1.2)	8 (5.4)	9	0.11*	0 (0.0)	5 (6.1)	5	0.02*
Ulcerative colitis[c]	N/A	N/A			N/A	N/A			5 (7.7)	1 (1.2)	6	0.08*
Total	175	167			81	147			65	82		

Table 5.5. The top ten conditions listed within past medical history (by n size) for [a]GDH positive patients that were recruited to the study , [b]that had at least one sample in the study, [c]PCDEs or [d]CDI patients, regardless of if they had samples in the study and if they had at least one sample included in the study. Those patients that had at least one GDH positive sample are divided into potential *C. difficile* excretors (PCDEs) and CDI cases based on the presence or absence of *C. difficile* toxins in the original routine sample. Full table of past medical history for all participants are shown in appendix table A3.2. Each participant could have more than one reason for admission recorded. *indicates where Fishers exact tests was used instead of χ^2

5.3.3 Participants with at least one sample in the study

The study data was only complete for participants that submitted at least one sample into the study, as these are the only participants who could have samples tested with the diagnostic assays. The following tables therefore only include these participants.

The proportion of participants that received at least one dose of antibiotics was significantly higher for GDH positive samples compared with control participants (table 5.6). There was also a significant difference between control participants and GDH positive participants for each number of doses of antibiotic received and for the total number of doses of antibiotic received (table 5.6). The proportion of CDI cases that had at least one antibiotic was significantly higher compared with PCDE's, and the total number of doses received was significantly higher for CDI cases compared with PCDEs. There was however no significant difference between the proportions of participants that received larger numbers of doses (table 5.6). The most commonly prescribed antibiotics for control and GDH positive participants are shown in tables 5.7 and 5.8. Control participants were significantly more likely to have been prescribed gentamicin compared with GDH positive participants. In contrast, GDH positive participants were significantly more likely to have the following antibiotics prescribed in the previous three months compared with control participants; ciprofloxacin, co-trimoxazole, fidaxomicin, meropenem, metronidazole, pipercillin-tazobactam, trimethoprim, vancomycin and the antifungal fluconazole (table 5.7). CDI cases were significantly more likely to have the following antibiotics prescribed in the previous three months compared with PCDEs; fidaxomicin, metronidazole and vancomycin (table 5.8). PCDEs were significantly more likely to have been prescribed the antifungal fluconazole in the previous three months compared with CDI cases.

	Had at least one sample in the study			Had at least one sample in the study and initial diagnostic specimen was GDH positive		
Number of antibiotic doses	Control n (%)	GDH positive n (%)	Significance (*p*)	PCDE n (%)	CDI n (%)	Significance (*p*)
One	41 (50.6)	134 (91.2)	<0.0001	55 (84.6)	79 (96.3)	0.03
Two	22 (27.2)	106 (72.1)	<0.0001	43 (66.2)	63 (76.8)	0.2
Three	11 (13.6)	64 (43.5)	<0.0001	25 (38.5)	39 (47.6)	0.4
Four	6 (7.4)	44 (29.9)	0.0002	21 (32.3)	23 (28.0)	0.7
Five	0 (0.0)	27 (18.4)	<0.0001	12 (18.5)	15 (18.3)	0.8
Six	0 (0.0)	16 (10.9)	0.005	7 (10.8)	9 (11.0)	0.8
Seven	0 (0.0)	7 (4.8)	0.04*	2 (3.1)	5 (6.1)	0.33*
Eight	0 (0.0)	1 (0.7)	0.6*	0 (0.0)	1 (1.2)	0.56*
Nine	0 (0.0)	1 (0.7)	0.6*	0 (0.0)	1 (1.2)	0.56*
Ten	0 (0.0)	1 (0.7)	0.6*	0 (0.0)	1 (1.2)	0.56*
Eleven	0 (0.0)	1 (0.7)	0.6*	0 (0.0)	1 (1.2)	0.56*
Twelve	0 (0.0)	1 (0.7)	0.6*	0 (0.0)	1 (1.2)	0.56*
Total number of doses	80	403	<0.0001	165	238	<0.0001

Table 5.6. Number of antibiotic doses each participant received in the three months prior to recruitment into the study

	Had at least one sample in the study			Had at least one sample in the study and initial diagnostic specimen was GDH positive		
	Control n (%)	GDH positive n (%)	Significance (p)	PCDE n (%)	CDI n (%)	Significance (p)
Aztreonam	3 (3.7)	11 (7.5)	0.20*	7 (10.8)	4 (4.9)	0.15*
Amoxicillin	4 (4.9)	12 (8.2)	0.52	5 (7.7)	7 (8.5)	0.92
Clarithromycin	3 (3.7)	7 (4.8)	0.50*	2 (3.1)	5 (6.1)	0.33*
Co- amoxiclav	12 (14.8)	27 (18.4)	0.62	11 (16.9)	16 (19.5)	0.84
Flucloxacillin	13 (16.0)	15 (10.2)	0.28	6 (9.2)	9 (11.0)	0.92
Gentamicin	5 (6.2)	0 (0.0)	0.005*	0 (0.0)	0 (0.0)	-
Linezolid	3 (3.7)	2 (1.4)	0.24	1 (1.5)	1 (1.2)	0.69
Metronidazole	3 (3.7)	82 (55.8)	<0.0001	26 (40.0)	56 (68.3)	0.001
Nitrofurantoin	4 (4.9)	8 (5.4)	0.57*	0 (0.0)	4 (4.9)	0.09*
Pipercillin tazobactam	5 (6.2)	46 (31.3)	<0.0001	22 (33.8)	24 (29.3)	0.68
Tazocin	5 (6.2)	18 (12.2)	0.22	8 (12.3)	10 (12.2)	0.86
Teicoplanin	5 (6.2)	3 (2.0)	0.11*	2 (3.1)	1 (1.2)	0.41*
Vancomycin	3 (3.7)	40 (27.2)	<0.0001	10 (15.4)	30 (36.6)	0.007

Table 5.7. Top ten (by n size) antibiotics taken by the control participants in the three months prior to the study, for all recruited participants that had at least one sample taken in the study. *indicates where Fishers exact tests was used instead of χ^2. Full table of antibiotic history for all participants is shown in appendix table A3.3

	Had at least one sample in the study			Had at least one sample in the study and initial diagnostic specimen was GDH positive		
	Control n (%)	GDH positive n (%)	Significance (p)	PCDE n (%)	CDI n (%)	Significance (p)
Aztreonam[a]	3 (3.7)	11 (7.5)	0.20*	7 (10.8)	4 (4.9)	0.15*
Co- amoxiclav[ab]	12 (14.8)	27 (18.4)	0.62	11 (16.9)	16 (19.5)	0.84
Co-trimoxazole[ab]	1 (1.2)	11 (7.5)	0.04*	6 (9.2)	5 (6.1)	0.34*
Flucloxacillin[ab]	13 (16.0)	15 (10.2)	0.28	6 (9.2)	9 (11.0)	0.92
Fluconazole[a]	0 (0.0)	10 (6.8)	0.01*	9 (13.8)	1 (1.2)	0.003*
Meropenem[ab]	1 (1.2)	16 (10.9)	0.0004	9 (13.8)	7 (8.5)	0.45
Metronidazole[ab]	3 (3.7)	82 (55.8)	<0.0001	26 (40.0)	56 (68.3)	0.001
Pipercillin tazobactam[ab]	5 (6.2)	46 (31.3)	<0.0001	22 (33.8)	24 (29.3)	0.68
Tazocin[ab]	5 (6.2)	18 (12.2)	0.22	8 (12.3)	10 (12.2)	0.86
Trimethoprim[a]	1 (1.2)	10 (6.8)	0.05*	6 (9.2)	4 (4.9)	0.24*
Vancomycin[ab]	3 (3.7)	40 (27.2)	<0.0001	10 (15.4)	30 (36.6)	0.007
Amoxicillin[b]	4 (4.9)	12 (8.2)	0.52	5 (7.7)	7 (8.5)	0.92
Ciprofloxacin[b]	1 (1.2)	9 (6.1)	0.009*	4 (6.2)	5 (6.1)	0.62*
Clarithromycin[b]	3 (3.7)	7 (4.8)	0.50*	2 (3.1)	5 (6.1)	0.33*
Fidaxomicin[b]	0 (0.0)	28 (19.0)	<0.0001	5 (7.7)	23 (28.0)	0.004

Table 5.8 Top ten (by n size) antibiotics taken by [a]PCDEs and [b]CDI participants in the three months prior to the study, for all recruited patients that had at least one sample taken in the study. *indicates where Fishers exact tests was used instead of χ^2. Full table of antibiotic history for all participants is shown in appendix table A3.3

Control participants were significantly more likely than GDH positive participants to have taken laxatives in the three months prior to recruitment in the study (p <0.0001) (table 5.9).

Of these laxatives, Docusate and Senna were significantly more likely to have been prescribed for control participants than for GDH positive participants (table 5.10). GDH positive participants were not significantly more likely than controls to be prescribed any particular class of drugs, however they were more likely to have been prescribed Omeprazole and Ranitidine than control participants (tables 5.9 And 5.10). There were no significant differences in the class or specific drugs prescribed for PCDEs and CDI participants in the three months prior to recruitment in the study (tables 5.9 And 5.10).

	Had at least one sample in the study			Had at least one sample in the study and initial diagnostic specimen was GDH positive		
	Control n (%)	GDH positive n (%)	Significance (*p*)	PCDE n (%)	CDI n (%)	Significance (*p*)
Analgesia	2 (2.5)	4 (2.7)	0.63*	3 (4.6)	1 (1.2)	0.23*
Anti-diarrhoeal	0 (0.0)	2 (1.4)	0.41*	0 (0.0)	2 (2.4)	0.31*
Anti-emetic	1 (1.2)	4 (2.7)	0.42*	2 (3.1)	2 (2.4)	0.60*
Anti-motility	1 (1.2)	4 (2.7)	0.42*	3 (4.6)	1 (1.2)	0.22*
Bowel prep	0 (0.0)	2 (1.4)	0.41*	0 (0.0)	2 (2.4)	0.31*
Enema	0 (0.0)	1 (0.7)	0.65*	1 (1.5)	0 (0.0)	0.44*
H2 receptor antagonist	0 (0.0)	3 (2.0)	0.27*	1 (1.5)	2 (2.4)	0.59*
Laxative	43 (53.1)	17 (11.6)	<0.0001	9 (13.8)	8 (9.8)	0.61
pH antagonist	3 (3.7)	17 (11.6)	0.08	6 (9.2)	11 (13.4)	0.60
PPI	33 (40.7)	70 (47.6)	0.39	33 (50.8)	37 (45.1)	0.61
Total	81	147		65	82	

Table 5.9 Class of drugs other than antibiotics, taken in the three months prior to the study, for all patients recruited to the study that had at least one sample taken. *indicates where Fishers exact tests was used instead of χ^2

	Had at least one sample in the study			Had at least one sample in the study and initial diagnostic specimen was GDH positive		
	Control n (%)	GDH positive n (%)	Significance (p)	PCDE n (%)	CDI n (%)	Significance (p)
Codeine	2 (2.5)	4 (2.7)	0.63*	3 (4.6)	1 (1.2)	0.23*
Docusate	4 (4.9)	0 (0.0)	0.01*	0 (0.0)	0 (0.0)	1
Esomeprazole	1 (1.2)	1 (0.7)	0.58	1 (1.5)	0 (0.0)	0.44*
Klean prep	0 (0.0)	1 (0.7)	0.65*	0 (0.0)	1 (1.2)	0.69*
Lactulose	4 (4.9)	2 (1.4)	0.12*	0 (0.0)	2 (2.4)	0.31*
Lansoprazole	24 (29.6)	34 (23.1)	0.36	16 (24.6)	18 (22.0)	0.89
Loperamide	1 (1.2)	8 (5.4)	0.11*	5 (7.7)	3 (3.7)	0.25*
Metaclopramide	1 (1.2)	4 (2.7)	0.42*	2 (3.1)	2 (2.4)	0.60*
Microlax	0 (0.0)	1 (0.7)	0.65*	1 (1.5)	0 (0.0)	0.44*
Movicol	14 (17.3)	12 (8.2)	0.06	6 (9.2)	6 (7.3)	0.92
Omeprazole	9 (11.1)	42 (28.6)	0.004	18 (27.7)	24 (29.3)	0.92
Pantoprazole	0 (0.0)	1 (0.7)	0.65*	0 (0.0)	1 (1.2)	0.56*
Phosphate enema	2 (2.5)	0 (0.0)	0.13*	0 (0.0)	0 (0.0)	1
Picolax	0 (0.0)	1 (0.7)	0.65*	0 (0.0)	1 (1.2)	0.56*
PO4 enema	1 (1.2)	0 (0.0)	0.35*	0 (0.0)	0 (0.0)	1
Rabeprazole	0 (0.0)	1 (0.7)	0.65*	1 (1.5)	0 (0.0)	0.44*
Ranitidine	3 (3.7)	26 (17.7)	0.005	10 (15.4)	16 (19.5)	0.66
Senna	16 (19.8)	7 (4.8)	0.0005	4 (6.2)	3 (3.7)	0.37*
Total	81	147		65	82	

Table 5.10. Name of drugs other than antibiotics, taken in the three months prior to the study, for all participants recruited to the study that had at least one sample taken. *indicates where Fishers exact tests was used instead of χ^2

5.3.4 Diagnostic assay results

Although there were 1173 sampling days results for each of the assays are not available for every sample; CCNA n = 1086, Culture N = 1129, CTC N = 1104, GDH EIA N = 1138, Toxin EIA N = 1135, Toxin gene B (PCR) N = 1086 (table 5.12). Reasons for discrepancies in the N sizes included lack of sample volume for processing by all assays, removal of any CCNA results that were 'indeterminate' and removal of Toxin gene PCR results that were 'invalid'.

There were three samples from control participants that were positive for *C. difficile* toxins by CCNA; two of which were also positive by the Toxin EIA (table 5.12). These three samples came from three different individuals; the sample for one of the individuals (participant 178) was positive by all assays and PCR ribotype 018 was isolated from the sample. The samples for the other two individuals were negative for all other assays. Participant 178 was withdrawn from the study as a control participant after this result.

There were fourteen samples from control participants that had *C. difficile* isolated from their sample; these were from seven individuals. Six of these seven participants had only one PCR ribotype isolated form their samples, one had two PCR ribotypes isolated (table 5.11). With the exception of participant 178 (discussed above) none of these participants had samples positive for *C. difficile* toxins. Of the 14 samples, 13 were positive by the GDH EIA assay, five were positive by CTC, and three positive by toxin B gene PCR. In addition to these 14 culture positive participants there were a further five samples positive for GDH

Study number	Number of culture positive samples (n)	Number of GDH EIA positive samples (n)	Number of toxin B gene PCR positive samples (n)	PCR ribotype isolated from the samples
1	0	1	1	N/A
2	0	0	1	N/A
9	5	5	0	067
27	1	1	0	015
63	0	1	0	N/A
76	2	2	0	026 and 716
82	1	0	0	073
99	0	0	1	N/A
117	3	6	3	020
157	1	1	0	039
178	1	1	1	018
Total	14	18	7	N/A

Table 5.11. Control participants that had *C. difficile* isolated from samples within the study and the PCR ribotype of that isolate.

Participants with CDI had a higher proportion of CCNA positive samples than PCDE's (24.7 vs 15.3% respectively) (table 5.12); however the majority of samples were CCNA negative. The proportion of samples positive for toxin by EIA was lower than those positive by CCNA for both participants with CDI and PCDE's (table 5.12). There were a similar number of samples that were culture positive and GDH EIA positive from CDI cases and PCDE's (48.5 vs 47.1% culture positive and 52.6 vs 51.8% GDH positive respectively), although there was a higher proportion of CTC positive samples and Toxin B gene PCR positive samples for CDI cases than for PCDE's (44.4% vs 29.2% CTC positive and 42.1 vs 30.4% PCR positive respectively).

For all samples that were CCNA positive 89.3% were also culture positive, 86.4% were CTC positive, 95.7% were GDH EIA positive, 64.5% were Toxin EIA positive and 90.1% were toxin B gene PCR positive. For all samples that were culture positive 39.9% were also CCNA positive, 83.0% were CTC positive, 89.3% were GDH EIA positive, 1.6% were toxin EIA positive and 65.4% were toxin B gene PCR positive. For all samples that were CTC positive 46.2% were also CCNA positive, 100% were culture positive (a pre-requisite for being CTC positive), 88.7% were GDH EIA positive, 35.4% were Toxin EIA positive and 74.4% were toxin B gene PCR positive.

When all submitted samples were included in the analysis, the most sensitive assay for detecting a CDI case was the GDH EIA assay (52.6% table 5.13). The majority of samples from CDI cases that were tested were not positive for toxin, as reflected in the sensitivity of 24.7% and 17.8% for CCNA and Toxin EIA respectively. The most sensitive assay for detecting a PCDE was also the GDH EIA (51.8%). There were fewer toxin positive samples in the PCDE group than in CDI cases leading to a lower sensitivity in the PCDE's compared to CDI cases for both CCNA and toxin EIA (15.3% vs 24.7% for CCNA, 13.0% vs 17.8% for toxin EIA respectively). When compared to reference methods the GDH EIA had the highest sensitivity when compared with CCNA (95.7%) followed by toxin gene PCR (90.1%) and culture (89.3%); GDH had the highest sensitivity when compared with culture (89.3%) followed by CTC (83.0%); Culture has the highest sensitivity when compared with CTC (100%), followed by GDH EIA (88.7%).

		Defined by routine diagnostic test result (used for recruitment into the study)				CCNA		Culture		CTC	
Assay		Control n (%)	GDH positive/toxin either positive or negative n (%)	PCDE n (%)	CDI n (%)	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)
CCNA	Positive	3 (1.3)	184 (21.6)	42 (15.3)	142 (24.7)	n/a	n/a	167 (39.9)	20 (0.3)	152 (46.2)	24 (3.3)
	Negative	232 (98.7)	667 (78.4)	233 (84.7)	434 (75.3)	n/a	n/a	252 (60.1)	642 (96.7)	177 (53.8)	704 (96.7)
	N size	235	851	275	576	n/a	n/a	419	662	329	728
Culture	Positive	14 (5.7)	424 (48.1)	132 (47.1)	292 (48.5)	167 (89.3)	252 (28.2)	n/a	n/a	341 (99.1)	70 (9.2)
	Negative	233 (94.3)	458 (51.9)	148 (52.9)	310 (51.5)	20 (10.7)	642 (71.8)	n/a	n/a	0 (0)	691 (90.8)
	N size	247	882	280	602	187	894	n/a	n/a	344	76
CTC	Positive	5 (2.0)	341 (39.8)	79 (29.2)	260 (44.4)	152 (86.4)	177 (20.1)	341 (83.0)	0 (0)	n/a	n/a
	Negative	242 (98.0)	516 (60.2)	192 (70.8)	326 (55.6)	24 (13.6)	704 (79.9)	70 (17.0)	691 (100)	n/a	n/a
	N size	247	857	271	586	176	881	411	691	n/a	n/a

	Positive	18 (7.2)	465 (52.3)	147 (51.8)	318 (52.6)	179 (95.7)	613 (68.3)	391 (89.3)	86 (12.5)	306 (88.7)	148 (19.6)
	Negative	231 (93.8)	424 (47.7)	137 (48.2)	287 (47.4)	8 (4.3)	285 (31.7)	47 (10.7)	603 (87.5)	39 (11.3)	608 (80.4)
GDH EIA	N size	249	889	284	605	187	898	438	689	345	756
	Positive	2 (0.8)	144 (16.2)	37 (13.0)	107 (17.8)	120 (64.5)	20 (2.2)	11 (1.6)	135 (30.8)	122 (35.4)	14 (1.9)
	Negative	246 (99.2)	743 (83.8)	248 (87.0)	495 (82.2)	66 (35.5)	876 (97.8)	675 (98.4)	303 (69.2)	223 (64.6)	739 (98.1)
Toxin EIA	N size	248	887	285	602	186	896	686	438	345	753
	Positive	7 (3.0)	326 (38.2)	85 (30.4)	241 (42.1)	163 (90.1)	161 (18.7)	274 (65.4)	55 (8.4)	247 (74.4)	64 (8.9)
	Negative	224 (97.0)	527 (61.8)	195 (69.6)	332 (57.9)	18 (9.9)	699 (91.3)	145 (34.6)	601 (91.6)	85 (25.6)	655 (91.1)
Toxin gene *tcdB* (PCR)	N size	233	853	280	573	181	860	419	656	332	719

Table 5.12. The results of each diagnostic assay for different categories of participants; controls, GDH positive (regardless of toxin status), PCDE or CDI case, and compared with each reference method, for all samples included in the study

		Case definition			Reference method		
		GDH positive/toxin either positive or negative (% (95% CI))	PCDE (% (95% CI))	CDI (% (95% CI))	CCNA (% (95% CI))	Culture (% (95% CI))	CTC (% (95% CI))
CCNA	Sensitivity	21.6 (18.9-24.6)	15.3 (11.3-20.2)	24.7 (21.2-28.4)	n/a	39.9 (35.1-44.7)	46.2 (40.7-51.8)
	Specificity	98.7 (96.0-99.7)	98.7 (96.0-99.7)	98.7 (96.0-99.7)	n/a	97.0 (95.3-98.1)	96.7 (95.1-97.8)
	PPV	98.4 (95.0-99.6)	93.3 (80.7-98.2)	97.9 (93.6-99.5)	n/a	89.3 (93.7-93.2)	86.4 (80.2-90.9)
	NPV	25.8 (23.0-28.8)	49.9 (45.3-54.5)	34.8 (31.2-38.6)	n/a	71.8 (68.7-74.7)	79.9 (77.1-82.5)
Culture	Sensitivity	48.1 (44.7-51.4)	47.1 (41.2-53.2)	48.5 (44.5-52.6)	89.3 (83.8-93.2)	n/a	100.0 (98.6-100)
	Specificity	94.3 (90.5-96.7)	94.3 (90.5-96.7)	94.3 (90.5-96.7)	71.8 (68.7-74.7)	n/a	90.8 (88.5-92.7)
	PPV	96.8 (94.6-98.2)	90.4 (84.1-94.5)	95.4 (92.3-97.4)	39.9 (35.2-44.7)	n/a	83.0 (78.9-86.4)
	NPV	33.7 (30.2-37.4)	61.2 (56.0-66.0)	42.9 (38.7-47.2)	97.0 (95.3-98.1)	n/a	100.0 (99.3-100)
CTC	Sensitivity	39.8 (36.5-43.2)	29.2 (23.9-35.0)	44.4 (40.3-48.5)	86.4 (80.2-90.9)	83.0 (78.9-86.4)	n/a
	Specificity	98.0 (95.1-99.3)	98.0 (95.1-99.3)	98.0 (95.1-99.3)	79.9 (77.0-82.5)	100.0 (99.3-100)	n/a
	PPV	98.6 (96.5-99.5)	94.0 (86.0-97.8)	98.1 (95.4-99.3)	46.2 (40.7-51.7)	100.0 (98.6-100)	n/a
	NPV	31.9 (28.6-35.4)	55.8 (50.9-60.5)	42.6 (38.5-46.8)	96.7 (95.1-97.8)	90.8 (88.5-92.7)	n/a

GDH EIA	Sensitivity	52.3 (50.0-55.6)	51.8 (45.8-57.7)	52.6 (48.5-56.6)	95.7 (91.4-98.0)	89.3 (85.9-91.9)	88.7 (84.8-91.7)
	Specificity	92.8 (88.6-95.5)	92.8 (88.6-95.5)	92.8 (88.6-95.5)	31.7 (28.7-34.9)	87.5 (84.8-89.8)	80.4 (77.4-83.2)
	PPV	96.3 (94.1-97.7)	89.1 (83.1-93.2)	94.6 (91.5-96.7)	22.6 (19.8-25.7)	82.0 (78.2-85.3)	67.4 (62.8-71.7)
	NPV	35.3 (31.6-39.1)	62.8 (57.6-67.7)	44.6 (40.3-49.0)	97.3 (90.4-94.6)	92.8 (90.4-94.6)	94.0 (91.8-95.6)
Toxin EIA	Sensitivity	16.2 (13.9-39.1)	13.0 (9.4-17.6)	17.8 (14.9-21.1)	64.5 (57.1-71.3)	1.6 (0.8-2.9)	35.4 (30.3-40.7)
	Specificity	99.2 (96.8-99.9)	99.2 (96.8-99.9)	99.2 (96.8-99.9)	97.8 (96.5-98.6)	69.2 (64.6-73.4)	98.1 (96.8-98.9)
	PPV	98.6 (94.6-99.8)	94.9 (81.4-99.1)	98.2 (92.9-99.7)	85.7 (78.6-90.9)	7.5 (4.0-13.4)	89.7 (83.0-94.0)
	NPV	24.9 (22.2-27.7)	49.8 (45.3-54.3)	33.2 (29.8-36.7)	93.0 (91.1-94.5)	31.0 (28.1-34.0)	76.8 (74.0-79.4)
Toxin gene tcdB (PCR)	Sensitivity	38.2 (35.0-41.6)	30.4 (25.1-36.2)	42.1 (38.0-46.2)	90.1 (84.5-93.8)	65.4 (60.6-69.9)	74.4 (69.3-78.9)
	Specificity	96.1 (93.6-98.7)	97.0 (93.6-98.7)	96.1 (93.6-98.7)	81.3 (78.5-93.8)	91.6 (89.1-93.6)	91.1 (88.7-93.0)
	PPV	97.9 (95.5-99.1)	92.4 (84.4-96.6)	97.2 (94.0-98.8)	50.3 (44.7-55.9)	83.3 (78.7-87.1)	79.4 (74.4-83.7)
	NPV	29.8 (26.6-33.3)	53.5 (48.6-58.3)	40.3 (36.2-44.5)	97.5 (96.0-98.4)	80.6 (77.5-83.3)	88.5 (85.9-90.7)

Table 5.13. Sensitivity, specificity, PPV and NPV for each assay compared with the case definition, CCNA, Culture and CTC, for all samples included in the study

The number of positive samples, sensitivity and specificity of each assay were also compared using only samples collected within the first 72 hours after recruitment into the study (table 5.14 and 5.15). CDI cases had a higher proportion of CCNA positive samples than PCDE's (24.4 vs 10.6% respectively) (table 5.14); however the majority of samples were CCNA negative. Although the proportion of CCNA positive samples in CDI cases within the first 72 hours was similar to the proportion of CCNA positive samples in CDI cases for all samples (24.4 vs 24.7% respectively), there were more CCNA positive samples in the PCDE group when all samples were included than when just those taken within the first 72 hours were analysed (15.3 vs 10.6% respectively) (table 5.14). The proportion of samples within the first 72 hours that were positive for toxin by EIA was lower than those positive by CCNA for both CDI cases and PCDE's (table 5.14). There were more samples within 72 hours that were culture positive and GDH EIA positive from PCDE's than CDI cases (53.1 vs 40.4% culture positive and 60.4 vs 56.5% GDH positive respectively), although there was a higher proportion of CTC positive samples and Toxin B gene PCR positive samples for CDI cases than for PCDE's (37.8% vs 25.0% CTC positive and 39.1 vs 23.4% PCR positive respectively).

For all samples that were CCNA positive 76.9% were also culture positive, 76.9% were CTC positive, 84.6% were GDH EIA positive, 50.0% were Toxin EIA positive and 92.3% were toxin B gene PCR positive. For all samples that were culture positive 24.4% were also CCNA positive, 67.0% were CTC positive, 88.1% were GDH EIA positive, 16.7% were toxin EIA positive and 52.4% were toxin B gene PCR positive. For all samples that were CTC positive 38.5% were also CCNA positive, 100% were culture positive (a pre-requisite for being CTC positive), 88.5% were GDH EIA positive, 2.7% were Toxin EIA positive and 80.8% were toxin B gene PCR positive.

A large number of CDI cases were negative by the time their first sample was tested in the study. The 16 CCNA positive samples that were tested within 72 hours of diagnosis were from 13 patients, however only 10 of these were CDI when recruited, rather than just GDH positive. This means that only 10/82 (12.2%) of patients that were recruited as CDI had another toxin positive sample during the 72 hours after initial diagnosis. This is reflected by the low sensitivity for CCNA of 24.4% and for Toxin EIA of 9.3% for detecting a CDI case with samples taken up to 72 hours after diagnosis (table 5.15). The GDH EIA had the highest sensitivity for detecting a CDI case within 72 hours of diagnosis (56.5%), followed by the culture (40.4%), PCR (39.1%) and CTC (37.8%). The assay with the highest sensitivity

for detecting a PCDE within 72 hours was the GDH assay (60.4%), followed by culture (53.1%), CTC (25.0)% and PCR (23.4%).

		Defined by routine diagnostic test result (used for recruitment into the study)				CCNA		Culture		CTC	
Assay		Control n (%)	GDH positive/toxin either positive or negative n (%)	PCDE n (%)	CDI n (%)	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)
CCNA	Positive	1 (1.6)	16 (17.4)	5 (10.6)	11 (24.4)	n/a	n/a	10 (24.4)	3 (2.6)	10 (38.5)	3 (2.3)
	Negative	62 (98.4)	76 (82.6)	42 (89.4)	34 (75.6)	n/a	n/a	31 (75.6)	114 (97.4)	16 (61.5)	126 (97.7)
	N size	63	92	47	45	n/a	n/a	41	117	26	129
Culture	Positive	2 (3.0)	45 (46.9)	26 (53.1)	19 (40.4)	10 (76.9)	31 (21.4)	n/a	n/a	26 (100)	13 (9.2)
	Negative	63 (97.0)	51 (53.1)	23 (46.9)	28 (59.6)	3 (23.1)	114 (78.6)	n/a	n/a	0 (0.0)	128 (90.8)
	N size	65	96	49	47	13	145	n/a	n/a	26	141
CTC	Positive	1 (1.5)	29 (31.2)	12 (25.0)	17 (37.8)	10 (76.9)	16 (11.3)	26 (67.0)	0 (0.0)	n/a	n/a
	Negative	64 (98.5)	64 (68.8)	36 (75.0)	28 (62.2)	3 (23.1)	126 (88.7)	13 (33.0)	128 (100)	n/a	n/a

	N size	65	93	48	45	13	142	39	128	n/a	n/a
GDH EIA	Positive	2 (3.0)	55 (58.5)	29 (60.4)	26 (56.5)	11 (84.6)	39 (27.1)	37 (88.1)	14 (11.1)	23 (88.5)	26 (18.7)
	Negative	63 (97.0)	39 (41.5)	19 (39.6)	20 (43.5)	2 (15.4)	105 (72.9)	5 (11.9)	112 (88.9)	3 (11.5)	113 (81.3)
	N size	65	94	48	46	13	144	42	126	26	139
Toxin EIA	Positive	1 (1.5)	9 (9.8)	5 (10.2)	4 (9.3)	6 (50.0)	1 (0.7)	7 (16.7)	0 (0.0)	7 (2.7)	0 (0.0)
	Negative	64 (98.0)	83 (88.0)	44 (89.8)	39 (90.7)	6 (50.0)	142 (99.3)	35 (83.3)	124 (100.0)	19 (73.1)	137 (100.0)
	N size	65	92	49	43	12	143	42	124	26	137
Toxin gene tcdB (PCR)	Positive	3 (3.0)	29 (31.2)	11 (23.4)	18 (39.1)	12 (92.3)	16 (11.6)	22 (52.4)	6 (5.1)	21 (80.8)	6 (4.6)
	Negative	62 (97.0)	64 (68.8)	36 (76.6)	28 (60.9)	1 (7.7)	122 (88.4)	20 (47.6)	111 (94.9)	5 (19.2)	124 (95.4)
	N size	65	93	47	46	13	138	42	117	26	130

Table 5.14. The results of each diagnostic assay for different categories of participants; controls, GDH positive (regardless of toxin status), PCDE or CDI case, and compared with each reference method, for all samples collected within 72 hours of the initial routine diagnostic sample (cases) or entry to the study (controls)

		Case definition			Reference method		
		GDH positive/toxin either positive or negative (% (95% CI))	PCDE (% (95% CI))	CDI (% (95% CI))	CCNA (% (95% CI))	Culture (% (95% CI))	CTC (% (95% CI))
CCNA	Sensitivity	17.4 (10.5-27.0)	10.6 (4.0-23.9)	24.4 (13.4-39.8)	n/a	24.4 (12.9-40.6)	38.5 920.9-59.20
	Specificity	98.4 (90.3-99.9)	98.4 (90.3-99.9)	98.4 (90.3-99.9)	n/a	97.4 (92.1-99.3)	97.7 992.8-99.4)
	PPV	94.1 (69.2-99.7)	83.3 (36.5-99.1)	91.7 (59.8-99.6)	n/a	76.9 950.0-93.8)	76.9 (50.0-93.80
	NPV	44.9 (36.5-53.6)	59.6 (49.5-69.0)	64.6 (54.1-73.9)	n/a	78.6 (70.9-84.8)	88.7 982.1-93.2)
Culture	Sensitivity	46.9 (36.7-57.3)	53.1 (38.4-37.2)	40.4 (26.7-55.7)	76.9 (46.0-93.9)	n/a	100.0 (84.0-100)
	Specificity	96.9 (88.4-99.5)	97.0 (88.4-99.5)	97.0 (88.4-99.5)	78.1 (70.9-84.8)	n/a	90.8 (84.4-94.8)
	PPV	95.7 (84.3-99.3)	92.9 (75.0-98.8)	90.5 (68.2-98.3)	24.4 (12.9-40.6)	n/a	66.7 (49.7-80.4)
	NPV	55.3 (45.7-64.5)	73.3 (62.4-82.0)	69.2 (58.6-78.3)	97.4 (92.1-99.3)	n/a	100.0 (96.4-100)
CTC	Sensitivity	31.2 (22.2-41.7)	25.0 (14.1-40.0)	37.8 (24.2-53.5)	76.9 (50.0-93.8)	66.7 (49.7-80.4)	n/a
	Specificity	98.5 (90.6-99.9)	98.5 (90.6-99.9)	98.5 (90.6-99.9)	88.7 (82.1-93.2)	100 (96.4-100)	n/a
	PPV	96.7 (80.9-99.8)	92.3 (62.1-99.6)	94.4 (70.6-99.7)	38.5 (20.9-59.3)	100 (84.0-100)	n/a
	NPV	50.0 (41.1-58.9)	64.0 (53.7-73.2)	69.6 (59.0-78.5)	97.7 (92.8-99.4)	90.8 (84.4-94.8)	n/a

GDH EIA	Sensitivity	58.5 (47.9-68.4)	60.4 (45.3-73.2)	56.5 (41.2-70.8)	84.6 (53.7-97.3)	88.1 (73.6-95.5)	88.5 (68.7-97.0)
	Specificity	96.9 (88.4-99.5)	96.9 (88.4-99.5)	96.9 (88.4-99.5)	72.9 (64.8-79.8)	88.9 (81.7-93.6)	81.3 (73.6-87.2)
	PPV	96.5 (86.6-99.4)	93.5 (77.2-98.9)	92.9 (75.0-98.9)	22.0 (12.0-36.3)	72.5 (58.0-83.7)	46.9 (32.8-61.6)
	NPV	61.8 (51.6-71.1)	76.8 (66.0-85.1)	75.9 (65.0-84.3)	98.1 (92.8-99.7)	95.7 (89.8-98.4)	97.4 (92.1-99.3)
Toxin EIA	Sensitivity	9.8 (4.8-18.2)	10.2 (3.8-23.0)	9.3 (3.0-23.1)	50.0 (22.3-77.7)	16.7 (7.5-32.0)	26.9 (12.3-48.1)
	Specificity	98.5 (90.5-99.9)	98.4 (90.6-99.9)	98.4 (90.6-99.9)	99.3 (95.6-99.9)	100.0 (96.3-100)	100.0 (96.6-100)
	PPV	90.0 (54.1-99.5)	83.3 (36.5-99.1)	80.0 (29.9-98.9)	85.7 (42.0-99.2)	100.0 (56.1-100	100.0 (56.1-100)
	NPV	43.5 (35.4-52.0)	59.3 (49.4-68.5)	62.1 (52.0-71.4)	95.9 (91.0-98.3)	78.0 (71.0-84.0)	87.8 (81.4-92.3)
Toxin gene tcdB (PCR)	Sensitivity	31.2 (22.2-41.7)	23.4 (12.8-38.4)	39.1 (25.5-54.6)	92.3 (62.1-99.6)	52.4 (26.6-67.7)	80.8 (60.0-92.7)
	Specificity	95.4 (86.2-98.8)	97.1 (86.2-98.8)	97.1 (86.2-98.8)	88.4 (81.6-93.0)	94.9 (88.7-97.9)	95.4 (89.8-98.1)
	PPV	90.6 (78.8-97.5)	78.6 (48.8-94.3)	85.7 (48.8-94.3)	42.9 (25.0-62.6)	78.6 (58.5-91.0)	77.8 (57.3-90.6)
	NPV	49.2 (40.2-58.2)	63.3 (52.9-72.6)	68.9 (52.9-72.6)	99.2 (94.9-99.9)	84.7 (77.2-90.2)	96.1 (90.7-98.6)

Table 5.15. Sensitivity, specificity, PPV and NPV for each assay compared with the case definition, CCNA, Culture and CTC, for all samples collected within 72 hours of the initial routine diagnostic sample (cases) or entry to the study (controls)

5.3.5 Potential C. difficile excretors

There were 65 participants whose initial routine diagnostic samples tested positive for GDH only; these were recruited as PCDE's. Of these 65, 18 (27.7%) went on to have at least one sample that tested positive by CCNA; median number of days to CCNA positive sample was 8 days (range 0-28) (table 5.16).

Participant number	Number of days until toxin positive result	PCR ribotype isolated from the sample
513	20	020
525	13	015
537	4	341
550	13	015
589	14	014
592	19	015
593	4	050
607	20	078
609	0	078
617	7	020
621	0	014
628	8	Culture negative[a]
634	8	Culture negative[a]
641	28	023
643	3	011
650	10	207
655	4	012
657	4	005

Table 5.16. The number of days from initial GDH positive sample until another sample was CCNA positive, and the PCR ribotype of *C. difficile* isolated from that sample. [a] Had treatment with fidaxomicin from day 0, all other participants were not on a concurrent antibiotic with activity against *C. difficile*

Both the mean white cell count and the mean serum creatinine level were higher in those PCDE that went on to have a toxin positive sample, compared to those PCDE that remained toxin negative; however only the serum creatinine was significantly higher ($p = 0.009$) (table 5.17).

	PCDE that become toxin positive	PCDE that stay toxin negative	*p* value[a]
White cell count	9.2	6.4	0.209
Serum albumin	32.8	33.0	0.900
Serum creatinine	160.1	94.1	0.009

Table 5.17 The mean white cell count, serum albumin and serum creatinine levels in PCDE participants that were either toxin positive or negative in later samples. [a] *t* test (two tailed)

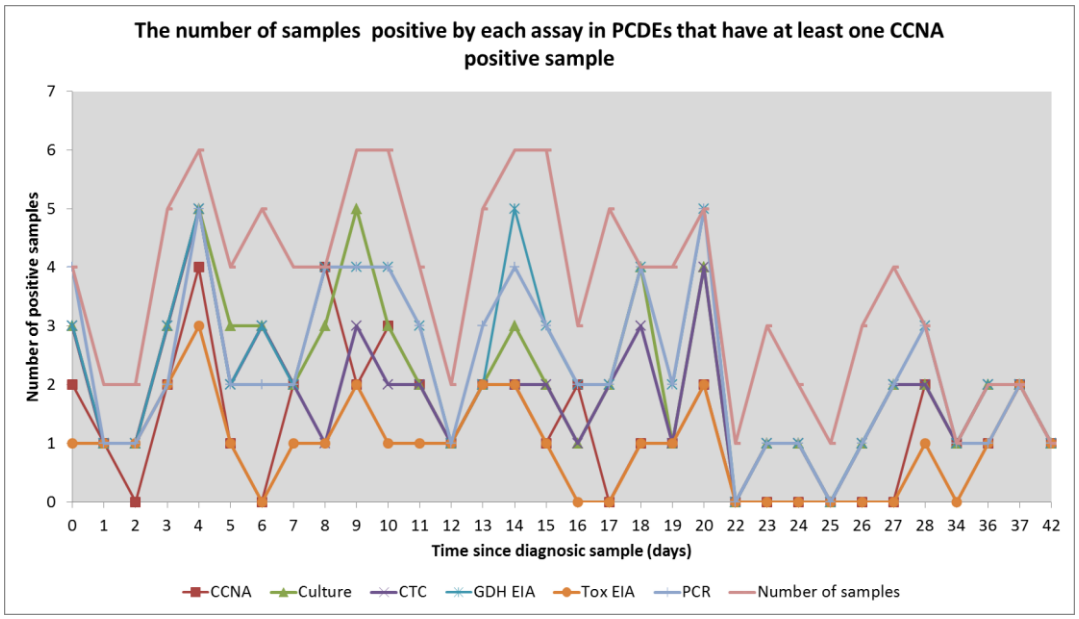


Figure 5.1 The number of samples that were positive for each assay on each day following the diagnostic specimen for PCDE's that go on to have at least one CCNA positive sample

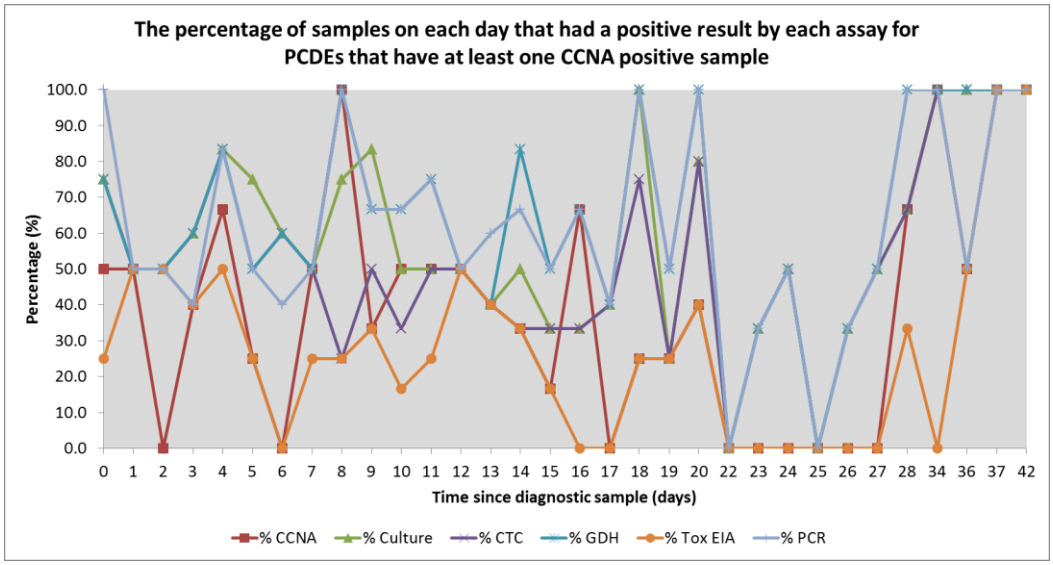


Figure 5.2 The percentage of samples that were positive for each assay on each day following the diagnostic specimen for PCDE's that go on to have at least one CCNA positive sample

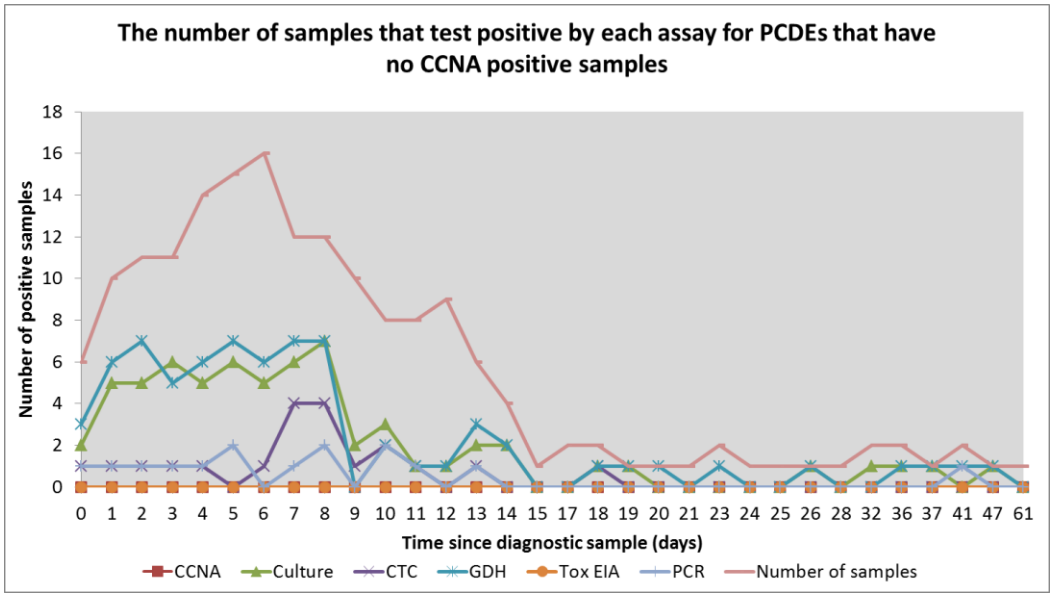


Figure 5.3 The number of samples that were positive for each assay on each day following the diagnostic specimen for PCDE's that do not go on to have at least one CCNA positive sample

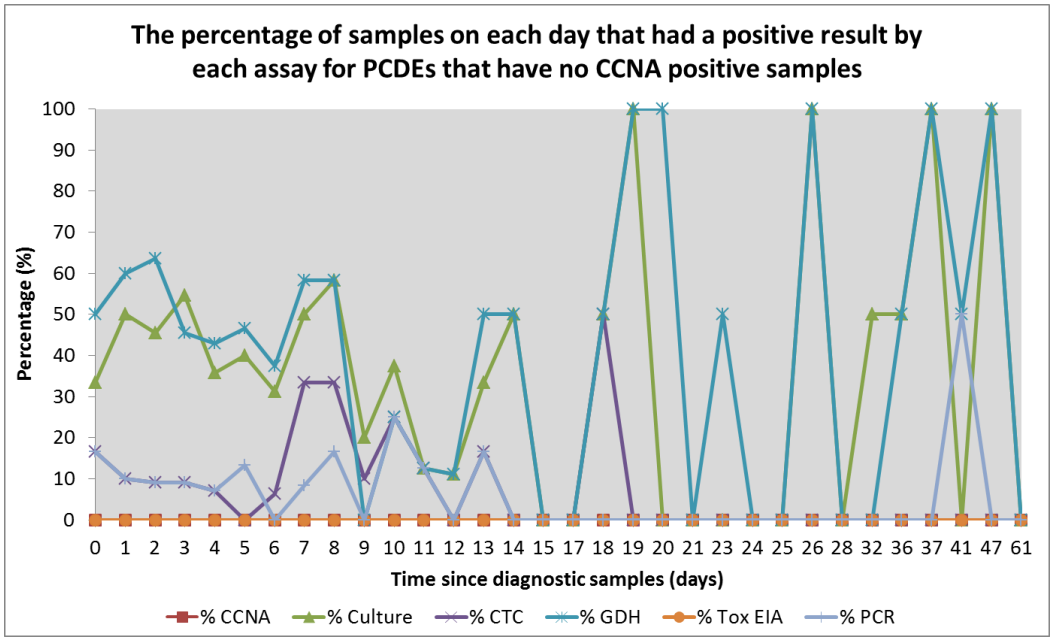


Figure 5.4 The percentage of samples that were positive for each assay on each day following the diagnostic specimen for PCDE's that do not go on to have at least one CCNA positive sample

For the PCDE that become toxin positive, the proportion of positive *C. difficile* diagnostic assays are all very similar, as are the proportion of positive *C. difficile* toxin diagnostic assays (figures 5.1 and 5.2). The proportion of positive GDH EIA and culture results in PCDE's that do not go on to have any CCNA positive samples, are similar, but are both higher than the proportion of positive CTC and PCR results (figures 5.3 and 5.4). Of those that were culture positive, 5/25 had a known non-toxigenic PCR ribotype isolated from the sample (table 5.18)

Participant number	PCR ribotype	Known non-toxigenic PCR ribotype[a]	Inferred toxinotype[b]	Inferred toxin status from toxinotype[c]
508	014/129/334		XVIII	$A^+B^+CDT^-$
517	051			
520	010	Yes		
542	081			
548	005			
552	Not available			
559	046			
563	018			
566	023		IV	$A^+B^+CDT^+$
573	010	Yes		
577	009			
588	078		V	$A^+B^+CDT^+$
595	009			
602	031			
603	327			
606	020			
608	012		I	$A^+B^+CDT^-$
611	031			
631	054	Yes		
645	026			
647	Not available			
652	027		III	$A^+B^+CDT^+$
656	039	Yes		
663	026			
666	039	Yes		

Table 5.18 The PCR ribotype of *C. difficile* isolated from PCDE's that did not have any CCNA positive samples, and known non-toxigenic status [a] from published literature (Fawley *et al*, 2016). [b] from toxinotype correlation website and [c] toxinotype characteristics website

5.3.6 Results of diagnostic tests on each day following the diagnostic sample for all recruited participants that were GDH positive (regardless of toxin status)

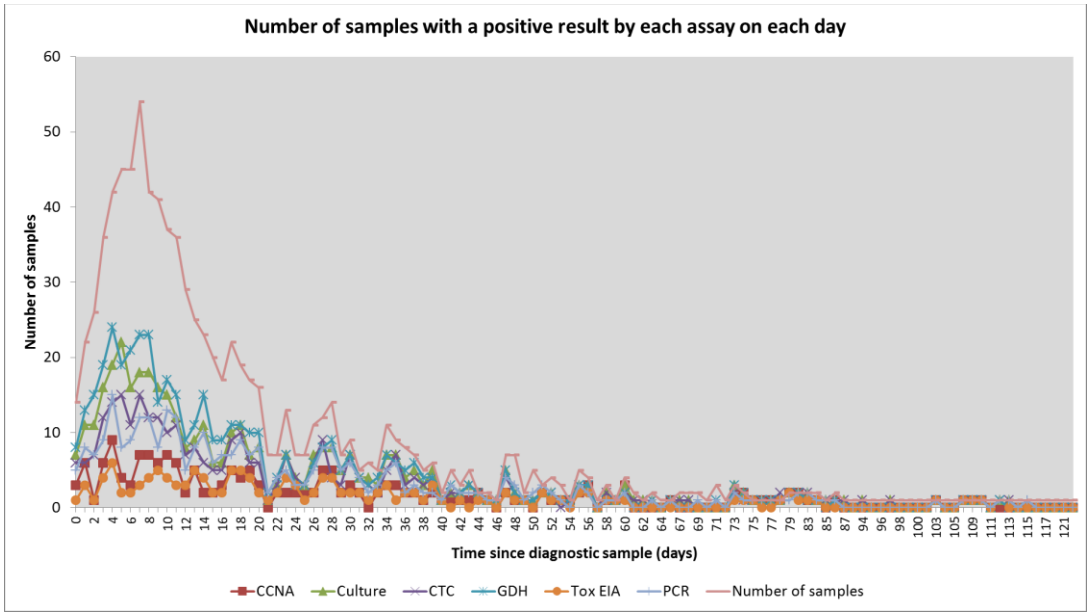


Figure 5.5. The number of samples with a positive result by each assay on each day for all participants with an initial GDH positive diagnostic sample

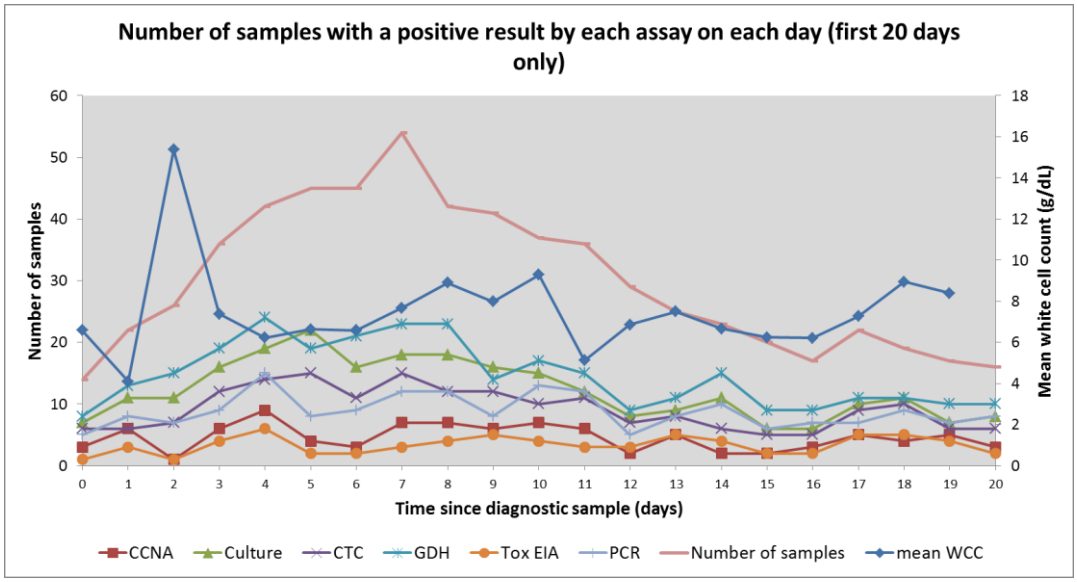


Figure 5.6. The number of samples with a positive result by each assay on each day of the first 20 days, for all participants with an initial GDH positive diagnostic sample

The number of samples collected was highest within the first 20 days after diagnosis, peaking at day seven (figure 5.5). To enable better visualisation of the patterns in the number of positive samples, the graphs were replotted for the first 20 days only, as not only were the highest number of samples collected during this time, this is also the period most likely to represent primary infection, not recurrence (figure 5.6). The GDH EIA consistently has the highest number of positive samples, followed by culture, then CTC. The pattern of the number of CTC positive samples is closely mirrored by the number of positive PCR samples. The CCNA and Toxin EIA tests have the lowest numbers of positive samples throughout the study period, with CCNA generally having more positive samples than the Toxin EIA. Peak white cell counts can be seen around day 2 after diagnosis (figure 5.6).

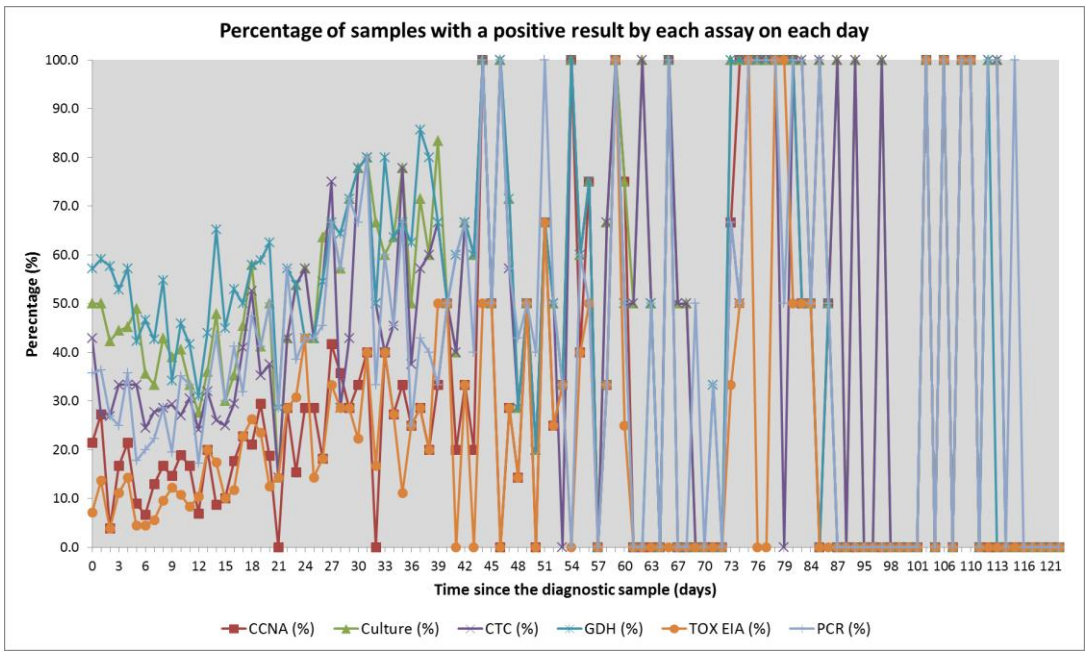


Figure 5.7. The percentage of samples with a positive result by each assay on each day for all participants with an initial GDH positive diagnostic sample

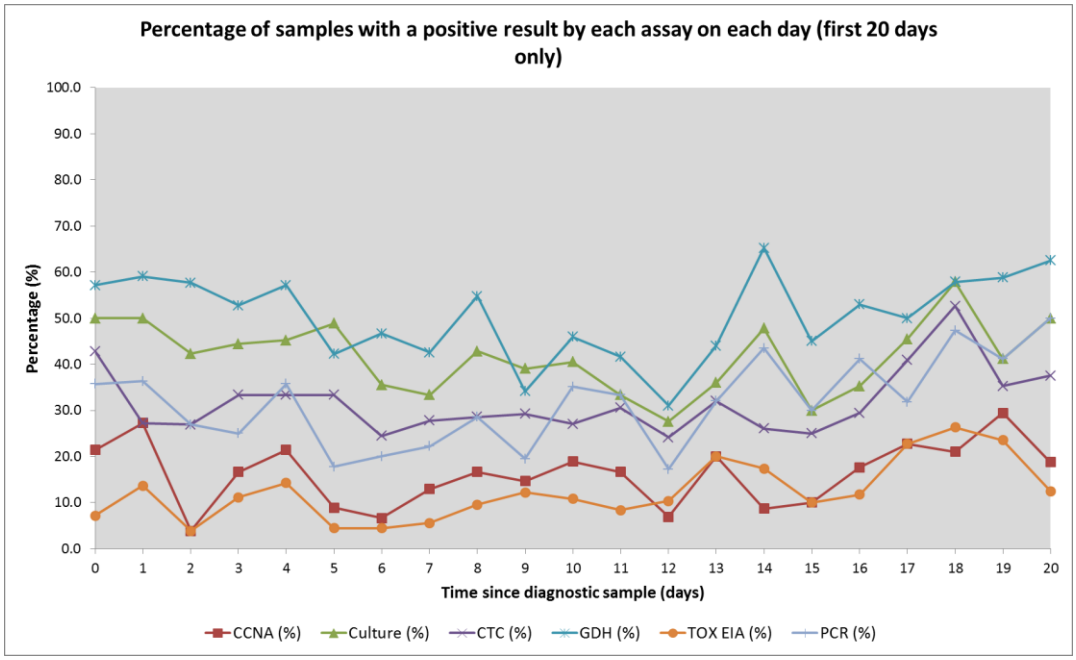


Figure 5.8. The percentage of samples with a positive result by each assay on each day of the first 20 days, for all participants with an initial GDH positive diagnostic sample

When converted to percentage of samples tested that had a positive result by each assay the same patterns as described above are observed. The GDH EIA had a consistently higher percentage of positive samples throughout the study period, up to around day 42, when the number of samples drops dramatically, so skewing the percentage data (figure 5.7). There is an increase in the positivity rate of all the tests between days 20 to 40 (figure 5.7).

When only viewing up to day 20 the patterns can be more easily seen (figure 5.8). The pattern of GDH positivity is mirrored by culture with a positivity rate about 5-10% lower than that of GDH. The pattern of culture positivity is then in turn mirrored by the CTC assay, again at a positivity rate about 10% lower than the culture positivity rate. The PCR assay closely follows the pattern and positivity rate of the CTC assay, occasionally showing a higher percentage of positive samples than CTC, and occasionally showing a lower positivity rate.

5.3.7 Results of diagnostic tests on each day following the diagnostic sample from either PCDE or CDI cases at recruitment

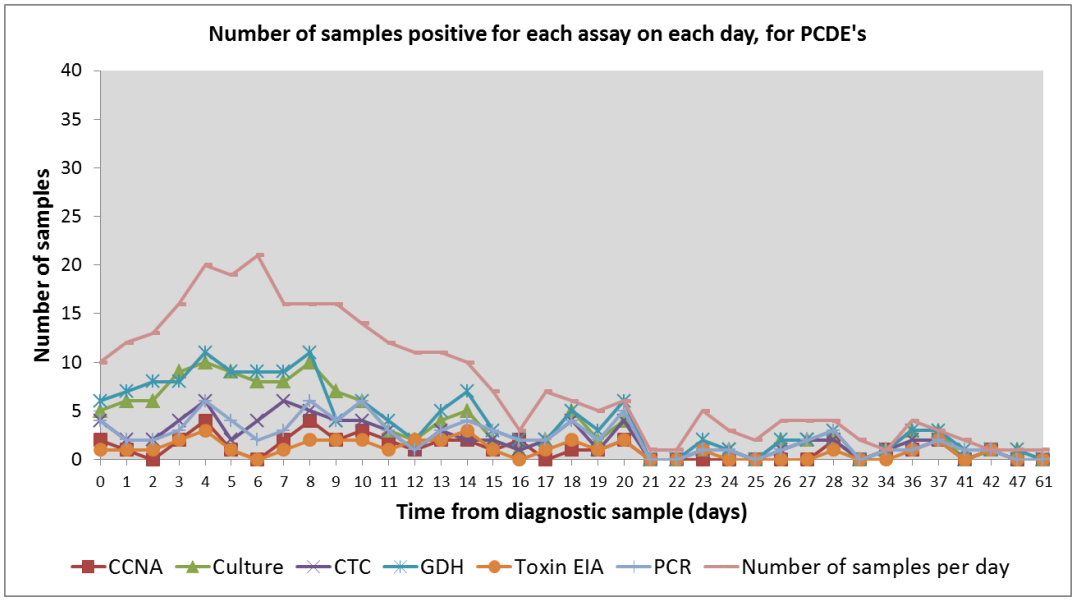


Figure 5.9. The number of samples with a positive result by each assay on each day for all PCDE participants

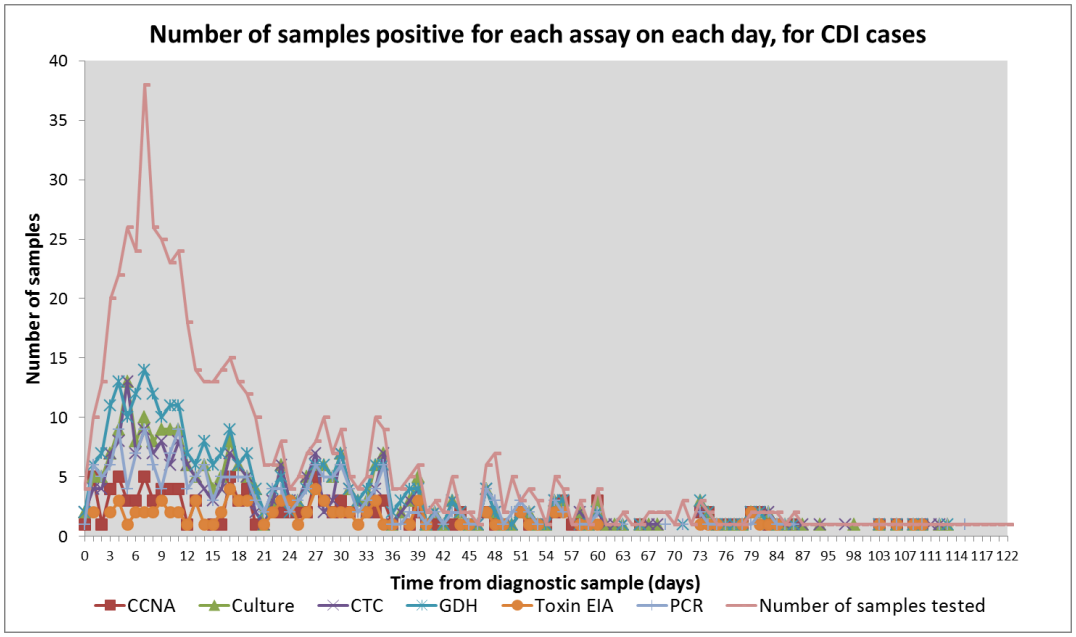


Figure 5.10. The number of samples with a positive result by each assay on each day for all CDI cases

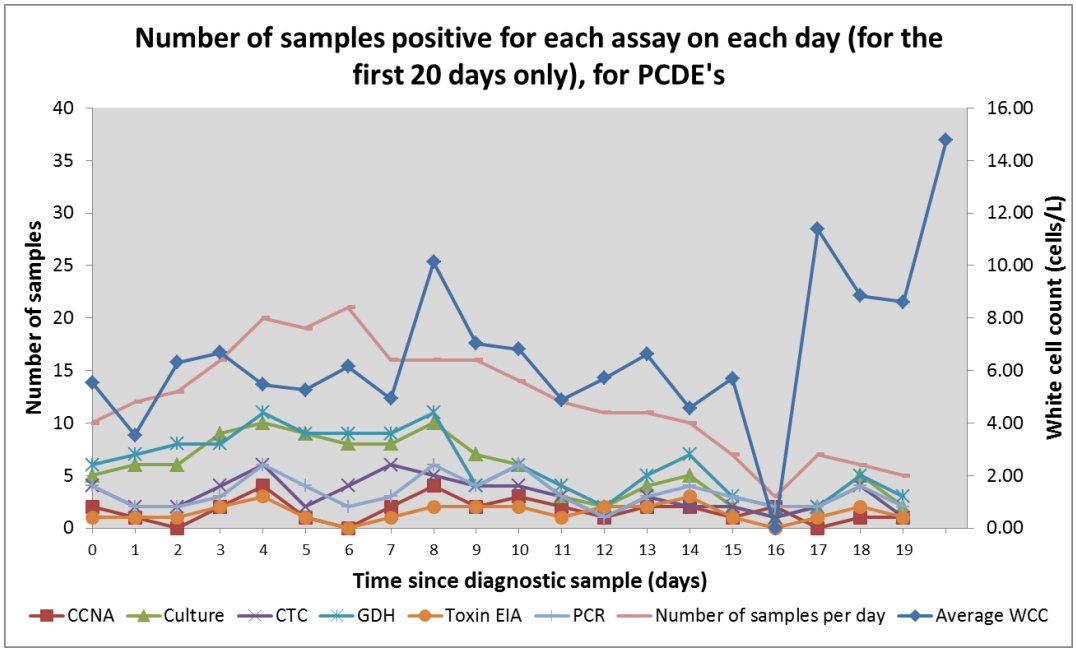


Figure 5.11. The number of samples with a positive result by each assay on each day, for the first 20 days only, for all PCDE participants

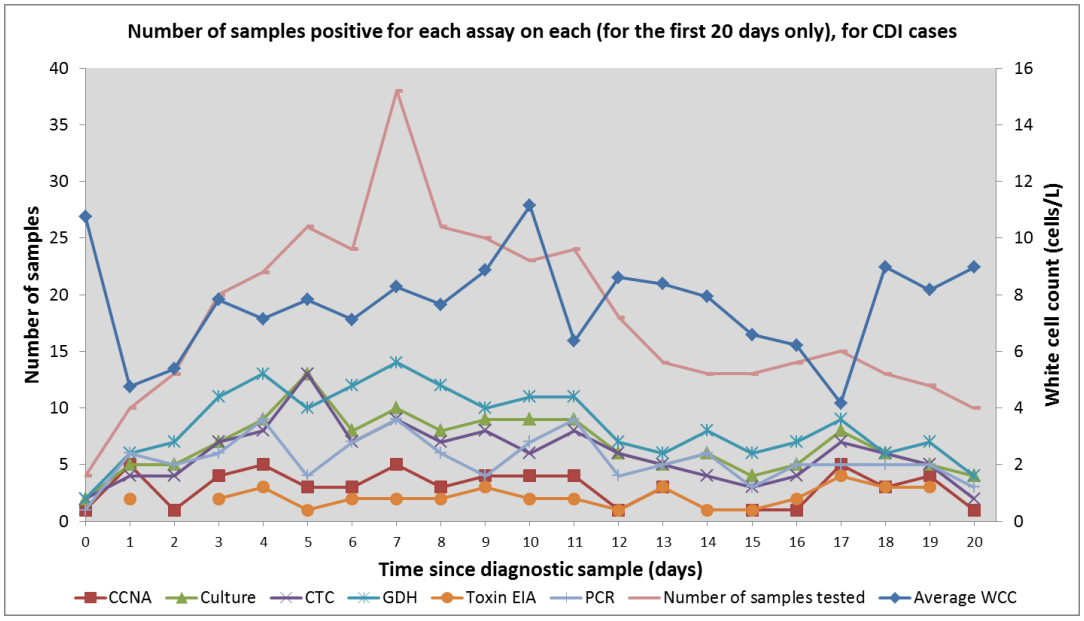


Figure 5.12. The number of samples with a positive result by each assay on each day, for the first 20 days, for all CDI cases

After splitting all GDH-positive participants into those recruited with GDH positive test only (potential *C. difficile* excretors (PCDE)) and those recruited with GDH/toxin positive (CDI cases), a similar pattern can be seen in the number of samples collected, with most of the

samples collected within the first 20 days and the highest peaks being on day five and day seven respectively (figures 5.9 and 5.10). The GDH EIA consistently has the highest number of positive samples in both groups, although this is closely mirrored by the number of culture positives in the PCDE group (figure 5.11). In the PCDE group there is a large difference in the number of samples positive by CTC compared to culture, whereas in the CDI case group culture and CTC are almost identical (figure 5.11 and 5.12). The pattern of the number of CTC positive samples is closely mirrored by the number of positive PCR samples, although in the PCDE group the number of PCR positive samples appears to be shifted by one-two days after CTC (figure 5.11). The CCNA and toxin EIA tests have the lowest numbers of positive samples throughout the study period, with CCNA generally having more positive samples than the toxin EIA (figure 5.12. There were more sample collection days in the CDI case group, although the latter samples were almost exclusively from one patient (figure 5.12). Peak white cell counts for the CDI cases are at day 0 (day of diagnosis) with a mean WCC of 10.7 cells/L. The PCDE group mean WCC at day 0 was 5.54 cells/L, with a peak of 10.13 cells/L on day nine after diagnosis.

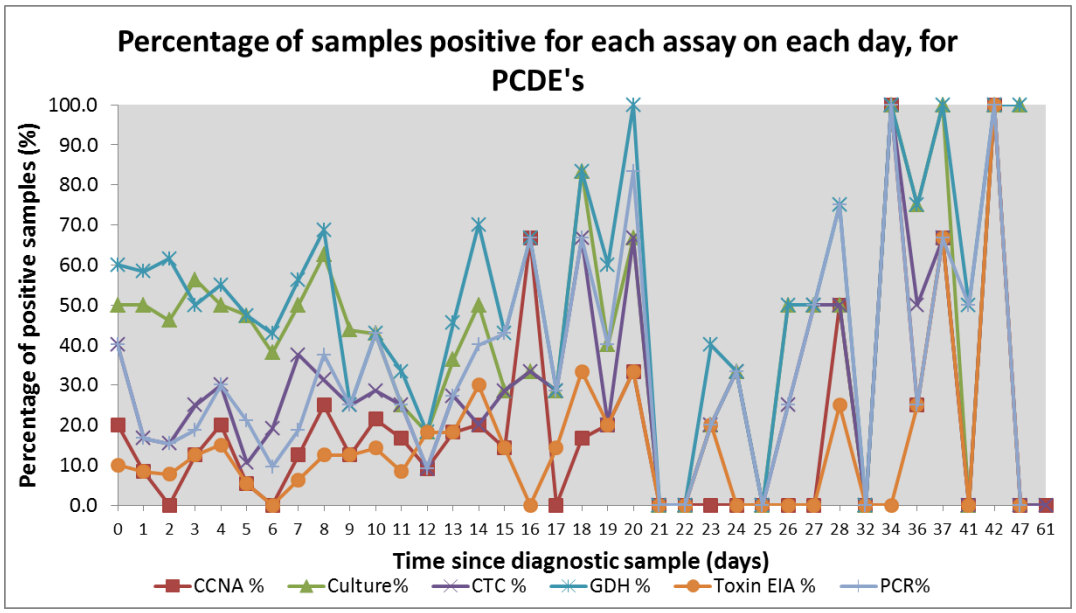


Figure 5.13. The percentage of samples with a positive result by each assay on each day for all PCDE participants

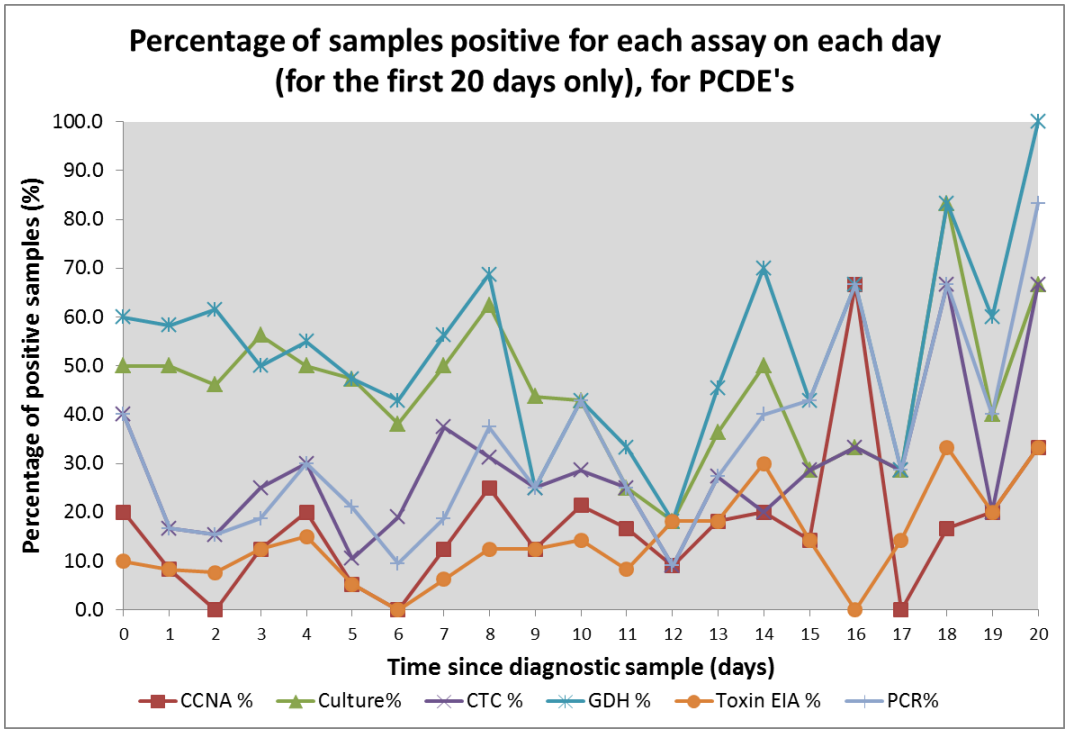


Figure 5.14. The percentage of samples with a positive result by each assay on each day, for the first 20 days only, for all PCDE participants

When converted to the proportion of samples tested that are positive by each assay, the GDH assay has a consistently higher positivity rate than any other assay for the PCDE group, although this is very closely matched by the culture positive rate (figures 5.13 and 5.14). There is a marked difference in the positive rate of CTC compared with culture, being some 5-40% lower at most time points. The PCR assay positivity rate is of a similar level to, and follows the pattern of, the CTC positivity rate However, at several time points the PCR rate appears to be shifted later than the CTC rate by one-two days. Interestingly this is in line with the pattern of the positivity of the CCNA test, which is also shifted approximately one-two days later than the CTC rate. The biggest peak in PCR positivity (day 16) coincides with the biggest peak of CCNA positivity; this occurs approximately two days after a large peak in GDH and culture positivity (figure 5.14). The toxin EIA assay follows the general pattern of CCNA positivity rate but often with a reduced positivity rate of 0-15% (figure 5.14).

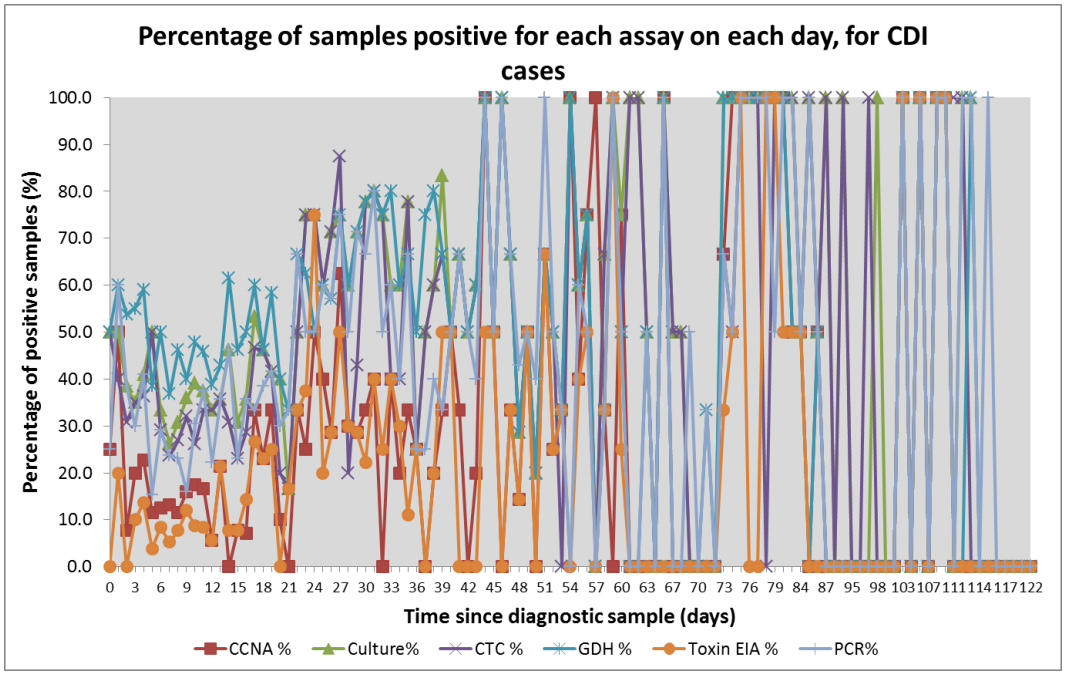


Figure 5.15 The percentage of samples with a positive result by each assay on each day, for all CDI cases

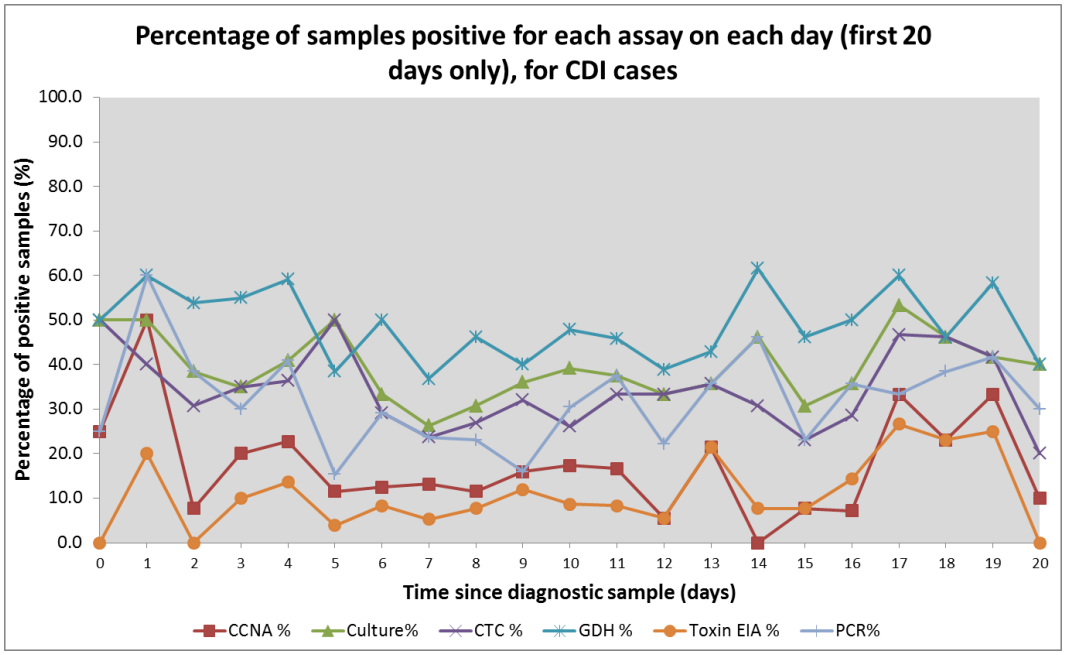


Figure 5.16 The percentage of samples with a positive result by each assay on each day, for the first 20 days only, for all CDI cases

The GDH assay positivity rate is lower for CDI cases than for the PCDE group, with 50% in the CDI cases and 60% in the PCDE group (figures 5.13 and 5.15). As with the PCDE group, the GDH assay positivity rate is consistently the highest of all the assays for the CDI cases; however the culture positive rate does not exactly mirror the GDH positive rate and can be from 0-20% lower on some days (figure 5.16). Unlike the PCDE group, the CTC positive rate more closely follows the culture positive rate for CDI cases than for PCDE's, and is only 0-10% lower, compared with 5-40% lower for PCDE's. Similar to the PCDE group the PCR positive rate is shifted behind that of the CTC positive rate by approximately two days at certain time points (figures 5.14 and 5.16). The CCNA and toxin EIA positive rates more closely follow each other in this dataset, but with a similar overall positivity rate to the PCDE group over the first 20 days of 16.8% and 17.4% for PCDE and CDI cases respectively (p = 0.52) (figures 3.14 and 3.16).

5.3.8 Ribotyping

There were 415/438 isolates from 102 participants available to be PCR ribotyped (seven controls and 95 GDH positive participants); 12 participants had two different ribotypes and one participant had three. The most common PCR ribotype isolated from samples was 002 (20.2% of all isolates), followed by 026 (8.7%), 014 (8.2% and 015 (8.2%) (figure 5.17). There were very few isolates of PCR ribotype 027 (n = 3, however), these three isolates came from three different participants.

The PCR ribotypes of isolates from controls, PCDEs and CDI cases are shown in figures 5.18, 5.19 and 5.20 respectively. There were some known non-toxigenic strain isolates, notably 010 (3.1% of the total isolates, two cases), 039 (1.0% of total isolates, one control and two PCDE's) and 054 (0.7% of total isolates, one PCDE and one CDI case). For the two participants with 010 isolates, both had only this PCR ribotype isolated from their samples; all of their samples were CCNA and CTC negative. For the three participants with 039 isolated from their samples, two (one control and one PCDE) had only one sample in the study, and were negative when tested by both CCNA and CTC. The third participant (PCDE) with 039 had three samples in the study, with 039 isolated from two of these; only one of the three was positive by CTC, none were positive by CCNA. Of the two GDH positive participants with 054 isolated from their samples, one (PCDE) had 054 isolated from 1/6 samples, which was positive by CTC but negative by CCNA. The second (CDI) with 054 had nine samples in the study, five of which were positive by CCNA and four of which were

positive by CTC; 054 was isolated from two samples and 002 was isolated from two other samples, suggesting a mixed infection.

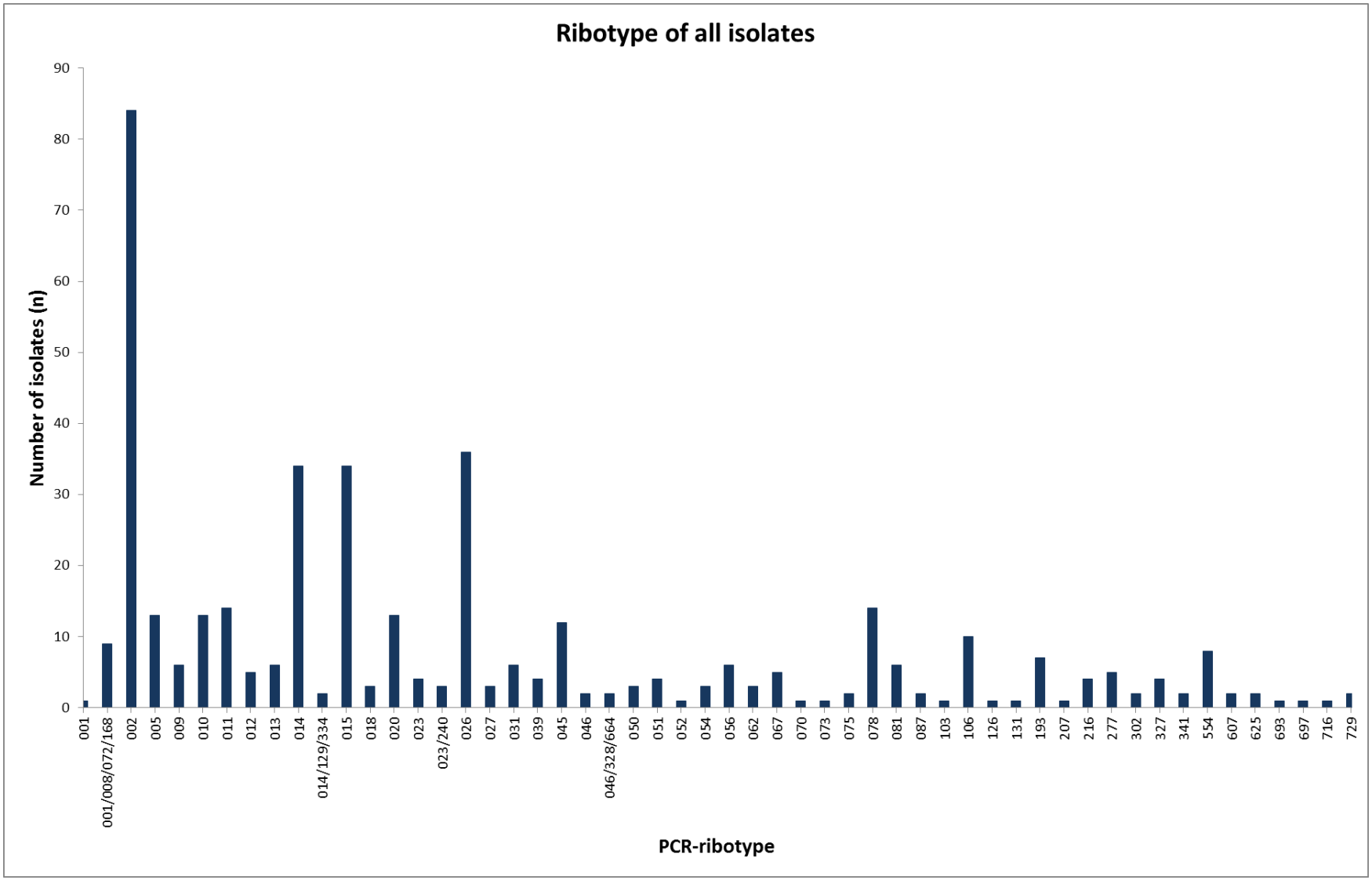


Figure 5.17. The PCR ribotypes of isolates from all participants and the number of isolates of each PCR ribotype.

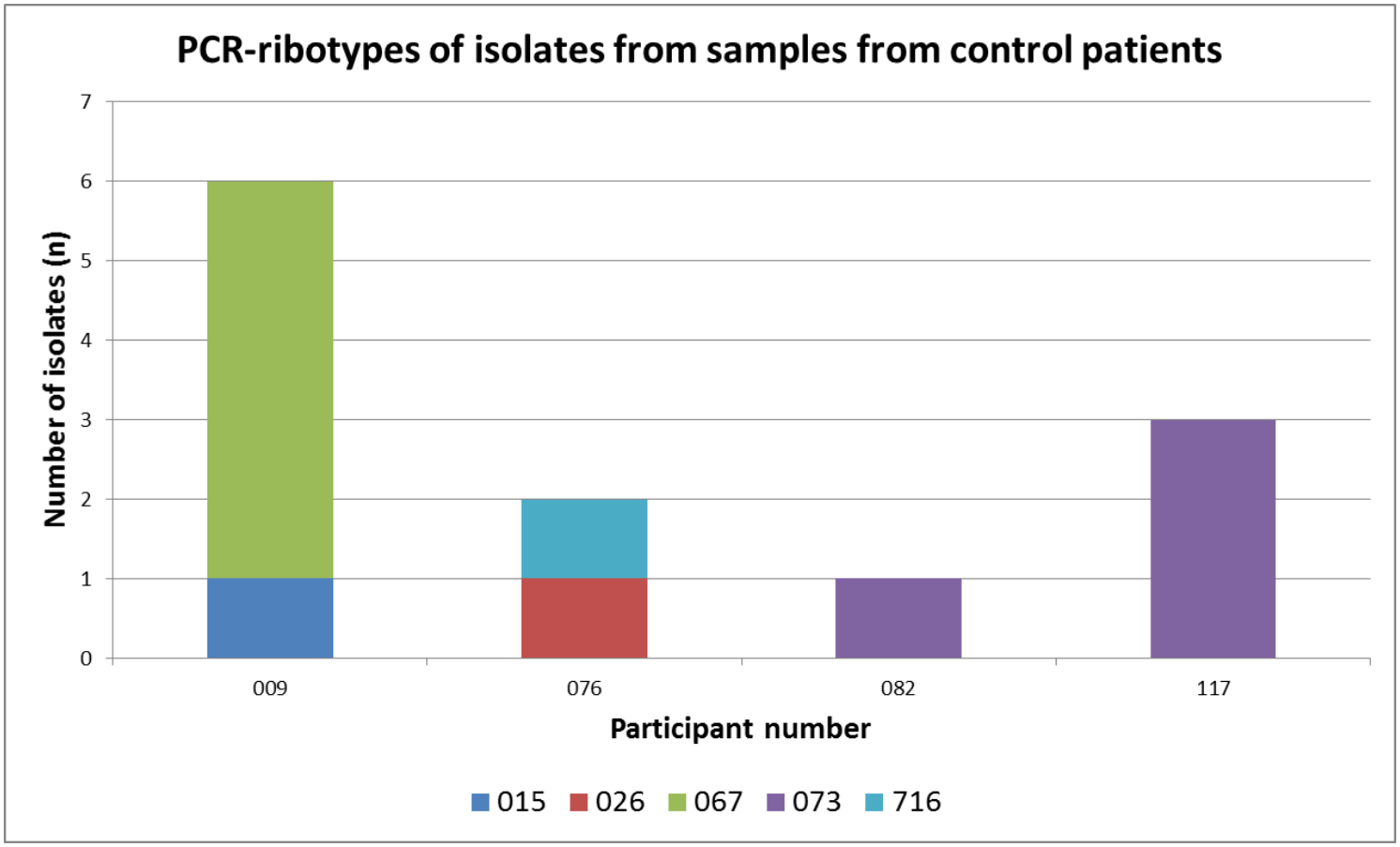


Figure 5.18. The PCR ribotypes of isolates from all control participants and the number of times each PCR ribotype was isolated from that participant.

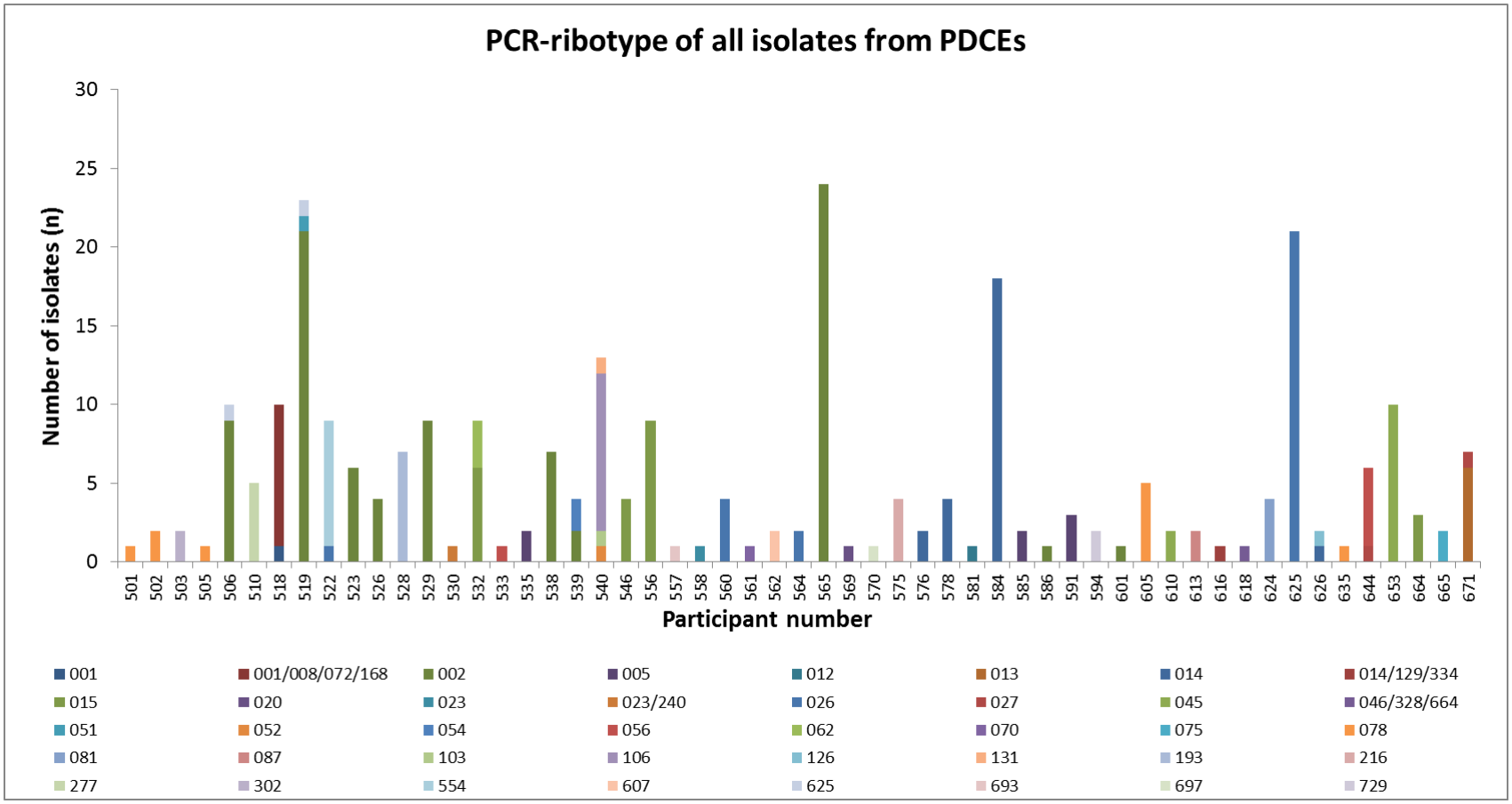


Figure 5.19. The PCR ribotypes of isolates from all PCDE participants and the number of times each PCR ribotype was isolated from that participant.

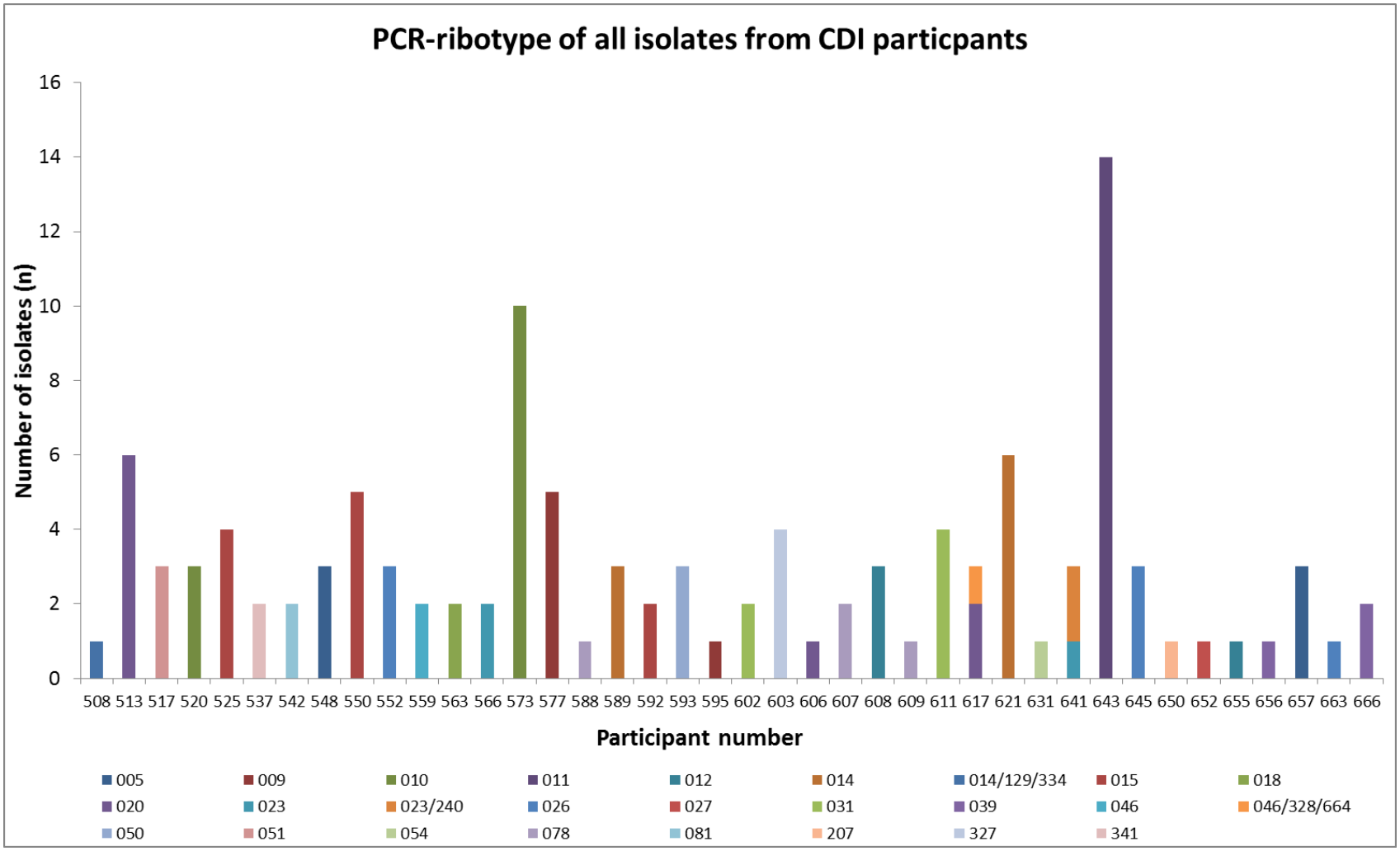


Figure 5.20. The PCR ribotypes of isolates from all CDI participants and the number number of times each PCR ribotype was isolated from that participant.

5.4 Discussion

The data from prospectively collected faecal samples from both control participants and those who were initially GDH positive has shown that the course of *C. difficile* infection in patients has similar patterns of proliferation and growth of the organism, as measured by diagnostic assays, as those seen in the gut model (Chapter 2). The GDH EIA consistently had the highest proportion of positive samples/day for all participants recruited as GDH positive, or for all participants when split into PCDE's and CDI cases, although in PCDE's this is closely mirrored by the proportion of culture positive samples. It would appear that there were more non-toxigenic strains in the PCDE vs CDI group, as there was a large difference in the number of samples positive by CTC compared with culture; by contrast, in the CDI cases, the proportion of samples positive by culture and CTC was almost identical. It should be noted however, that only 5/25 of PCDE's that did not go on to have a *C. difficile* toxin positive sample, had a known non-toxigenic PCR ribotype isolated from their samples (table 5.18). This suggests therefore that the difference in CTC and culture positive rates is due to factors other than just the presence of non-toxigenic PCR ribotypes. For all of the assays, there is an increase in the proportion of positive samples between days 20 to 40 (figure 5.7). This may represent recurrence, which will be investigated further in Chapter 4.

The proportion of PCR assay positives follows the pattern of, and is at a similar level to, the CTC positivity rate; however, at several time points the PCR rate appears to be shifted later than the CTC rate by one-two days (figures 5.11 and 5.12). Interestingly this shift is also seen in the pattern of the positivity of CCNA. Certainly, for CCNA, this mirrors the patterns seen in the gut model, where toxin production follows proliferation by a shift of a few days (Chapter 2). It is unclear why the PCR assay is shifted to follow the pattern of CCNA rather than CTC, as the PCR assay detects the toxin B gene and it could therefore, be hypothesised that the gene should increase at the same rate as proliferation. It may be explained by the fact that toxin production is not constitutive, but influenced by external factors. This is easily demonstrated within the laboratory, as *C. difficile* growing in BHI will produce toxin, whereas *C. difficile* growing in media with additional biotin will not (Yamakawa *et al*, 1998). In addition, the PCR assay used in this study did not perform as well as the GDH assay, with a sensitivity of 74.4% vs 88.7% respectively when compared to cytotoxigenic culture (table 5.13). This is in contrast to the data from the Department of Health study, where PCR and GDH assays had comparable sensitivities compared to CTC of 93.2 and 93.6%, respectively (Planche *et al*, 2013). It should be noted however, that a different PCR assay was used in the present study to that used previously, which may have lower sensitivity. Unfortunately,

this PCR assay also does not allow the user to collect cycle threshold (CT) values, which may have provided more information than the binary 'positive' and 'negative' result. CT values are discussed further in Chapter 4.

There was a similar daily CCNA positivity rate for PCDE and CDI cases (16.8%and 17.4% respectively, p = 0.52) over the first 20 days (figures 5.14 and 5.16). Interestingly, of the 65 PCDE, 18 (27.7%) went on to have at least one sample that tested positive by CCNA; median number of days to CCNA positive sample was eight days (range 0-28) (table 5.16). Peak white cell counts for the PCDE group were on day nine (10.13 cells/L), compared with day 0 (day of diagnosis) for CDI cases (10.7 cells/L). In addition both the mean white cell count and the mean serum creatinine level were higher in those PCDE that went on to have a toxin positive sample, compared to those PCDE that remained toxin negative; however only the serum creatinine was significantly higher (p = 0.009). Concomitant antibiotics with activity against *C. difficile* may have supressed growth of the organism and therefore toxin production in these PCDEs, however, only two of these participants were on an antibiotic with activity against *C. difficile* at the time of their initial sample. Does this perhaps suggest that the organism had not yet reached a level of growth where a) it had started to produce toxin, or b) had produced toxin but at a level below the limit of detection of CCNA? Testing these samples with the newly developed ultra-sensitive *C. difficile* toxin detection assays may have resolved this question; however such assays were not available at the time of this study (Pollock, 2016, Banz *et al*, 2018). The detection of these PCDEs that go on to have a toxin positive sample does not provide evidence that repeat testing of GDH positive/toxin negative samples is warranted. Repeat testing is currently not recommended in the *C. difficile* diagnostic guidelines, due to the issues of increasing the likelihood of false positive results (Crobach *et al*, 2016; McDonald *et al* 2018). Further data would be required to fully understand this group before any such change to the guidelines could be suggested. Indeed, this is a single centre study, and could be biased by the patient population studied; multicentre studies would be needed to confirm that this is not only a local phenomenon. Lactoferrin, a marker of gut inflammation, has been suggested as an adjunctive test for CDI (Wren *et al*, 2009). Recent work (Davies *et al*, 2019b) has demonstrated a correlation between lactoferrin level and level of toxin (as measured semi-quantitatively). Future studies are required to determine if raised lactoferrin in these GDH positive/toxin negative samples may provide an indication of gut inflammation, and potentially therefore, presence of toxin.

Overall, for all samples, participants with CDI had a significantly higher proportion of CCNA positive samples than PCDE's (24.7 vs 15.3% respectively, p = 0.003) (table 5.12); however the majority of samples were CCNA negative. A large number of CDI cases were negative by the time their first sample was tested in the study, and only 10/82 (12.2%) had another toxin positive sample within 72 hours of their initial diagnosis (table 5.14). This is reflected by the low sensitivity for CCNA of 24.4% and for toxin EIA of 9.3% for detecting toxin in samples taken up to 72 hours after diagnosis (table 5.15). The GDH EIA had the highest sensitivity in samples from both PCDEs and CDI cases within 72 hours of diagnosis (60.4% and 56.5% respectively), followed by culture (53.1 % and 40.4% respectively). In addition, the sensitivities of the GDH EIA and culture were higher in samples from PCDEs than CDI cases, while CTC and PCR were more sensitive in samples from CDI cases than PCDEs (CTC 37.8% vs 25.0%, and 39.1% vs 23.4%, respectively) (table 5.15). These data suggest that GDH remains detectable within patient faecal samples for much longer than other *C. difficile* markers, mirroring the patterns of detection seen in the gut model (Chapter 4). In patients that have been toxin positive, detection of GDH could therefore, indicate that the organism is no longer proliferating, but also that it has also not been cleared from the gut; potentially making that patient vulnerable to recurrence, especially if exposed to further antibiotic therapy.

The most common PCR ribotype isolated from samples was 002 (20.2% of all isolates), followed by 026 (8.7%), 014 (8.2% and 015 (8.2%)) (figure 5.17). There were very few isolates of PCR ribotype 027 (only n=3); however, these three isolates came from three different participants. This is not unexpected as there has been a shift in proportion of cases caused by PCR ribotype 027 in the UK, with 027 rate declining since 2007 (Dingle *et al*, 2017). There was some evidence of mixed infections (12.7% of all culture positive participants), with 12 participants having two different PCR ribotypes and one participant having three different PCR ribotypes isolated from their samples. One recent study found that patients with more than one PCR ribotype of *C. difficile* were more likely to go on to have a recurrent episode (Seekatz *et al*, 2018). The proportion of patients who had mixed infections in that study were higher than those seen in the PlaciD study (12.7%); 78.6% in the group who went on to have recurrence *vs* 18.1% in the group who did not develop recurrence. The reasons behind the lower mixed infection rate in the PlaciD study are unclear, although it is plausible that it may be linked to different infection control practices between the study sites.

CDI cases were significantly older than PCDEs (median age 75 *vs* 62 years respectively, P <0.0001), and there was a significantly higher proportion of females in the PCDEs than in the CDI cases (60% *vs* 40.2% respectively, P = 0.03, table 5.1). Increasing age is a well-known risk factor for CDI (McDonald *et al*, 2006, Miniño et al 2011), but this is the first time that the age of GDH-positive/toxin-negative and GDH-positive/toxin-positive patients has been compared. Participants in the control group were significantly older than GDH positive patients (median age = 80 years and 70 years respectively, p <0.0001, table 5.1). As CDI is more likely in those >65 years old (McDonald *et al*, 2006, Miniño et al, 2011), the control groups were selected from potential participants >50 years old; however, those that were recruited were actually significantly older. The rate of asymptomatic carriage of *C. difficile* in hospitalised patients in the literature is 0.6-15% (Furuya-Kanamori *et al*, 2015); however, it was at the lower end of this range in this study at 8.6% (table 5.12). The reasons for this are unclear but may have been due to the way the cohort was selected.

Another well characterised risk factor for CDI is prior antibiotic therapy (Bartlett and Gerding, 2008). In this study we were able to compare GDH positive participants (rather than just CDI) with controls, and a significantly higher proportion of patients recruited as GDH positive had at least one antibiotic in the previous eight weeks when compared to patients recruited as controls (90.7% *vs* 47.7%, P <0.0001, table 5.1). This was also true for all participants with at least one sample in the study (controls = 50.6% vs GDH positive 91.9%, p <0.0001, table 5.1). There was also a significant difference between control participants and GDH positive participants for each number of doses of antibiotic received and for the total number of doses of antibiotic received (table 5.6). GDH positive participants were significantly more likely to have the following antibiotics prescribed in the previous three months compared with control participants; ciprofloxacin, co-trimoxazole, fidaxomicin, meropenem, metronidazole, pipercillin-tazobactam, trimethoprim, vancomycin and the antifungal fluconazole (table 3.7). Exposure to ciprofloxacin and pipercillin-tazobactam has previously been shown to be a risk factor for CDI (Bartlett and Gerding, 2008). A previous prescription for fidaxomicin, must indicate previous CDI treatment, as this antibiotic has no other indication for use, while vancomycin and metronidazole may represent previous CDI treatment in this group.

Of the GDH positive participants, those with CDI were significantly more likely to have had an antibiotic in the previous eight weeks than PCDEs, although the proportions in both groups were high (96.3% Vs 84.6% respectively, p = 0.03, table 3.1). This demonstrates that

prior antibiotic therapy is a risk factor for acquisition of *C. difficile*, not just the development of CDI, in similarity to a previous study where prior use of antibiotics was significantly associated with carriage of *C. difficile* (Bignardi, 1998). However, the proportion of CDI cases that had at least one antibiotic and the total number of doses received by CDI cases were significantly higher compared with PCDEs, suggesting that increased antibiotic exposure changes the risk from just acquisition of *C. difficile* to development of CDI ($p = 0.03$ and <0.0001, respectively, table 5.6). Importantly, increasing risk of CDI from increasing does of antibiotic has been demonstrated previously (Stevens *et al*, 2011; Davies *et al*, 2016c) while the shift from risk of acquisition to risk of CDI with increasing antibiotic doses is a novel finding. However, only CDI treatment antibiotics were significantly more likely to have been prescribed to CDI cases than PCDEs in the previous three months; presumably to treat a previous case of CDI (table 5.8). A single type of antibiotic could not therefore be linked to the shift from acquisition to risk of CDI; indeed this study was not designed for such analysis and may be under powered to answer this question.

GDH positive participants were not significantly more likely than controls to be prescribed any particular class of drugs, such as PPIs; however, they were more likely to have been prescribed omeprazole and ranitidine than control participants (tables 5.9 and 5.10).The risk of developing CDI while on PPIs is disputed in the literature, and indeed maybe derived from the selection of the cohort studied (Dial *et al*, 2004; Davies *et al*, 2016c). It should be noted that there was no significant difference in the number of participants taking PPIs in the prior three months between PCDEs and CDI cases (table 5.9).

We wanted to investigate the possibility of using rectal swabs in future studies, as often faecal samples can be difficult to obtain. To this end, we asked participants in the study to also have a rectal swab taken when they were recruited into the study. This was declined by 90.9% of participants, and interestingly, a significantly higher proportion of GDH positive patients refused the rectal swab than control patients (91.2% Vs 79.0%, $p = 0.03$, table 5.1). Although there was a higher rate of refusal in the CDI cases than PCDEs this was not significant (96.3% Vs 84.6%, $p = 0.19$, table 5.1). This may suggest that those who are more sick, or those with diarrhoea, are more likely to refuse to have a rectal swab taken. If this is true, using rectal swabs for diagnosis may not be a viable option, as these are the very patients you would want to sample. There is obviously a difference in opting out of a voluntary rectal swab and having to have one for diagnosis however, but this does indicate

patient preference; they were willing to donate faecal samples to the study but not have a rectal swab taken.

There were significant differences between the control group and GDH positive group, with control participants significantly more likely to have been admitted following a fall, or with a fractured neck of femur, fracture of the knee, fracture of the shaft of the femur and septic arthritis (all p <0.01) (table 5.2). Control participants were also significantly more likely to have dementia than GDH positive participants (p = 0.01), while GDH positive participants were significantly more likely to have a history of alcoholism or asthma (p = 0.02 and 0.002 respectively, table 5.4). Participants recruited into the GDH positive arm were significantly more likely than control participants to have been admitted with gastrointestinal symptoms (diarrhoea, diarrhoea and vomiting, abdominal pain) or with pyrexia, or for being 'unwell' (table 5.3). GDH positive participants with CDI were significantly more likely to be admitted for being 'unwell' than PCDE participants (p = 0.03, table 5.3).

6. Prediction of poor outcome or recurrence using commercial assays

6.1 Introduction

Although the use of direct PCR detection of *C. difficile* toxin gene detection for CDI diagnosis is still contested within the literature (Fang *et al*, 2017) it is possible that the cycle threshold value of such assays may provide additional information for the clinician. The point at which a PCR assay becomes positive, the cycle threshold value (CT value), is determined by the amount of starting material. The more starting material (target) there is, the faster the assay will become positive, and therefore the lower the CT value will be. A low CT value has been associated with poorer patient outcomes since the pathogen load will be high. However, previous studies are limited by the use of composite outcomes, lack of reference methods, small samples size and single centre design (Kamboj *et al*, 2018; Reigadas *et al*, 2016b)

Recurrence of infection occurs in ~20% of CDI cases (Cornely *et al*, 2012; Kelly *et al*, 2008), however predicting such occurrences is extremely difficult. Prediction models have attempted, to overcome this issue (Escobar *et al*, 2017; Reveles *et al*, 2018), but a diagnostic assay that could predict recurrence would enable clinicians to use antibiotic therapies that are known to provide greater protection to the gut microbiome, such as fidaxomicin or immunological adjunctive therapies such as bezlotoxumab (Cornely *et al*, 2012, Wilcox *et al*, 2018). The high cost of such treatments often precludes their use, however: cost-effectiveness studies demonstrate that fidaxomicin may be the most cost-effective option, due to the reduction in recurrence cases, and on-going care costs (Lapointe-Shaw *et al*, 2016). A predictive diagnostic assay would therefore strengthen the justification for use.

Here, the data gathered from the Department of Health (DoH) study to optimise laboratory diagnosis of CDI, and data from the PlaciD study (Chapter 5) have been interrogated to investigate both the utility of CT values to predict patient outcomes, and alternative assays that could predict CDI recurrence. The key findings from this chapter are;

- The CT value of a PCR test for detection of toxin gene was significantly associated with a toxin-positive result, presence of PCR ribotype 027 and mortality
- The positive predictive value of low CT (≤25) for toxin detection was 84%

- The relative risk of mortality for patients with low CT (≤25) was 1.45, and increased to 2.18 if patients also had infection caused by PCR ribotype 027
- GDH OD value was related to the bacterial load of *C. difficile* within the sample
- A cohort of patients were discovered that were constantly positive for GDH in their sequential faecal samples, despite resolution of symptoms. All of these patients went on to have a recurrent episode of CDI following additional antibiotic therapy

6.2 Methods

6.2.1 Can the cycle threshold value of commercial molecular assays for C. difficile toxin genes provide additional information to the diagnosis of a toxigenic C. difficile strain?

6.2.1.1 Analysis of existing data set to determine if the cycle threshold value of commercial molecular assays for *C. difficile* toxin genes can provide additional information to the diagnosis of a toxigenic *C. difficile* strain

Hypothesis

A lower cycle threshold (CT) value from a molecular assay for the detection of a toxigenic strain of *C. difficile* will correlate with mortality, toxin status and recurrence. This work has been published; Davies K, Planche T and Wilcox M. The predictive value of quantitative nucleic acid amplification detection of *Clostridium difficile* toxin gene for faecal sample toxin status and patient outcome. PLoS One 13(12): e0205941 2018. The concept of the work was devised by Kerrie Davies, the analysis and initial draft of the manuscript was completed by Kerrie Davies with review by Dr Tim Planche and Prof Mark Wilcox.

Methods

Diarrhoeal faecal samples, collected as part of the Department of Health, multicentre evaluation of CDI laboratory diagnostics, as described in Chapter 3, were tested with two reference methods; cell cytotoxicity and cytotoxigenic culture, and by three enzyme immunoassays (EIAs); one for detection of glutamate dehydrogenase (*C. diff CHEK-60*™, Techlab, USA) and two for the detection of toxins A and B (*Toxin AB II*™, Techlab, USA and Premier Toxins A & B™, Meridian, USA) (Planche *et al*, 2013). In addition, samples were also tested using the commercial molecular assay Cepheid geneXpert® C. diff (Cepheid, Sunnyvale, CA, US), for the detection of *C. difficile* toxin genes (*tcdB*, binary toxin and a

truncated form of *tcdC*); as described in methods section 2.2.4. All isolates of *C. difficile* were typed by CDRN using PCR-ribotyping, as described in methods section 2.1.6.

The samples were collected between October 2010 and September 2011 from Leeds Teaching Hospitals, Leeds; St George's University of London, London; University College Hospital NHS Foundation Trust, London and Oxford University Hospitals NHS Trust, Oxford. Assays and reference methods were performed on the same day by the same evaluator at three sites (Leeds and both London sites) or two evaluators in one site (Oxford). In total, one evaluator worked at each of the two London sites, two at Leeds, and three at Oxford. Kerrie Davies was one of the evaluators at the Leeds site and scientific coordinator for the entire study. Ethical approval for the extended analysis of the study data was given by the NHS REC, approval number 12/EE/0495. The initial study ethical approval number was 10/H0715/34.

Commercial enzyme immunoassays

All of the commercial EIAs were performed on an automated platform (DS2, Dynex Magellan Biosciences, USA) to reduce operator error, following manufacturer's protocols given in the kit insert, with minor changes listed within the Materials and Methods chapter section 2.2, and as discussed with the manufacturer. The optical density (OD) of each test was recorded as well as the result defined by the assay (using manufacturer's set cut-offs).

Reference methods

The cell-cytotoxicity neutralisation assay, culture and cytotoxigenic culture assay were performed as described in the Materials and methods chapter section 2.1.

Commercial molecular test

The commercial molecular test was performed on a Cepheid geneXpert® instrument following the protocol in the manufacturer's kit insert, as described in the Material and Methods chapter section 2.2.4.

PCR ribotyping

The *C. difficile* isolates identified from the study were typed using PCR ribotyping by the *C. difficile* Ribotyping Network of England and Northern Ireland (CDRN) as previously described (Stubbs *et al*, 1999) and as expounded in the Materials and Methods chapter section 2.1.6.

Analysis

Only those patients who had at least one PCR-positive sample were included in the analysis. The Area Under Receiver Operator Curve (AUROC) was calculated for those samples that were positive by the PCR assay (as defined by the algorithm within the assay software of the Cepheid Xpert® platform) to determine if there was an association between low cycle threshold (CT) and patient mortality, recurrence and the presence of a toxin-positive faecal sample. Recurrence was defined as a sample that tested positive for toxin (by CCNA) at least 14 days after the initial CCNA-positive sample for that same patient was tested. Recurrence could only be assessed in those patients who had repeat samples submitted within the study period. Once low CT was defined, associations between low CT (≤25) and markers of CDI severity and outcome were investigated by univariate analysis; *t* test was used to compare means, Mann-Whitney for Medians (LOS) and χ^2 test for categorical variables.

6.2.2 Can diagnostic assays be used to predict recurrence?

6.2.2.1 Analysis of existing data set to determine if there is an association between a positive primary *C. difficile* diagnostic assay and recurrent infection

Hypothesis

There is an association between laboratory assays used to diagnose the presence of *C. difficile* or *C. difficile* infection on a primary sample, and the likelihood of that patient suffering from a recurrent infection.

Methods

Diarrhoeal faecal samples, collected as part of the Department of Health, multicentre evaluation of CDI laboratory diagnostics, as described in Chapter 3, were tested with two reference methods; cell cytotoxicity and cytotoxigenic culture, and by three enzyme immunoassays (EIAs); one for detection of glutamate dehydrogenase (*C. diff CHEK-60*™, Techlab, USA) and two for the detection of toxins A and B (*Toxin AB II*™, Techlab, USA and Premier Toxins A & B™, Meridian, USA) (Planche *et al*, 2013). In addition, samples were also tested using the commercial molecular assay Cepheid geneXpert® C. diff (Cepheid, Sunnyvale, CA, US), for the detection of *C. difficile* toxin genes (*tcdB*, binary toxin and a

truncated form of *tcdC*); as described in methods section 2.2.4. All isolates of *C. difficile* were typed by CDRN using PCR-ribotyping, as described in methods section 2.1.6.

The samples were collected between October 2010 and September 2011 from Leeds Teaching Hospitals, Leeds; St George's University of London, London; University College Hospital NHS Foundation Trust, London and Oxford University Hospitals NHS Trust, Oxford. Assays and reference methods were performed on the same day by the same evaluator at three sites (Leeds and both London sites) or two evaluators in one site (Oxford). In total, one evaluator worked at each of the two London sites, two at Leeds, and three at Oxford. Kerrie Davies was one of the evaluators at the Leeds site and scientific coordinator for the entire study. Ethical approval for the extended analysis of the study data was given by the NHS REC, approval number 12/EE/0495. The initial study ethical approval number was 10/H0715/34.

Commercial enzyme immunoassays

All of the commercial EIAs were performed on an automated platform (DS2, Dynex Magellan Biosciences, USA) to reduce operator error, following manufacturer's protocols given in the kit insert, with minor changes listed within the Materials and Methods chapter section 2.2 and as discussed with the manufacturer. The optical density (OD) of each test was recorded as well as the result defined by the assay (using manufacturer's set cut-offs).

Reference methods

The cell-cytotoxicity neutralisation assay, culture and cytotoxigenic culture assay were performed as described in the Materials and Methods chapter section 2.1.

Commercial molecular test

The commercial molecular test was performed on a Cepheid geneXpert® instrument following the protocol in the manufacturer's kit insert, as described in the Material and Methods chapter section 2.2.5.

PCR ribotyping

The *C. difficile* isolates identified from the study were typed using PCR ribotyping by the *C. difficile* Ribotyping Network of England and Northern Ireland (CDRN) as previously described (Stubbs *et al*, 1999) and as expounded in the Materials and Methods chapter section 2.1.6.

Analysis

Recurrence was assessed in those patients that had repeat samples submitted within the study period and was defined as a sample that tested positive for toxin (by CCNA) at least 14 days after the initial CCNA positive sample for that same patient was tested (Group 1). Odds ratios (OR) for recurrence were calculated for each assay type. Due to the association of CCNA with recurrence, CCNA positives were removed from the sample pool for the other assay types to leave CTC positive/CCNA negative (Group 2), PCR positive/CCNA negative (Group 3) and CT<25/CCNA negative (Group 4). A binary logistic regression model was constructed for recurrence, with all data, and with group data as defined above, with a cut off of 0.5.

6.2.2.2 Analysis of data from the PlaciD dataset to determine if there is an association between the bacterial bioload during primary infection and recurrence

Hypothesis

There is an association between bacterial bioload (as measured by EIAs) during the primary infection and recurrence?

Methods

Patients with GDH positive faecal samples (diagnosed by the routine microbiology laboratory) and controls (≥50 year olds from the same hospital but who had no diarrhoeal symptoms) were recruited and followed during their hospital admission; faecal samples were collected daily where possible with routine clinical data. Samples were tested using culture for *C. difficile*, cell-cytotoxicity assay (CCNA), cytotoxigenic culture (CTC), enzyme immunoassays (EIAs) for glutamate dehydrogenase (GDH) and toxins (Chek-60 and ToxABII, Techlab, USA) and PCR for toxin genes (BD Max™, BD). All samples were tested by Kerrie Davies, except for a four month period of maternity leave, when samples were tested by Claire Berry, Clinical Scientist within the Healthcare Associated Infections research group. A full description of the PlaciD study is given in Chapter 5. Ethical approval for PlaciD was given by NHS REC, approval number 13/NE/0255.

Commercial enzyme immunoassays

All of the commercial EIAs were performed on an automated platform (DS2, Dynex Magellan Biosciences, USA) to reduce operator error, following manufacturer's protocols

given in the kit insert, with minor changes listed within the Materials and Methods chapter section 2.2, and as discussed with the manufacturer. The optical density (OD) of each test was recorded as well as the result defined by the assay (using manufacturer set cut-offs).

Reference methods

The cell-cytotoxicity neutralisation assay, culture and cytotoxigenic culture assay were performed as described in the Materials and Methods chapter section 2.1.

Commercial molecular test

The commercial molecular test was performed on a BD Max™ instrument following the protocol in the manufacturer's kit insert, as described in the Material and Methods chapter section 2.2.6.

PCR ribotyping

The *C. difficile* isolates identified from the study were typed using PCR ribotyping by the *C. difficile* Ribotyping Network of England and Northern Ireland (CDRN) as previously described (Stubbs *et al*, 1999) and expounded in the Materials and Methods chapter section 2.1.6.

Analyses

Of 228 patients recruited (with samples collected), 82 were cases and 81 were controls (totally contributing 1173 sampling days). Cases were categorised into single cases (n=62) and recurrent cases. Recurrence was defined as more than one CCNA-positive sample in a case >14 days after they had a CCNA-negative sample (n=20).

Optical density (OD) values for EIAs, white cell counts, serum albumin and serum creatinine values were compared between cases and controls, and between cases with a single episode and those with recurrent episodes. Antibiotic and proton pump inhibitor (PPI) use in those patients with recurrent episodes were also examined.

6.3 Results

6.3.1 Can the cycle threshold value of commercial molecular assays for C. difficile toxin genes provide additional information to the diagnosis of a toxigenic C. difficile strain?

6.3.1.1 Analysis of existing data set to determine if the cycle threshold value of commercial molecular assays for *C. difficile* toxin genes can provide additional information to the diagnosis of a toxigenic *C. difficile* strain

The study collected 8853 samples from 7335 patients across the four sites; 1281 of these samples tested positive using the PCR assay and were therefore included in the analysis. Of the 1281, 713 were CCNA positive and 917 were CTC positive, with a positive agreement between the two reference assays of 51.2% (figure 4.1). The median CT value for *tcdB* gene detection by the PCR assay for samples from patients who died was 25.5 *vs* 27.5 for those who survived (p = 0.021), 24.9 *vs* 31.6 for CCNA positive samples *vs* CCNA negative faecal samples respectively (p <0.001) and 25.6 *vs* 27.3 for samples from patients who went on the have a recurrent episode *vs* those who had a single episode of CDI (p = 0.111). The AUROC was plotted for CT value against death, CCNA-positive result and recurrence (figure 4.2). The highest AUROC was for toxin positivity (0.831, 95% CI 0.808-0.853, P<0.001) (figure 4.2b) followed by mortality (0.572, 95% CI 0.519-0.624, p = 0.009) (Figure 4.2a) and recurrence (0.557, 95% CI 0.490-0.624 p = 0.11) (figure 4.2c). In addition, box plots of CT value for mortality/survival, CCNA positive/negative and recurrence/single infection were plotted to show the differences between the median CT values for each group (Figure 4.3).

CT values of ≤24 (to optimise for CCNA positivity) and ≤25 (to optimise for mortality) were both investigated as possible cut-off values for the PCR assay. The agreement between the reference assays using each of these CT value cut-offs was investigated. Using a cut-off of ≤25, there were 436 samples, 366 of which were CCNA positive and 399 of which were CTC positive, with a positive agreement of 78.4%. Using a cut-off of ≤24 there were 145 samples, 120 of which were CCNA positive and 131 of which were CTC positive, with a positive agreement of 76.6% (figure 4.1).The positive predictive value for recurrence with both cut-off values were 49.3% and 18.3% for CT≤25 and CT≤24 respectively.

Low CT

Univariate analysis demonstrated that a CT cut-off of ≤25 was significantly associated with a toxin-positive sample result, the presence of PCR ribotype 027 in the sample, and patient mortality with a positive predictive value of 83.9% for the detection of toxin from the sample by CCNA (table 6.1). A CT cut-off of ≤24 was also found to be significantly associated with a toxin positive sample result and the presence of PCR ribotype 027 in the sample, with a positive predictive value of 86.1% for the detection of toxin from the sample by CCNA (table 6.1). The cut-off of CT ≤24 was not, however, significantly associated with patient mortality, in contrast to the cut-off of CT ≤25. Due to the slight advantage of using CT ≤25 rather than ≤24 as the cut-off, further analyses defined 'low CT' as ≤25.

Patients with a low CT (≤25) had higher mean white cell count, a higher mean baseline serum creatinine, lower mean serum albumin and longer length of hospital stay, however none of these differences were significant, with the exception of serum creatinine (p = 0.04, table 6.1).

As both low CT and the presence of PCR ribotype 027 were associated with mortality and length of stay, the combined effect of these two variables was examined. Mortality and increased length of stay were both significantly associated with samples that had both a low CT and the presence of PCR ribotype 027 combined (table 6.1.). The positive predictive value of low CT for mortality in this population was 17.4% (95% CI 13.5-22.2%) with a negative predictive value of 88.0% (95% CI 85.0-90.5%). The relative risk of mortality in patients with low CT was 1.45 (95% CI 1.0-2.0, p = 0.04), however this increased to 2.18 (95% CI 1.2-4.0 p = 0.03) in those cases that were due to PCR ribotype 027 (in addition to the low CT result). The relative risk of mortality in those patients with a sample with a low CT result and a positive binary toxin gene test was 1.72 (95% CI 1.1-2.8, p = 0.05). In addition, there was a significant difference in the mortality rates between those patients with versus without binary toxin gene detection from their samples (25.3 *vs* 14.7%, p = 0.049, table 6.1). Importantly, however, there was no difference in the rate of toxin positive (CCNA) samples between those with or without detection of the binary toxin gene from their samples (table 6.1). Patients with a low CT and whose samples tested positive for binary toxin had higher mean WCC, a higher mean serum creatinine, lower mean serum albumin and had a shorter length of stay in hospital. Again, the only significant difference was between the serum creatinine levels of the two groups (p = 0.006) (table 6.1). Unsurprisingly, a positive binary toxin gene result was significantly associated with samples containing PCR ribotype 027 (p<0.001) (table 6.1.).

Variable	CT≤25 (n/N (%))	CT>25 (n/N (%))	*p* value*	CT≤24 (n/N (%))	CT>24 (n/N (%))	*p* value*	CT≤25 and Binary toxin PCR positive (n/N [%])	CT≤25 and Binary toxin PCR negative (n/N [%])	*p* value*
All PCR-positive patients									
CCNA positive	366/436 (83.9)	347/838 (41.4)	<0.001	278/323 (86.1)	435/523 (45.4)	<0.001	100/112 (89.3)	266/324 (82.1)	0.10
Died	54/310 (17.4)	69/575 (12.0)	0.032	41/233 (17.6)	82/652 (12.6)	0.52	20/79 (25.3)	34/231 (14.7)	0.049
Recurrence	36/189 (19.0)	42/307 (13.7)	0.111	27/145 (18.6)	51/351 (14.5)	0.70			
Had PCR ribotype 027	46/272 (16.9)	41/387 (10.6)	0.025	35/323 (10.8)	52/958 (5.4)	0.003	42/79 (53.2)	1/189 (0.5)	<0.001
Median length of stay (days)	28	23	0.77	n/a[c]	n/a[c]	n/a[c]	20	29	0.11
Mean white cell count ($x10^9$/L)	12.1	10.9	0.3	n/a[c]	n/a[c]	n/a[c]	13.2	11.8	0.13
Mean serum creatinine	120.0	110.7	0.04	n/a[c]	n/a[c]	n/a[c]	112.9	111.8	0.006

(mg/dL)									
Mean serum albumin (g/L)	31.3	32.4	0.34	n/a[c]	n/a[c]	n/a[c]	31.0	31.3	0.46
All PCR positive patients with PCR ribotype 027									
Median length of stay (days)	32.5	28	0.018	n/a[c]	n/a[c]	n/a[c]	N/A	N/A	
Died	11/33 (33.3)	26/170 (15.3)	0.024[b]	n/a[c]	n/a[c]	n/a[c]	11/32 (34.4)	0/1 (0)	0.66[b]

* *t* test for comparisons of means, Mann Whitney for comparisons of medians, χ^2 for categorical comparisons. [b] Fisher's exact probability. [c] CT ≤ 24 not investigated further as low CT defined as ≤5 for further analyses.

Table 6.1 Outcomes and severity markers for patients with CT values ≤ 25 or > 25. Reproduced from PLOS One with permission

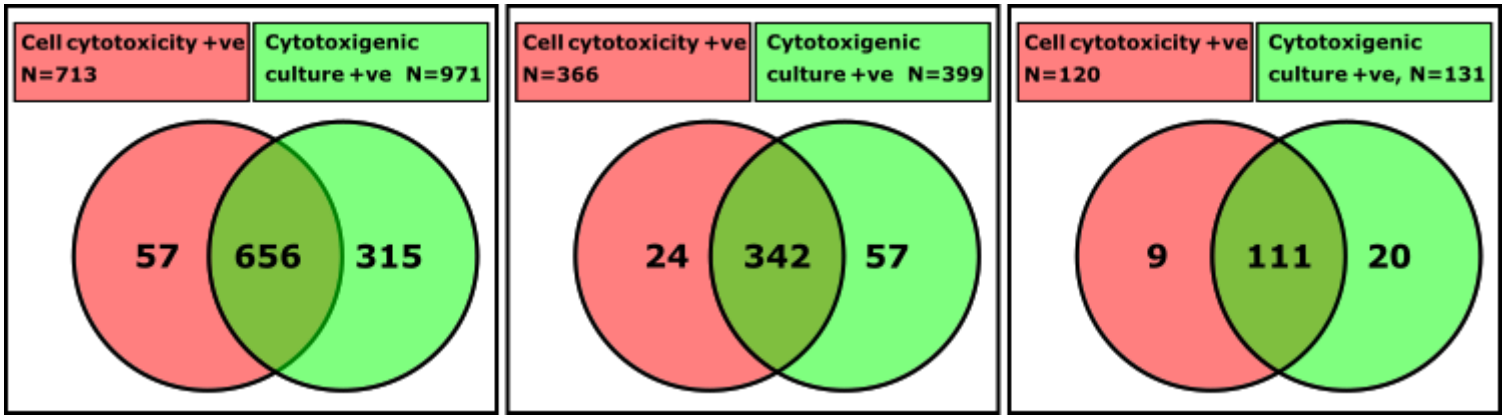


Figure 6.1 Results of *C. difficile* reference methods A) all PCR positive samples (n = 1281) positive agreement 51.2%; (B) those with only CT < 25 (n = 436) positive agreement 78.4%; (C) those with only CT ≤ 24 (n = 145) positive agreement 76.6%. Reproduced from PLOS One with permission

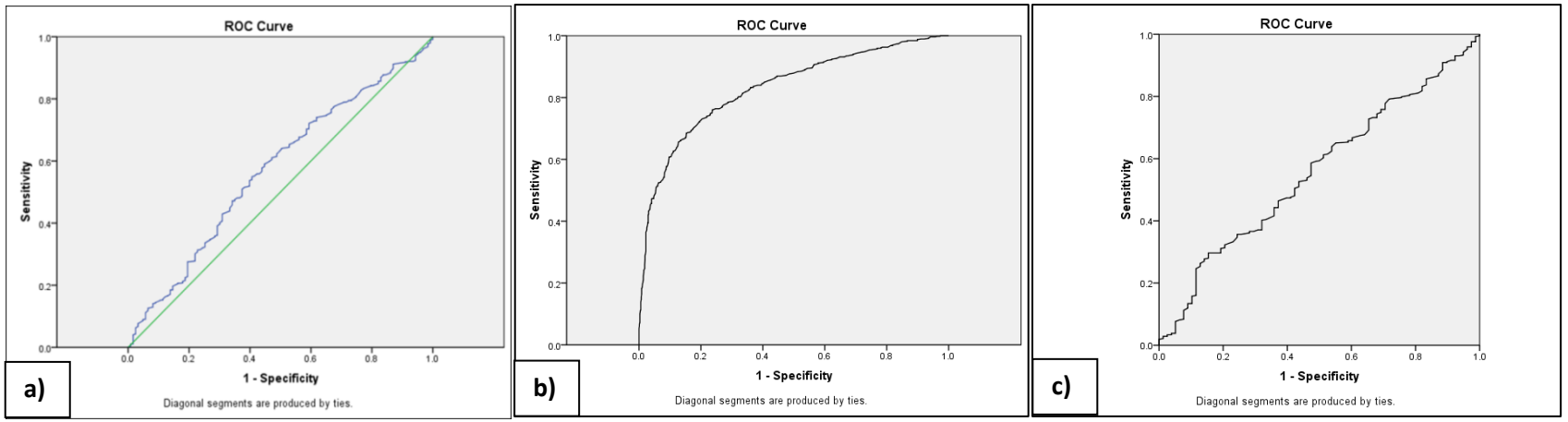


Figure 6.2 ROCs for PCR *tcdB* CT value against a) mortality (AUROC 0.569) and b) CCNA (0.83) and c) recurrence (0.557). Reproduced from PLOS One with permission

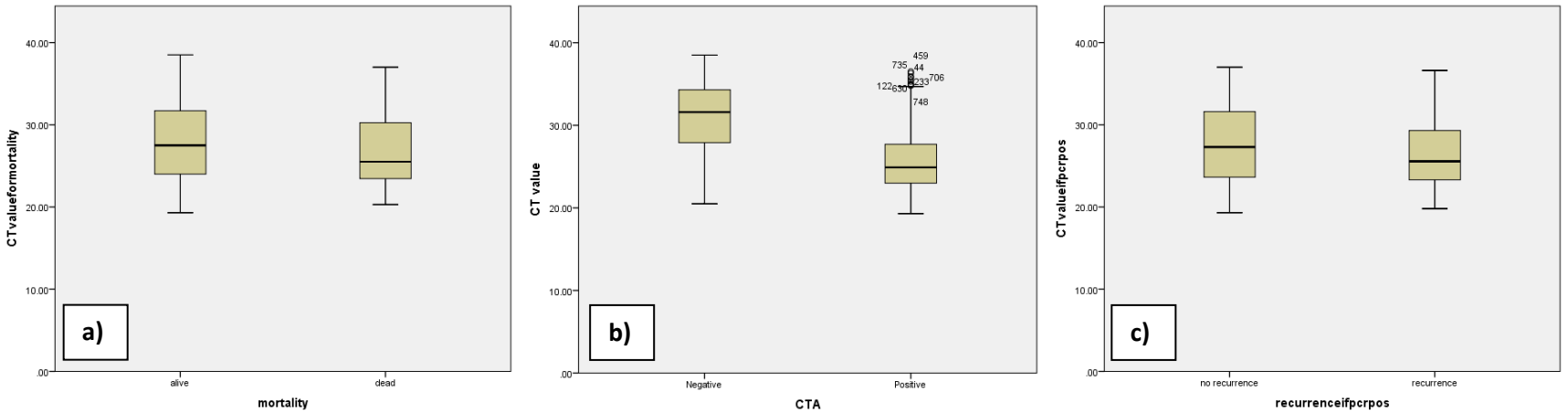


Figure 6.3 Boxplot of PCR *tcdB* CT value against a) mortality b) CCNA result and c) recurrence. Values clustered around the top bar indicate outlier values. Reproduced from PLOS One with permission

6.3.2 Can diagnostic assays be used to predict recurrence?

6.3.2.1 Analysis of existing data set to determine if there is an association between a positive primary *C. difficile* diagnostic assay and recurrent infection

There were 1578 patients that had with more than one sample submitted during the period of the study, equating to a total of 3808 samples that could be used for the analysis of recurrence. The highest risk of recurrence occurred in those patients who had a previous sample that was CCNA positive (recurrence rate 20.3%, OR 8.04, 95% CI 5.69-8.18, p <0.001, table 6.2.). However, having a sample that had been previously positive by any of the assays was significantly associated with recurrence (all p <0.011, table 6.2.). Although the OR for recurrence if the previous sample was positive by PCR was 6.84, this analysis also contained samples that were also CCNA positive. The data were therefore adjusted to contain only those that were PCR positive (Group 3) and the OR decreased to 2.52 (95% CI 1.56-4.07, p <0.001, table 6.2.). The same pattern was seen for recurrence risk in those patients whose previous sample had been CTC positive; the OR decreased from 5.86 (95% CI 4.21-8.18, p <0.001) to 2.13 (95% CI 1.18-3.84, p = 0.011, Table 6.2.) once CCNA-positive samples were removed from this group (Group 2). Whilst the ORs were similar between all those with an initial positive PCR test *vs* those with a PCR test with *tcdB* CT≤25 (6.84 *vs* 6.31 [95% CI 4.96-9.43 and 4.21-9.44, respectively]), it should be noted that the rate of recurrence in the *tcdB* PCR CT≤25 group was higher than that in the whole group that was positive by PCR, although this was not significant (19% *vs* 15.7%, p = 0.35) (table 6.2.). There was however a significant difference between group 3 (PCR positive/CCNA negative) and Group 4 (PCR positive with CT≤25/CCNA negative) (9.6% *vs* 19.0%, p = 0.009, table 6.2.). There was also a higher rate of recurrence in those patients with *tcdB* PCR-positive samples that were also positive for binary toxin gene, compared with those without binary toxin gene (24.2% *vs* 13.6%, OR 2.03 95% CI 1.18-3.49, p = 0.009, table 6.2.1). However, the difference in recurrence rates between those with and without binary toxin gene in those samples with *tcdB* PCR CT≤25 (23.7 *vs* 17.9%, OR 1.43 95% CI 0.61-3.35, p = 0.42, table 6.2.1) was not significant.

The relationship between initial samples that were baseline positive by any assay, followed by a CCNA-positive sample within 14 days was also analysed (table 6.2.) Interestingly, the CCNA positive rate in subsequent samples within 14 days when the initial positive result was by CCNA (Group 1), CTC only (Group 2) or PCR with low CT only (Group 4) was

approximately half that of the positive number after 14 days. However, the positive rate for those in Group 3 (initial sample positive by PCR only) was the same (9.6 *vs* 9.9%) within both periods.

In a binary logistic regression model for recurrence (n = 3804 samples), with the variables; initial test positive by CTC, CCNA, GDH, PCR or *tcdB* PCR CT ≤25, having an initial sample that was positive by CCNA or PCR was significantly associated with recurrence (p <0.001, table 6.2.). However, as the PCR group also contained samples that were positive by CCNA, the binary logistic regression analysis was also repeated for the variables CCNA (Group 1, Group 2, Group 3 and Group 4, n=496 samples). Following this analysis, recurrence was only significantly associated with having an initial sample that was positive by CCNA (p = 0.046, table 6.2.).

		Patient had another CCNA positive sample after 14 days							
Initial test	Initial test result	No	Yes	Total	Recurrence %	OR	95% CI for OR	*p* value (χ^2)	Binary logistic regression *p* value
Cytotoxigenic culture	negative	3298	103	3401	3.0				
	positive	344	63	407	15.5	5.86	4.21-8.18	<0.001	0.55
Cell cytotoxicity neutralisation assay (Group 1)	negative	3414	108	3522	3.1				0.001 (0.046 in second model)
	positive	228	58	286	20.3	8.04	5.69-8.18	<0.001	
Group 2	negative	3502	153	3655	4.2				
	positive	140	13	153	8.5	2.13	1.18-3.84	0.011	0.840
GDH	negative	3113	90	3203	2.8				
	positive	530	76	606	12.5	4.96	3.61-6.82	<0.001	0.56
PCR	negative	3225	88	3313	2.7				
	positive	418	78	496	15.7	6.84	4.96-9.43	<0.001	<0.001
Group 3	negative	3445	145	3590	4.0				Dropped from model
	positive	198	21	219	9.6	2.52	1.56-4.07	<0.001	
CT<25	CT>25	3486	130	3616	3.6				
	CT<25	153	36	189	19.0	6.31	4.21-9.44	<0.001	0.73
Group 4	CT>25	265	42	307	13.7				
	CT<25	153	36	189	19.0	1.49	0.91-2.4	0.111	0.76
PCR and Binary toxin PCR	Positive	24	75	99	24.2				
	Negative	54	343	397	13.6	2.03	1.18-3.49	0.009	N/A
CT<25 and Binary toxin PCR	Positive	9	29	38	23.7				N/A
	Negative	27	124	151	17.9	1.43	0.61-3.35	0.42	
		Patient had another CTA positive sample within 14 days							
Initial test	Initial test	No	Yes	Total	CCNA positive	OR	95% CI for OR	*p* value (χ^2)	

	result				%				
Cell cytotoxicity neutralisation assay (group 1)	Negative	3431	17	3522	0.5				
	positive	363	44	407	10.8	24.5	13.8-43.2	<0.001	N/A
Group 2	Negative	3600	55	3655	1.5				
	Positive	147	6	153	3.9	2.67	1.13-6.3	0.045	N/A
Group 3	Negative	3225	88	3313	2.8				
	Positive	437	49	496	9.9	4.1	2.9-5.9	<0.001	N/A
Group 4	Negative	237	70	307	22.8				
	Positive	166	23	189	12.6	0.47	0.28-0.78	0.005	N/A

Odds ratios and χ^2 results for categorical comparisons. Initial binary logistic regression included the variables CTC, CCNA, GDH, PCR or PCR *tcdB* CT<25, second model included the variables CCNA (Group 1), CTC positive/CCNA negative (Group 2), PCR positive/CCNA negative (Group 3) and CT<25/CTA negative (Group 4).

Table 6.2 The risk of recurrent CDI according to prior *C. difficile* test results in patients who had more than one sample

6.3.2.2 Analysis of data from the PlaciD dataset to determine if there is an association between the bacterial bioload during primary infection and recurrence

GDH OD values correlated with bacterial load in the sample, as measured by semi-quantitative culture (figure 6.4a-c) with a significant Spearman's correlation of 0.74 (p <0.001) suggesting that GDH OD values could be used to measure the faecal bacterial burden. There were significant differences between the GDH OD values for cases and controls (median GDH OD for cases = 0.182 *vs* 0.005 for controls; Mann Whitney p <0.001), and between cases with a single episode and those with recurrent episodes (median GDH OD for recurrent cases = 0.778 *vs* 0.058 for controls; Mann Whitney p <0.001), with those with recurrence having the highest median GDH OD values of all.

There were 20 patients (mean age 70) with CDI who had a recurrent infection with a total of 25 recurrent episodes. In 16 of these 25 recurrent episodes (64%), which occurred in 14 of the 20 patients with CDI, GDH was detected before the CCNA positive sample that signalled the recurrent CDI episode (median 8.5 days before). In five of the 14 patients (equating to six out of the 16 episodes where GDH was detected first) individuals (median age 77) had consistent GDH-positive results, despite having a resolution of their diarrhoea symptoms. In total, these five patients had 70 samples tested; 69/70 samples were positive for GDH, 55/70 were positive by cytotoxigenic culture and 59/70 were positive for toxin genes by PCR assay (figures 6.5a-e). Interestingly, the toxin gene PCR assay was not positive on as many occasions as the GDH assay, suggesting that the GDH assay potentially has a lower limit of detection. We were unable to recover *C. difficile* by culture from any samples from one of the patients; the patient had, however, been treated with fidaxomicin, which has been shown to reduce bacterial recovery from clinical samples (Chilton, PLOS one 2016).

Six of the six recurrence episodes in these five patients were precipitated by antibiotic therapy, for four of the six antibiotic therapies these were not given to treat CDI (figures 6.5a-e). In addition, four of the five patients were using concomitant PPIs. These five patients appear to have been more ill than those who cleared the bacteria below the limit of detection for the GDH assay between CDI episodes, with higher WCC's, higher serum albumin levels and lower serum creatinine levels (table 6.3).

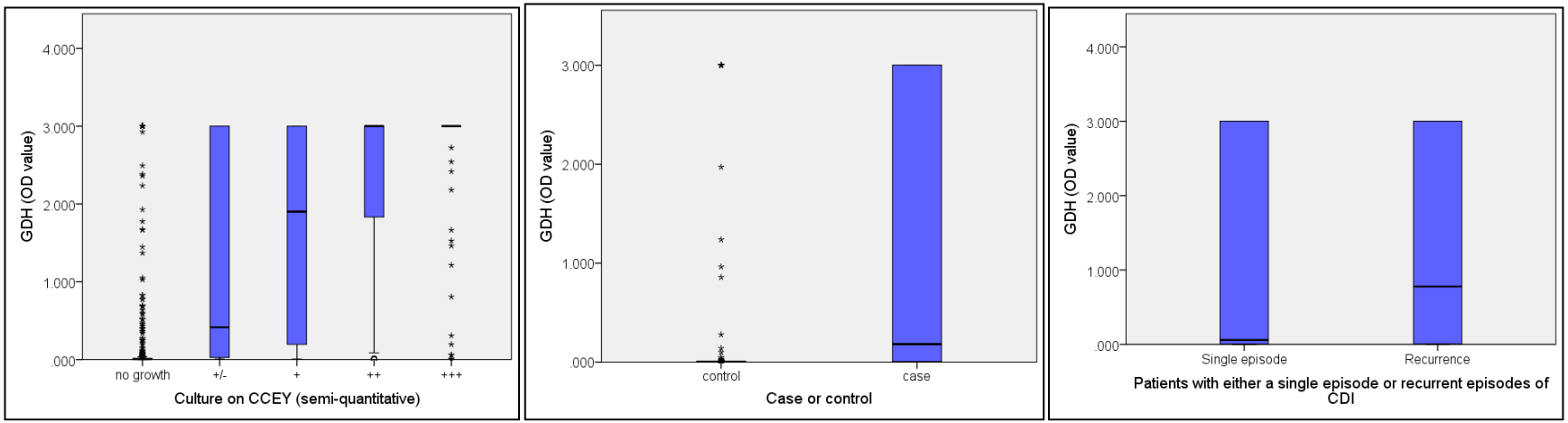
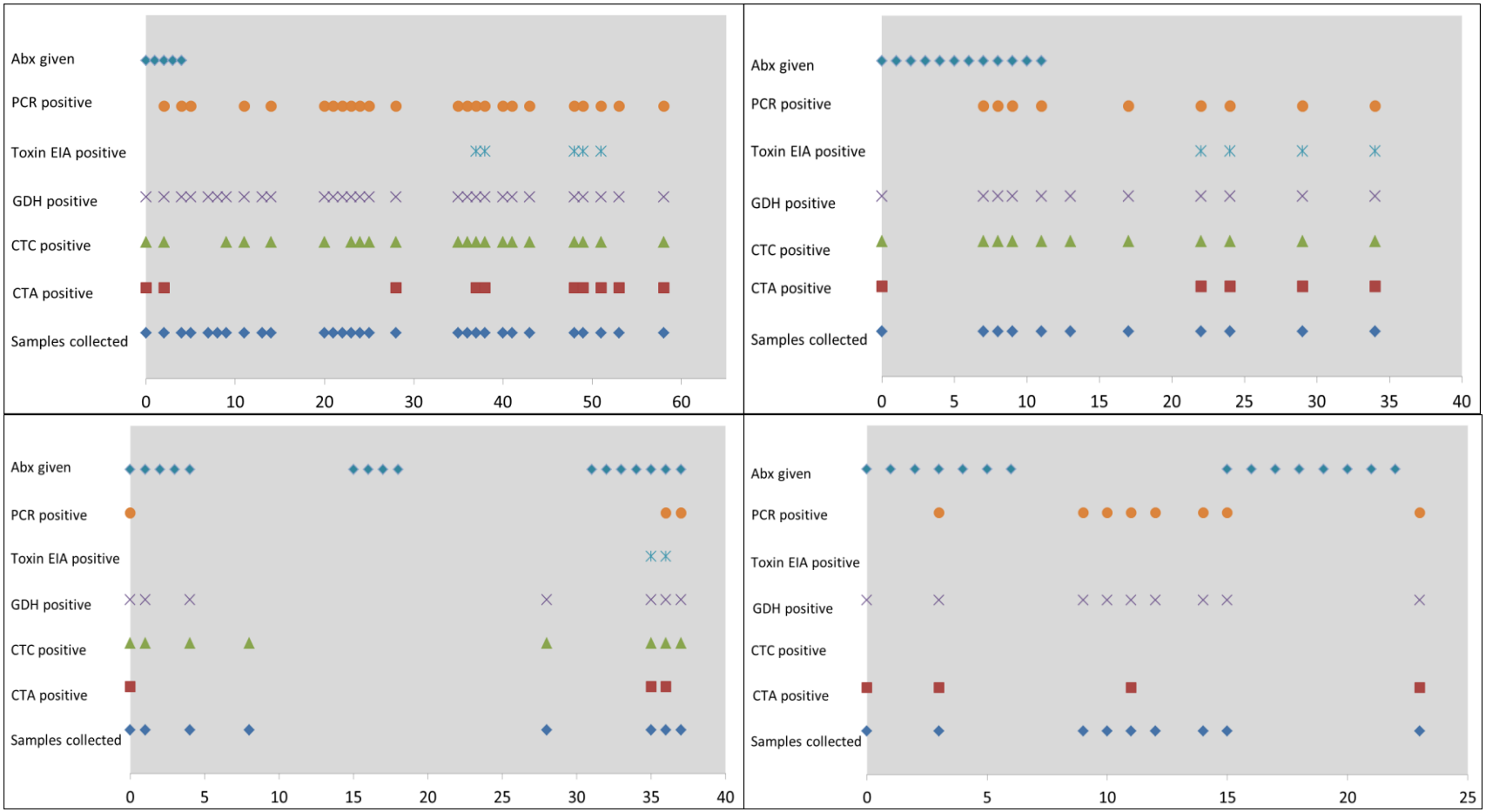
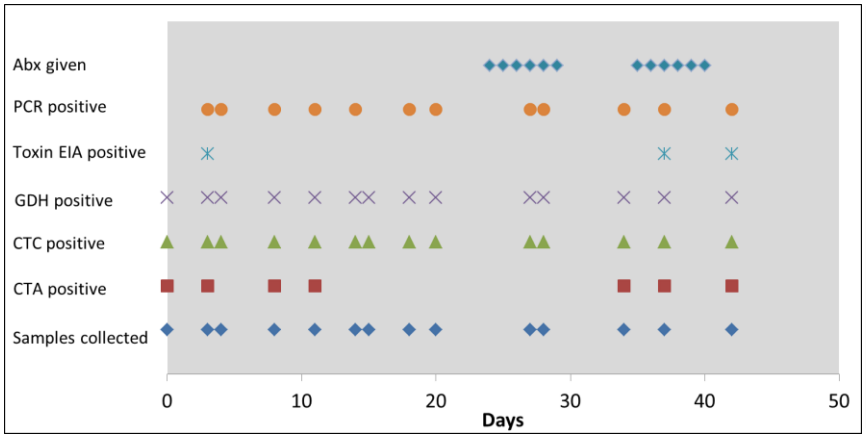


Figure 6.4 a) GDH OD values for different bacterial loads, as measured by semi-quantitative culture. Spearman's = 0.74, *p* <0.001); b) GDH OD values for cases and controls. Median GDH OD for cases = 0.182 *vs* 0.005 for controls (U *p* <0.001); c) GDH OD values for recurrent cases and single CDI cases. Median GDH OD for recurrent cases = 0.778 *vs* 0.058 for controls (U *p* <0.001)


Abx given
PCR positive
Toxin EIA positive
GDH positive
CTC positive
CTA positive
Samples collected
0 10 20 30 40 50 60
Abx given
PCR positive
Toxin EIA positive
GDH positive
CTC positive
CTA positive
Samples collected
0 5 10 15 20 25 30 35 40
Abx given
PCR positive
Toxin EIA positive
GDH positive
CTC positive
CTA positive
Samples collected
0 5 10 15 20 25 30 35 40
Abx given
PCR positive
Toxin EIA positive
GDH positive
CTC positive
CTA positive
Samples collected
0 5 10 15 20 25



Key:	
Horizontal axis	Days
Abx given	Day on which antibiotics were given to the patient
PCR positive	Sample tested positive for *C. difficile* toxin genes
Toxin EIA positive	Sample tested positive for *C. difficile* toxin using toxin EIA
GDH positive	Sample tested positive for GDH using GDH EIA
CTC positive	Sample tested positive for a toxigenic strain of *C. difficile*
CTA positive	Sample tested positive for *C. difficile* toxin using cell-cytotoxicity neutralisation assay
Sample collected	Days on which faecal samples were collected

Figures 6.5a-e. The days on which the five recurrent cases had samples taken and tested, and the results of each of those tests

		WCC (10.8×10^9 cells per litre)	Serum albumin (g/L)	Serum creatinine (μmol/L)
	mean	7.34	32.77	93.51
	min	0.13	23	26
Controls	max	16.81	42	552
	mean	10.92	31.12	105.67
	min	0.01	5.8	23
Cases	max	1135	45	752
p value (case/ control)		0.47	0.1	0.44
	mean	11.14	32.07	110.79
	min	0.01	18	31
Single	max	1135	45	752
	mean	10.34	28.66	93.38
	min	3.45	5.8	23
Recurrent	max	45.35	44	747
p value (single/ recurrent)		0.28	<0.001	0.21
	mean	10.18	28.36	99.9
Recurrence without constant GDH	min	3.45	5.8	27
	max	45.35	44	747
	mean	10.97	30.07	69.12
Recurrence with constant GDH	min	6.99	21	23
	max	15.03	38	181
p value (inconstant/constant)		0.05	0.2	0.04

Table 6.3 The white cell count, serum albumin and serum creatinine results for different groups of patients within the study. Comparison between groups were made using *t* tests

6.4 Discussion

Utilising the largest dataset collected on CDI diagnostic assays and patient outcomes enabled analysis to examine the potential of CT value to inform on the diagnosis and outcomes of CDI, and. Importantly, this dataset comes from the only study which tested all samples with both of the CCNA and CTC reference assays. A low CT value for a *C. difficile tcdB* PCR assay was significantly associated with a toxin-positive result, presence of PCR ribotype 027, and mortality (table 6.1). In addition, recurrence was also associated with low CT, although this was not a significant finding, possibly due to the low number of recurrent cases within the dataset. There was a significant AUROC for *tcdB* PCR CT value against sample toxin positivity, as measured by CCNA of 0.83 (p <0.001) with a median *tcdB* PCR CT for toxin positive samples of 24.9 *vs* 31.6 for toxin negative samples (p <0.001). In addition, the median *tcdB* PCR CT value for patients who died was significantly lower than for those who survived (25.5 *vs* 27.5 p = 0.021) with a significant AUROCC for *tcdB* PCR CT positives against mortality; 0.572 p =0.009. In order to optimise the CT cut-off both ≤24 and ≤25 cycles were further investigated, but there was little difference between the two cut-off values (table 6.1); a slighter higher PPV for toxin positivity using CT ≤24 was offset by a lack of significant association with mortality seen with CT≤25 . A 'low CT' was therefore defined as *tcdB* PCR CT of ≤25. This cut-off was slightly higher than cut-offs used in two smaller studies which reported CT values. Indeed, one of these studies reported an AUROC of 0.857 when using a cut-off of <23.5 cycles; however, this was for a composite end-point of 'poor outcome' which included recurrent infection, treatment failure or progressing to severe complicated CDI (Reigadas *et al*, 2016b). The large dataset in this study, 7.5 times larger than that of the previous study, has highlighted the association of low CT with single outcome measures, rather than a composite end-point. Indeed, with a positive predictive value of low CT (≤25) for toxin detection of 83.9% it is possible that a low *tcdB* PCR CT value could be used as a proxy for toxin detection as suggested from a recent small study (Kamboj *et al*, 2018).

The risk of mortality was higher in patients with a low CT (≤25) compared with those with a PCR CT value >25 (relative risk [RR] 1.45) (table 6.2). The risk of mortality was further increased in those patients who had a low CT and had an infection caused by *C. difficile* PCR ribotype 027 (RR 2.18). Indeed, in those patients with a low CT (≤25), mortality and longer length of stay were also significantly associated with the presence of *C. difficile* PCR ribotype 027 in their sample, compared to other PCR ribotypes, highlighting a possible 'at risk' group. There is conflicting evidence regarding the role of the presence of the binary

toxin gene in CDI outcomes (Reigadas *et al*, 2016a, Berry *et al*, 2017), however in this dataset the relative risk for mortality for those patients with low CT increased from 1.45 to 1.72 when the presence of binary toxin gene was taken into account.

Patients with a low CT appeared to be sicker than those with a *tcdB* PCR CT value >25, as they had a higher mean WCC, higher mean serum creatinine, lower mean serum albumin and longer length of stay, but only the difference in serum albumin levels was significant (table 6.2). This was possibly due to the small sample size in these subgroups, as data could only be collected when patients had routine blood tests, they were not tested systematically. The same pattern of differences in CDI severity markers was seen in patients who had a low CT and whose samples tested positive for binary toxin gene detection, with the exception that these patients had a shorter length of stay than binary toxin negative patients. The predictive value of *tcdB* PCR low CT to determine those patients who are more sick warrants further investigation. Any future study would need to be larger, and use prospective, systematic sampling of patients.

Prevention of recurrence is a key goal of patient management in cases of CDI, indeed the antibiotic fidaxomicin is recommend in first recurrent cases to prevent further episodes (Debast *et al*, 2014, McDonald *et al*, 2018) due to the reduction in recurrence risk with this antibiotic, compared with vancomycin. The high cost of this treatment does, however, impact on a hospital's decision to fund this drug. A diagnostic tool that could indicate those patients in which it would be most cost-effective to use, *i.e.* those at biggest risk of recurrence, such as in those patients with low CT, may help to guide antibiotic management. Although recurrence was associated with a low CT in this dataset, this was not a significant finding, possibly due to the low number of recurrent cases within the PCR-positive samples that were used for the CT value analysis (table 6.1 and figures 6.2 and 6.3). Risk of recurrence has also previously been shown to be higher in PCR ribotype 027 (Marsh *et al*, 2012). In order for this to be a timely measure however, clinicians cannot rely on traditional PCR-ribotyping methods; in this regard a rapid assay with a 'presumptive 027' result would be of importance. The Cepheid geneXpert® C. diff assay has a high NPV for 'presumptive RT027' but does overcall the number of samples that truly contain a strain of PCR ribotype 027, as demonstrated by the low PPV (69.9%). The presence of the gene target used to identify PCR ribotype 027 by the Cepheid geneXpert® C. diff assay in other ribotypes related to PCR ribotype 027 can confound the results produced by this assay (Spigaglia and Mastrantonio, 2002, Rupnik *et al*, 1998).

In order to investigate recurrence further, all patients who had more than one sample taken during the study were included, regardless of PCR positivity. Using this larger dataset recurrence was significantly associated with a sample that had tested positive by any of the diagnostic assays used; however, the highest risk was in those with a previous CCNA-positive sample (OR 8.04), PCR-positive sample (as defined by the software algorithm within the Cepheid geneXpert®) (OR 6.84) or low *tcdB* PCR CT value (OR 6.31) (table 6.2). Importantly, however, the OR for both PCR and low CT decrease when samples within these groups that were also CCNA positive were removed from the analysis; CCNA was the only significant variable associated with recurrence after binary logistic regression (Table 6.2). In addition, the rate of subsequent CCNA-positive samples within 14 days of the initial sample is the same for those that are PCR positive only (9.9% *vs* 9.6% for those >14 days), whilst for CCNA, CTC only (Group 2) and low CT only (Group 4) the incidence is approximately half within that 14-day period (table 6.2). This suggests that CCNA and low CT are better predictors of a true recurrence, as they are diagnosing the first sample with a 'true' case of CDI, whereas a positive toxigenic *C. difficile* PCR result (with any CT) could be detecting a patient without toxin and therefore without 'true' disease. Without a 'true' initial CDI episode, can there be a 'true' recurrence, or are these patients developing a toxin positive disease for the first time?

There are some limitations associated with this retrospective data analysis of a pre-existing dataset, such as the lack of a validation dataset. It should be noted, however, that this dataset was from the largest ever study of *C. difficile* diagnostic assays, and was therefore a valuable resource, in terms of sheer numbers, for this analysis. In order to analyse the data for recurrence, only those patients who had more than one sample submitted could be included in the analysis. Unfortunately, in the original study some recurrent samples may have been missed as there was a break in sample collection of a few months, as the study changed from a derivation to a validation testing set (Planche *et al*, LID 2012), and indeed the study was not designed to prospectively capture recurrence data. Therefore, a prospective study to investigate the impact of CT value information provided to clinicians on the rate of recurrence and any potential changes in patient management is warranted. In addition, these data only relate to one NAAT assay (Cepheid geneXpert® C. diff); it cannot be assumed that all assays will perform in the same way; however, there is some evidence emerging from other groups using different NAATs about the potential value of the CT value (Crobach *et al*, 2018).

If the theory that recurrence is more likely in those patients who have a higher bacterial burden of disease during their primary infection, are there assays, other than PCR CT values, that could be used to predict recurrence? A study in 2017 found that a higher bacterial burden, as measured by cfu of toxigenic *C. difficile*/g of stool, was associated with a poor outcome (composed of recurrence, or treatment failure or severe complicated CDI) (Reigadas *et al*, 2017). The GDH OD values from the PlaciD dataset correlated with the bacterial load in the sample, as measured by semi-quantitative culture of *C. difficile*, suggesting that GDH OD values could be used to measure the bacterial burden with faecal samples (figure 6.4). As discussed in Chapter 1, the GDH EIA is a quantitative assay, and increasing OD values correlate with increasing bacterial burden within the gut model. The highest median GDH OD values were seen in those patients with recurrent infection, with significant differences seen between cases and controls and those patients with single *verses* recurrent CDI (figure 6.4). This appears to confirm that a higher bacterial burden, as measured by either GDH OD could be used to predict recurrence. In addition, GDH could be detected from samples a median of 8.5 days before samples were produced that were toxin positive (as measured by CCNA).

In addition to examining the GDH OD values of all patients, five patients were identified who had consistent GDH-positive samples throughout their hospital stay, despite resolution of their diarrhoeal symptoms. This would appear to indicate that this group of patients were unable to clear the organism from their gut, but were colonised until they had another clinical recurrence. In all six of the recurrent infections that occurred in this group of five patients, their recurrent episode was precipitated by antibiotic therapy (figures 6.5a-e). In addition, four of these five patients were also using concomitant PPIs, although the potential role of PPIs as a risk factor for CDI is contentious (Faleck *et al*, 2016, Dial *et al*, 2004, Vesteinsdottir *et al*, 2012).

Interestingly, for these five patients, culture and the toxin gene PCR assay were not positive on as many occasions as the GDH assay, perhaps suggesting that the GDH assay has a lower limit of detection than both culture and the PCR assay. This appears to contradict the data from the Department of Health (DoH) study, where PCR was as sensitive as the GDH assay, however the PCR assay used in the DoH study was the Cepheid geneXpert® C. diff, whereas the PCR assay used here was the BD Max™ C.diff assay (Planche *et al*, 2012). This could therefore highlight differences between molecular platforms, as shown by Gilbreath *et al*, where the BD Max™ assay had the lowest sensitivity of the platforms tested (2014).

C. difficile could not be cultured from any of the samples from one patient; this patient had, however, been treated with fidaxomicin, which has been shown to reduce bacterial recovery from clinical samples (Biswas *et al*, 2015).

The reasons behind the persistent carriage of *C. difficile* in these five patients are unclear. They appear to be more ill than those patients who cleared the bacteria below the limit of detection for the GDH assay between CDI episodes, as they had higher WCCs, higher serum albumin levels and lower serum creatinine levels (Table 6.3). However, whether these deranged values are due to the presence of the bacteria, or are a demonstration of the frailty of the patient, and therefore, perhaps a compromised immune system that cannot clear the infection, is unclear.

7. Final Discussion

CDI laboratory diagnostic methods have been shown to have variable performance (introduction section 1.13), but what are the reasons for this? The PCR ribotype has been cited as a factor in reduced sensitivity for both the GDH and toxin EIAs (Tenover *et al*, 2010), while in this study, none of the EIAs tested were significantly more likely to miss any one PCR ribotype over another. Importantly, however, there were significant differences between the median OD values of PCR ribotypes 027 and 015 for both of the toxin EIAs tested (figure 3.1). Given that the GDH EIA has been shown to be quantitative (Chapter 4), it could be theorised that the same is true for the toxin assays, and that the different median values reflect differences in the level of toxin produced by different PCR ribotypes. This is supported by the data from batch culture experiments, where the cytotoxin titre for PCR ribotype 002 was lower than the titre for other PCR ribotypes tested, even though total viable counts remained consistent between the strains (figures 3.2 and 3.5). There were also significant differences between median OD values of the two toxin EIA for the same PCR ribotypes (table 3.2), further highlighting the variability between toxin EIAs.

The GDH EIA does not appear have the same variation in performance with different PCR ribotypes (table 3.1, figures 3.2 and 3.5). Indeed, the GDH assay appeared to be the most sensitive method for detection of *C. difficile* from sequential faecal samples, compared with culture, CTC and PCR (BD Max™) (tables 5.13 and 5.15). In contrast, the PCR assay (Cepheid GeneXpert®) had comparable sensitivity to the GDH assay in an earlier study (Planche *et al*, 2012), suggesting that different PCR platforms have different sensitivity for detecting *C. difficile* from faecal samples (Gilbreath *et al*, 2014). It should also be noted that the earlier study was testing faecal samples from patients in whom a diagnosis of CDI was suspected, while in the present study the samples were tested on sequential days after an initial GDH positive sample had been identified. Testing sequential samples, however, gives an indication of how long markers of *C. difficile* or CDI remain detectable within patient samples, and therefore also provides an indication about the limit of detection of these assays. In addition, GDH was detectable from samples from the gut-model experiments when no organisms could be cultured from the samples (figures 4.1, 4.2, 4.4, 4.5, and 4.7-4.12), demonstrating that GDH remained within the system after the organism had washed out. The reasons for the extended detection of GDH both *in vitro* and *in vivo* are unclear; it may be related to the large amounts of GDH produced, sequestration within biofilm, and/or release from dead cells. There is not, however, an additional peak in GDH after cell death within the gut models, just a continual decline, in-line with the

reduction in cell numbers, suggesting that GDH is not released after cell death. This is supported by the data from growth curve experiments, which show that GDH was produced during the exponential phase of growth, then declined as vegetative growth slowed and there was conversion from vegetative cells to spores (figures 3.8 and 3.9). Dilution of gut model samples allowed visualisation of peak GDH levels produced during the gut model experiments, which in later experiments reached very high levels (Model F figures 4.11a-c). It is possible therefore that the high sensitivity of the GDH EIA could be related to the large amounts of the protein produced by the organism. Further studies are required to investigate the copy number of the *gluD* gene within *C. difficile*, expression of the gene and perhaps what promoter genes are associated with *gluD*, in order to fully understand the production of GDH by the organism.

In addition to increased GDH levels within Model F, the highest peak total viable count and toxin titre was also observed within this model (Model F figures 4.11a-c), suggesting that levels of both GDH and toxin are related to the bioload during infection. This correlates with a recent study where poor outcome was associated with higher bacterial burden, as measured by cfu of toxigenic *C. difficile*/g of stool (Reigadas *et al*, 2017). Within faecal samples in the current study, GDH EIA OD values also correlated with culture, as measured semi-quantitatively (figure 6.4a), further supporting the theory that GDH EIA OD values can be used as a measure of bioload within the sample. In addition, the median OD value of cases was significantly higher than control participants, and the median OD value of recurrent cases was significantly higher than those participants who only had one episode of CDI (figures 6.4b-c). The GDH OD value may therefore, potentially offer the opportunity to predict recurrence. Similarly, the CT value of PCR assays, specifically the Cepheid geneXpert®, may also provide information to help predict those participants who are toxin-positive, have an infection caused by PCR ribotype 027, and are at greater risk of mortality (table 6.1). Although low CT (<25) was associated with recurrence, this was not a significant finding; the only assay significantly associated with recurrence after binary logistic regression was CCNA (table 6.2). It should be noted, however, that not all commercial PCR systems provide CT values to the user, meaning that this 'extra' analysis could not take place. In addition, not all PCR systems may be comparable; the BD Max™ C. diff assay used in the sequential faecal sample testing for the present study appeared to have decreased sensitivity compared with the Cepheid geneXpert® C. diff assay used in our earlier study (Planche *et al*, 2012), although this was not a true head-to-head comparison. This potential variability was highlighted in a recent comparison of four molecular assays, with sensitivity

ranging from 90-100%, but with 95% CI ranging from 82-100% (Caulfield *et al*, 2018), although this comparison was not against a recognised reference method. A second smaller study found sensitivities of 92-99%% with 95% CI ranging from 84.6-100.0, compared with toxigenic culture for six different molecular assays (Paitan *et al*, 2017).

The current *C. difficile* diagnostic guidelines in the UK and Europe (Crobach *et al*, 2016) state that a two-stage algorithm should be used for the diagnosis of CDI, to counter the lack of sensitivity of stand-alone toxin EIAs. The most commonly used option in the UK (Davies *et al*, 2016b) is GDH/toxin. This does, however, highlight a 'new' patient group, denoted potential *C. difficile* excretors (PCDEs), i.e. those patients who have a sample that tests positive for GDH but negative for toxin (Planche *et al*, 2012). It could be argued that some of these PCDE's are 'missed' infections, due to the use of a toxin EIA (with reduced sensitivity) as the second test. However, using CCNA to test sequential samples demonstrates that toxin cannot be detected, even by this more sensitive method. Samples could be further investigated by the use of novel ultra-sensitive toxin detection assays (Pollock, 2016; Banz *et al*, 2018). It is possible that failure to detect toxin from a *C. difficile* positive sample is because the patient is carrying a non-toxigenic strain, which is therefore incapable of producing toxin. However, of the 25 PCDEs that had no samples with detectable toxin, 20/25 had a toxigenic PCR ribotype of *C. difficile* isolated from their sample. This is an important point, as CDI is a toxin mediated disease (Bartlett *et al*, 1978). Interestingly, 27% of PCDEs went on to have a toxin positive sample, after a median of 8 days. Does this indicate that the initial samples were taken before the organism had started to proliferate and produce toxin, or that the level of toxin was not yet detectable by CCNA, so yielding a GDH-positive/toxin-negative result? Indeed, the converse could be true; only 12.2% of CDI cases went on to have another toxin positive sample within 72 hours of the initial sample (table 5.14).

The data from gut models demonstrate the fast decline of toxin following treatment with an appropriate antibiotic (figures 4.1, 4.2, 4.4, 4.5, and 4.7-4.12). The gut model cannot, however, simulate the effect of the immune system on the levels of toxin within the gut and how fast levels decline *in vivo*. It is therefore possible that a GDH-positive/toxin-negative result could be obtained for a sample collected at either end of the episode of infection. Current guidance does not support repeated testing of patients, due to the potential for increased false-positives, and the data from this study are currently not strong enough to contradict that advice. Further studies are required to determine if

ultra-sensitive toxin detection methods could detect low levels of toxin, below those currently detected by CCNA, in these GDH-positive/toxin-negative (by CCNA) patient samples, and indeed to determine the clinical importance of these results. In addition, as there has been a correlation demonstrated between lactoferrin (Davies *et al*, 2019b), and toxin level in a faecal samples, tests for gut inflammation, such as lactoferrin or calprotectin could be used to provide an indication of toxin damage to the gut mucosa in patients with GDH-positive/toxin-negative results. Further studies are also needed to determine the utility of lactoferrin and calprotectin when used as an adjunct to ultra-sensitive toxin detection assays.

Antibiotic stewardship is important to reduce the risk of emergence and spread of antibiotic resistance, and so increase the chance that the current portfolio of antibiotics remains effective. Within the context of CDI, antibiotic stewardship is additionally important, as a method of protecting the vital gut microbiome. Almost all classes of antibiotic have been shown to damage the microbiome, allowing a niche for *C. difficile* to proliferate (Bartlett and Gerding, 2008), including some used to treat CDI itself, such as vancomycin. A subset of patients has been highlighted in this study, which was consistently GDH positive in almost every sample (69/70, 98.5%) despite resolution of symptoms. The reasons that they were unable to clear the infection are unclear; however, all of these patients went on to have a recurrent CDI following antibiotic treatment for another suspected or proven (non-CDI) infection (figures 6.5 a-e). Risk prediction models are not currently reproducible (Escobar *et al*, 2017), and so, if the patients had been tested before the new antibiotic prescription had been written, and found to be GDH positive, would this have changed the decision of the prescriber to use a more gut-protective agent? These data, taken with those discussed above mean that diagnostic assays may therefore, be able to provide more information than a binary positive/negative result, and thereby, augment existing testing methodology, by providing guidance for opportunities for antibiotic stewardship or the use of therapies such as bezlotoxumab to help prevent recurrence. Alternatively, there are investigational drugs that are designed to reduce the negative impact of antibiotics on gut microbiota (and so reduce the risk of CDI) i.e. SYN-004 (ribaxamase) (Kokai-Kun *et al*, 2017b) and DAV-132 (de Gunzberg *et al*, 2015). The novel GDH results from the present study offer a potential way to target the use of such preventative therapies to those patients most likely to benefit.

8. References

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Appendix 1. List of suppliers for materials and methods

Applied Maths NV
Keistraat 120
9830 Sint-Martens-Latem,
Belgium

Brighton Systems Ltd
Unit K Quarry Road Industrial Estate
Newhaven
East Sussex
BN9 9DG
United Kingdom

Cepheid UK Limited
Neptune House
Mercury Park
Wooburn Green
Buckinghamshire
HP10 0HH
United Kingdom

Cole Palmer Ltd
9 Orion Court
Ambuscade Road
Colmworth Business Park
St Neots
Cambridgeshire
PE19 8YX
United Kingdom

Copan Diagnostics,
26055
Jefferson Avenue Murrieta,
CA 92562
USA

Don Whitley
Victoria Works
Victoria St
Bingley
BD16 2NH
United Kingdom

Dynex Technologies
14340 Sullyfield Circle
Chantilly,
VA 20151-1621
USA

European collection of animal cell cultures
Public Health England
Porton Down
Salisbury
SP4 0JG
United Kingdom

Grant Instruments Ltd
29 Station Rd
Shepreth
Royston
SG8 6GB

United Kingdom

Fisher Scientific UK Ltd (now Thermofisher Scientific Ltd)
Bishop Meadow Road
Loughborough
LE11 5RG
United Kingdom

Fisons Scientific Equipment (formerly Gallenkamp and now part of Fisher Scientific)
Bishop Meadow Rd
Loughborough
LE11 5RG
United Kingdom

Invitrogen Europe limited
3 fountain drive
Inchinnan Business Park
Paisley
Renfrewshire
PA4 9RF
United Kingdom

Leica Microsystems (UK)
Larch House
Woodlands Business Park
Breckland
Linford Wood
Milton Keynes
MK14 6FG
United Kingdom

Life Technologies
Lingley House
120 Birchwood Blvd,
Birchwood,
Warrington
WA3 7QH
United Kingdom

Meridian Bioscience, Inc. Europe
Unit 16 The Edge Business Centre
Humber Road
London
NW2 6EW
United Kingdom

Microgen Bioproducts Ltd,
Unit 1 Watchmoor Point,
Watchmoor Road,
Camberley,
Surrey,
GU15 3AD
United Kingdom

New Brunswick Scientific co (part of Eppendorf)
Eppendorf Austria GmbH
Ignaz Köck Straße 10/2.
OG 1210
Vienna
Austria

Oxoid Ltd
Wade Road
Basingstoke
Hampshire
RG24 8PW
United Kingdom

Panasonic
1006, Oaza Kadoma
Kadoma-shi
Osaka 571-8501
Japan

Parker Balston
Tachbrook Park Drive
Tachbrook Park
Warwick
CV34 6TU
United Kingdom

Pro-lab Diagnostics Ltd,
Stanhope House,
Mark Rake,
Bromborough,
Merseyside,
CH62 2DN
United Kingdom

Qiagen Ltd
Skelton House,
Lloyd St N,
Manchester
M15 6SH
United Kingdom

Sarstedt Inc
68 Boston Rd,
Leicester
LE4 1AW
United Kingdom

Seward Ltd
8 Platinum Rd
Urmston
Stretford
Manchester
M41 7LJ
United Kingdom

Sigma-Aldrich Company Ltd.
The Old Brickyard
New Rd
Gillingham
Dorset
SP8 4XT
United Kingdom

Soham Scientific
37 Mildenhall Rd
Fordham
Ely
CB7 5NP
United Kingdom

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Appendix 2. Publications during the period of the PhD

Directly related to work in this PhD: Most recent shown first. (Presenting author shown in bold for conferences presentations).

1. Davies K A, Planche T, Wilcox M H. The predictive value of quantitative nucleic acid amplification detection of *Clostridium difficile* toxin gene for faecal sample toxin status and patient outcome. *PLoS One*. 2018 Dec 5;13(12):e0205941. doi: 10.1371/journal.pone.0205941.
2. **Davies K A**, Berry C E, Heritage J, Wilcox M H. Predicting those at risk of recurrence; should we be monitoring *Clostridium difficile* infection patients with diagnostic assays*? Poster presentation at 28th European Congress of Clinical Microbiology and Infectious Diseases in Madrid, Spain, 21st-24th April 2018*
3. **Davies K A**, Planche T, Shetty N, Wren M, Crook D, Wilcox M H. Toxin gene nucleic acid amplification test cycle threshold result is associated with severity of *C. difficile* infection and poor patient outcomes. *Poster presentation at Infectious Disease (ID) week, San Diego 2015*
4. **Davies K A**, Chilton C, Crowther G, Todhunter S, Heritage J, Wilcox M H. Can a human gut model explain GDH positive/toxin negative faecal samples seen in clinical practice? *Poster presentation at 24th European Congress of Clinical Microbiology and Infectious Diseases in Barcelona, Spain, 10–13 May 2014*
5. **Davies K.A**., Planche T., Crook D., Shetty N., Wren M. and Wilcox M.H Performance of enzyme immunoassays is affected by PCR-ribotype of *C. difficile* in faecal samples. *Poster presentation at 23rd European Congress of Clinical Microbiology and Infectious Diseases in Berlin, Germany, 26-30th April 2013*

Publications not directly related to the work in this PhD, but about closely related topics: Most recent shown first.

1. Banz A, Lantz A, Riou B, Foussadier A, Miller M, Davies K, Wilcox M. Sensitivity of Single-Molecule Array Assays for Detection of *Clostridium difficile* Toxins in Comparison to Conventional Laboratory Testing Algorithms. J Clin Microbiol. 2018 Jul 26;56(8). pii: e00452-18. doi: 10.1128/JCM.00452-18. Print 2018 Aug.

2. Berry C, Davies K, Woodford N, Wilcox M, Chilton C. Survey of screening methods, rates and policies for the detection of carbapenemase producing Enterobacteriaceae in English hospitals. J Hosp Infect. 2018 Aug 6. pii: S0195-6701(18)30416-X. doi: 10.1016/j.jhin.2018.08.005.
3. Davies K, Freeman J, Mayor S. Update on *Clostridium difficile* from the European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna, Austria, April 22-25, 2017. J Hosp Infect. 2018 Jan;98(1):1-3. doi: 10.1016/j.jhin.2017.11.016. Epub 2017 Dec 1.
4. Martin J S H, Eyre D W, Fawley W N, Griffiths D, Davies K, Mawer D P C, Peto T E A, Crook D W, Walker A S, Wilcox M H. Patient and strain characteristics associated with *Clostridium difficile* transmission and adverse outcomes. 2018. *Clinical Infectious Disease*. 67(9):1379-1387. doi: 10.1093/cid/ciy302
5. Davies K, Davis G, Barbut F, Eckert C, Petrosillo N, Pisapia R, Herrmann M, Berger F, Reigadas E, Bouza E, Wilcox M H. Multivariate analysis of factors affecting reported *Clostridium difficile* infection rates; the more you look, the more you find; but should you believe what you see? Submitted for publication
6. Davies K, Lawrence J, Berry C, Davis G, Yu H, Cai B, Gonzalez E, Prantner I, Kurcz A, Macovei I, Pituch H, Nováková E, Nyč O, Gärtner B, Berger F, Oleastro M, Cornely O, Vehreschild M, Pedneault L, Wilcox M . Consistency of Risk factors for *Clostridium difficile* infection; results from the Observational study of Risk factors for *Clostridium difficile* infection in Hospitalised patients with Infective Diarrhoea (ORCHID). Submitted for publication
7. Eyre D W, Davies K A, Davis G, Fawley W N, Dingle K E, De Maio N, Karas A, Crook D W, Peto T E A, Walker A S, Wilcox M H; EUCLID Study Group. Two Distinct Patterns of *Clostridium difficile* Diversity Across Europe Indicating Contrasting Routes of Spread. 2018. *Clinical Infectious Disease*. 67(7):1035-1044. doi: 10.1093/cid/ciy252
8. Morris K A, Davies K A, Wilcox M H. Impact of *Clostridium difficile* toxin gene PCR result on decisions to de-isolate patients: Do the ends justify the means*? Journal of Infection Prevention* 2018.
9. Couturier J , Davies K, Gateau C, Barbut F. Ribotypes and new virulent strains across Europe. Chapter in Updates on *Clostridium difficile* in Europe. 2018. *Advances in Experimental Medicine and Biology* .

10. Pituch H, Obuch-Woszczatyński P, Lachowicz D, Kuthan R, Dzierżanowska-Fangrat K, a Mikucka A, a Jermakow K, Pituch-Zdanowska A, Davies K and Polish EUCLID *C. difficile* Study Group. Prevalence of *Clostridium difficile* infection in hospitalized patients with diarrhea: results of a Polish prospective, multicenter, bi-annual point prevalence study. 2018. *Advances in Medical Sciences*. 63(2).
11. Berry CE, Davies KA, Owens DW, Wilcox MH. Is there a relationship between the presence of the binary toxin genes in *Clostridium difficile* strains and the severity of *C. difficile* infection (CDI)? *European Journal of Clinical Microbiology and Infectious Disease*. 2017 Aug 5. doi: 10.1007/s10096-017-3075-8.
12. Davies K, Davis G, Barbut F, Eckert C, Petrosillo N, Wilcox M H. Variability in testing policies and impact on reported *Clostridium difficile* infection rates: results from the pilot Longitudinal European *Clostridium difficile* Infection Diagnosis surveillance study (LuCID). 2016. *European Journal ofClinical Microbiology and Infectious Disease*. 35(12):1949-1956. doi: 10.1007/s10096-016-2746-1
13. Davies K A, Ashwin H, Longshaw C M, Burns D A, Davis G L, Wilcox M H, on behalf of the EUCLID Study Group. Diversity of *Clostridium difficile* PCR ribotypes in Europe: results from the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID), 2012 and 2013. 2016. *Eurosurveillance* . 21(29). doi: 10.2807/1560-7917.ES.2016.21.29.30294 http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=225356
14. Fawley WN, Davies KA, Morris T, Parnell P, Howe R, Wilcox MH; Clostridium difficile Ribotyping Network (CDRN) Working Group. Enhanced surveillance of *Clostridium difficile* infection occurring outside hospital, England, 2011 to 2013. 2016. *Eurosurveillance*. 21(29). doi: 10.2807/1560-7917.ES.2016.21.29.30295 http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=22535
15. Davies KA, Berry CE, Morris KA, Smith R, Young S, Davis TE, Fuller DD, Buckner RJ, Wilcox MH. Comparison of VIDAS® *C. difficile* GDH automated enzyme linked fluorescence immunoassay (ELFA) with another commercial EIA (QUIK CHEK-60), two selective media and a PCR assay for GluD for the detection of *Clostridium difficile* in faecal samples. 2015. *Journal of Clinical Microbiology*. 53(6):1931-4. doi: 10.1128/JCM.00649-15
16. Davies K A, Longshaw C M, Davis G L, Bouza E, Barbut F, Barna Z, Delmée M, Fitzpatrick F, Ivanova K, Kuijper E, Macovei IS, Mentula S, Mastrantonio P, von

Müller L, Oleastro M, Petinaki E, Pituch H, Norén T, Nováková E, Nyč O, Rupnik M, Schmid D, Wilcox M H. Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). 2014. *Lancet Infectious Disease*. 14(12):1208-19. doi: 10.1016/S1473-3099(14)70991-0

17. Pankhurst L, Macfarlane-Smith L, Buchanan J, Anson L, Davies K, O'Connor L, Ashwin H, Pike G, Dingle K E, Peto T E, Wordsworth S, Walker A S, Wilcox M H, Crook D W. Can rapid integrated polymerase chain reaction-based diagnostics for gastrointestinal pathogens improve routine hospital infection control practice? A diagnostic study. 2014. *Health Technology Assessments*. 18(53):1-167. doi: 10.3310/hta18530
18. Planche T D., Davies K A., Coen P G., Finney J M., Monahan I M., Morris K A., O'Connor L., Pope C F., Wren M W., Shetty N P., Crook D W. and Wilcox M H. 2013. Differences in outcome according to C. difficile testing method: a prospective multicentre diagnostic validation study of *C. difficile* infection. 2013. *Lancet Infectious Diseases*. 936-45. doi: 10.1016/S1473-3099(13)70200-7

Posters and oral presentations not directly related to the work in this PhD, but about closely related topics (Presenting author highlighted in bold);

1. **Davies K**, Moura I, Boone J, Clark E, Owen K, Wilcox M H. Correlation between faecal lactoferrin and *C. difficile* toxin; a semi-quantitative analysis. 2019. *Accepted for poster presentation at ASM Microbe, San Francisco, USA.*
2. **Davies K**, Viprey V, Ewin D, Spittal W, Vernon J, Fawley W, Davis G, Wilcox M H. Detection of *Clostridium difficile* infection across whole healthcare economies in Europe: results from COMBACTE-CDI. *Poster presented at 29th European Congress on Clinical Microbiology and Infectious Disease (ECCMID), Amsterdam, The Netherlands*, 13-16th April 2019.
3. **Davies K**, Viprey V, Banz A, Ewin D, Spittal W, Vernon J, Davis G, Benson A, Frager F, Cleuziat P, Miller M, Wilcox M H. Investigation of *Clostridium difficile* positive and negative samples using an ultra-sensitive toxin detection assay and BIOFIRE

FILMARRAY Gastrointestinal Panel. *Poster presented at 29th European Congress on Clinical Microbiology and Infectious Disease (ECCMID), Amsterdam, The Netherlands*, 13-16th April 2019.

4. **Davies K,** Mawer D, Walker A S, Berry C, Planche T, Stanley P, Goldenberg S, Sandoe J, Wilcox M H. Fidaxomicin, unlike conventional treatment options, reduces *Clostridium difficile* environmental contamination during and at the end of therapy. *Poster presented at 29th European Congress on Clinical Microbiology and Infectious Disease (ECCMID), Amsterdam, The Netherlands*, 13-16th April 2019.
5. **Davies K**, Viprey V, Tkalec V, Ewin D, Spittal W, Vernon J, Fawley W, Benson A, Davis G, Rupnik M, Wilcox M, on behalf of the COMBACTE-CDI consortium. Comparison of PCR-ribotypes and Toxinotypes causing community versus hospital *C. difficile* infection (CDI). *Oral presentation at 29th European Congress on Clinical Microbiology and Infectious Disease (ECCMID), Amsterdam, The Netherlands*, 13-16th April 2019.
6. **Davies K**, Moura I, Owen K, Bates C, Carr H, Wilcox M. Do the results of membrane and well-based EIAs for the diagnosis of *Clostridium difficile* infection (CDI) correlate? Poster presentation at 28th *European Congress of Clinical Microbiology and Infectious Disease in Madrid, Spain, 21st-24th April 2018*
7. **Rooney C**, Clark E, Chilton C, Wilcox M, Davies K. Using a human in-vitro gut model to evaluate carbapenamase-producing Enterobacteriaceae screening methods. *Poster presented at Federation of Infection Society, Birmingham, 30th Nov-2nd Dec 2017.*
8. **Rooney C**, Clark E, Davies K, Walker AS, Wilcox MH, Chilton C. Investigating colonisation of the intestinal microbiota with Carbapenemase Producing Enterobacteriaceae using a clinically reflective in vitro gut model. *Poster presentation at 27th European Congress of Clinical Microbiology and Infectious Disease in Vienna 22nd-25th April 2017.*
9. **Davies K**, Davis G, Barbut F, Eckert C, Petrosillo N, Pisapia R, Gärtner B, Berger F K, Reigadas E, Bouza E, Demont C, Wilcox M H. Multivariate analysis of factors affecting reported *Clostridium difficile* infection rates; the more you look, the more you find; but should you believe what you see? 2017. *Oral presentation at 27th European Congress on Clinical Microbiology and Infectious Disease (ECCMID), Vienna, Austria. 22nd-25th April 2017.*

10. **Davies K**, Davis G, Barbut F, Eckert C, Petrosillo N, Pisapia R, Reigadas-Ramirez E, Bouza E, Berger F, Herrmann M, Wilcox M. Seasonality of *C. difficile* Infection (CDI) Case Rates: Myth or Reality? *Oral Presentation at ASM MICROBE Boston, 16th-20th June 2016*.
11. **Davies K,** Lawrence J, Berry C, Davis G, Yu H, Cai B, Gonzalez E, Prantner I, Kurcz A, Macovei I, Pituch H, Nováková E, Nyč O, Gärtner B, Berger F, Oleastro M, Cornely O, Vehreschild M, Pedneault L, Wilcox M Observational Study of Risk factors for Clostridium difficile Infection in Hospitalised Patients with Infective Diarrhoea (ORCHID): Consistency of Risk Factors. *Poster presentation at ID week New Orleans, 26th-30th October 2016.*
12. **Davies K**, Davis G, Barbut F, Eckert C, Spigaglia P, Barbanti F, Herrman M, Berger F, Bouza E, Reigadas-Ramirez E, Wilcox M. Adverse outcomes for missed cases of CDI; results from retrospective data collection on patients with samples received during the EUropean, multi-centre, prospective bi-annual point prevalence study of *CLostridium difficile* Infection in hospitalised patients with Diarrhoea (EUCLID2). *Mini-Oral presentation at 26th European Congress of Clinical Microbiology and Infectious Disease in Amsterdam, 9th-12th April 2016*
13. **Davies K**, Davis G, Barbut F, Eckert C, Petrosillo N, Wilcox M. Longitudinal European *Clostridium difficile* Infection Diagnosis Surveillance Study (LuCID) Shows Effects of Place, Patient Age and Testing Method on CDI Reporting . *Oral presentation at ID week in San Diego, 4th-8th October 2015*.
14. **Davies K**, Davis G, Barbut F, Eckert C, Petrosillo N, Wilcox M. Variability in testing policies and impact on *Clostridium difficile* infection rates; First report from the Longitudinal European *Clostridium difficile* Infection Diagnosis Surveillance Study (LuCID). *Oral presentation at 25th European Congress of Clinical Microbiology and Infectious Disease in Copenhagen, 25th-28th March 2015.*
15. **Davies K A**, Longshaw C M, Davis G L, Ashwin H, Lee F, Wilcox M H. Increased diversity of *C. difficile* PCR-ribotypes across European countries and disparity of 027 prevalence; Results of the European, multi-centre, prospective bi-annual point prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). *Oral presentation* at *24th European Congress of Clinical Microbiology and Infectious Diseases in Barcelona, Spain, 10–13 May 2014).*
16. **Davies K A**, Longshaw C M, Davis G L, Ashwin H, Lee F, Wilcox M H. Second report from the EUropean, multi-centre, prospective bi-annual point prevalence study of

CLostridium difficile Infection in hospitalised patients with Diarrhoea (EUCLID). *Poster presentation at 24th European Congress of Clinical Microbiology and Infectious Diseases in Barcelona, Spain, 10–13 May 2014*

17. **Davies K A**, Longshaw C M, Davis G L, Ashwin H, Lee F, Wilcox M H. *C. difficile* toxin detected in children with hospital associated diarrhoea; should we be looking for CDI in children? Data from EUropean, multi-centre, prospective bi-annual point prevalence study of *CLostridium difficile* Infection in hospitalised patients with Diarrhoea (EUCLID). *Poster presentation at 24th European Congress of Clinical Microbiology and Infectious Diseases in Barcelona, Spain, 10–13 May 2014*
18. **Ashwin H**, Davies K A, Davis G L, Lee F, Longshaw C M, Wilcox M H. Optimised diagnosis of *Clostridium difficile* infection; is there still room for improvement? Results of a European point prevalence study of C. difficile infection (EUCLID). Poster presentation at *24th European Congress of Clinical Microbiology and Infectious Diseases in Barcelona, Spain, 10–13 May 2014*
19. **Macfarlane-Smith, L.R.**, Davies, K.A., Ashwin H., Pankhurst L., Walker A.S., Crook D.W., Wilcox, M.H. Multiplex PCRs for the diagnosis of gastrointestinal pathogens are unreliable for the detection of *Salmonella enterica*. *eposter at 24th European Congress of Clinical Microbiology and Infectious Diseases in Barcelona, Spain, 10–13 May 2014*
20. **Davies K. A**., Planche T., Crook D., Monahan I., Pope C. and Wilcox M. H. Comparison of Premier *C. difficile* GDH Enzyme immunoassay and Illumigene loop mediated isothermal amplification assay with two reference methods for the laboratory detection of *C. difficile. Poster presentation at 23rd European Congress of Clinical Microbiology and Infectious Diseases in Berlin, Germany, 26-30th April 2013*
21. **Davies K A**, Longshaw C M, Davis G L, Ashe S, Ashwin H, Lee F, Wilcox M H. First report from EUropean, multi-centre, prospective bi-annual point prevalence study of *CLostridium difficile* Infection in hospitalised patients with Diarrhoea (EUCLID). *Poster presentation at 23rd European Congress of Clinical Microbiology and Infectious Diseases in Berlin, Germany, 26-30th April 2013*
22. **Davies K A**, Longshaw C M, Davis G L, Ashe S, Ashwin H, Lee F, Wilcox M H. UK report from the EUropean, multi-centre, prospective bi-annual point prevalence study of *CLostridium difficile* Infection in hospitalised patients with Diarrhoea

(EUCLID). *Poster presentation at Federation of Infection Societies Annual Conference, Birmingham, UK, 11-13th November 2013.*

23. **Houston A**, Davies K A, Crook D, Shetty N, Wren M, Monahan I, Wilcox M H, Planche T. Use of faecal lactoferrin assay in the diagnosis and assessment of *Clostridium difficile* infection. *Poster presentation at Federation of Infection Societies Annual Conference, Birmingham, UK, 11-13th November 2013.*
24. Davies K A, **Planche T**, Coen P, Crook D, Shetty N, Wren M, Wilcox MH. The largest ever study to define a testing algorithm to optimise the laboratory diagnosis of *C. difficile* infection. *Poster presentation at 22nd European Congress of Clinical Microbiology and Infectious Diseases in London, UK, 31st March - 3rd April 2012*
25. **Davies K A**, Bosomworth C E, Carricajo A, Adam T, Wilcox M H. Comparison of VIDAS® GDH automated immunoassay with Cepheid GeneXpert® *C. difficile* PCR assay and an in-house PCR assay for GluD, for the detection of *C. difficile* in faecal samples. *Poster presentation at 22nd European Congress of Clinical Microbiology and Infectious Diseases in London, UK, 31st March - 3rd April 2012*
26. **Planche T**, Davies K A., Coen P, Crook D, Shetty, N, Wren M, Wilcox M H. Clinical validation of *C. difficile* infection (CDI) diagnostics: Importance of toxin detection. *Poster presentation at 52nd ICAAC Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, USA, September 9-12 2012*

Invited speaker

- Factors affecting reported CDI rates, International *Clostridium difficile* symposium, Slovenia 2018
- All about *C. difficile*. British Infection Association training day, London 2016.
- Postgraduate researcher of the year competition, Leeds University 2016.
- A detailed look at the performance of *C. difficile* toxin enzyme immunoassays. Astellas Pharma Europe Ltd training day, London, 2014.
- Are we still talking about *C. difficile* Infection (CDI) diagnosis? Microbe 2012, Sheffield, UK.

Recent relevant research projects where Kerrie Davies has been the study lead

NRES or University of Leeds ethical approval codes in brackets

1. Innovative Medicines Initiative (part of Horizon 2020) grant award: COMBACTE-CDI, Combatting Bacterial Resistance in Europe - *Clostridium difficile* Infections. Part of 7 academic and 7 industry partners consortium. K. Davies an academic partner and co-investigator. **(18/W/)**
2. Laboratory investigation of Bezlotoxumab. K Davies scientific lead. **(18/W/)**
3. Comparison of membrane and well-based enzyme immune assays for the detection of *C. difficile* glutamate dehydrogenase and *C. difficile* toxins. **(SoMREC16-)**
4. Development of a cell-cytotoxin assay with lower limit of detection. K Davies scientific lead. Ethics not required.
5. Investigation of new highly sensitive *C. difficile* toxin detection assay in comparison with cell-cytotoxin neutralisation assay. K Davies scientific lead. **(16//)**
6. Healthcare Infection Society grant award: Development of a rapid, cost-effective algorithm to improve detection of intestinal Carbapenamase Producing Enterobacteriaceae. K Davies Co-investigator. **(SoMREC16-168)**
7. Clinical course of Patient's infection determined by Laboratory Diagnostic Assays for *C. difficile* Detection from Faecal Samples (PLaciD). K Davies PI. **(13/NE/0255).**
8. Longitudinal European *Clostridium difficile* Infection Diagnosis Surveillance Study (LuCID). K Davies scientific lead. **(SoMRECC13032).**
9. Observational study of Risk factors for *Clostridium difficile* infection in Hospitalised patients with Infective Diarrhoea (ORCHID). K Davies scientific lead. **(SoMREC14085)**
10. Retrospective data collection on patients with samples received during the EUropean, multi-centre, prospective bi-annual point prevalence study of *CLostridium difficile* Infection in hospitalised patients with Diarrhoea (EUCLID2). K Davies scientific lead. **(15/SW/0125)**
11. European, multi-centre, prospective bi-annual point prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). K Davies scientific lead. **(HSLTLM/12/001)**
12. Sample factors affecting the performance of laboratory diagnostic assays for *C. difficile* detection from faecal samples. K Davies PI **(12/EE/0495)**
13. *C. difficile* diagnostic testing algorithm evaluation for the Department of Health. K Davies scientific lead. **(10/H0715/34)**

Appendix 3. Supplementary tables for Chapter 3

	Recruited to the study			Had at least one sample in the study			Had at least one sample in the study and initial diagnostic specimen was GDH positive		
	Control	GDH positive	Total	Control	GDH positive	Total	PCDE	CDI	Total
	n (%)	n (%)	n	n (%)	n (%)	n	n (%)	N (%)	n
Abdominal pain	1 (9.1)	10 (90.9)	11	1 (9.1)	9 (90.0)	10	4 (44.4)	5 (66.6)	9
Abdominal aortic aneurysm	1 (100)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Acute kidney injury	1 (20.0)	4 (80.0)	5	1 (20.0)	4 (80.0)	5	1 (25.0)	3 (75.0)	4
Alcohol withdrawal	0 (0)	2 (100)	2	0 (0)	2 (100)	2	1 (50.0)	1 (50.0)	2
Appendix	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Bacteraemia	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Biliary sepsis	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Bladder cancer	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Community acquired pneumonia	0 (0)	2 (100)	2	0 (0)	2 (100)	2	1 (50.0)	1 (50.0)	2
CDI	0 (0)	3 (100)	3	0 (0)	3 (100)	3	1 (33.3)	2 (66.7)	3
Cellulitis	1 (33.3)	2 (66.7)	3	1 (33.3)	2 (66.7)	3	1 (50.0)	1 (50.0)	2
Chest infection	1 (100)	0 (0)	1	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Chest pain	1 (100)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Cholangitis	1 (100)	0 (0)	1	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Chronic osteomyelitis	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Chronic kidney disease	1 (100)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Collapse	0 (0)	1 (100)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Confusion	2 (25.0)	6 (75.0)	8	0 (0)	4 (100)	4	2 (50.0)	2 (50.0)	4
Constipation	1	1	2	1	1	2	0	1	1

	(50.0)	(50.0)		(50.0)	(50.0)		(0)	(100)	
COPD	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Crohn's	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Diarrhoea and vomiting	0 (0)	14 (100)	14	0 (0)	14 (100)	14	7 (50.0)	7 (50.0)	14
Decreased mobility	1 (100)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Dementia	1 (100)	0 (0)	1	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Dental abscess	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Diabetic foot	3 (60.0)	2 (40.0)	5	2 (50.0)	2 (50.0)	4	1 (50.0)	1 (50.0)	2
Diarrhoea	1 (4.5)	21 (95.5)	22	1 (5.0)	19 (95.0)	20	6 (31.6)	13 (68.4)	19
Discitis	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Dislocated ankle	1 (100)	0 (0)	1	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Dislocated hip	1 (100)	0 (0)	1	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Dizziness	0 (0)	2 (100)	2	0 (0)	2 (100)	2	1 (50.0)	1 (50.0)	2
Dysphagia	1 (100)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
DVT	1 (50.0)	1 (50.0)	2	1 (50.0)	1 (50.0)	2	1 (100)	0 (0)	1
Elective chemotherapy	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Elective Stem cell Transplant	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	0 (0)	1
Emergency surgery	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Encephalopathy	0 (0)	2 (100)	2	0 (0)	2 (100)	2	1 (50.0)	1 (50.0)	2
Endocarditis	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Epigastric pain	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Fracture of the ankle	3 (100)	0 (0)	3	2 (100)	0 (0)	2	0 (0)	0 (0)	0
Fracture of the elbow	1 (50.0)	1 (50.0)	2	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Fracture of the fibula	1 (100)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Fracture of the foot	0 (0)	1 (100)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Fracture of the	4	0	4	2	0	2	0	0	0

humorous	(100)	(0)		(100)	(0)		(0)	(0)	
Fracture of the knee	7 (100)	0 (0)	7	5 (100)	0 (0)	5	0 (0)	0 (0)	0
Fracture of the shaft of the femur	7 (100)	0 (0)	7	5 (100)	0 (0)	5	0 (0)	0 (0)	0
Fracture of the tibia	4 (100)	0 (0)	4	2 (100)	0 (0)	2	0 (0)	0 (0)	0
Fracture of the wrist	1 (100)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Fall	87 (87.0)	13 (13.0)	100	36 (76.6)	11 (23.4)	47	3 (27.3)	8 (72.7)	11
Fatigue	0 (0)	2 (100)	2	0 (0)	2 (100)	2	0 (0)	2 (100)	2
Fits	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Flu	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Fractured neck of femur	58 (96.7)	2 (3.3)	60	17 (89.5)	2 (10.5)	19	0 (0)	2 (100)	2
Foot ulcer	1 (100)	0 (0)	1	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Gallstones	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Haematuria	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Hepatic	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Hip pain	5 (83.3)	1 (16.7)	6	3 (75.0)	1 (25.0)	4	0 (0)	0 (0)	0
Hypercalcaemia	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Hyperkalaemia	0 (0)	2 (100)	2	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Inflammatory bowel disease	0 (0)	1 (100)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Ischaemia	1 (100)	0 (0)	1	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Leg pain	3 (75.0)	1 (25.0)	4	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Leg swelling	1 (100)	0 (0)	1	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Leg ulcers	1 (100)	0 (0)	1	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Lower respiratory tract infection	3 (37.5)	5 (62.5)	8	1 (20.0)	5 (80.0)	6	3 (42.9)	4 (57.1)	6
Multi organ failure	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Necrotising fasciitis	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Neutropenic	2	0	2	2	0	2	0	0	0

	(100)	(0)		(100)	(0)		(0)	(0)	
Neutropenic sepsis	0 (0)	4 (100)	4	0 (0)	2 (100)	2	1 (50.0)	1 (50.0)	2
Not known	7 (100)	0 (0)	7	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Pain in ankle	1 (100)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Pancreatitis	0 (0)	3 (100)	3	0 (0)	3 (100)	3	2 (66.7)	1 (33.3)	3
Planned surgery	9 (32.1)	19 (67.9)	28	7 (28.0)	18 (72.0)	25	11 (61.1)	7 (38.9)	18
Planned Treatment	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Pyrexia	0 (0)	8 (100)	8	0 (0)	7 (100)	7	2 (28.6)	5 (71.4)	7
Recurrent CDI	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Reduced Glasgow coma scale	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Sepsis	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Septic arthritis	6 (100)	0 (0)	6	4 (100)	0 (0)	4	0 (0)	0 (0)	0
Shortness of breath	5 (41.7)	7 (58.3)	12	3 (30.0)	7 (70.0)	10	5 (71.4)	2 (28.6)	7
Stem cell transplant	0 (0)	2 (100)	2	0 (0)	2 (100)	2	2 (100)	0 (0)	2
Stoma leak	0 (0)	1 (100)	1	0 (0)	1 (100)	1	1 (100)	0 (0)	1
Stroke	2 (40.0)	3 (60.0)	5	1 (25.0)	3 (75.0)	4	0 (0)	3 (100)	3
Subdural haematoma	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Tachycardia	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Transfusion	1 (100)	0 (0)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Ulcerative colitis	0 (0)	3 (100)	3	0 (0)	3 (100)	3	3 (100)	0 (0)	3
Unsteady	0 (0)	1 (100)	1	0 (0)	1 (100)	1	0 (0)	1 (100)	1
Unwell	0 (0)	7 (100)	7	0 (0)	6 (100)	6	0 (0)	6 (100)	6
Upper GI bleed	0 (0)	1 (100)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Urinary sepsis	0 (0)	4 (100)	4	0 (0)	4 (100)	4	3 (75.0)	1 (25.0)	4
Upper respiratory tract infection	1 (50.0)	1 (50.0)	2	1 (50.0)	1 (50.0)	2	0 (0)	1 (100)	1
Urinary tract	5	4	9	1	4	5	1	3	4

infection	(55.6)	(44.4)		(20.0)	(80.0)		(25.0)	(75.0)	
Vertigo	0 (0)	1 (100)	1	0 (0)	0 (0)	0	0 (0)	0 (0)	0
Vomiting	4 (44.4)	5 (55.6)	9	2 (33.3)	4 (66.7)	6	1 (25.0)	3 (75.0)	4
Whipple's	0 (0)	2 (100)	2	0 (0)	2 (100)	2	0 (0)	2 (100)	2
Total	252	206	458	112	187	299	78	109	187

Table A3.1 The reasons for admission for control patients that were recruited to the study, regardless of if they had samples in the study and if they had at least one sample included in the study. Those patents that had at least one GDH positive sample are divided into potential *C. difficile* excretors (PCDEs) and CDI cases based on the presence or absence of *C. difficile* toxins in the original routine sample. Each participant could have more than one reason for admission recorded. Summary table of the top ten conditions listed in admission for all participants are shown in tables 5.2 and 5.3

	Recruited to the study			Had at least one sample in the study			Had at least one sample in the study and initial diagnostic specimen was GDH positive		
	Control	GDH positive	Total	Control	GDH positive	Total	PCDE	CDI	Total
	n (%)	N (%)	n	n (%)	n (%)	n	n (%)	n (%)	n
Abdominal sepsis	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Abdominal aortic aneurysm	1 (33.3)	2 (66.7)	3	1 (33.3)	2 (66.7)	2	1 (50.0)	1 (50.0)	2
Acute kidney injury	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2	1 (50.0)	1 (50.0)	2
Alcoholic	4 (22.2)	14 (77.8)	18	1 (7.7)	12 (92.3)	13	5 (45.5)	6 (54.5)	11
Alcoholic liver disease	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Alzheimer's	3 (75.0)	1 (25.0)	4	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Acute myeloid leukaemia	2 (28.6)	5 (71.4)	7	2 (28.6)	5 (71.4)	7	2 (40.0)	3 (60.0)	5
Anaemia	5 (62.5)	3 (37.5)	8	2 (40.0)	3 (60.0)	5	1 (33.3)	2 (66.7)	3
Angina	8 (57.1)	6 (42.9)	14	3 (50.0)	3 (50.0)	6	4 (66.7)	2 (33.3)	6
Ankylosing spondylitis	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Arthritis	1 (100.0)	0 (0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Asthma	5 (20.0)	20 (80.0)	25	2 (10.0)	18 (90.0)	20	9 (50.0)	9 (50.0)	18
Atrial fibrillation	15 (53.6)	13 (46.4)	28	10 (43.5)	13 (56.5)	23	3 (23.1)	10 (76.9)	13
Autism	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Atrial ventricular fibrillation	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
B12 deficiency	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Biliary sepsis	1 (33.3)	2 (66.7)	3	1 (33.3)	2 (66.7)	3	2 (100.0)	0 (0)	2

Bone marrow transplant	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2	2 (100.0)	0 (0)	2
Bronchiectasis	0 (0)	1 (100.0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Bronchitis	1 (33.3)	2 (66.7)	3	0 (0)	2 (100)	2	0 (0)	2 (100.0)	2
Cancer	28 (40.6)	41 (59.4)	69	14 (26.4)	39 (73.6)	53	1 (33.3)	2 (66.7)	3
CABG	10 (66.7)	5 (33.3)	15	5 (62.5)	3 (37.5)	8	22 (56.4)	17 (43.6)	39
Cardiac failure	2 (100.0)	0 (0)	2	0 (0)	0 (0)		0 (0)	0 (0)	0
CDI	1 (100.0)	0 (0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Cellulitis	1 (50.0)	1 (50.0)	2	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Cerebral palsy	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2	2 (100.0)	0 (0)	2
Child C	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Cirrhosis	1 (50.0)	1 (50.0)	2	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Cholecystectomy	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2
Chronic kidney injury	10 (76.9)	3 (23.1)	13	5 (62.5)	3 (37.5)	8	0 (0)	3 (100)	3
Cystic fibrosis	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Chronic lymphocytic leukaemia	1 (50.0)	1 (50.0)	2	1 (50.0)	1 (50.0)	2	0 (0)	1 (100.0)	1
Coeliac	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Colostomy	1 (100.0)	0 (0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Confusion	2 (100.0)	0 (0)	2	2 (100.0)	0 (0)	2	0 (0)	0 (0)	0
Constipation	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
COPD	12 (54.5)	10 (45.5)	22	4 (28.6)	10 (71.4)	14	6 (60.0)	4 (40.0)	10

Crohn's	1 (25.0)	3 (75.0)	4	1 (33.3)	2 (66.7)	3	1 (50.0)	1 (50.0)	2
Cystectomy	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Dementia	15 (83.3)	3 (16.7)	18	6 (66.7)	3 (33.3)	9	0 (0)	3 (100)	3
Diabetes	33 (52.4)	30 (47.6)	63	19 (42.2)	26 (57.8)	45	9 (32.1)	19 (67.9)	28
Diverticulitis	1 (14.3)	6 (85.7)	7	1 (14.2)	6 (85.7)	7	2 (33.3)	4 (66.7)	6
Deep vein thrombosis	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2	1 (50.0)	1 (50.0)	2
Dyspepsia	1 (100.0)	0 (0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Emphysema	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Enteritis	1 (50.0)	1 (50.0)	2	1 (50.0)	1 (50.0)	2	1 (100.0)	0 (0)	1
Epilepsy	5 (41.7)	7 (58.3)	12	4 (36.4)	7 (63.6)	11	5 (71.4)	2 (28.6)	7
Fibromyalgia	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100)	0 (0)	1
Gall stones	1 (33.3)	2 (66.7)	3	1 (33.3)	2 (66.7)	3	1 (50.0)	1 (50.0)	2
Gastritis	0 (0)	4 (100.0)	4	0 (0)	3 (100)	3	1 (33.3)	2 (66.7)	3
Gastro-oesophageal reflux disease	3 (100.0)	0 (0)	3	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Gout	2 (50.0)	2 (50.0)	4	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2
Heart disease	14 (63.6)	8 (36.4)	22	7 (50.0)	7 (50.0)	14	1 (14.3)	6 (85.7)	7
Heart failure	3 (37.5)	5 (62.5)	8	1 (11.1)	8 (88.9)	9	0 (0)	5 (100)	5
Heart Transplant	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100)	1
Heart valve	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2
Hepatitis C	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2	0 (0)	1 (100.0)	1
Hepatitis	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Heart failure	1	0	1	1	0	1	0	0	0

	(100.0)	(0)		(100.0)	(0)		(0)	(0)	
HIV	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Hodgkin's lymphoma	0 (0)	2 (100.0)	2	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Home parenteral nutrition	1 (100.0)	0 (0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Hypercholesterolemia	2 (66.7)	1 (33.3)	3	1 (50.0)	1 (50.0)	2	1 (100.0)	0 (0)	1
Hypertension	55 (61.8)	34 (38.2)	89	22 (42.3)	30 (57.7)	52	13 (41.9)	18 (58.1)	31
Hyponatraemia	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100)	1
Hypothyroid	10 (66.7)	5 (33.3)	15	3 (42.9)	4 (57.1)	7	1 (25.0)	3 (75.0)	4
Inflammatory bowel disease	0 (0)	2 (100.0)	2	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Irritable bowel syndrome	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Incontinence	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Intravenous drug user	1 (50.0)	1 (50.0)	2	0 (0)	1 (100.0)	1	0 (0)	0 (0)	0
Knee replacements	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Laryngectomy	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Leg amputation	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	
Leg ulcers	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Leukaemia	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100)	0 (0)	1
Liver disease	0 (0)	6 (100.0)	6	0 (0)	5 (100)	5	4 (80.0)	1 (20.0)	5
Liver Transplant	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2	2 (100.0)	0 (0)	2
Lobectomy	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Left ventricular	2	0	2	2	0	2	0	0	0

failure	(100.0)	(0)		(100)	(0)		(0)	(0)	
Lymphoma	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Memory loss	1 (100.0)	0 (0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Myocardial infarction	15 (65.2)	8 (34.8)	23	8 (50.0)	8 (50.0)	16	5 (62.5)	3 (37.5)	8
Motor neurone	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Multiple sclerosis	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Myeloma	0 (0)	3 (100.0)	3	0 (0)	2 (100.0)	2	0 (0)	2 (100.0)	2
None	7 (77.8)	2 (22.2)	9	3 (60.0)	2 (40.0)	5	0 (0)	2 (100.0)	2
Nausea and vomiting	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Neurogenic bladder	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Neurofibromatosis	1 (100.0)	0 (0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Not known	3 (100.0)	0 (0)	3	2 (100.0)	0 (0)	2	0 (0)	0 (0)	0
Obesity	3 (75.0)	1 (25.0)	4	1 (50.0)	1 (50.0)	2	0 (0)	1 (100.0)	1
Osteoarthritis	15 (53.6)	13 (46.4)	28	6 (33.3)	12 (66.7)	18	5 (41.7)	7 (58.3)	12
Osteomyelitis	0 (0)	2 (100.0)	2	0 (0)	2 (100)	2	0 (0)	2 (100.0)	2
Osteoporosis	11 (73.3)	4 (26.7)	15	4 (50.0)	4 (50.0)	8	0 (0)	4 (100)	4
Pacemaker	1 (33.3)	2 (66.7)	3	1 (50.0)	1 (50.0)	2	0 (0)	2 (100.0)	2
Pancreatectomy and splenectomy	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Pancreatitis	1 (100.0)	7 (87.5)	8	0 (0)	5 (100)	5	3 (60.0)	2 (40.0)	5
Pancytopenia	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Paralysis	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0

Parkinson's	2 (40.0)	3 (60.0)	5	1 (25.0)	3 (75.0)	4	1 (33.3)	2 (66.7)	3
Pneumocystis pneumonia	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	
Pulmonary embolism	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Pericardial effusion	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Pneumonia	0 (0)	2 (100.0)	2	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Prostate	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Pulmonary embolism	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Pulmonary fibrosis	2 (100.0)	0 (0)	2	2 (100.0)	0 (0)	2	0 (0)	0 (0)	0
Peripheral ventricular disease	3 (50.0)	3 (50.0)	6	3 (50.0)	3 (50.0)	6	2 (66.7)	1 (33.3)	3
Renal disease	1 (16.7)	5 (83.3)	6	0 (0)	4 (100)	4	2 (50.0)	2 (50.0)	4
Renal failure	2 (20.0)	8 (80.0)	10	1 (11.1)	8 (88.9)	9	4 (50.0)	4 (50.0)	8
Renal Transplant	0 (0)	5 (100.0)	5	0 (0)	5 (100)	5	4 (100)	0 (0)	4
Rheumatoid	9 (52.9)	8 (47.1)	17	6 (42.9)	8 (57.1)	14	4 (50.0)	4 (50.0)	8
Scoliosis	1 (100.0)	0 (0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Sepsis	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Sleep apnoea	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	
Splenectomy	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Spondylitis	1 (100.0)	0 (0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Stent for CBD	0 (0)	1 (100.0)	1	0 (0)	0 (0)		0 (0)	0 (0)	0
Stroke	17 (53.1)	15 (46.9)	32	6 (35.3)	11 (64.7)	17	3 (27.3)	8 (72.7)	11
Trauma	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Ulcerative colitis	0 (0)	6 (100.0)	6	0 (0)	6 (100)	6	5 (83.3)	1 (16.7)	6

Urinary sepsis	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Urinary stent	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Ventricular fibrillation	1 (100.0)	0 (0)	1	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
VP shunt	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Vancomycin resistant Enterobacteriaceae	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Whipple's	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Total	377	402	779	181	366	547	160	206	366

Table A3.2 Past medical history for control patients that were recruited to the study regardless of if they had samples in the study and if they had at least one sample included in the study. Those patents that had at least one GDH positive sample are divided into potential *C. difficile* excretors (PCDEs) and CDI cases based on the presence or absence of *C. difficile* toxins in the original routine sample. Each participant could have more than one reason for admission recorded. Summary table of the top ten conditions listed in past medical history for all participants are shown in tables 5.4 and 5.5

	Had at least one sample in the study			Had at least one sample in the study and initial diagnostic specimen was GDH positive		
	Control n (%)	GDH positive n (%)	total n	PCDE n (%)	CDI n (%)	tota l n
Amikacin	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Amoxicillin	4 (25.0)	12 (75.0)	16	5 (41.7)	7 (58.3)	12
Anidulafungin	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Aztreonam	3 (21.4)	11 (78.6)	14	7 (63.6)	4 (36.4)	11
Benzyl/Penicillin	0 (0)	3 (100.0)	3	2 (66.7)	1 (33.3)	3
Caspofungin	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Cefotaxime	0 (0)	2 (100.0)	2	1 (50.0)	1 (50.0)	2
Cefuroxime	0 (0)	6 (100.0)	6	4 (66.7)	2 (33.3)	6
Cephalexin	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Ciprofloxacin	1 (10.0)	9 (90.0)	10	4 (44.4)	5 (55.6)	9
Clarithromycin	3 (30.0)	7 (70.0)	10	2 (28.6)	5 (71.4)	7
Clindamycin	0 (0)	2 (100.0)	2	1 (50.0)	1 (50.0)	2
Co- amoxiclav	12 (30.8)	27 (69.2)	39	11 (40.7)	16 59.3)	27
Co-Fluampicil	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Cotrimazole	1 (100)	0 (0)	1	0 (0)	0 (0)	0
Co-trimoxazole	1 (8.3)	11 (91.7)	12	6 (54.5)	5 (45.4)	11
Daptomycin	0 (0)	1 (100.0)	1	1 (100.0)	0 (0)	1
Doxycycline	0 (0)	4 (100.0)	4	0 (0)	4 (100.0)	4
Ertapenem	1 (50.0)	1 (50.0)	2	0 (0)	0 (0)	0
Erythromycin	0 (0)	2 (100.0)	2	2 (100.0)	0 (0)	2
Fidaxomicin	0 (0)	28 (100.0)	28	5 (17.9)	23 (82.1)	28
Flucloxacillin	13	15	28	6	9	15

	(46.4)	(53.6)		(40.0)	(60.0)	
Fluconazole	0 (0)	10 (100.0)	10	9 (90.0)	1 (10.0)	10
Fucidin	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Gentamicin	5 (100.0)	0 (0)	5	0 (0)	0 (0)	0
Itraconazole	0 (0)	5 (100.0)	5	3 (60.0)	2 (40.0)	5
Levofloxacin	1 (10.0)	9 (90.0)	10	5 (55.6)	4 (44.4)	9
Linezolid	3 (60.0)	2 (40.0)	5	1 (50.0)	1 (50.0)	2
Meropenem	1 (5.9)	16 (94.1)	17	9 (56.3)	7 (43.7)	16
Metronidazole	3 (3.5)	82 (96.5)	85	26 (31.7)	56 (38.3)	82
Nitrofurantoin	4 (33.3)	8 (66.7)	12	0 (0)	4 (100.0)	4
Nystatin	0 (0)	3 (100.0)	3	2 (66.7)	1 (33.3)	3
Pipercillin tazobactam	5 (9.8)	46 (90.2)	51	22 (47.8)	24 (52.2)	46
Pivmecillinam	1 (12.5)	7 (87.5)	8	3 (42.8)	4 (57.1)	7
Posaconazole	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Tazocin	5 (21.7)	18 (78.3)	23	8 (44.4)	10 (55.6)	18
Teicoplanin	5 (62.5)	3 (37.5)	8	2 (66.7)	1 (33.3)	3
Temicillin	0 (0)	1 (100.0)	1	0 (0)	1 (100.0)	1
Tigecycline	1 (100.0)	0 (0)	1	0 (0)	0 (0)	0
Trimethoprim	1 (9.1)	10 (90.9)	11	6 (60.0)	4 (40.0)	10
Vancomycin	3 (7.0)	40 (93.0)	43	10 (25.0)	30 (75.0)	40
Number of participants in each group	81	147	228	65	82	147

Table A3.3 Antibiotics taken by controls, GDH positive, PCDEs and CDI participants in the three months prior to the study, for all recruited patients that had at least one sample taken in the study. Summary tables of antibiotic history for all participants are shown in tables 5.7 and 5.8

Appendix 4. Ethical approval for extended use of Department of Health study data

NHS
Health Research Authority
NRES Committee East of England - Cambridge Central
Victoria House
Capital Park
Fulbourn
Cambridge
CB21 5XB

Telephone: 01223 597685
Facsimile: 01223 597645

24 October 2012

Mrs Kerrie Davies
Clinical Scientists Office, Microbiology department,
Leeds Teaching Hospitals NHS Trust
Old medical School
Thoresby Place, Leeds
LS1 3EX

Dear Mrs Davies

Study title:	**Sample factors affecting the perfomance of C.difficile diagnostic assays**
REC reference:	**12/EE/0495**
Protocol number:	**N/A**

The Proportionate Review Sub-committee of the NRES Committee East of England - Cambridge Central reviewed the above application on 23 October 2012.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated

Appendix 5. Ethical approval for PlaciD study

NHS
Health Research Authority
NRES Committee North East - Newcastle & North Tyneside 2
Room 002
TEDCO Business Centre
Rolling Mill Road
Jarrow
NE32 4BW

Tel: 0191 428 3565

18 October 2013

Mrs Kerrie Davies
Clinical Scientist
Leeds Teaching Hospitals NHS Trust
Clinical Scientists Office, Microbiology Dept
Leeds Teaching Hospitals NHS Trust
Old medical School
Thoresby Place
Leeds LS1 3EX

Dear Mrs Davies

Study title:	**Clinical course of Patient's infection determined by Laboratory Diagnostic Assays for C. difficile Detection from Faecal Samples (PLaciD)**
REC reference:	**13/NE/0255**
Protocol number:	**N/A**
IRAS project ID:	**108081**

Thank you for your letter of 9 October 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Gillian Mayer, nrescommittee.northeast-newcastleandnorthtyneside2@nhs.net

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a **Favourable** ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Mental Capacity Act 2005

I confirm that the committee has approved this research project for the purposes of the Mental Capacity Act 2005. The committee is satisfied that the requirements of section 31 of the Act will be met in relation to research carried out as part of this project on, or in relation to, a person who lacks capacity to consent to taking part in the project.

Appendix 6. Protocol for PlaciD study

Protocol version 2.8 30-07-13

Chief Investigator: Kerrie Davies

Clinical course of Patient's infection determined by Laboratory Diagnostic Assays for *C. difficile* Detection from Faecal Samples (PLaciD)

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Background:

Clostridium difficile is still the major cause of nosocomial diarrhoea in the developed world and rapid and accurate diagnosis is paramount for patient care and infection prevention[1]. Furthermore, infection reduction targets have been imposed on healthcare facilities in England. Patients with *C. difficile* Infection (CDI) often have protracted periods of hospital stay, usually in isolation facilities with specialist nursing care, resulting in a huge financial burden on the NHS. One UK study attributed costs of approximately £6986 per case of CDI (2010 prices)[2]. There has been an increase in the measured incidence of CDI in countries with active surveillance programmes, and a marked shift in epidemiology over the last decade[3]. Outbreaks caused by a strain of *C. difficile* known as PCR-ribotype 027 have been reported worldwide since 2002-03. This fluoroquinolone resistant strain has been associated with increased morbidity and mortality[4]. Recurrent infection is a known sequela of CDI; a recent study showed that the risk of re-infection, as distinct from relapse, increased by >58% every time the interval between positive samples doubled; mixed infections (>1 genotype) were found in 7% of CDI cases[5]. Although the 'classical' picture of *C. difficile* transmission has been that it is spread from other patients and or the environment within hospitals, recent data have shown that only 20-25% of inpatient cases can be linked to another in-patient case, leaving the 'source' of infection unknown in the majority of cases[6-7]. It is known however that the rate of asymptomatic carriage in hospitalised patients (21%) is higher than in the community (1.6%)[20].

The anaerobic, spore-forming bacterium *Clostridium difficile* is a well-recognised nosocomial pathogen, which produces two toxins; an enterotoxin, A, and a cytotoxin, B. *Clostridium difficile* is the aetiological agent of pseudomembranous colitis and is implicated in most cases of antibiotic associated diarrhoea[1]. Symptoms can range from mild to severe diarrhoea and may even be fatal; either from CDI alone, or due to exacerbation of co-morbidities[1]. Elderly hospitalised patients and patients previously treated with antibiotics (especially 3rd generation cephalosporins, and protracted courses) are at an increased risk for CDI. The investigation of CDI in the elderly, including risk factors, such as waning immunity, comorbidities, frequent hospitalisations and exposure to antibiotics, is a growing field. Indeed, a recent review of CDI in the elderly noted that the number of published articles per year on the subject doubled between 2006 and 2008[8]. In the USA in 2006 patients >65 years old were disproportionately affected by CDI compared with other age groups, and in 2008 93% of CDI deaths were aged >65 years old[9-10]. Advanced age has also been linked to the risk of recurrent *C. difficile* disease. A recent study looking at the effect of age on treatment outcomes in CDI developed a mathematical model that predicted lower clinical cure (17%), greater recurrence (17%) and lower sustained clinical response (13%) for each increased decade of life[11]. Accurate, rapid

diagnosis of CDI is therefore imperative to ensure early recognition and treatment of this disease.

Sub-optimal laboratory diagnostics have impacted on both patient care and epidemiological data, meaning that the true burden of CDI remains unclear[12-15]. Laboratory diagnosis has largely relied on the detection of *C. difficile* toxins within a faecal sample by direct cytotoxin assay or immunoassays (EIA). A large study in 2009 highlighted the poor performance of these immunoassays, and Department of Health (DH) guidance published subsequently suggested that no toxin immunoassay should be used in isolation for the diagnosis of CDI[15-16]. Alternative methods of detection have now been introduced, including detection of *C. difficile* specific Glutamate dehydrogenase (GDH) a cell surface enzyme and Polymerase Chain Reaction (PCR) assay for the detection of the ToxB gene, or the TcdC deletion suggestive of PCR-ribotype 027 (table 1, appendix 1). Guidance from the DH has now been updated following a large multicentre study, recommending GDH EIA/toxin EIA or cytotoxin as the optimal method for diagnosis of CDI[15, 17]. Using this algorithm patients can be divided into those with CDI, those likely with *C. difficile* and those without CDI/*C. difficile*[15, 17]. Patients identified as potential *C. difficile* excretors (GDH positive/toxin negative) may pose a possible infection control risk, requiring source isolation. Crucially, whilst the variability in performance of diagnostics assays is now well documented[15, 17], there are few data on the effect of sample, patient and organism factors on the performance of these assays. Recent studies have shown that the presence of toxin in the faecal sample is closely correlated with mortality and severity of infection, whilst the presence of a toxigenic strain of *C. difficile* or its DNA in the sample is not[18-19]. Initial data from a Human Gut model shows that *C. difficile* GDH remains detectable in the system for longer than toxin after treatment of CDI (data unpublished), highlighting the fact that each of the targets may reflect a different point in the disease.

There is a significant knowledge gap regarding the clinical course of CDI as determined by the different diagnostic markers of *C. difficile*; organism, toxin or DNA. The aim of this study is to elucidate the clinical course of *C. difficile* infection detected by these different diagnostic assays and match this with severity markers. In order to determine this we will need to follow known CDI positive patients to examine what happens to these tests during disease; similarly, in order to understand the natural history of how these tests perform, we will need to follow all patients from selected wards from admission. Elderly medical wards have been selected for this purpose as these patients are the group most at risk from CDI and its sequelae.

The serial samples collected on symptomatic and asymptomatic patients as part of this study will also prove invaluable for investigating asymptomatic carriage and potential *C. difficile* excretors and the relatedness of isolates from these different patient groups. The role of diagnostic assays in detecting these two potential disease states, along with their use as admission surveillance tools is as yet unclear.

Hypothesis:

Current commercial CDI diagnostic tests detect different targets: the bacterium, DNA or toxin. We hypothesise that each test and target are optimal at different points in the course of CDI infection.

Objectives:

Primary objective:
To determine the time course of *C. difficile* test results during *C. difficile* infection in relation to severity markers.

Secondary objectives:
To determine the accuracy of laboratory diagnostic assays in detecting asymptomatic colonisation/carriage on admission to hospital, different testing methodologies will be compared.

To compare the PCR-ribotype of isolates from patients with CDI, asymptomatic carriage of *C. difficile*, recurrent CDI and new infections with CDI.

To determine the utility of rectal swabs compared with faecal samples for detecting asymptomatic colonisation/carriage at time of admission.

To examine the utility of determining CD colonisation status on admission as a predictor of CDI risk in this patient cohort.

Methodology for objectives:

Primary objective:

The clinical course of infection will be examined for each patient (diarrhoea status, temperature, white cell count, serum albumin, serum creatinine, antibiotic therapy) and analysed with results of each of the *C. difficile* test results to demonstrate if different assays are more likely to be positive at different time points during episodes of *C. difficile* infection (CDI).

Secondary objectives:

Asymptomatic carriage will be defined as a positive test for *C. difficile* in a patient without symptoms of diarrhoea.

Molecular typing analysis will be used to elucidate any relationship between the isolates of *C. difficile* from patients and whether they had asymptomatic carriage, recurrent CDI or a new infection with CDI.

In addition to collecting a faecal sample on admission from a patient, the patient will be asked if they can be sampled using a rectal swab (for the first sample only). The results from the rectal swab by different testing methodologies would be compared to those from the faecal sample. There are a paucity of data on the use of rectal swabs for detection of *C. difficile,* as most assays are not CE marked for this sample type.

.If patients with asymptomatic carriage on admission are treated with antibiotics do they go on to develop CDI? Our null hypothesis is that the rate of CDI is equal between patients screened positive and negative for CDI on admission. If we have a 4:1 ratio of negatives:carriers then we would need 101 positives and 404 negatives to detect a difference between 2% CDI (negatives) and 10% CDI (positives) with 90% power and a 5% threshold of significance.

Study design:

Proposed study duration: 01/08/2013 -31/07/2014

Proposed duration of patient recruitment: 6 months (01/10/2013-31/03/2014)

Following patients from admission:

To take place on two elderly medicine wards (ward 17/7 at SJUH) at LTHT.

Potential eligible patients will be identified by the clinical care staff to the research nurse. Patients will be enrolled in the study on admission, once informed consent is obtained. Baseline data (age, gender, previous exposure of antibiotics, co-morbidities) will be collected using a data capture form. A stool chart will be kept for the patient daily. A faecal sample and/or an optional rectal swab will be collected on enrolment. Further faecal samples will be collected as often as produced (max 1 per day) and sent to the R&D laboratory for testing. Samples can be self-collected or by the medical staff, as a large proportion of these patients will be using a bed pan or commode. The research nurse will visit the ward every day to ensure that there are adequate supplies of samples containers and to remind staff to save samples where possible.

Samples received in the R&D lab will be stored at 5°C and tested within 5 days using GDH EIA, GDH PCR, Toxin EIA, Toxin PCR, cell-cytotoxicity assay and cytotoxigenic culture. Isolates will be stored (spore preparation in 10% glycerol broth) at -80°C before typing using PCR-ribotyping. If the patient has had routine blood samples taken these will be collected, once requested laboratory diagnostics have been completed, and stored at -20°C before testing for antibodies to *C. difficile* and *C. difficile* toxin. No additional blood samples will be taken. Once patients have been discharged their medical records will be reviewed and further clinical data collected using a second data capture form. Patients from each ward will be recruited during a 6 month period, and followed until discharged or until 30 days after the end of the recruitment period.

Following known positive patients:

To take place at LTHT.

Potential eligible patient will be identified by the laboratory staff to the research nurse. In-patients (>16 years old) that have had a routine faecal sample with a positive GDH EIA result will be enrolled on the study, once informed consent is obtained. Baseline data (age, gender, previous exposure of antibiotics, co-morbidities) will be collected using a data capture form. A stool chart will be kept for the patient daily. Faecal samples and/or an optional rectal swab will be collected on enrolment. Further faecal samples will be collected as often as produced (max 1 per day) and sent to the R&D laboratory for testing. Samples can be self-collected or by the medical staff, especially if the patient is using a bed pan or commode. The research nurse will visit the ward every day to ensure that there are adequate supplies of samples containers and to remind staff to save samples where possible.

Samples received in the R&D lab will be stored at 5°C and tested within 5 days using GDH EIA, GDH PCR, Toxin EIA, Toxin PCR, cell-cytotoxicity assay and cytotoxigenic culture. Isolates will be stored (spore preparation in 10% glycerol broth) at -80°C before typing using PCR-ribotyping. If the patient has had routine blood samples taken these will be collected, once requested laboratory diagnostics have been completed, and stored at -20°C before testing for antibodies to *C. difficile* and *C. difficile* toxin. No additional blood samples will be taken. Once patients have been discharged their medical records will be reviewed and further clinical data collected using a second data capture form. Patients from LTHT will be recruited during a 6 month period, and followed until discharged or until 30 days after the end of the recruitment period.

Inclusion criteria for patient enrolment:

Following from admission:

Any patient admitted to ward 17 (female elderly care) or 7 (male elderly care) at SJUH during the study period

Following known positive patients:

Any in-patient(>16 years old) with a positive GDH result on a routine faecal sample not already enrolled on the study, during the study period

Exclusion criteria for patient enrolment:

Patients aged <16 years.

Unable/unwilling to give informed consent and unable to gain consultee approval.

Patient recruitment and consent

All patients will be assessed by the research nurse, who has training in mental capacity, in combination with the clinical care team to ensure they have mental capacity to give informed consent. In line with the Mental Capacity Act all patients will be assumed to have capacity unless judged by the research nurse and clinical care team to lack capacity to understand and consent to the study. As capacity is decision specific the patient involvement in the study has been made as simple as possible to maximise the chance that the patient will have capacity to consent. Where patients are judged to be unable to give informed consent the next of kin will be approached to give consultee approval. Where possible, if consent can be taken we will still ask for a consultee to be present to support the participant through the process. There are insufficient funds to provide an interpreter for non-English speaking patients in this study. An interpreter would need to be present every day for the research nurse visit to ask about bowel habits since the last visit. Exclusion of non-English speakers due to cost is in line with the exclusion guidelines on the NRES website, especially in the context of student led research.

This study does not involve any invasive interventions or risk to the patient, simply the collection of faecal samples. In many cases these frail patients will be bed-bound and use bed pans or commodes, which makes collection of a sample by the clinical care team simpler and with little burden upon the patient. Where patients have mental capacity and are mobile, they will be supplied with commode bowls to fit over the toilet bowl to aid collection of their sample. They will be able to do this themselves in privacy in the toilet. Sample pots will also be available in the toilets for the participant to use. Previous donors have found using larger samples pots and tongue depressors makes saving samples easier than when using standard sample pots, so these will be supplied. Again, the burden on the patient and the invasion of their privacy would be minimal.

Potential participants will be visited by the study research nurse and presented with a patient information leaflet either upon admission to one of the study wards or after the identification of *C. difficile* GDH from their routine faecal sample by the study research nurse. The research nurse will explain the study and 24 hours after the initial contact informed consent will be sought by the research nurse, who has training in mental capacity. The nurse will consent, recruit, perform initial data gather, re-data gather after discharge, and collect samples (daily or when produced).Full ethical approval will be sought, and adoption by the NIHR portfolio.

A copy of the consent form/consultee approval and the patient information sheet will be filed in the patient's notes and the original copy held by the research team. The patient/consultee will also have a copy of the patient information sheet and consent form/consultee approval to keep which includes contact details for the research team.

Loss to follow up/patient withdrawal

If a patient wishes to withdraw from the study then they are free to do so. This may be because they have changed their mind or because they have gained the mental capacity to make informed decisions and wish to overturn the consultee consent. The research nurse will ask

them to complete a withdrawal form, a copy of which will be added to the patient's notes, a copy given to the participant and the original held by the research team. The original consent process will make it clear that any samples already collected before withdrawal will still be included in the study, along with any clinical data already collected.

Sample storage:

Anonymised faecal samples will initially be stored at 5°C for up to 5 days before testing with the commercial assays is completed. For longer term storage samples will be stored at -20°C to enable further investigation. Isolates will also be stored at -20°C in 10% glyercol broths before typing using PCR-ribotyping. Blood samples will also be stored anonymously at -20°C.

Clinical data:

Clinical data will be collected on enrolment and at the end of the study period on patient demographics (age, gender, ethnicity), patient length of stay, patient movement (between wards/clinics), antibiotic treatment, co-morbidities, white cell count, albumin, creatinine, presence/absence of diarrhoea, evidence of colitis and clinical team diagnosis. All data will be recorded on specific study clinical report forms (CRFs) for both initial data capture and follow up, which will then be input onto an electronic secure password protected database.

Data storage

Patients will be assigned a unique study number; which along with the patient's hospital number will be used to identify that patient in the study, to allow searching of electronic clinical records. The informed consent/consultee approval form will be held separately. All data will be held on security protected encrypted servers at Leeds Teaching Hospitals NHS Trust. All CRFs will be held in a secure lockable cabinet in the research office of the Microbiology Department in the Old Medical School. Only those involved with the project will have access to both the written and electronic data collected for the study. Patient confidentially will be preserved throughout. Data will be stored for 10 years in accordance with local policy.

Reporting:

This is an observational study. Results will not be reported back to clinicians. Patients will continue only to be treated for CDI when symptomatic. Furthermore, results obtained on those samples obtained for the study will not be available in a timescale that is conducive to patient intervention. Patients will continue to get routine samples sent if CDI is suspected in-line with LTHT policy.

Statistical considerations:

Following patients from admission:

Based on testing everyone as soon as they are admitted to the selected wards and testing daily until they leave:

Admissions for study wards last year:

Ward17 (SJH), 16 beds in bays plus 6 side rooms, female elderly medicine; bed turnover ~530 patients/year
Ward 7 (SJH), 16 beds in bays, 0 side rooms, male elderly medicine: bed turnover ~550 patients/year

In LTHT current cytotoxin +ve rate = 4-6%
Current GDH +ve rate = 13.6%

520 patients/6 months (wards 17 and 7))
520 x 4.6% = 20.8 toxin positive patients in 6 months
520 x 13.6% = 70.72 GDH positive patients in 6 months

If each patient roughly stays for one week then there will be 3640 samples to test in total
Expected enrollment of 50% = 1820 samples

It is noted that there will be a lot of 'negative' test results in this cohort but it is important to be able to follow the course of infection from the very early stages and this will only be possible by following people from admission. It will also allow the detection of asymptomatic carriers and potential *C. difficile* excretors.

Following known positive patients:

Based on testing all GDH positive patients for follow up (each patient stays for roughly one week). The figures are based upon the trust as a whole initially, as there may be issues with getting enough people to consent. If numbers appear unwieldy the study will centre of specific locations, e.g. elderly medicine.

No of GDH/toxin positive patients last year 185 (2011-2012),

In 6 months there were 300 GDH positive samples

300 GDH positive patients' daily samples = 2100 samples to test in total

Expected enrollment of 50% = 1050 samples

Statistical analysis

Statistical analyses will be carried out on STATA 12; using descriptive statistics to monitor the experience of patients as they are admitted to the target wards, such as Gantt Charts, percentages and histograms (for carriage, CDI incidence and for ribotype distributions). Diagnostic assays will be assessed by sensitivity, specificity, negative and positive predictive value and Area Under Receiver Operator Curve (AUROCs). Hypotheses tests will be done via standard methods such as Fisher's exact tests (for comparing percentages); for AUROCs we will use the Boostrap method for 95% confidence intervals and for testing the significance of

differences between tests as described elsewhere[19]. Multivariate unconditional logistic regression analysis will be used to address some of the secondary objectives, so as to control for confounders

Project management

This project will be managed by the Chief Investigator (Kerrie Davies), who has previously managed the Department of Health study 'The optimum algorithm for laboratory diagnosis of *C. difficile',* and is the European coordinator for the EUropean, multi-centre, prospective bi-annual point prevalence study of CLostridium difficile Infection in hospitalised patients with Diarrhoea (EUCLID). The research study team will consist of the Chief Investigator, a research nurse (Claire Brown), and a data entry clerk (Frank Lee). Additional supervision will be provided by Prof. Mark Wilcox.

This study will comply with the principals of Good Clinical Practice, the Mental Capacity Act, the Data Protection Act and NHS research Governance.

Publication

Analyses will be submitted for publication as poster or oral presentations at international microbiology conferences and in a peer reviewed journal. All patient data will be fully anonymised. A summary sheet will be available for study participants if requested. This study will also be included in a PhD thesis (Kerrie Davies).

Finances:

This study is fully funded by Public Health England.

Staff:

Clinical Scientist (80% full time) 12 months	£38,473.60
Research Nurse (100% full time) 6 months	£22,976.52

Total: £61,450.12

Non staff:

Consumables (Kits including collection/sample pots)

GDH EIA
Toxin EIA
Cytotoxin & Cytotoxigenic Culture
GDH PCR (in-house)

Toxin PCR (commercial)

£18.50 per sample
3195 estimated samples

Sub total: £59,672.00

PCR-Ribotyping

£6.36 per sample
1400 estimated samples (positives only)

Sub total: £8,904

Total: £68,576.00

TOTAL FUNDING: £130,026.12

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Appendix 1

Table 1. Current *C. difficile* assays and their differences

Assay	Target	Pros	Cons
C. difficile culture	*C. difficile* in the faeces	Good sensitivity	Cannot distinguish between toxigenic and non-toxigenic strains
Cell-cytotoxicity	*C. difficile* toxin in the faeces	Good sensitivity and specificity Detects toxin direct from the sample	Negative result can take up to 48 hours
Cytotoxigenic culture	*C. difficile* in the	Good sensitivity	Cannot detect toxin in

	faeces capable of producing toxin		the sample, only that the *C. difficile* in the sample has the potential to produce toxin
C. difficile GDH EIA	*C. difficile* in the faeces	Good sensitivity	Cannot distinguish between toxigenic and non-toxigenic strains
C. difficile toxins EIA	*C. difficile* toxin in the faeces	Good specificity Detects toxin directly from the sample	Poor sensitivity
C. difficile toxin genes PCR	*C. difficile* DNA in the faeces	Good sensitivity	Cannot detect toxin or organism in the sample, only the toxin gene DNA; lacks specificity

Appendix 7. Ethical approval for gut model studies up to April 2016

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UNIVERSITY OF LEEDS

18 April 2013

Dr Caroline Chilton
Post-doctoral research fellow
Molecular Gastroenterology
Old Medical School
Leeds General Infirmary
LS1 3EX

Dear Caroline

Re ref no: **HSLTLM/12/061 - Blanket approval**
(re: prior project approval HSLTLM/12/056 and HSLTLM/12/048)

Titles (proposed): "research involving the in vitro human gut model"

I am pleased to inform you that "blanket" ethics approval has been granted for future research in respect of the above project title relating to gut model experiments. The "blanket" ethical approval request has been reviewed by the Leeds Institute of Health Sciences and Leeds Institute of Genetics, Health and Therapeutics and Leeds Institute of Molecular Medicine (LIHS/LIGHT/LIMM) joint ethics committee. I can confirm that this approval negates the necessity for individual ethical review applications. However the following is understood to be agreed between the Committee and the Applicant:

- "Blanket" cover expires 30 April 2016.
- The researcher utilises the templates and protocols which has been approved in HSLTLM/12/056 and HSLTLM/12/048
- Should any ethical concerns be raised, a request for an amendment and/or separate ethical approval will be made.
- The applicant shall be responsible for reviewing and monitoring proposals to ensure that no personal harm shall come to the researchers.
- Compliance with any governance requirements within participating NHS Trust and with any requirements of the Human Tissue Act 2004 will be met.
- The blanket approval covers the processes for collection of samples to maintain the in vitro human gut model. It does not specifically cover the research that utilises that model. Where such research is compatible with research previously considered and approved by the Ethics Committee, then a new ethics application would not be required. However, the onus is on the researchers to discuss with the Ethics Committee any research that is not in keeping with this understanding.

Please notify the committee if you intend to make any amendments to the original research as submitted at date of this approval. This includes recruitment methodology and all changes must be ethically approved prior to implementation. Please contact the Faculty Research Ethics and Governance Administrator for further information FMHUniEthics@leeds.ac.uk

Ethical approval does not infer you have the right of access to any member of staff or student or documents and the premises of the University of Leeds. Nor does it imply any right of access to the premises of any other organisation, including clinical areas. The committee takes no responsibility for you gaining access to staff, students and/or premises prior to, during or following your research activities.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited.

It is our policy to remind everyone that it is your responsibility to comply with Health and Safety, Data Protection and any other legal and/or professional guidelines there may be.

I wish you every success with the project.

Yours sincerely

Professor Darren Shickle
Acting Chair, LIHS/LIGHT/LIMM Joint REC, University of Leeds

Appendix 8. Ethical approval for gut model studies after April 2016

UNIVERSITY OF LEEDS

Faculty of Medicine and Health Research Office
School of Medicine Research Ethics Committee (SoMREC)

Room 10.111b, level 10
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Clarendon Way
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04 April 2016

Dr Caroline Chilton
University Academic Fellow
Molecular Gastroenterology, LIBACS
Medicine and Health
Microbiology R&D
Old Medical School
Leeds General Infirmary
Thoresby Place
LEEDS LS1 3EX

Dear Caroline

Ref no: MREC15-070

Title: Investigation of the Interplay between Commensal Intestinal Organisms and Pathogenic Bacteria

Your research application has been reviewed by the School of Medicine Ethics Committee (SoMREC) and we can confirm that ethics approval is granted based on the following documentation received from you:

Document	*Version*	*Date Submitted*
Chilton_2016_gut model ethics application_revised	2.0	02/03/2016
The Human Gut Model v2.1_revisions highlighted	2.1	02/03/2016
uni_loneworking_risk_assessment_form	1.0	26/01/2016

Please notify the committee if you intend to make any amendments to the original research ethics application or documentation. All changes must receive ethics approval prior to implementation. Please contact the Faculty Research Ethics Administrator for further information (fmhuniethics@leeds.ac.uk)

Ethics approval does not infer you have the right of access to any member of staff or student or documents and the premises of the University of Leeds. Nor does it imply any right of access to the premises of any other organisation, including clinical areas. The committee takes no responsibility for you gaining access to staff, students and/or premises prior to, during or following your research activities.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited.

It is our policy to remind everyone that it is your responsibility to comply with Health and Safety, Data Protection and any other legal and/or professional guidelines there may be.

Appendix 9. Study protocol for Department of Health Study

C. difficile diagnostic testing algorithm evaluation 2010

Authors

Kerrie Eastwood
Mark Wilcox
Tim Planche

Version 1.4

Date – 6th December 2010

Study manual

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1. Sample processing

1.1 Performing assays for evaluation

Every sample submitted to the routine hospital laboratory for investigation for *C. difficile* is to be included in this study. Each sample is allocated a study number; supplied in the study pack. The numbers run consecutively and start with the code for the study site at which the testing is taking place.

Each sample will be tested by each assay:

Cytotoxin assay	Reference assays
Cytotoxigenic culture	
Premier Toxin A+B EIA assay	Commercial assays
TechLab Toxin A/B II EIA assay	
TechLab C. diff Chek 60 EIA assay	
Cepheid C. difficile GeneXpert PCR assay	

The routine assay of the testing hospital site will be performed first. The samples will then be collected from the routine lab and the other assays performed. If there is insufficient sample to complete all of the assays they will be performed in the following order until the sample runs out:

Cytotoxin assay
Cytotoxigenic culture assay
GDH EIA
Toxin EIA

1.2 Storage of specimens

The routine laboratory will routinely store any sample positive by the standard method used at that hospital site. For the study, each site will also need to save any samples that are positive by either of the two reference methods or by the two toxin detection EIA's. Samples should be stored at 4°C for one week after testing, then at -20°C. Negatives need not be stored beyond one week post testing, or longer if dictated by routine laboratory practice.

1.3 Safety considerations

All work should be carried out in containment level two (or higher) facilities. Any process that may give rise to infectious aerosols must be performed in a microbiological safety cabinet.

Safety goggles are required when using UV fluorescent lamps.

COSHH and risk assessments should be performed locally.

2. Cytotoxin testing

2.1 Materials

Tissue culture flasks
Tissue culture trays
Automatic pipettes
Microcentrifuge tubes
Universals
Bijoux bottles
Syringes and filters (if needed)
Inverted microscope
Microcentrifuge
CO_2 Incubator

2.2 Reagents

- Trypsin + EDTA. 20 ml bottle is distributed into 0.5ml amounts in sterile plastic bijou, then stored at -20°C.

- Hanks Balanced Salt Solution (HBSS). 100 ml bottle stored at room temperature.

- Calf serum. 100 ml bottle arrives frozen. Thaw and distribute into 25 ml amounts in sterile universals. Label the universals including the date made & freeze at -20°C. Thaw for use when required.

- G.A.G. Contains:

- L-Glutamine. Arrives in 20 ml amounts (100x strength). Stored at -20°C. Thaw out & pipette 5 ml into each of 4 sterile universals.

- Antimycotic/antibiotic mix. Arrives in 20 ml amounts (100x strength). Stored at -20° C. Thaw out and pipette 5 ml into each of the universals containing L-glutamine.

- Gentamicin. Arrives as 10 ml bottle at a concentration of 50 mg/ml. Place 2 mls into a sterile universal and make up to 25 mls with sterile distilled water. This gives a final concentration of 4 mg/ml. Pipette 5 ml of this solution into each of the universals containing L-glutamine/antimycotic/antibiotic.

- Label the universals including the date made and store at -20^{o}C. Thaw for use when required.

- Phosphate buffered saline (PBS). Store at 4^{o}C.

- Dulbecco Medium (DMEM). Arrives as 500 ml bottles. Store in the fridge.

IT IS ONLY USED WITH THE ADDITIVES.

- Add one thawed universal containing calf serum and one thawed universal containing GAG to each 500 ml bottle of Dulbecco prior to use. Date and label the bottle to which additives have been added.

Ensure Lot numbers and expiry dates of new reagents are recorded on the reagent log in the laboratory data folder (Appendix 11.2).

2.3 Method

2.3.1 Tissue culture

Receiving cell lines

Follow instructions received with cell lines from ECACC on how to process received growing cells lines

Cell harvesting

1. Examine flasks using the inverted microscope to confirm satisfactory growth of cells. Flasks are usually used after 4 days growth (i.e. make Monday, use Friday).

2. Remove one bijou (per flask) containing 0.5 ml trypsin + EDTA from the -20°C freezer, allow to thaw, and then aseptically add 4.5 ml Hanks BS solution.

3. Open the flask and pour off the culture medium aseptically into a waste container.

4. Aseptically add half of the Trypsin/Hanks solution from a bijou to the flask.

5. Recap flask and wash the solution over the cell sheet. Decant aseptically into a yellow waste sharps container.

6. Aseptically add the remaining half of the Trypsin/Hanks solution to the flask.

7. Recap the flask and lay it flat on the bench so that the cell sheet is covered with the solution. Leave for one minute.

8. Decant aseptically into a waste container.

9. Lay the flask flat in a 37°C CO_2 incubator for approximately 5 minutes, after which time the cells will have detached from the bottom of the flask.

Note. Before the cell sheet has detached from the bottom of the flask it will appear opaque/hazy. A gentle tap on the flask may be sufficient to detach the cells.

10. Aseptically add 10 ml of Dulbecco Medium to the flask and use a 1 ml automatic pipette to gently mix the cell suspension until smooth.

Preparing flasks and trays

1. Add 750 µl of cell suspension to each of 3 flasks and make up to 10 ml with Dulbecco medium. Record date and passage number (goes up by 1 each time) on the side of the flask and incubate flat side down at 37^{o}C in a plastic box in the CO_2 incubator.

2. Label bottom of each tray with the day, order of use, passage number and date made. (E.g. Monday 1, 2, 3 – pass 211 – 3.6). Label lid with day and order of use.

3. Each tray requires 17 ml of diluted cell suspension (1 ml of cells diluted in 17 ml Dulbecco).

Therefore for a set of
3 trays - dilute 3 ml of cells with 2 universals (approx. 50 ml) of Dulbecco in a sterile Petri dish.
4 trays – dilute 4 ml of cells with 3 universals (approx 65 ml) of Dulbecco in a sterile Petri dish.

4. Using a 8 channel multi pipette and sterile tips, mix the diluted cells well. Add 160 µl of suspension to each well of the tray. Repeat for each tray.

5. Use a clean universal and Petri dish for each set of trays.

6. Incubate the trays at 37^{o}C in a plastic box in a CO_2 incubator.

Antitoxin
Clostridium sordelli antitoxin is purchased commercially. The 5 ml stock bottle is stored at 4^{o}C. The antitoxin is diluted before use; 0.1 ml antitoxin in 4.9 ml sterile distilled water in a sterile plastic bijou. The lot number, expiry date and date made up is written on each bottle of diluted antitoxin.

Positive control
Pool the supernatant from several recent known positive samples. Filter as described in sample processing 2.3.2. Test the filtrate alongside the current control and dilute to give +/- result in well 3 or 4 (filtrate may need diluting with PBS). Label bijou with lot letter (A-Z) and date made.

2.3.2 Sample processing

Label microcentrifuge tubes with sample number.

Add 1 ml of PBS to each tube.

Add sufficient faeces to make a 1 in 5 dilution and mix thoroughly.

Centrifuge at maximum rpm (10,000 rpm or greater) for 10 minutes in a microcentrifuge.

If the supernatant is turbid transfer it to a 1 ml syringe. Fix a syringe filter onto the syringe and apply gentle pressure to pass the supernatant through the filter into a new (labelled) microcentrifuge tube.

If microcentrifuge preparations are not to be tested immediately store in the fridge at 4^{o}C.

Select a tray and using the inverted microscope check that there is a reasonable monolayer of cells with a low proportion of dead (rounded) cells and no microbial contamination.

Add 20 µl of *C. sordelli* antitoxin to each well of rows B, D, F & H.

Take a blank template (Appendix 11.6) and fill in the tray ID and date, the positive control batch number used, the antitoxin batch number used and the rows in which the

antitoxin has been added. Then label the worksheet with the study numbers of the test samples to be added to the tray. Store template in laboratory data folder, use later to record result.

Add 20 µl of the positive control to well A1, mix and transfer 20 µl to well A2, mix again and transfer 20 µl to well A3, mix again and transfer 20 ul to well A4. Repeat this process with the positive control in wells B1 to B4.

For the test samples start with well A5 – adding 20 µl of supernatant, mix well and transfer 20 µl to well A6. Repeat the process in wells B5 to B6. Continue with the test supernatants using two pairs of wells for each test, following template.

Incubate the tray at 37^{o}C in a plastic box in the CO_2 incubator.

Store microcentrifuge tube at 4 ^{o}C until after result is recorded, in case the supernatant needs to be filtered and retested.

2.3.3 Results

Read using an inverted microscope at both 24 and 48 hours.

Cells can be read after 72 hours (when set up over a weekend). Negatives should be recorded as negative. Positives can be recorded as positive as long as the cells in the protected wells look healthy. A negative control should be set up on the plate along with the positive control to ensure healthy cells after 72 hours. If cells are poor, positives should be repeated.

Toxin detected (positive) – indicated by the rounding up of 50% of the cells in the upper row of the set of wells for that sample, this effect has to be neutralised by the antitoxin. (If only first well positive, record as weak positive)

Toxin not detected – no rounding up of the cells in the upper row of the set.

If bacterial contamination is noted in the wells then the supernatant is passed through a syringe filter and the test set up again as above.

If the cells in the wells all show signs of rounding up (i.e. both rows in the set) the supernatant is passed through a syringe filter and the test set up again diluting it over 4 wells.

Where the rounding up occurs after filtering and diluting the test is reported as inconclusive.

- -

-	-	Negative
+	+	
-	-	Positive
+	-	
-	-	Weak Positive
+	+	
+	-	Positive (plus non-specific cpe in first wells)
+	-	
+	-	Toxic, filter and retest
+	+	
+	+	Toxic, filter and retest

3. *C. difficile* culture

3.1 Materials

Industrial alcohol (Absolute ethanol 95%)
Sterile distilled water
Plastic universal
Cotton tip swab
Plastic inoculation loop
Braziers agar plate (Oxoid)

3.2 Method

3.2.1 Alcohol shock

Label one universal for each sample

Dispense 500 µl industrial ethanol into a sterile plastic universal container. Add 500 µl sterile distilled water. Mix well.

Remove a pea-sized amount of faecal sample with a swab and immerse in ethanol/water in the universal container.

Vortex each universal and leave at room temperature for a minimum of 1 hr.

3.2.2 Culture

After 1 hr, take a swab and immerse in the alcohol shock sample. Use this swab to inoculate a Braziers agar plate and streak out for single colonies.

Incubate plates at 37^{o}C in an anaerobic cabinet for 48 hours.

3.2.3 Identification

Examine plates for growth after 48 hours.

Suspect isolates (with classic *C. difficile* appearance of grey-brown, irregular edge, horse manure odour) should be checked for green/yellow fluorescence under longwave (365 nm) UV light and latex agglutination for somatic antigen. ID organism based on the following table.

	C. difficile	*C. bifermentans* *C. sordellii* *C. glycolicum*	*C. innocuum*
Fluorescence under UV at 365 nm	+	-	+
Latex agglutination	+	+	-

Record the ID on the laboratory data worksheet. All isolates identified as *C. difficile* must be saved (see section 5. saving isolates). ID only needs to be to *C. difficile* or non-*C.difficile* level.

4. Cytotoxigenic culture

4.1 Materials

Brain Heart Infusion broth
Cotton tip swab
Microcentrifuge tube
Automatic pipette
Syringe and syringe filter (if needed)

4.2 Method

Cytotoxigenic culture is to be carried out when *C. difficile* is isolated by culture from a sample.

4.2.1 Broth culture

Pre-reduce BHI broths (one per *C. difficile* culture positive sample to be tested) by incubating in an anaerobic cabinet for at least 48 hours before use.

Inoculate one BHI broth per sample from the Brazier's agar plate using a cotton tip swab.

Incubate broths for 48 hours in an anaerobic cabinet at 37^{o}C.

4.2.2 Cytotoxin testing

Label one microcentrifuge tube per sample

Remove 1 ml supernatant from the BHI broth and place into a sterile microcentrifuge tube.

Test for cytotoxin following method given in section 2 of this manual (cytotoxin testing).

5. Saving isolates

5.1 Materials

Columbia blood agar plate
Inoculation loop
Cotton tip swab
Cryogenic microtube
Cryogenic freezer box

5.2 Method

Label a Columbia blood agar plate for each *C. difficile* culture positive sample (toxigenic and non-toxigenic strains) with specimen study number and date.

Using a sterile loop, transfer a colony from the Braziers agar plate onto the Columbia blood agar plate and streak out for single colonies.

Incubate plates at 37^{o}C in an anaerobic cabinet for 7 days to encourage growth of spores.

Label a cryogenic microtube for each plate and add 1 ml nutrient broth with 10% glycerol.

Harvest all growth (if pure) from the Columbia blood agar plate using a cotton tip swab and inoculate the broth.

Store in nalgene cryobox in -70^{o}C freezer. Record freezer box number and position of the isolate on the laboratory data worksheet (appendix 11.3).

Note: If nutrient broth with glycerol unavailable please store using local protocols.

6. Inter-laboratory Quality control – Sending laboratory

6.1 Materials

6 study samples (1 or 2 positives, 4 or 5 negatives) with at least 4 ml remaining after normal study testing

Bijou bottle
Cotton tip swab
Hayes DX plastic container and box

6.2 Method

Label 3 bijoux bottles per sample with QC number (next in numerical sequence) and transfer 1ml of the sample to each bottle.

Record original sample study number, and QC number on the QC worksheet.

For each set of 6 samples:

- Wrap parafilm around the lid of each bottle
- Wrap bottles in paper towel

Place in specimen bag, seal and place inside Hayes DX plastic container with a list of the samples included
Place in Hayes DX box

Send a set of 6 samples to each of the other 3 study sites. Hayes DX codes for each site are included in this study manual (appendix 11.7.)

7. Inter-laboratory Quality control – Receiving laboratory

Process each QC sample in the same way as a study sample but record the results on the Quality control Excel spreadsheet. This spreadsheet must be emailed at the beginning of **each month** to kerrie.eastwood@leedsth.nhs.uk.

8. Commercial assays

8.1 Materials

Premier Toxin A+B EIA assay
TechLab Toxin A/B II EIA assay
TechLab C. diff Chek 60 EIA assay
Cepheid *C. difficile* GeneXpert PCR assay

8.2 Equipment

DS2 for automated performance of EIA's
Cepheid GeneXpert for performance of PCR

8.3 Method

8.3.1 DS2

All assays are carried out according to manufacturers' instructions in the product insert, with the following exceptions;

1. The Premier Toxin A + B assay is run with a larger volume (800µL diluent with 200µL sample) to enable processing on the DS2; supporting information will be given at training.

2. The same sample diluent is used for the two Techlab assays. The sample can therefore be diluted once, in 2x diluent (i.e. 100µL of sample in 400µL of diluent) and used for both assays, to reduce sample processing time. This is supported by the manufacturer.

For plate ID on the DS2's please use the following notation:

Premier toxin A +B	Ptab date initials of operator	e.g. Ptab20/10/10KE
Techlab Toxin A B II	ToxAB date initials of operator	e.g. ToxAB20/10/10KE

Teclab GDH GDH date initials of operator e.g. GDH20/10/10KE

All operators must receive training before using the DS2's. Daily washes must be completed on both machines.

8.3.2 GeneXpert

Training will be provided for the Cepheid GeneXpert. All users must receive training before using the Instrument.

If sample result is 'invalid' repeat the test once. The result of the second test should be recorded in the study database (even if it is invalid again).

Only use the Copan swabs supplied for performing the GeneXpert assay as these are the only swabs to have been validated for the assay.

Please archive all data daily, and place into another folder in the C:drive of the laptop attached to the machine. Down load this data onto your office pc daily (using memory stick) and save in secure folder. Data can then either be transcribed in to the study database by hand

For any problems with instruments or training requirements please contact kerrie.eastwood@leedsth.nhs.uk.

9. Data Collection

9.1 Laboratory data

A laboratory folder containing laboratory data sheets and templates should be kept at each site.

All printouts from the DS2 and the Cepheid GeneXpert are to be kept in the correct section in the laboratory folder. Each sheet should be signed by the operator; date is added by the computer.

All backing sheets for the cytotoxin assay, culture plates and cytotoxigenic culture are to be kept in the correct section in the laboratory folder. Each sheet should be signed and dated by the operator.

At the end of each day the laboratory results are entered onto the laboratory data Excel spreadsheet (see Appendix 11.3-11.5 for laboratory data sheets).

Paper records should be kept for 5 years after the completion of the study.

9.2 Clinical data

Please collect the data listed in table 9.2.1 on **every sample** and enter onto the clinical data Excel spreadsheet, in the 'all' section;

Table 9.2.1 Data to be collected for EVERY specimen

Variable	Definition	units
Patient study number	Number allocated to each patient on the study (The patient number can only be allocated once but a patient can have more than one sample included in the study)	e.g. PLT0001
Study specimen number	Number allocated to each specimen (The number can only be allocated once and each specimen can only be included in the study once)	e.g. LTH0001
Patient Name	Name of patient	
Date of sample	Date sample taken	dd/mm/yy
Processing hospital laboratory number		
Gender	Gender of patient	M= male F=female
Age	Age of patient at time of sample	Years (whole number)
Ward name/number or GP	Ward name or if GP give name	N/A
Recurrent or new diagnosis (R/N)	Whether the patient has had a previous diagnosis within the previous 12 months	R=recurrent N=new N/A=not applicable
White cell count	Peripheral white cell count on the day of stool sample (or the nearest one within 3 days) – If equal time then take sample AFTER stool sample	$x10^9$/L
Serum creatinine	Serum creatinine concentration on the day of stool sample (or the nearest one within 3 days)	µmol/L

	– If equal time then take sample AFTER stool sample	
baseline creatinine	Lowest Serum creatinine concentration taken within the 6 months before the stool sample	µmol/L
Serum albumin	Serum albumin concentration on the day of stool sample (or the nearest one within 3 days) – If equal time then take sample AFTER stool sample	g/L
Date of hospital admission	Date of this admission to hospital	dd/mm/yy
Date of hospital discharge/death	Date patient discharged from hospital or died	dd/mm/yy
Survival at 30 days or discharged	Survival to day 30 as an inpatient or discharge home before day 30	Survived Died Discharged

N.B. please answer N/A – not available/not applicable if there is no value available for any field

N.B. – the day of the stool sample is defined as day 1

Please collect the extra data listed in table 9.2.2. on samples **positive for *C. difficile*** by at least one of the gold standard methods or by PCR (GeneXpert). Enter this data onto the clinical data Excel spreadsheet in the 'Pos extended clinical data' section;

Table 9.2.2. Extra data to be collected on all positive samples (Gold standard or PCR)

Variable	**Definition**	**units**
Highest serum lactate within 3 days of the sample	Highest serum lactate concentration within 3 days of the first positive stool sample – If equal time then take sample AFTER stool sample	mmol/L NA – not available
Admission onto ITU	Admission to intensive care unit for any reason within 3 days before the first positive stool specimen or 10 days after the first positive stool	Y - Yes N = No

	specimen	
Is there evidence of colitis as reported on CT scan	Evidence of the colitis noted on any Computerised tomography scan (CT scan)	Y = Yes N = No N/A – not applicable
Is *C. difficile* mentioned on either part 1 or part 2 of the death certificate	The mention of *C. difficile*, or a synonym on the death certificate, if the person has died	Y = Yes N = No N/A – not applicable

N.B. please answer N/A – not available/not applicable if there is no value available for any field

N.B. – the day of the stool sample is defined as day 1

Please collect the extra data listed in table 9.2.3 on samples that are **positive by ONE, but NOT BOTH, of the Gold standard methods,** and enter onto the clinical data Excel spreadsheet in the 'discrepant sample clinical data' section;

Table 9.2.3 Extra data to be collected for discrepant samples

Variable	**Definition**	**units**
Treating team clinical diagnosis	Diagnosis for the cause of diarrhoea recorded by the attending clinical team	
Is treatment given for *C. difficile* infection (CDI)	Was treatment for *C. difficile* started within 3 days before the first positive stool specimen or 10 days after the first positive stool specimen	Y = Yes N = No
What agent is given to treat CDI	Name of *C. difficile* antibacterial chemotherapy	Vancomycin Metronidazole
Did the patient have diarrhoea documented	Documented diarrhoea (>3 loose (Bristol stool ≥5) stools in a 24 hour period) in the medical or nursing notes	Y = Yes N = No
How many days did the patient have diarrhoea for	Number of days of recorded diarrhoea from start of symptoms until stool sample	days
How many stools did the patient pass on the day of sample	How many stools did the patient pass on the day of sample – as recorded in the nursing or medical notes	
What was the Bristol stool	(if not documented please	1-7

chart rating for that sample	record for nearest stool sample in same episode of diarrhoea)	

N.B. please answer N/A – not available/not applicable if there is no value available for any field

N.B. – the day of the stool sample is defined as day 1

Quick guide:
Negative samples will therefore have all the data from table 9.2.1 collected.

Samples positive by gold standard or by PCR will have the data from tables 9.2.1 AND 9.2.2 collected.

Samples positive by ONLY ONE of the gold standards will have the data from tables 9.2.1, 9.2.2 AND 9.2.3 collected.

10. Data management

10.1 Storage of data

All electronic data must be kept on a secure password protected (N3 compliant) server and should only be accessible to those personnel involved in the study. All paper copies with patient identifiable details should be kept in a secure office.

10.2 Movement of data

At the beginning of each month the Quality control spreadsheet should be emailed to kerrie.eastwood@leedsth.nhs.uk

At the end of the training set (first 4 months) the laboratory data set should be checked for accuracy, locked and emailed to kerrie.eastwood@leedsth.nhs.uk

At the end of the training set (first four months) the clinical data set should be checked for accuracy and the **patient name and processing hospital laboratory number removed** and the spreadsheet resaved under the name clinical data Sitev1, e.g. clinical

data Leedsv1**.** The spreadsheet can then be locked and emailed to kerrie.eastwood@leedsth.nhs.uk. The original spreadsheet with patient details on must remain on the local secure server.

New databases will be supplied for the testing set (second 4 months). The same policies for the storage and management of data will apply to these new databases.

11. Appendix

11.1 Order sheet for Gold standard reagents

To be ordered by processing laboratory (budget supplied). All other kits/media/cell lines will be ordered centrally and delivered to you.

Consummables order sheet

	Unit	volume/number	List price	Supplier	Item code	Number to order Leeds/Oxford	Number to order SGH/UCH
Reference assays							
Industrial alcohol	Bottle	2.5l	23.24	SLS	CHE1938	1	1
Brain Heart Infusion broth	10ml Bottle	50	31.00	E&O	BM0070	4	3
Tissue culture							
Antibiotic/Antimycotic 100X	bottle	20ml	10.05	Invitrogen	15240-096	2	1
L-Glutamine (100x 20.2mg/ml)	bottle	20ml	3.71	Invitrogen	25030-032	2	1
Newborn Calf serum	bottle	100ml	5.67	Invitrogen	16010-167	2	1
Hanks balanced salt solution	bottle	100ml	3.61	Invitrogen	14170-070	1	1
Dulbecco Modified Eagles medium	bottle	500ml	8.23	Invitrogen	10938-025	6	3
Gentamicin (50mg/mll)	bottle	10ml	51.10	Sigma	G1397-10ml	1	1
C. sordellii antitoxin	bottle	5ml	60.60	Prolab	PL6507	1	1
Trypsin-EDTA 0.5% (10x)	bottle	100ml	25.80	Invitrogen	15400-054	1	1
Flasks nunclon, filter cap	pk	300	190.52	Sarstedt	83.1810.002	1	1
TC microwell 96F sterile	pk	50	102.00	VWR	402032808	4	2
Bijoux	pk	700	161.00	Sterilin VWR	215-0328	1	1
Microcentrifuge tubes 1.5ml	pk	1000	30.90	Alpha	LW2075	4	2
Syringe filter Nalgene (CA membrane)	pk	50	111.00	VWR	513-1911	2	1
Syringes 2ml	pk	100	13.67	Fisher Scientific	SZR-160-	1	1

					019L		
phosphate buffered saline	pk	1L	16.00	sigma	D8662	4	2
Consumables							
Universals	Pk	500	71.55	Sarstedt	63.9922.252	7	4
10 uL Culture loops	Pk	1920	78.68	Sarstedt	86.1562.050	2	1
Cryogenic tubes 1.2 ml	case	500	95	Sarstedt	72.377	1	1
Microtube 2.0ml skirted with cap	case	5000	491.9	Sarstedt	72.694	3	2
Storage boxes (cryo) 9x9	each (min 5)	1	39.45	Sarstedt	93.873.481	1 set of 5	1 set of 5
Blue tips with filter Racked1000uL	case	1000 (10 boxes of 100)	76.38	Sarstedt	70.762.211	4	2
cotton tipped swabs	pk	5000	88.00	Fisher Scientific	TS10	2	1
latex agglutination kit	box	50 tests	203.74	Microgen bioproducts	M41CE	1	1

11.2 Tissue culture reagent log

Please record the Lot number, expiry date and date of all reagents used.

DATE		DMEM	TRYPSIN	HBSS	CALF SERUM	L-GLUTAMINE	ANTIBIOTIC MYCOTIC	GENTAMYCIN
	LOT No							
	Expiry date							
	LOT No							
	Expiry date							
	LOT No							
	Expiry date							
	LOT No							
	Expiry date							
	LOT No							
	Expiry date							
	LOT No							
	Expiry date							
	LOT No							
	Expiry date							
	LOT No							
	Expiry date							
	LOT No							
	Expiry date							

11.3 Gold Standards Laboratory data sheet

Specimen study number	Date of sample	Cytotoxin assay (Pos/Neg)	Culture (Pos/Neg)	Cytotoxigenic culture (Pos/Neg)	QC number	Isolate storage (Freezer box/position)

Operator……. **Date**…….

11.4 Commercial EIA Laboratory data sheet

Specimen study number	Date of sample	Premier Toxin A+B result	Premier Toxin A + B OD value	TechLab Tox A/B II OD value	TechLab Quik Chek 60 OD value

Operator…… **Date**…….....

11.5 GeneXpert Laboratory data sheet

Specimen study number	Date of sample	Cepheid GeneXpert result	Cepheid geneXpert Tox B cycle No.	Cepheid GeneXpert Binary Toxin cycle No.	Cepheid GeneXpert Tcd C cycle No.

Operator……. **Date**…….

11.6 Cytotoxin assay template

Tray/Date ……..Positive control batch no. …… 24hr tray read by ……
Antitoxin batch no. …… Antitoxin in rows …… 48hr tray read by

	1	2	3	4	5	6	7	8	9	10	11	12	
A	·	·	·	·	·	·	·	·	·	·	·	·	A
	POSITIVE CONTROL												
B	·	·	·	·	·	·	·	·	·	·	·	·	B
C	·	·	·	·	·	·	·	·	·	·	·	·	C
D	·	·	·	·	·	·	·	·	·	·	·	·	D
E	·	·	·	·	·	·	·	·	·	·	·	·	E
F	·	·	·	·	·	·	·	·	·	·	·	·	F
G	·	·	·	·	·	·	·	·	·	·	·	·	G
H	·	·	·	·	·	·	·	·	·	·	·	·	H
	1	2	3	4	5	6	7	8	9	10	11	12	

Tray/Date ……Positive control batch no. …… 24hr tray read by …
Antitoxin batch no. ……...Antitoxin in rows …… 48hr tray read by

	1	2	3	4	5	6	7	8	9	10	11	12	
A	·	·	·	·	·	·	·	·	·	·	·	·	A
	POSITIVE CONTROL												
B	·	·	·	·	·	·	·	·	·	·	·	·	B
C	·	·	·	·	·	·	·	·	·	·	·	·	C
D	·	·	·	·	·	·	·	·	·	·	·	·	D
E	·	·	·	·	·	·	·	·	·	·	·	·	E
F	·	·	·	·	·	·	·	·	·	·	·	·	F
G	·	·	·	·	·	·	·	·	·	·	·	·	G
H	·	·	·	·	·	·	·	·	·	·	·	·	H
	1	2	3	4	5	6	7	8	9	10	11	12	