"Development of novel molecular methods for the detection of *C. difficile* infections"

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Abstract

BACKGROUND: C. *difficile*-associated infection (CDI), particularly in hospital patients has led to an increase in mortality and morbidity rate in US, UK and Europe. Virulence is mainly dependent on the expression of two key *C. difficile*-specific proteins, toxin A (TcdA) and toxin B (TcdB). Current CDI diagnostic is by ELISA or polymerase chain reaction (PCR); the former is limited in terms of sensitivity the latter in terms of clinical relevance, as detection of bacterial DNA is not informative about viability or whether the bacteria express toxins. Hence the development of this project, which aims to combine the clinically relevant information provided by an antibody-based test with the sensitivity of a PCR assay by using the proximity ligation assay (PLA) for detection of C. *difficile* TcdA and TcdB. PLA detects proteins via their interaction with pairs of antibodies coupled to noncomplementary DNA oligonucleotides. The binding of both antibodies to their target protein brings the oligonucleotides into proximity, allowing them to be bridged by a third oligonucleotide with complementarity to the other two. This facilitates their ligation and the detection of the resulting amplicon by real-time quantitative PCR (qPCR) acts as a surrogate marker for the protein of interest. Hence PLA has potential as a clinically relevant diagnostic tool for the detection of pathogens where nucleic acid based tests are inconclusive proof of infection.

METHODS: We prepared monoclonal and polyclonal PLA probes targeting purified *C. difficile* toxins A (TcdA) and B (TcdB) and also targeting TcdA and TcdB spiked in canine faeces. Further evaluation of the assay was also done targeting TcdA and TcdB in clinical faeces and swab samples. Hydrolysis probe-based qPCR as well as digital PCR (dPCR) assays were used to detect antibody/antigen interactions.

RESULTS: The performance of the PLA assays was antibody-dependent but both TcdA and TcdB assays were 10X more sensitive than comparable ELISAs in either single or duplex format when detecting purified toxins and spiked canine faeces shows sensitivity similar to ELISA performed in our lab. But the assay did not show sufficient sensitivity when evaluating the clinical faeces and swab samples. Both PLAs could be performed using single monoclonal antibodies coupled to different oligonucleotides. Finally, we used digital PCR to demonstrate accurate and reliable quantification of TcdA by digital PLA (dPLA).

CONCLUSIONS: PLA has potential as new diagnostic applications for the detection of C. *difficile*. Further optimization of an assay is required to develop the assay for the detection

of TcdA and TcdB in clinical samples. Once this assay is developed into a diagnostic kit for C. *difficile* TcdA and TcdB, PLA can be used for further development of an assay for other pathogenic organisms where nucleic acid based tests do not indicate viability or expression of toxins, resulting in more targeted clinical decision-making, helping reduce the mortality rate for high-risk individuals. Importantly, since it is not always necessary to use two different antibodies, the pool of potential antibodies useful for PLA diagnostic assays is vastly enhanced. Finally, in the future, the combined testing of DNA and protein targets from the same sample on the same analytical platform (i.e. qPCR) may further improve the sensitivity and specificity of disease diagnosis leading to improved clinical outcomes, patient satisfaction and reduced associated costs.

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DECLARATION OF ORIGINALITY

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Harvinder Singh Dhillon

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LIST OF PUBLICATIONS ARISING FROM THIS THESIS

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1.0 Overview:

Clostridium *difficile* (*C. difficile*) is a gram positive spore forming bacillus responsible for *C. difficile*-associated infection (CDI) in hospital patients (Shah et al., 2010) and has become one of the most common health care-associated pathogens (Gerding and Lessa, 2015b). Its severity in terms of mortality and morbidity is associated with several epidemic strains (Bauer et al., 2011), although more than half of the infected patients do not present with any symptoms (Loo et al., 2011). The clinical symptoms of CDI are rather variable ranging from diarrhoea to pseudomembranous colitis (Janarthanan et al., 2012) due to the variability in the interaction between the bacterial pathogen virulence factors and the hosts' immune response (Solomon et al., 2013).

The primary virulence factors of *C. difficile* are enterotoxins A (TcdA) and B (TcdB), which are specified by two genes, tcdA and tcdB, respectively**.** Most pathogenic strains are toxin A-positive, toxin B-positive (A+B+) although some variants are toxin A-negative, toxin B-positive (A-B+) (Voth and Ballard, 2005). There are also non-pathogenic strains that do not express either toxin (Natarajan et al., 2013). New strains of *C. difficile* for example, the North American pulsed-field type 1, restriction-endonuclease analysis group type BI, and PCR ribotype 027 have emerged which are more virulent than the normal *C. difficile* strains (Goorhuis et al., 2008a) and have contributed to the increase in the morbidity and mortality rate associated with CDI in US, UK and Europe (Ghose, 2013). Therefore, the critical and timely intervention of CDI is required, which depends on faster and more accurate diagnosis of infectious agents. Numerous diagnostic tools have been developed over the last few decades for the detection of CDI, ranging from selective anaerobic culture method, Cell Cytotoxicity Neutralisation Assay (CCNA) to Enzyme Linked Immuno Sorbent Assay (ELISA) for the detection of proteins. Until recently, CCNAs were considered the gold standard method for detection of CDI but the development of molecular test such as the polymerase chain reaction (PCR) has replaced this method. Although the PCR-based assays target bacterial toxin genes with high sensitivity, PCR positive results cannot confirm the viability of the bacteria or their ability to produce toxins, suggesting that PCR positive results may not always accurately reflect the clinical disease (Platts-Mills, Liu and Houpt, 2013)

Therefore, there exists a gap in the methods used for the diagnosis of CDI. Currently, clinical laboratories such as Public Health England, UK recommend the use of a two-step method for CDI diagnosis including an initial screening ELISA for the presence of Glutamate Dehydrogenase (GDH) antigen followed by testing of positive samples using CCNA or PCR (Goldenberg et al., 2010a). Since this approach to *C. difficile* diagnosis is costly, laborious and time consuming, there is an urgent need for the development of the single diagnostic test for CDI that is sensitive and specific and which can address the drawbacks of the current diagnostic methods.

2.0 Clostridium *difficile*

Clostridium *difficile* (*C. difficile*) is a Gram – positive, spore forming, anaerobic bacillus which was first described by Halle and O' Toole in 1935 (Hall and O'Toole, 1935) and has become most common health care associated pathogen (Gerding and Lessa, 2015a). It is the cause of *C. difficile* infection in hospital patients, causing *C. difficile* associated diarrhoea (CDAD) and can lead to a severe life-threatening condition called pseudomembranous colitis (PMC) which results in inflammation of the large intestine (Janarthanan et al., 2012).

2.1 *C. difficile* Infection

2.1.1 Clinical disease

C. difficile spores exist in the environment but can also be found in the normal gastrointestinal tract of animals and humans (Burnham and Carroll, 2013). Nearly 1-3% of healthy adults and 20-40% of hospitalised patients are expected to carry *C. difficile* spores but show no disease symptoms (Hookman and Barkin, 2009). However, once *C. difficile* has colonised a host there are several factors that may result in the development of severe CDI. These include chronic underlying disease, impaired immune response against infection, prolonged use of antibiotics and an increased length of stay in hospital (Kuipers and Surawicz, 2008). The use of antibiotics such as clindamycin, cephalosporin, penicillin and fluoroquinolone (Hensgens et al., 2012) has been associated with CDI. It is presumed that the use of multiple antibiotics or prolonged course of antibiotics can disrupt the normal gut microbiota, which may cause germination and proliferation of *C. difficile* followed by production of *C. difficile* toxins (Owens et al., 2008). Symptoms of CDI are dependent upon

the production of secreted *C. difficile* toxins, TcdA and/or TcdB (Rupnik, Wilcox and Gerding, 2009). There are also non-pathogenic strains that do not express either toxin (Natarajan et al., 2013) thus cause no illness.

Clinical features and complications of CDI are variable and depend on the severity of the disease which can range from mild diarrhoea, dehydration, nausea, fever, abdominal cramps to fulminant pseudomembranous colitis (PMC) (Bartlett and Gerding, 2008). Roughly, 4-10% of CDI patients develop fulminant PMC, which is characterised by hypotension, increased level of lactic acid, Ileus or toxin megacolon, sepsis, multi-organ failure leading to death (Greenstein et al., 2008).

One of the most serious and problematic features of CDI is its recurrence or relapse. In this condition, the CDI infection reappears even after the successful treatment of the first CDI infection. The recurrence may occur with the same *C. difficile* strain or as a result of reinfection with a different strain (Williams and Spencer, 2009). The reason for relapse is still unclear but it may be caused by the antibiotic treatment of the initial CDI which may result in germination and proliferation of *C. difficile* spores on the GI tract.

2.1.2 *C. difficile* virulence factors

A) Toxins

C. difficile strains produce three toxins, TcdA, TcdB and a binary toxin (CDT). Both TcdA and TcdB are glycosyltransferase toxins encoded by tcdA and tcdB genes respectively and are found in single open reading frames located within a 19.6-kb pathogenicity locus (PaLoc)(Rupnik, Wilcox and Gerding, 2009). PaLoc also contains three additional regulatory open reading frames tcdC, tcdD and tcdE (Figure1). tcdC and tcdD are the regulatory genes in which tcdC is a negative regulator of toxin (tcdA and tcdB) production and tcdD is a positive regulator of tcdA and tcdB expression. The gene encoding the tcdE (a putative holine protein) is speculated to facilitate the release of large toxin molecules (TcdA and TcdB) through the permeabilization of the pathogen cell wall (Carter et al., 2014).

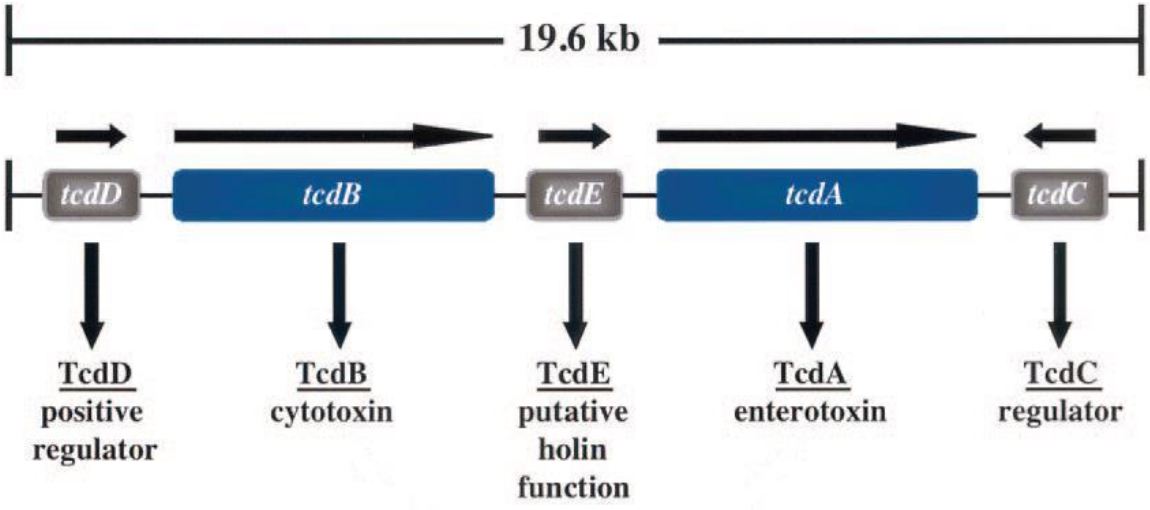


Figure 1: Pathogenicity Locus (PaLoc) encodes for two large toxins, TcdA and TcdB in the pathogenic strain of *C. difficile*. It is absent in non-pathogenic strain of *C. difficile* ($TcdA^-$ $TcdB^-$). PaLoc comprises of fives gene *tcdD, tcdB, tcdA, tcdE and tcdC*. Figure taken from (Voth and Balllard, 2005)

In non-toxigenic strains of *C. difficile* ($TcdA^-$ $TcdB^-$) the PaLoc sequence is replaced by 127 bp non –coding sequence (Tan, Wee and Song, 2001).

TcdA (308 kDa) and TcdB (270kDa) consist of three functional domains as seen in *Figure 2.* The enzymatic domain or N-terminal Gylcosylatransferase (GT) domain is located at the amino-terminus , the receptor binding domain (RBD) is present at the carboxy-terminus and there are hydrophobic(HR) amino acids that act as a putative transmembrane segment, which is responsible for the translocation of toxin into the cytosols of the host cells (Chumbler et al., 2012). The enzyme domain is responsible for glycosylation of small GTPases of Rho and Ras families in host cells causing their inactivation leading to cytoskeletal variation in host cells. Since Rho was identified as a regulator of cell contraction, adhesion, division, and motility , TcdA and TcdB act as a glucosyltransferases which affects the molecular function of Rho thus leading to inflammation, angiogenesis, and/or atherogenesis of the host cells in CDI (Jank, Giesemann and Aktories, 2007). The RBD contains multiple repetitive oligopeptides known as clostridial repetitive oligopeptide (CROPs). Sequence and crystralographic analysis of this region reveal that tcdA contains between 30 and 38 contiguous repeats whereas tcdB contains between 19 and 24 residues (Ho et al., 2005). These CROP regions may play a putative role in initial target cell interaction and binding of the toxin to the cell surface carbohydrates (Ho et al., 2005).In the study done by *Ho et al,* terminal 127 and 255 residues of receptor binding domain (RBD) of TcdA were crystallised which showed that TcdA forms a solenoid like structure, which is proposed to increase the surface area of proteins and thus causes protein-protein or protein-carbohydrate interaction.

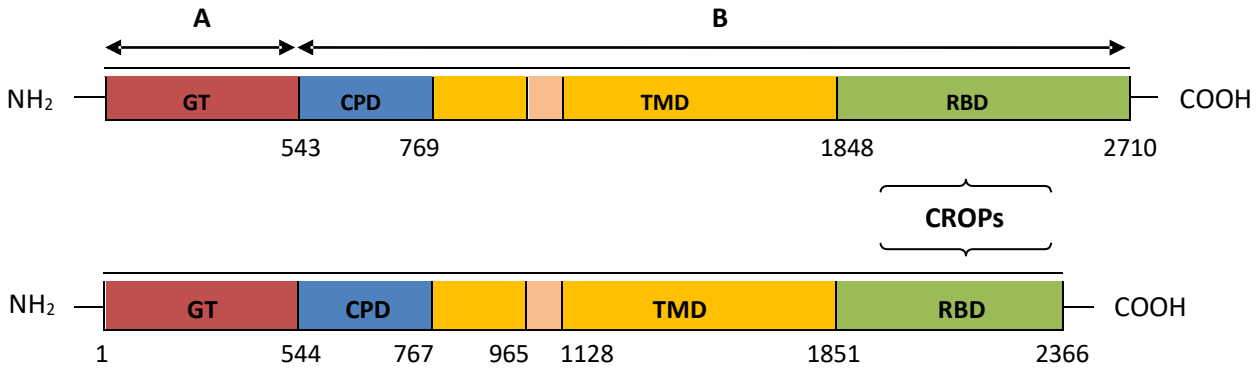


Figure 2 : Structure of TcdA and TcdB: TcdA and TcdB consist of four domains: The enzymatic A component is an N-terminal glucosyltransferase domain (GT) (red). The B component contains an autocatalytic cysteine protease domain (CPD) (blue) , a central translocation domain (TMD) (yellow) covering a hydrophobic region (orange) and a receptor binding domains consisting of clostridial repetitive oligopeptides (CROPs) (green).

TcdA and TcdB are primary determinants of virulence and pathogenicity and produce classical symptoms of CDI (Jank, Giesemann and Aktories, 2007). The cytotoxic effect of both TcdA and TcdB cause disruption of the actin cytoskeleton and tight junctions, which leads to decrease in transepithelial resistance, fluid accumulation and destruction of the intestinal epithelium (Carter, Rood and Lyras, 2012). This process of disruption initially involves translocation of the toxins in the cytosols followed by glycosylation of Rho GTPases as the results of enzymatic activity of the toxins. Finally due to the inactivation of Rho proteins, down regulation and inactivation of numerous cell functions occurs such as actin cytoskeleton regulation, epithelial barrier functions, wound repairs, cell deaths and phagocytosis (Pruitt and Lacy, 2012a) (Figure 3).

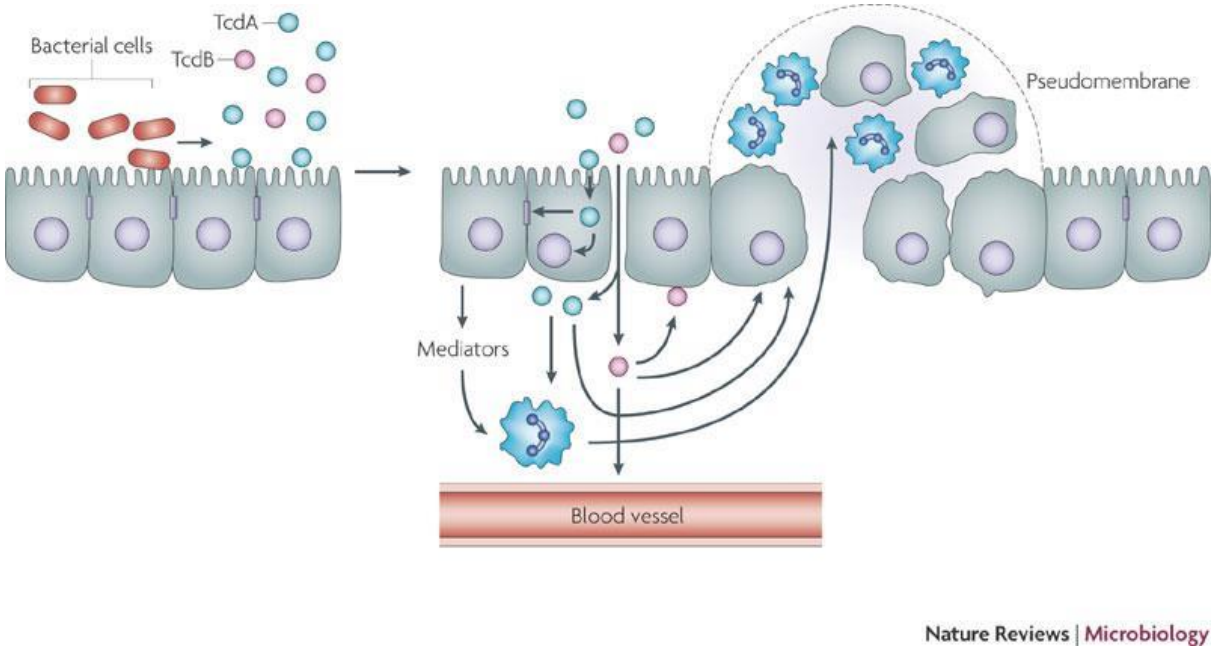


Figure 3 Action of TcdA and TcdB toxins from C. *difficile* in the intestine: The *C. difficile* bacterial cells can be seen in red attached to the host cells. The bacterial strains which are toxigenic produce the TcdA and TcdB as shown in blue and pink respectively. TcdA binds to the pointed side of the epithelial cells and once it enters the cells it causes cytoskeleton changes to the cells which results in disruption and loosening of the epithelial barrier, production of inflammatory mediators attracting neutrophils (light blue), cell death and also allows both TcdA and TcdB to cross the epithelium. TcdB binds to the basolateral cell membrane and destroys the epithelial integrity of monolayer. Accumulation of neutrophils takes place due to the cytotoxic effect of both TcdA and TcdB. (Rupnik, Wilcox and Gerding, 2009)

The contributions of toxins TcdA and TcdB to pathogenesis has been assessed in animal models. Lyras et al in 2009 used a hamster model and with the help of isogenic tcdA and tcdB (encoding TcdA and TcdB respectively) mutants of *C. difficile* strains they revealed that purified TcdB is more virulent that TcdA (Lyras et al., 2009). Genetic inactivation of tcdA and *tcdB* genes showed that the absence of TcdA and TcdB results in the absence of disease in a hamster model of infection (Kuehne et al., 2010).

B) Binary toxin (CDT)

Binary toxin or CDT is produced by hypervirulent strains of *C. difficile* strains which is the least well understood of the toxins and exact association of the toxin and disease is still unknown (Cloud and Kelly, 2007; Ananthakrishnan, 2011). It belongs to the family of ADP-ribosylating toxins consisting of two separate toxins known as CdtA and CdtB encoded by their genes cdtA and cdtB respectively as seen in Figure 4. The *cdt* genes are located in the binary toxin locus known as CdtLoc which includes cdtR, encoding a regulator of toxin synthesis (Perelle et al., 1997).The function of CdtA is to induce the production of ADP-ribosyltransferase which causes the breakdown of actin cytoskeleton followed by the cell cytopathy, whereas CdtB binds to the host cells and helps in translocation of binary toxin A into the cytosol (Gerding et al., 2014).

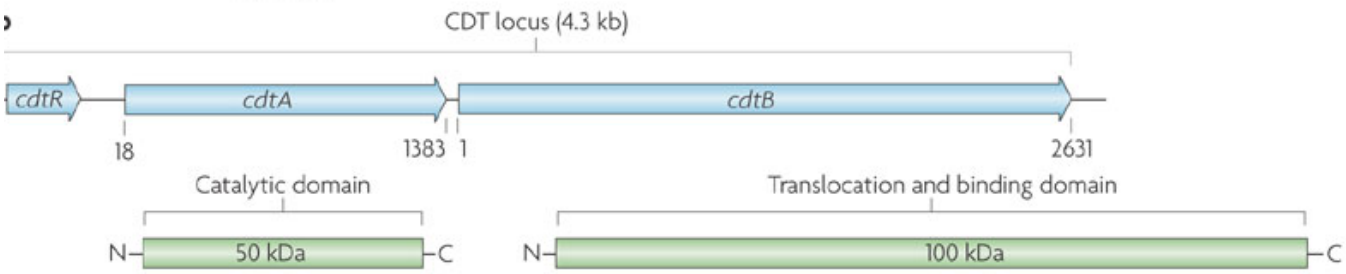


Figure 4 Binary toxin or CDT: It is encoded by the cdt Loc pathogenicity locus comprised of three genes cdtA, cdtB and cdtR.cdtA and cdtB encodes for two proteins. cdtB binds to the cells and is a translocation component while cdtA is an enzymatic component which helps in enzymatic activity of the toxin. The orphan response regulator or cdtR help in the expression of cdtAB genes. Figure taken from Rupnik *et al.,* 2009

2.1.3 Additional virulence factors

Other virulence factors produced by *C. difficile* include proteolytic and hydrolytic enzymes, a capsule and fimbriae. Hydrolytic and proteolytic enzymes play an important role in providing the important nutrients for the growth of bacteria within the gut. These enzymes also cause the breakdown of the host tissues to help in adherence and colonisation of the colon (Janoir et al., 2007). In about a third of *C. difficile* isolates fimbriae have been proven in an attachment of the pathogen to the gut. The polysaccharide capsule of *C. difficile* makes the pathogen more virulent as it prevents the opsonisation by neutrophils (Haiko and Westerlund-Wikström, 2013)2.2 Hypervirulent (HV) strains of C. *difficile*

The emergence of new HV strains of *C. difficile* has resulted in higher incidence of CDI, increased severity of disease and higher mortality rates (McDonald et al., 2005). HV strains cluster into a distinct phylogenetic groups but the most prominent of them is the strain that belongs to the ribotype 027, identified as toxinotype III , North American pulsed-field gel electrophoresis type 1 (NAP1), and restriction endonuclease analysis group BI (BI/NAP1/027)(Cookson, 2007). Ribotype 027 is characterised by increased sporulation and toxin production. This hypervirulence may be due to an 18 base pair deletion and a single nucleotide mutation at position 117 in the toxin regulatory gene (tcdC), the latter resulting in a frameshift and premature stop codon leading to a cropped tcdC gene (Curry et al., 2007). Since TcdC is a negative regulator of TcdA and TcdB expression, these alterations lead to increased expression of both toxin proteins and result increased virulence (Spigaglia and Mastrantonio, 2002). This BI/NAP1/027strain produces 16 times more TcdA and 23 times more TcdB than normal *C. difficile* strain (Warny et al., 2005). This strain also produces CDT and increased level of proteolytic and hydrolytic enzymes, which causes increased colonisation in the gut by increasing adherence to mucosal epithelial cells (Deneve et al., 2009).

A second hypervirulent strain of *C. difficile* is NAP8/078, isolated from calves and pigs. This strain is different from the BI/NAP1/027, because this strain shows the 18-bp deletion in tcdC which causes down-regulation of toxin management and then additional 21-bp deletion in the same gene (Angione et al., 2014). This strain is usually causing CDI in the

human population in the rural areas where pigs and calves are raised (Goorhuis et al., 2008).

2.3 Epidemiology

The mortality rate and the incidence of CDI have increased considerably over the last two decades in both the community and hospital settings; probably due to the improper administration of antibiotics and spread of the hypervirulent C. *difficile* strain (Huttunen et al., 2012). According to US epidemiological reports, C. *difficile* has replaced the methicillin-resistant *Staphylococcus aureus* (MRSA) as the commonest cause of the infection associated in the healthcare system (Miller MD et al., 2011). Several reports from Europe, US and Canada, show a 2 to 4 fold increase in the incidence of CDI since the last decade, mainly affecting elderly patients who are exposed in the health care settings such as hospitals and long-term healthcare facilities (Khanna et al., 2012). In the US, alone annually 250,000 CDI cases are reported and 14000 deaths are associated with CDI (Centres for Disease Control and Prevention 2013, (Lessa et al., 2015)

According to another study conducted by a research group in Europe names European study group on Clostridium *difficile* (ESGCD), the mean incidence of CDI associated with the healthcare system is 4.1 per 10000 hospital patient days (Bauer et al., 2011). Figure 5, shows that in the UK alone, a significant increase in CDI was seen between 1990 and 2007. In 2007, over 50,000 cases of CDI were reported out of which 20% belong to the younger age group of less than 30 years. Due to these increase in incidences, recurrence and mortality, reporting of all CDI cases was made mandatory by Public Health England and the *C. difficile* Ribotyping Network (CDRN) was created to analyse the faecal samples collected from NHS laboratories across the UK. After 3 years of analysis (2008- – 2011) performed by the CDRN reference laboratory, it was found that the majority of the samples contained ribotype 027 and most of these samples belonged to patients above 65 years of age (Wilcox et al., 2012). However the current data shows that the prevalence of *C. difficile* ribotype 027 has fallen to <5%, showing the ribotype 027 is no longer the prominent strain of *C. difficile* causing CDI in England (PHE, Biennial report 2013-2015). Decline in these ribotypes led to the compensatory rise in other ribotypes such as R002, R005, R014/020, R015, R023 (Public Health England, 2014).

The current situation with CDI in the UK is that improvement in the incidence of CDI has been achieved because of strict CDI management regime such as antibiotic stewardship, mandatory reporting of the CDI, financial penalties on the CDI outbreak within hospitals and disinfecting the hospital environment and hand hygiene (Wilcox et al., 2012). Although there has been an overall decline in the CDI reported cases has been seen in the UK approx. 44500 cases in 2004 to approx. 14000 cases in 2014, but there is still an urgent need for the development of accurate diagnostic method (England, 2015)

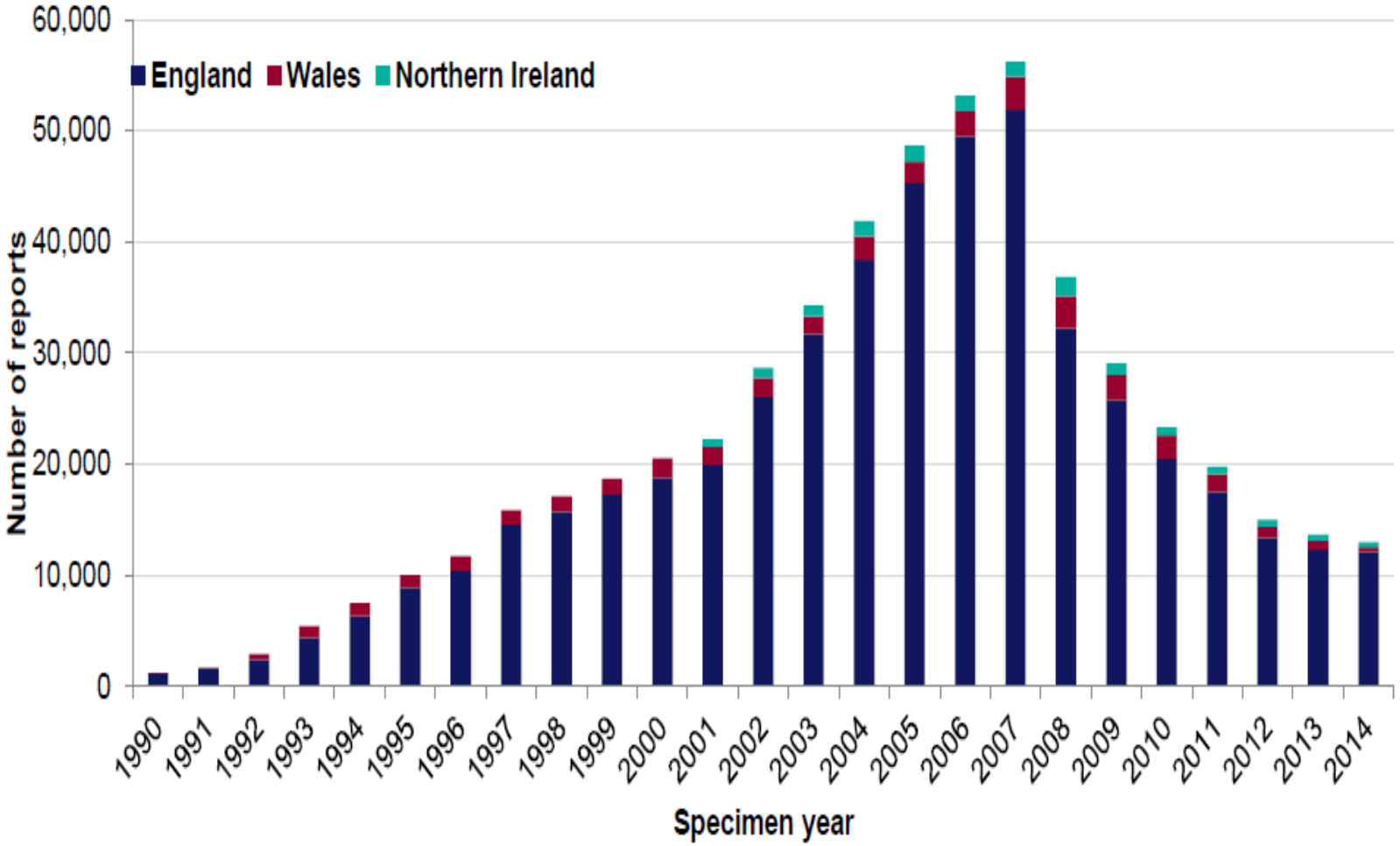


Figure 5 Laboratory reports of the *C. difficile* positive faecal specimens in UK (Reference: Health Protection Report (England, 2015))

2.4 Current diagnostic techniques for *C. difficile*

CDI is diagnosed based upon clinical symptoms such as diarrhoea, fever, abdominal pain, leucocytosis, a history of antibiotic administration (Kazanowski et al., 2014) and followed by laboratory confirmation. Currently, there are many different assays available that can be used for CDI diagnosis; however the best diagnostic method for CDI has still not been clearly established (Surawicz et al., 2013). The current diagnostic tests for CDI can be divided into three main categories as shown in Figure 6:

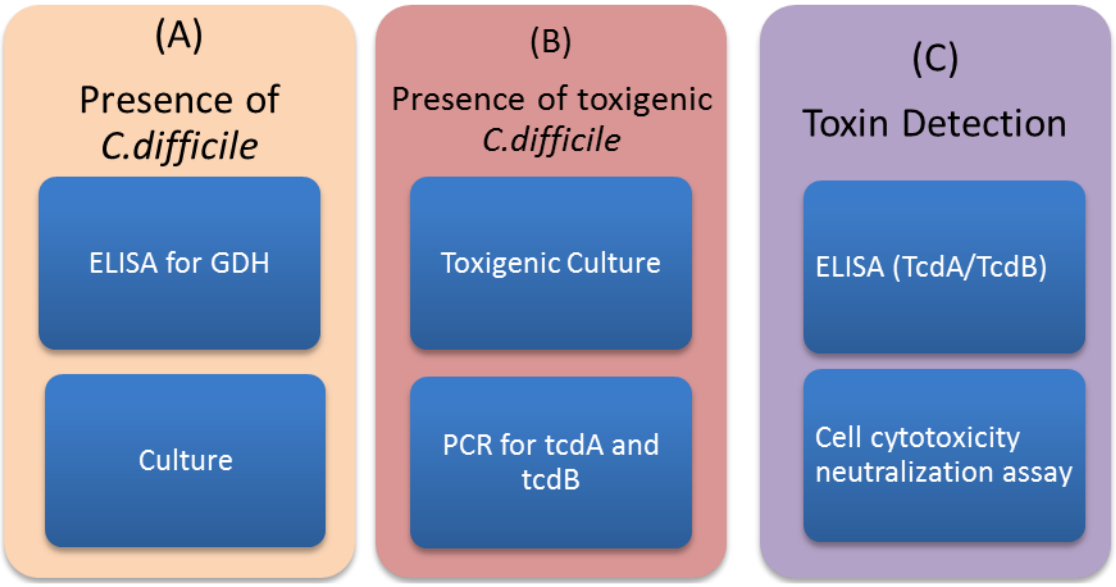


Figure 6 Different diagnostic methods used for CDI diagnosis are divided into: detection of TcdA and TcdB (yellow), detection of *C. difficile* (purple) and determination of toxigenic *C. difficile* (red)

A. Presence of *C. difficile*

Culture Method:

George et al., in 1979 developed the first culture method *for C. difficile* using selective cycloserine-cefoxitin fructose agar (CCFA) (George et al., 1979). In order to detect the presence of *C. difficile,* infected stool samples were inoculated to culture media in anaerobic conditions. After 48 hours of growth, the C. *difficile* can be recognised as white grey colonies which produce a characteristic smell like horse manure. This is a very sensitive method for the detection of *C. difficile* but limitations in the technique include that it cannot distinguish between toxin and non-toxin producing strains (Arroyo et al., 2005), moreover, it is laborious and time-consuming.

Glutamate Dehydrogenase Antigen Detection:

C. difficile strains produce a relatively large amount of a cell wall associated metabolic enzymes known as glutamate dehydrogenase (GDH). Among *C. difficile* ribotypes, GDH appears to be highly conserved and independent of PaLoc structure (Carman et al., 2012). Hence, GDH can act as a biomarker for the presence of the *C. difficile* pathogen in stool samples. A rapid and simple immune-enzymatic method such as enzyme immunoassay (EIA) (mainly well or membrane type ELISA) is used to detect the GDH enzyme. EIA for GDH is 80 to 90% more sensitive when compared with the culture method (Crobach et al., 2009). These tests also allow the CDI diagnosis to be ruled out by negative results as they have a highly negative predictive value range between 94% and 100% (Shetty, Wren and Coen, 2011) which confirms and excludes the patients that truly don't have the disease. Like the bacterial culture method, a positive result for the GDH test means the presence of *C. difficile* pathogen only, but it does not predict the toxicity of the *C. difficile* which is the main drawback of this test. The GDH test can be used as a sensitive screening test in a dual testing algorithm in which only the GDH positive sample are further tested for confirmation tests to differentiate between the toxins producing and non-toxin producing *C. difficile* strains (Fenner et al., 2008). However, there is a debate on the choice of confirmation test which should be used. The tests that could be combined with the stool GDH test for toxin detection on the GDH positive samples are solid – phase toxin A/B EIAs, Cell Cytotoxicity Assay and PCR. PCR is the most sensitive and fastest method to confirm

the presence of toxigenic *C. difficile* strain in GDH positive sample (Doing et al., 2010)(Doing et al., 2010) (Goldenberg et al., 2010b). (Gilligan, 2008) showed that ELISA's are less sensitive than cell cytotoxicity test, therefore, proposed the use of cell cytotoxicity test as a confirmatory test.

B. Presence of toxigenic *C. difficile*

Toxigenic culture method

The toxigenic culture method is a two-step gold standard method for the diagnosis of *C. difficile* infection. The test is based on the isolation of *C. difficile* in a selective culture media followed by toxin determination by cell cytotoxicity neutralization assay (CCNA), ELISA or PCR-based assay. Although, the toxigenic culture method is very sensitive but the long turnaround time (2-5 days) and labour intensive procedure makes it difficult to use in a routine laboratory environment. Therefore, this method can be best used as a reference method for the evaluation of any new diagnostic tests, new therapies and for epidemiological purposes (Planche and Wilcox, 2011).

C. Toxin Detection

Cell Cytotoxicity Assay (CTA)

The CTA is an FDA (US) approved method which was first described by Chang et al. in 1978 for the diagnosis of CDI (Chang, Gorbach and Bartlett, 1978). The method is based on the detection of the biological properties of toxicogenic *C. difficile* in stool samples. The first step of the method involves a 24 – 48 hours' incubation of the diluted and filtered stool sample onto cultured cell monolayers. After incubation, a specific cytopathic effect can be observed in the cells due to the cell cytoskeleton disruption which results in rounding of the cell (Pancholi et al., 2012). This cytopathic effect is caused by the cytotoxic activity associated with the TcdB. Toxin B is 1000 to 10000 times more potent to cause cytopathic effects than Toxin A (Sullivan, Pellett and Wilkins, 1982). The cytotoxic effect of the cell is neutralised or reversed by *C. difficile* antitoxin and if the effect is neutralized this confirms that the faecal sample is *C. difficile* positive. The test is very specific and sensitive as it can detect *C. difficile* toxins (particularly *C. difficile* toxin B) at a picrogram level (Aldeen et al., 2000). However, there are several drawbacks associated

with this test, for example as slow turnaround time as the results are not available before 24 to 48 hours. CTA requires the supply of cultured cell monolayers which makes the method expensive and requires a high level of technical expertise to perform which restricts this method for easy use in the reference laboratories. Finally, this method is not standardised as the results depend on dilution of a stool sample, incubation period and type of cell lines used for the monolayer (René et al., 2012)

ELISA for TcdA and TcdB:

ELISA is a rapid and easy to perform assay for detection of *C. difficile* TcdA and TcdB in stool samples. In 2009, Crobach et al., did a comparison study in which diagnostic accuracy of ELISA for TcdA/ TcdB, GDH ELISA and real-time PCR for diagnosis of *C. difficile* TcdB was evaluated and compared with CCNA and toxigenic culture method. ELISAs gave high specificity but relatively low sensitivity in detecting CDI. However, due to the lower relative sensitivity to detect the toxigenic *C. difficile* , Society for Healthcare Epidemiology of America and Infectious disease of America CDI guidelines state that ELISA for *C. difficile* toxins are sub optimal and are not suitable for use as a single standalone test for CDI diagnosis (Cohen et al., 2010).

Molecular Methods

Rapid molecular methods such as the PCR and loop-mediated isothermal amplification (LAMP) can be used for CDI diagnosis (Surawicz et al., 2013).The primary targets for these methods are the *C. difficile* tcdA and tcdB and *PaLoc* accessory genes (Spigaglia and Mastrantonio, 2002). As compared to other non-culture based methods (EIAs and LFDs), molecular methods have a higher sensitivity but they can only detect the presence of *C. difficile* toxin genes and not the toxin (protein) itself, therefore, they provide no information on toxin expression levels or pathogen viability (Platts-Mills, Liu and Houpt, 2013). According to Crobach et al 2009, although PCR-based assays are highly sensitive they cannot be used as a single standalone test because of their low positive predictive values. Crobach et al recommended using them as a screening test in the endemic situation emphasising mainly on the negative test results. For instance, if a sample tested with the PCR gives the negative result than CDI can be excluded but if the sample gives a positive

result than a confirmatory test has to be performed in order to recognise the sample a truly positive.

There are currently FDA approved qPCR assays available commercially; for detection of tcdA genes, 1) Xpert® *C. difficile* , Cepheid , Sunnyvale, USA, 2) illumogene®, Meridian Bioscience, OH, USA and 3) AmpliVue® and 4) QUIDEL Molecular, CA, USA are available. Whereas, few methods are designed to target the gene tcdB for instance , 1) BD GeneOhm C diff Assay, 2) BD Diagnostics ,NJ, USA; 3) D MAX Cdiff , BD Diagnostics and 4) Simplexa™ *C. difficile* universal Direct, France).

Two-Step laboratory testing algorithm

Limitations in sensitivity and specificity of a common rapid diagnostic test have led to the development of several two - step as well as a three-step algorithm method to improve the diagnostic accuracy for CDI. The two-step algorithm method includes an initial screening ELISA for the presence of GDH antigen followed by testing of positive samples using CCNA (Goldenberg et al., 2010a). *Table1.1,* interprets the outcomes of the recommended two step algorithm workflow for CDI. Figure 7 shows a two-step algorithm method for diagnosis that has been standardised and adopted by different societies and is used in different hospitals in US, UK and Europe. The societies that recommend the usage of this method are for example American Society of Microbiology in the US, European Society of Clinical microbiology and infectious diseases and NHS laboratories in England. This two-step algorithm method can further be extended by adding third confirmatory testing method of toxin gene PCR (Nucleic acid amplification test - NAAT) which now makes this method a three-step algorithm method. According to the department of health, third step can be used as an optional step and is not mandatory according to the guidelines.

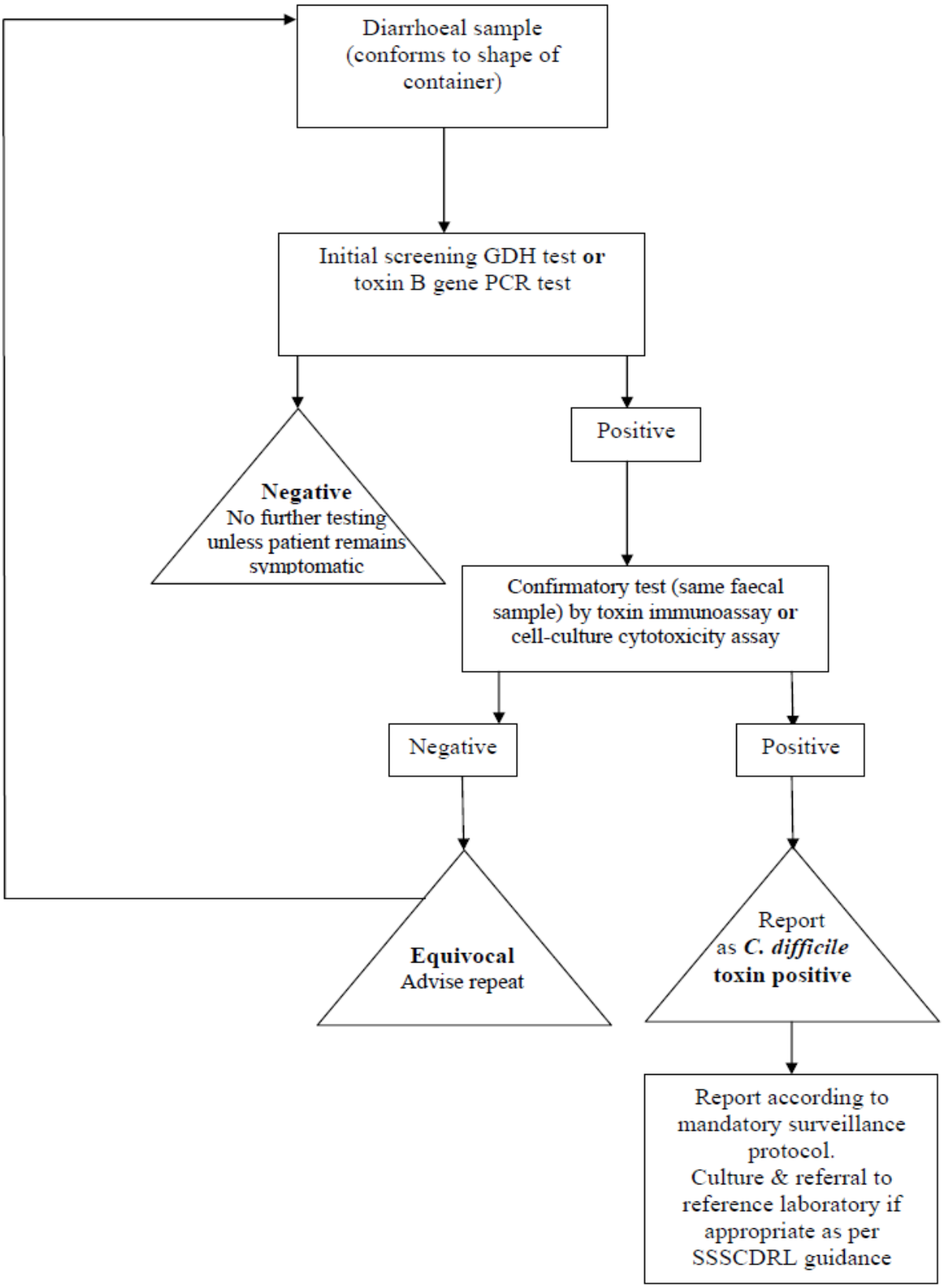


Figure 7: Two step algorithm method for CDI diagnosis (figure taken from PHE, 2014) Different combinations of diagnostic methods are used for accurate diagnosis of CDI, but for increased accuracy, PCR assay are also performed as a third step (optional) for confirmation of CDI.

Results of 2 step Algorithm	Interpretation
GDH EIA (or NAAT*) positive, toxin EIA positive	CDI is likely to be present
GDH EIA(or NAAT) positive, toxin EIA negative	*C. difficile* could be present; patient may be carrying the pathogen without any symptoms. Patient could be potential *C. difficile* excretor.
GDH EIA (or NAAT) negative , toxin EIA negative	*C. difficile* or CDI is very unlikely to be present.

Table 1.1 Interpretation of two step-algorithm method for CDI diagnosis

(Table modified from *PHE*, 2014).

*NAAT: Nucleic acid amplification test.

This approach of two-step algorithm *C. difficile* diagnosis is costly, laborious and time-consuming therefore, there is an urgent need for the development of the single diagnostic test for CDI that is sensitive and specific and which can address the drawbacks of the current diagnostic methods. We aim to develop the rapid diagnostic methods for CDI based on proximity ligation assay. Such an assay will be more physiologically relevant than PCR and will provide the specificity of an ELISA whilst making use of the sensitivity of the PCR.

3. Proximity Ligation Assay

3.1 Introduction

The proximity ligation assay (PLA) is a technology that is used for detection, quantification and localization of proteins. The method requires two DNA tagged antibodies which bind to the same protein or protein complex, allowing their attached DNA molecules to come into close proximity which are then hybridised by a connector oligonucleotide (by enzymatic ligation) to form a DNA template. The amplification of the DNA template can be done by either real-time PCR or isothermal amplification.

PLA was first demonstrated in 2002 (Fredriksson et al., 2002). In the beginning, two DNA aptamers (ssDNA that can bind to the target proteins) were used for PLA (Famulok, Mayer and Blind, 2000), which bind their target antigen and have target binding specificity and affinity comparative to monoclonal antibodies (Pai, Roberts and Ellington, 2008). However, difficulties in designing aptamers and availability of large range of commercial antibodies, resulted in the development of antibody-based PLA (Fredricks and Relman, 1999); (Gullberg et al., 2004). PLA combines the specificity of antibody-based assays with the sensitivity and broad dynamic range of real-time PCR together with a simplified workflow and faster turnaround time (Ke et al., 2013). Currently, different types of PLA has been developed for many applications such as analysis of cellular protein/protein interaction (Gajadhar and Guha, 2010), cancer biomarker analysis and other proteomic studies (Söderberg et al., 2007) but there has been only a single report for demonstration of proof of principle on development of PLA for detection of a pathogen, *Lawsonia intravellularis* and porcine parvovirus. The PLA demonstrated the same sensitivity as nucleic acid based tests (Gustafsdottir et al., 2006).

3.2 Type of PLAs

1. The homogenous PLA: The assay uses two proximity probes (3' and 5'), prepared by non-covalent binding of biotinylated antibodies with two different streptavidin modified oligonucleotides which are non-complementary to each other (Fredriksson et al., 2002). The proximity probes are incubated with the target antigen and a connector oligonucleotide, which can hybridise to both proximity probes if the probes bind to adjacent epitopes on the target antigen. A ligation step joins the 3'-end of one of the two streptavidin-linked oligonucleotides to the 5'-end of the other, generating a DNA molecule that can be amplified and detected real-time PCR amplification. The workflow for homogenous PLA is illustrated in Figure 8. This method has many advantages over current molecular and antibody-based assay such as the accurate detection of target molecules with significantly reduced problems of cross-reactivity in complex samples, high sensitivity due to low background noise, faster turnaround time (Gustafsdottir et al., 2005) and a higher dynamic range than ELISA.

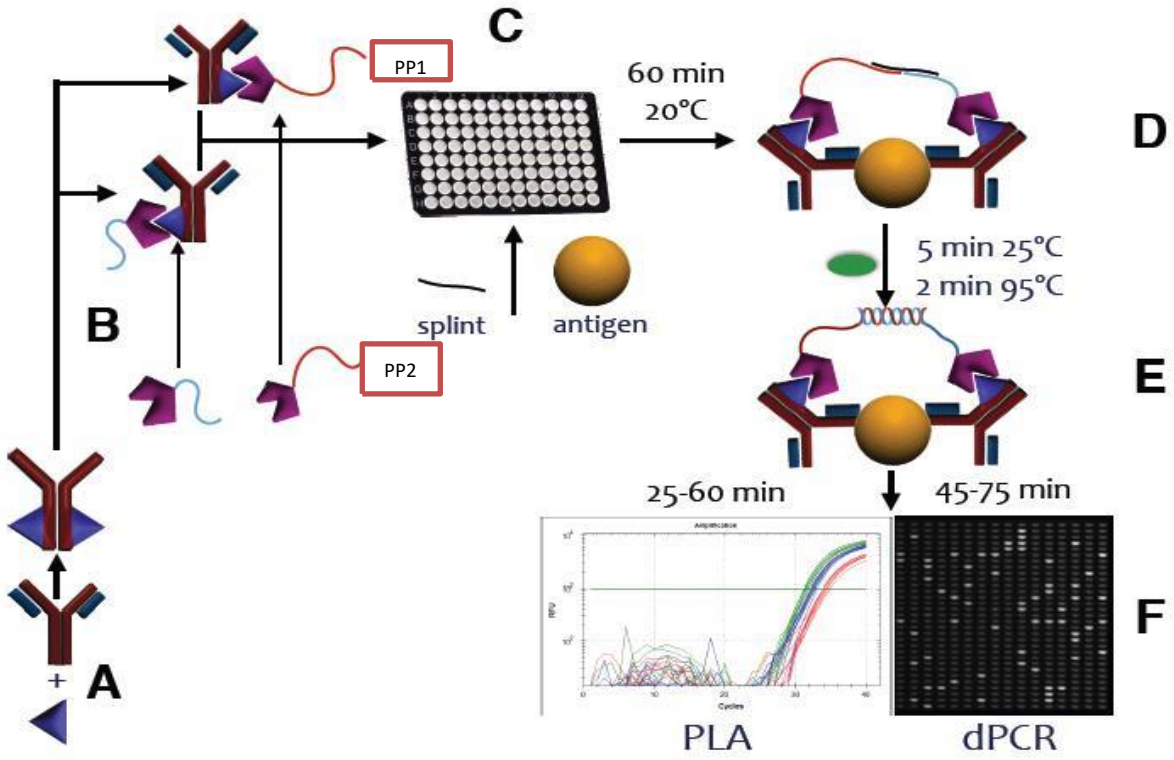


Figure 8: Homogenous PLA workflow: A) Biotinylation of antibodies: Antibodies are attached to biotin (purple cone) and excess biotin is removed by dialysis. The efficiency of biotinylation is determined by Forced Probe Proximity Test (FPPT) (not shown in diagram). B) Preparation of proximity probes: Two proximity probes (PP1 and PP2) are prepared by noncovalent binding of two non-complementary streptavidin- modified oligonucleotides to biotinylated antibodies C) PP1 and PP2, a connector oligonucleotide ('splint') (complementary to the 3' end of one and the 5' end of the other oligonucleotide) and the sample containing the target antigen are mixed together and incubated for an hour at 20˚C. D) Incubation helps in binding of probes with the antigen and hybridization to the connector E) A ligation step joins the 3'-end of one of the two streptavidin-linked oligonucleotides to the 5'-end of the other, generating Ligation product as DNA molecule. F) Finally these ligation products can be detected and quantified by real-time PCR. *Ref: Greenwood, Christina 2015*

The homogenous PLA was further developed to create a more specific assay that requires the binding of three independent affinity reagents to the same target molecule thus enhancing the specificity of the signal generated. This method is known as triple specific proximity ligation assay (3PLA). Despite showing high sensitivity than standard homogenous PLA, no further development and published research has been done using 3PLA. This may be due to the need for three probes which increases the complexity and price of the assay.

2. Solid phase PLA (SP-PLA)

SP-PLA is another form of PLA which is dependent on three binding events of the antibody (see figure 9). It uses the capture antibody in order to immobilise target protein onto a solid phase. Firstly, the sample is combined with a capture antibody the unbound antigen is removed by washed with buffer. The capture antibody and antigen complex is then incubated with the proximity probes and thus target antigen is sandwiched between the proximity probes and the capture antibody. After the probe binding step, a further wash step removes the unbound proximity probes. Finally, the ligation and qPCR steps are carried out as with homogenous PLA (Nong et al., 2013). This method shows more sensitivity, specificity and has greater dynamic range than the homogenous PLA, (Darmanis et al., 2010).

The sensitivity and specificity of sp-PLA are increased due to the additional binding event through the capture antibody. Moreover, sensitivity and specificity are also increased by washing steps in this assay which causes the removal of unbound antigen, proximity probes and excess reagent reducing the risk of cross-reactive detection of the antigen other than target antigen and reducing background amplification. The method may also be very useful for detecting proteins directly from bio-fluids such as blood and faeces as the washing step may remove ligation or PCR inhibitors present in them.

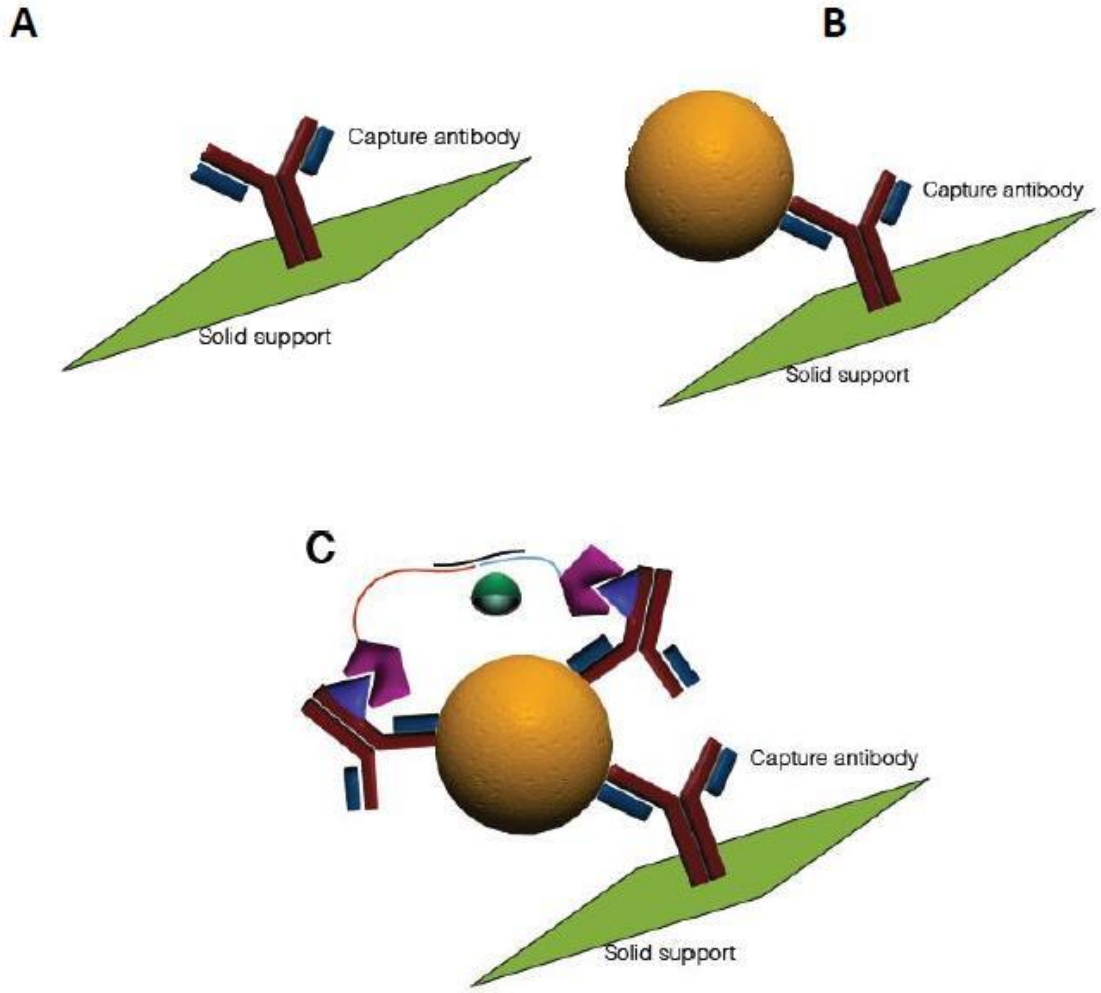


Figure 9: Solid Phase PLA (SP-PLA) is similar to standard sandwich immunoassay in which A) antibody specific to an antigen (yellow) is captured on a solid surface B) antigen is combined to capture antibody followed by washing off the unbound antigens. C) Binding of the antigen antibody complex is detected by qPCR-PLA. *Greenwood et al 2015*

3. *In situ* proximity ligation assay (*in situ* PLA):

Another modification of PLA is, *in situ* PLA which uses rolling circle amplification (RCA) for the detection of individual proteins and protein-protein interaction in cell lines and tissues (Söderberg et al., 2006). In this method, cell or tissues are fixed on a slide and proximity probes are added causing binding of two proximity probes to the same protein complex in the sample. Oligonucleotides conjugated to the antibodies come in proximity which is hybridised by the addition of two connector oligonucleotides. The addition of ligase to this complex causes the ligation and seals the gap to form a circular DNA molecule. This newly formed circular DNA molecule is then amplified by isothermal amplification method known as RCA. (Figure 10)

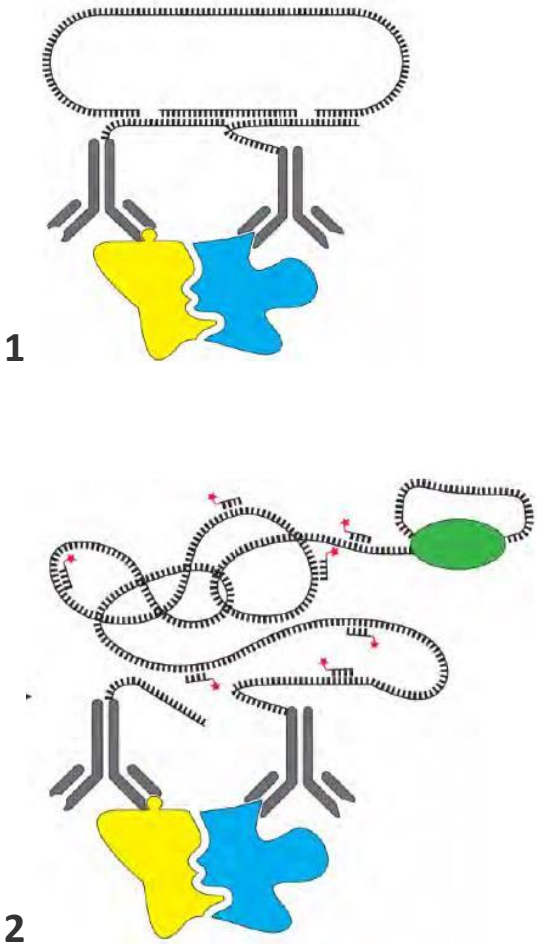


Figure 10: In situ PLA: 1) the antigen complex (yellow and blue**)** is probed with one oligonucleotide coupled antibody for each protein. To the oligonucleotides, two pieces of single stranded DNA (the short one is named splint and the long one is termed the backpiece) can hybridise. 2) After ligation of them, a DNA polymerase (green) uses the circle as a template, producing a long strand of ssDNA to which fluorescently labelled detection probes are able to hybridise. The analysis is then continued by fluorescence microscopy. *(Gabriele et al 2009)*

The RCA uses phi29 DNA polymerase for amplification of circular DNA because if its proofreading activity with low error rate of 1 in 10^6 Bp thus making the RCA more accurate and efficient (Yang et al., 2007). The RCA is initiated by using one of the proximity probes as a primer and after the first cycle of amplification the phi 29 polymerase displaces to the newly created strand and repeated cycles of amplification results in the formation of long single stranded DNA molecules which consists of multiple copies of the same DNA sequences linked in series. The amplified product is then detected through hybridization of fluorescence – labelled oligonucleotide complementary to a tag sequence in the RCA product (Söderberg et al., 2008)

3.3 Applications of PLA

PLA has been used to quantify and evaluate proteins in diverse sample types and applications. The use of the low volume of sample in homogenous PLA and the ability of solid phase PLA to investigate larger sample volumes made this technology a useful tool for proteomic studies ranging from detection of cancer biomarkers, stem cell proteins protein-protein complex (Swartzman et al., 2010), protein-mRNA correlation.

With regard to pathogen detection very few studies have been done such as avian influenza virus (Schlingemann et al., 2010) and bacterial markers have also been identified using proximity assays. PLA for the detection of pathogens was first demonstrated with the development of both homogenous and solid phase PLA for *Lawsonia intracellularis* and porcine parvovirus (Gustafsdottir et al., 2006) showing 100 times more sensitivity than standard ELISAs and similar sensitivity to qPCR. Foot and mouth disease virus has also been detected using homogenous PLA with sensitivity 100 fold more than standards ELISAs and comparable analytical sensitivity to reverse transcription-qPCR (Nordengrahn et al., 2008). PLA using a different detection method other than qPCR has also been used for identification of pathogen such as isothermal loop-mediated amplification for detection of *Brucella abortus* responsible for causing brucellosis (Zhu, Deng and Shi, 2009) and RCA – PLA for detection of RNA viruses such as human and avian influenza virus (Schlingemann et al., 2010) which does not require any nucleic acid extraction procedure and costly equipment as in standard PCR method. Moreover, due to the high variability of RNA virus, nucleic acid based methods are not consistent, thus, detection of protein using PLA may provide information about the on-going infection.

PLAs have been used to analyse functional differences between mutations, which may help with the development of mutation-specific targeted therapies. For example in glioblastoma multiforme which is the most common primary brain tumour, *in situ* PLA helps in identification of mutant epidermal growth factor receptor (EGFR) dimer configuration which is capable of evading the blockade caused by anti-EGFR therapeutics (Gajadhar et al., 2012). Therefore, PLA method can also be very useful in the detection of pathogens, that shows frequent antigenic shift or drift such as influenza virus A and B. The genetic variation can interfere with current diagnostic assays but the use of antibodies that target the highly conserved nucleoproteins could allow proximity assays to be more robust to genetic variation. Sensitive and specific multiplex assays for pathogen detection is very important, therefore, if appropriate antibodies or aptamers are identified for the pathogens, development of PLA for detection of multiple pathogens in a single reaction will be very useful in diagnostic and monitoring of bio warfare agents and also differentiate between the pathogenic and non-pathogenic strain of pathogen in a single PLA reaction.

3.4 Limitations of PLA

Although PLAs have many advantages over standard diagnostic methods such as ELISAs or PCR, there are some drawbacks which are restraining the wider use of PLA. One of the main limitations is that it is highly dependent upon the quality of the antibody used in the probe. Therefore, in order to get successful analytical and diagnostic sensitivity and specificity of PLA, a source of suitable antibodies is needed which are difficult to generate as compared to the generation of oligonucleotides for nucleic acid based test (NATs). Moreover, the performance of antibodies varies from batch to batch, therefore, adding to the variation in the PLA results and may require reoptimisation of the assay with every new batch of antibodies (Marx, 2013). The recent innovations in aptamers technology add to the range of binding reagents that complement the vast pool of antibodies that can be used for this assay.

A further limitation in applying PLA as a clinical diagnostic method is the detection of background signal due to nonspecific ligation of oligonucleotides in the absence of antigen (Nong et al., 2013). This nonspecific background signal can be minimised by the use of solid phase PLA, which uses magnetic beads as solid supports for the capture and

separation of the target molecule from unbound probes and antibodies. However, it requires multiple washing steps making the method laborious and time consuming (Jiang et al., 2014). The triple specific PLA (3PLA) also has limitations as it is complex due to the use of three probes hence makes it difficult to be used as the clinical diagnostic method.

In conclusion, PLA provides an integrated approach to the quantification of protein, protein/protein interaction and pathogen detection using the specificity of antibody- based assay and sensitivity and broad dynamic range of PCR. The broad dynamic range of PLA (up to 6 logs) is an additional advantage in case of pathogen detection as the individual sample is likely to contain both abundant and the scarce target antigen. Availability of proximity assays in numerous variants provides flexibility and adaptability of an assay in the detection, quantification and localization of the protein. Finally, PLAs have a great potential to be developed as fast, ultra-sensitive and highly convenient assay for diagnosis of pathogens and proteins following further advancement in instrumentation and reagents.

Chapter 2

Materials and Methods

4.0 Materials

4.1 Equipment

The equipment used in the study is listed in Table 2.1 below.

Equipment	Name of Equipment	Company
Pipettes	mLine pipettes 0.1µL - 3µL 0.5µL - 10µL 2µL - 20µL 10µL - 100µL 20µL - 200µL 100µL - 1000µL	Sartorius Ltd, Epsom, UK
Centrifuge	Rotina 380 R centrifuge	Hettich Zentrifuge, Tutttlingen, Germany
Microfuge	5424 Microfuge	Eppendorf, Stevenage, UK
ELISA Reader	iMark Microplate Absorbance Reader	160-1130, Biorad, Hemel Hemstead, UK
QPCR	Biorad CFX Connect	Biorad, Hemel Hempstead, UK
	Eco48 PCR	PCRMax, Stone, UK
	TC9639 Flatbed thermal cycler	Denville Scientific Inc. South Plainfield, USA
Digital PCR (dPCR)	Constellation dPCR instrument	Formulatrix, Bedford, MA, USA
Water Bath	Grant Sub Aqua pro	Grant Instruments Ltd., Cambridgeshire, UK
Western Blotting Electrophoresis Unit	miniVE Integrated Vertical Electrophoresis Unit	Fisher Scientific UK Ltd., Loughborough, UK
Electrophoresis power supply	EPS3501 XL	GE Healthcare Life Sciences Ltd., Buckinghamshire, UK

Table 2.1 Equipment used in the study

4.2 Antigens

Purified and lyophilised *C. difficile* TcdA and TcdB, (The Native Antigen Company, Upper Heyford, UK) bought contained 0.05M Hepes, 0.15M NaCl and 5% sucrose. Details such as molecular weight, concentration, and strain and catalogue number are shown in table 2.2. The lyophilised antigens were reconstituted in 250µL of sterile distilled water (10245203, Thermo Scientific, Loughborough, UK), giving final concentrations of 0.4µg/µL and 0.4µg/µL of antigen, respectively. 10 aliquots of antigen (25µL) were stored at -80°C. Once the antigen was taken out of the -80°C and thawed, the aliquot was stored at 4°C for up to 1 month.

Antigen Type	Molecular Weight	Strain	Amount	Concentration	Source
***C. difficile* TcdA**	308kDa	VPI 10463	100µg	0.4mg/mL	The Native Antigen Company, Upper Heyford, UK)
***C. difficile* TcdB**	270kDa	VPI 10463 (toxinotype 0)	100µg	0.4mg/mL	The Native Antigen Company, Upper Heyford, UK)

Table 2.2 List of *C. difficile* toxins used in the study

4.3 Antibodies

The anti-*C. difficile* TcdA and TcdB antibodies that were utilised in this thesis, the company from which they were supplied and the concentration they were used at and the antigens they were raised against are shown in table 2.3.

Antibody	Host	Specificity	Immunogen	Final Concentration	Source
***Anti-Clostridium difficile* toxin A IgG2a (PCG4)**	Mouse monoclonal	*C. difficile* toxin A only	Full length Protein (*C. difficile*)	1.160mg/mL	#Ab19953, Abcam, Cambridge, UK
***Clostridium difficile* toxin B IgG1 Antibody(5A8-E11)**	Mouse monoclonal	*C. difficile* toxin B only	Full length *C. difficile* Toxin B Protein	1.14mg/mL	#ABIN234836, Antibodies-online, Aachen, Germany)
***Clostridium difficile* toxin B Antibody (IgY)**	Chicken Polyclonal	*C. difficile* toxin B	Full length *C. difficile* Toxin B Protein with Freund's adjuvant	2mg/mL	#PAB29154, Abnova, Tapei, Taiwan

Table 2.3 List of *C. difficile* antibodies used in the study

5.0 Methods

5.1 Biotinylation of antibodies

Biotinylation of the antibodies was performed using two different methods, EZ-Links Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation kit (21327, Thermo Scientific, Loughborough, UK) and APEX Biotin-XX Ab labelling method (Thermo Scientific, Loughborough, UK).

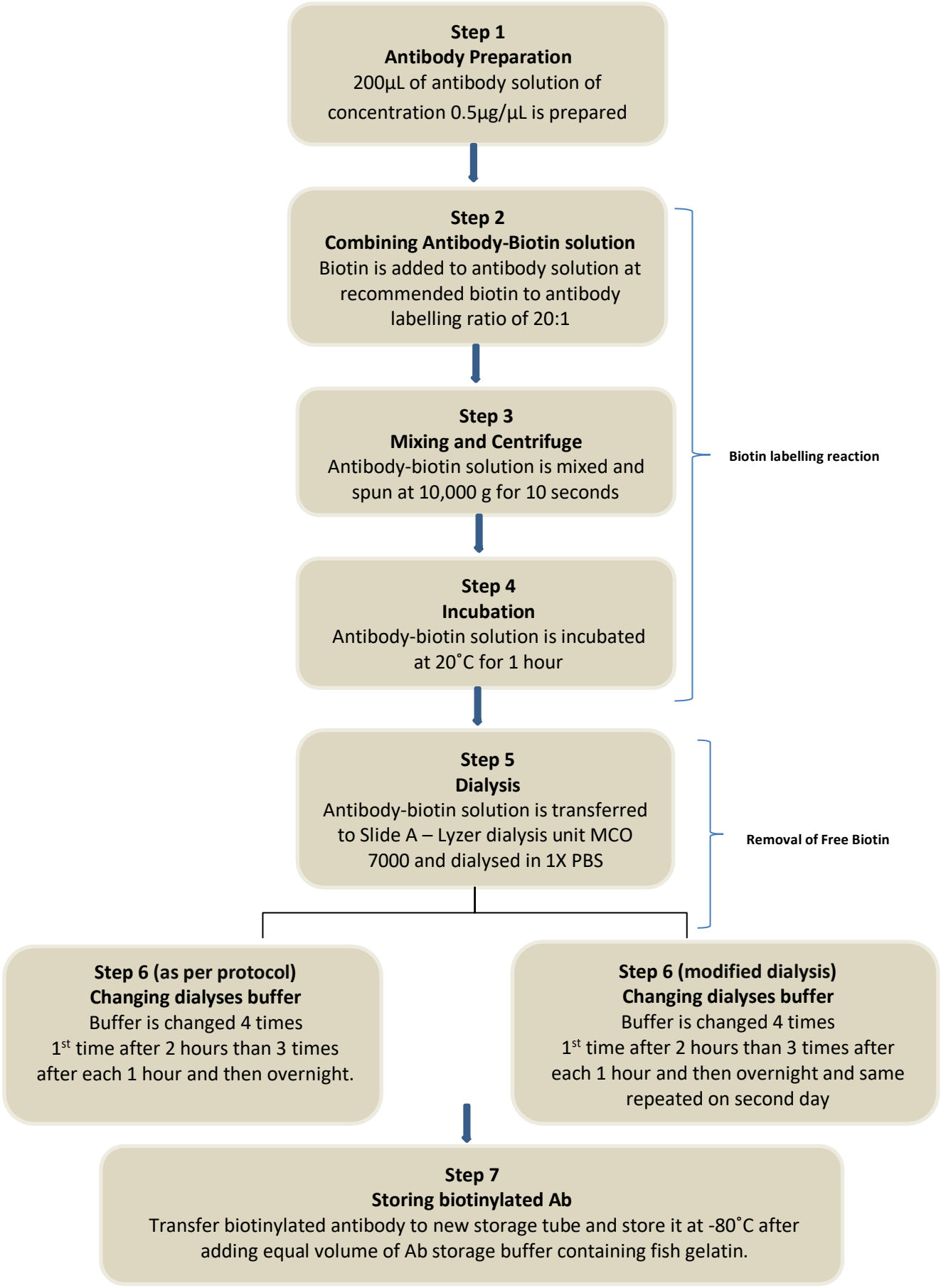
5.1.1 EZ-Links Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation method

Component	Name of Component	Company
Antibodies	50µg of each antibody Anti-clostridium difficile toxin A IgG2a (PCG4)	#Ab19953, Abcam, Cambridge, UK
	Clostridium difficile toxin B IgG1 antibody(5A8-E11)	#ABIN234836, Antibodies-online, Aachen, Germany)
	Clostridium difficile toxin B antibody (IgY)	#PAB29154, Abnova, Tapei, Taiwan
Buffer	1X PBS , pH 7.4	10051163, Thermo Scientific, Loughborough, UK
Dialysis Unit	Slide A –Lyzer mini dialysis units MCO 7000	69562, Thermo Scientific, Loughborough, UK

Table 2.4 EZ-Links Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation components

The components for EZ-Links Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation method are described in Table 2.4. In this method, 50 µg of antibody was added to 1x PBS, pH7.4 making a final volume of 200µL to which 0.67µL of 10 nM biotin was added. Tubes were centrifuged at 10,000 g for 10 seconds and incubated at 20°C for 1 hour. Two times 100µL of each antibody-biotin solution were transferred to two Slide A –Lyzer mini dialysis units with MCO 7000 KDaltons (pore size) per antibody and free biotin was removed by dialysis in 500 mL of 1x PBS, pH 7.4 at 4°C. The buffer was changed 5 times; 1st after 2 hours and then 3 times after every one hour followed by overnight dialysis against 1 litre of a buffer.

A modified dialysis step was also performed by doing using the same dialysis process as described above for 2 consecutive days. The flow chart F1 below describes the steps involved in the EZ-Links Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation method.



Flowchart F1 : Steps involved in the EZ-Links Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation method.

5.1.2 APEX Biotin-XX Ab labelling kit

The table 2.5 describes the components used for Apex Biotin-XX Ab labelling kit is as follows:

Component	Name of Component	Company
Biotinylation kit	APEX Biotin-XX Ab labelling kit	A10495, Invitrogen Ltd, Paisely, UK
Antibodies	50µg of each antibody Anti-clostridium difficile toxin A IgG2a (PCG4)	#Ab19953, Abcam, Cambridge, UK
Buffer	1X PBS , pH 7.4	10051163, Thermo Scientific, Loughborough, UK
Dialysis Unit	Slide A –Lyzer mini dialysis units MCO 7000	69562, Thermo Scientific, Loughborough, UK

Table 2.5 Components for Apex Biotin-XX Ab labelling kit

The flow chart F2 below shows that steps involved in the Apex Biotin-XX Ab labelling method for biotinylation. The APEX antibody-resin was hydrated by applying 100µl of wash buffer (Component C) to the resin in the labelling tip. 10µL of antibody solution was applied to the top of the resin. The antibody solution was gently pushed onto the resin using the elution syringe (Component H), any drops that eluted from the tip were discarded as waste to the vial of reactive dye (Component A), and following were added to the tube;

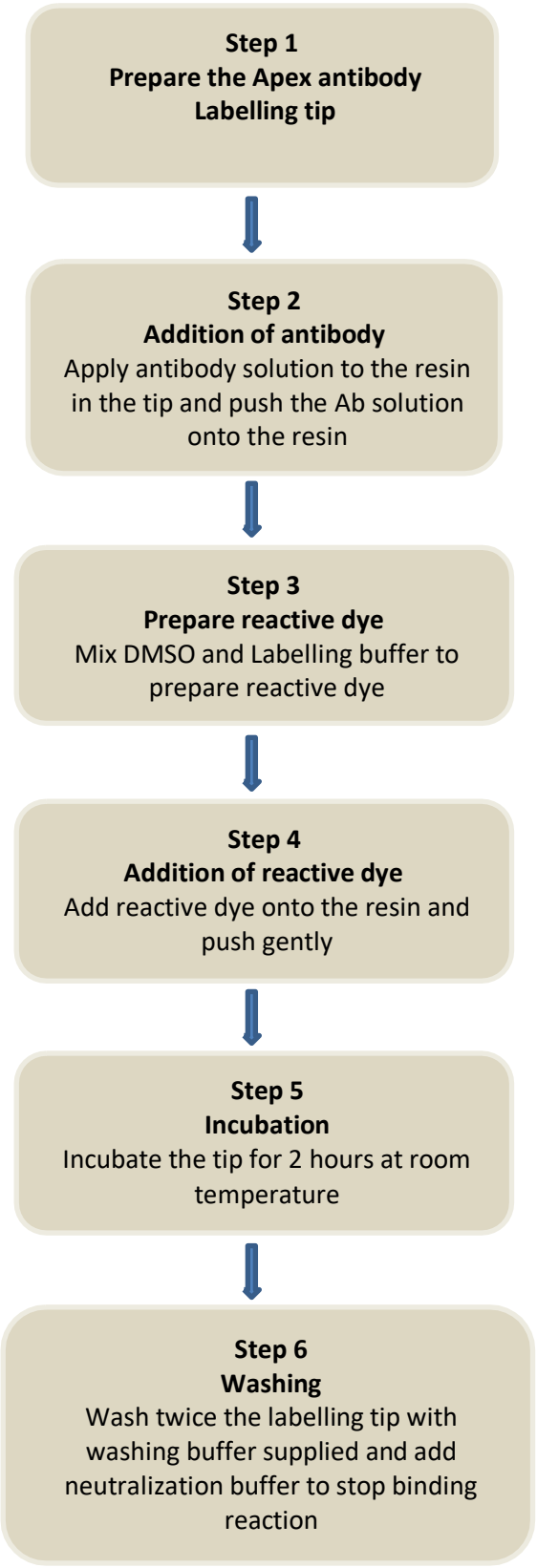
a. 2µl Dimethyl sulfoxide (DMSO), (Component D); then pipetted up and down to dissolve.

b. 18µl Labelling buffer (Component E); pipetted up and down to dissolve. 10µl of this was added to the top of the resin, and the solution was gently pushed through.

Any dye that eluted from the tip was discarded as waste. The tip was incubated at room temperature for 2 hours. The APEX antibody labelling tip was washed twice with 50µL of wash buffer (Component C) by applying 50µL to the top of the resin, pushing through the tip

into the microcentrifuge tube. 10µL of neutralisation buffer (Component F) was added to a clean microcentrifuge tube and the APEX antibody labelling tip was transferred to this tube. 40µl of elution buffer (Component G) was applied to the top of the resin. This was pushed through the tip to elute the labelled antibody into the microcentrifuge tube containing neutralisation buffer. The 50µL of eluted solution was mixed to ensure neutralisation and the tube placed on ice.

The biotin-labelled Ab solution was extensively dialysed in cold PBS (pH 7.4) using the Thermo Slide-A-Lyzer MINI Dialysis Unit.



Flowchart F2: Steps involved in the Apex Biotin XX Ab labelling

5.2 Forced Proximity Probe Test (FPPT)

Reagents kit	Reagents	Company
TaqMan® Protein Assays Oligo Probe Kit	3' Prox-Oligo, 200 nM 5' Prox-Oligo, 200 nM	#4453745 Life Technologies, USA
TaqMan® Protein Assays Open Kit	Antibody Dilution Buffer II Assay Probe Storage Buffer II Assay Probe Dilution Buffer II	#4483013 Life Technologies, USA
TaqMan® Protein Assays Core Reagents Base Kit	DNA Ligase II (250X) Universal PCR Assay II (20X) Fast Master Mix, 2×	#4483013 Life Technologies, USA

Table 2.6 Components for FPPT

A forced proximity probe test (FPPT) was performed according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA), to determine whether the antibodies to be used in the PLA were adequately biotinylated. The components used for the FPPT are shown in Table 2.6 and the steps involved in a FPPT are shown in flowchart F3. The concentration of biotinylated antibody stored at -80˚C was 0.25mg/mL. Biotinylated antibodies were diluted to 200nM by adding 44µL of Antibody Dilution Buffer II to 6µL of biotinylated antibody. Prox-Oligo mix was prepared by combining 5µL 200nM of 5' Prox-Oligo (5' Streptavidin linked oligonucleotide) and 200nM of 3' Prox-Oligo (3' Streptavidin linked oligonucleotide). The prox-oligo mix was mixed gently and centrifuged at 10,000g for 10 seconds.

The Forced Proximity Probe (FPP) as shown in table 2.7 was made by combining 2µL of diluted 200nM biotinylated antibody to a 2µL of the prox-oligos mix. A negative control was also included in which 2µL of Antibody Dilution Buffer II was added to the 2µL of 200nM of prox-oligo mix. The negative control (NC) does not contain biotinylated antibody.

Reagents	Forced Proximity Probes	No Protein Control
200nM oligo mix	2µL	2µL
200nM biotinylated antibody	2µL	-----
Antibody Dilution Buffer II	-----	2µL

Table 2.7 Forced Proximity Probe mix

The FPP and negative control were centrifuged (10,000g for 10 seconds) and incubated at 20°C for 1 hour to bind the streptavidin-linked oligonucleotide to the biotinylated antibody. Assay Probe Dilution Buffer II (36µL) was added to both forced proximity probe and NC and incubated for 30 minutes at 20°C. Following the incubation 98µL of Assay Probe Dilution Buffer II was added to both the forced proximity probe and negative control and was mixed and centrifuged at 10,000g for 10 seconds.

Diluted FPP and negative control (4µL each) were aliquoted in quadruplicate to a 96 wells PCR plate. To each well containing FPP (4 wells) and NC (4 wells), 16µL of ligation/PCR mixture was added (Table 2.8). The fluorescently labelled connector oligonucleotide and the primers are present in Universal PCR Assay II (20X).

Components	Final Concentration/20µL Rxn	Volume (µL) per reaction
Fast Master Mix II (2X)	1X	10
DNA Ligase II (250X)	1X	0.076
Universal PCR Assay (20X)	1X	1
Nuclease-free water		4.92
Total		16

Table 2.8 Reagents used for single PCR/Ligation reaction for the FPPT

Real-time PCR was performed using CFX Connect real-time PCR detection system using thermal conditions as shown in Table 2.9.

Function	Temperature (°C)	Time	Cycles
Ligation Step	25	5 minutes	1
Ligation deactivation and Denaturation	95	2 minutes	1
Denaturation	95	5 seconds	40
Amplification	60	30 seconds	40

Table 2.9 Thermal Cycling Conditions used in the FPPT

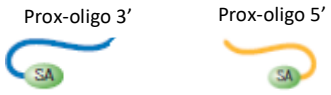
Real-time PCR data were analysed using threshold setting at 10^3 and an automatic baseline. This resulted in Cq (quantification cycle) values. The ΔC_q is calculated as the difference in the average of the C_q values obtained for the forced proximity probe and NC.

$$\Delta C_q = \text{average } C_{q\,(NC)} - \text{average } C_{q\,(FPP)}$$

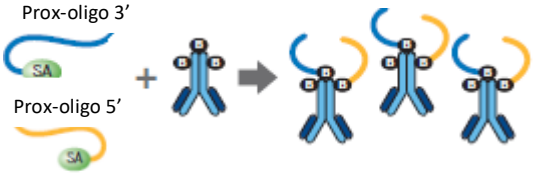
The biotinylation efficiency is measured as a ΔC_qvalue and if ΔC_q >8.5 this indicates that the antibody has passed the FPPT and can be used further for TaqMan Protein Assay II.

Step 1
Antibody Dilution
Dilute the biotinylated antibody to 200nM with ADB II

Step 2
Prepare Prox-Oligo Mix
Prepare Prox-Oligo mix by combining equal volume of each 3'oligo and 5' oligo followed by mixing and centrifugation



Step 3
Probe Preparation
Prepare forced proximity probe by combining prox-oligos and B-Ab and negative control by combining prox - oligo with ADB II



Step 4
Binding reaction
Prepared forced proximity probe and negative control is than incubated at 20˚C for 1 hour.

Step 5
Probe Dilution
Forced proximity probe and negative control are diluted with APDB II and incubated at 20˚C for 20 minutes.

Step 6
Ligation/PCR reaction
Ligation/PCR mix is performed

PCR

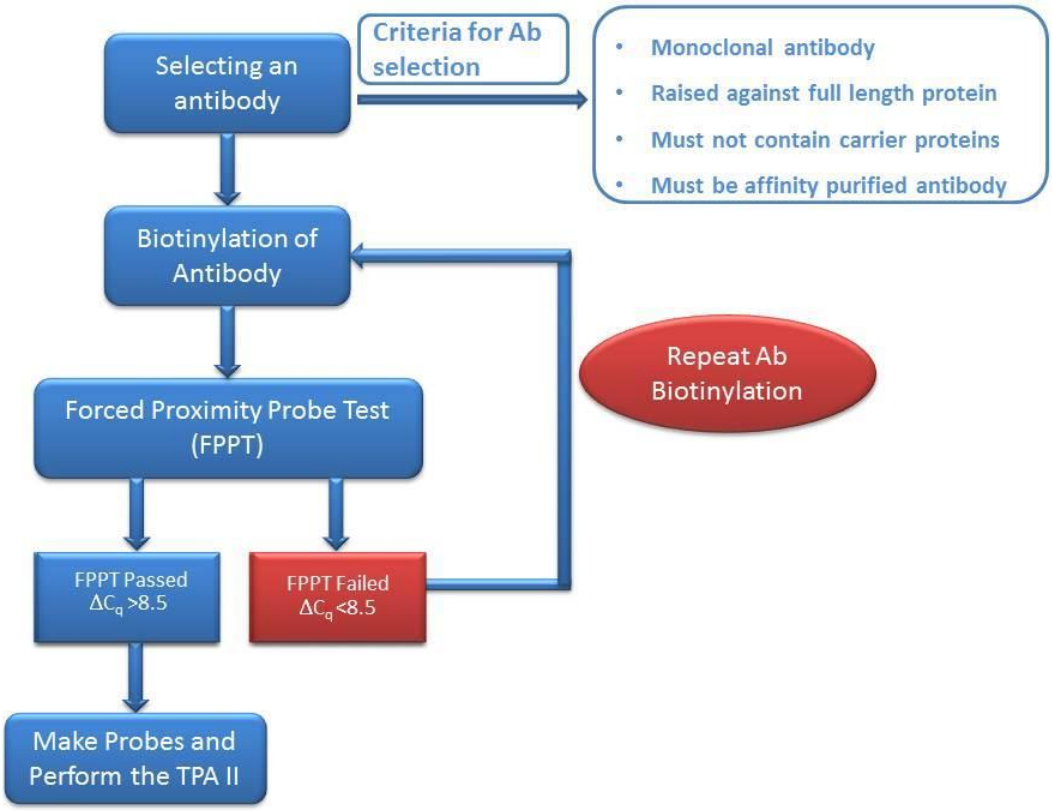
Flowchart F3: The steps involved in a Forced proximity Probe test (FPPT

5.3 Storage of biotinylated antibodies

Biotinylated antibodies were stored at -80°C, -20°C or 4°C after adding an equal volume of Antibody Dilution Buffer II (supplied in TaqMan® Protein Assays Open Kit (# 4453745, Life Technologies ,USA) which helps in the stability of the antibodies. The final concentration of the entire stored biotinylated antibody after adding an equal volume of Antibody Dilution Buffer II is 0.125mg/mL.

5.4 PLA

To perform a robust and accurate PLA several preparatory steps were performed to ensure all the components are optimised. These steps are shown in Flowchart F4.



Flowchart F4 Preparation Steps for Proximity Ligation Assay

The components used for performing Proximity ligation assay are as follows (Table 2.10)

Reagents kit	Reagents	Company
TaqMan® Protein Assays Oligo Probe Kit	3' Prox-Oligo, 200 nM 5' Prox-Oligo, 200 nM	#4453745 Life Technologies, Carlsbad, CA, USA
TaqMan® Protein Assays Open Kit	Antibody Dilution Buffer II Assay Probe Storage Buffer II Assay Probe Dilution Buffer II Serum Dilution Buffer II	#4483013 Life Technologies, Carlsbad, CA, USA
TaqMan® Protein Assays Core Reagents Base Kit	DNA Ligase II (250X) Universal PCR Assay II (20X) Fast Master Mix, 2 X	#4483013 Life Technologies, Carlsbad, CA, USA

Table 2.10 List of reagents for PLA

5.4.1 Preparing Proximity Probes (3' and 5')

Two proximity probes for each antibody were prepared by combining the streptavidin-linked oligonucleotides with the biotinylated antibodies. 5µL of biotinylated antibody (200nM) was added to both 5µL of 3' prox-oligo (200nM) to generate probe A and 5µL of 5' prox-oligo (200nM) to generate Probe B, respectively. The 3' and 5' proximity probe mix was mixed and centrifuged at 10,000g for 10 seconds and incubated for 1 hour at 20°C. Following the incubation 90µL of Assay Probe Storage Buffer II was added and incubated at 20°C for further 30 minutes. Ten aliquots of 10µL of probes A and B were made and stored at -20°C.

5.4.2 qPCR – PLA

The PLA was performed in steps as shown in the flowchart F5. A probe mix was prepared by adding both proximity probes (A and B) to the Assay Probe Dilution Buffer II. For a 20µL of ligation reaction, the components used for preparing the probe mix are shown in Table 2.11

Reagents for Probe Mix	1X (µL)
Assay Probe Dilution Buffer II	1.92
3' proximity probe	0.04
5' proximity probe	0.04
Total volume	2

Table 2.11 Reagents and Volume for Probe Mix

A probe mix was prepared in large volume by combining 2.5µL each of probes A and B with 125µL probe dilution buffer and placing the mixture on ice. For each PLA, 2µL of this probe mix was placed in a single well of a 96 well plate, followed by 2µL of the target antigen, which was appropriately diluted with 1x Serum Dilution Buffer II. No protein controls (NPC) consisted of 2µL of proximity probe mix and 2µL of 1x Serum Dilution Buffer II. The plate was sealed, centrifuged at 780 g for 2 minutes and incubated for 1 hour at 20°C. Following removal of the seal, 16µL of PCR/ligation solution II (as in Table 2.12) was added to each well, the plate was sealed again, spun as before and the ligation was performed on a CFX Connect qPCR instrument with the conditions described in Table 2.13.

Components	Final Concentration/20µL Reaction	Volume (µL) per reaction
Fast Master Mix II (2X)	1X	10
DNA Ligase II (250X)	1X	0.076
Universal PCR Assay (20X)	1X	1
Nuclease-free water		4.92
Total		16

Table 2.12 Reagents used for single PLA reaction

Function	Temperature (°C)	Time	Cycles
Ligation Step	25	5 minutes	1
Ligation deactivation and Denaturation	95	2 minutes	1
Denaturation	95	5 seconds	40
Amplification	60	30 seconds	40

Table 2.13 PLA cycling conditions for ABI on CFX connect qPCR instrument

Alternatively, the PLA assay was carried as described, but using 48 well plates suitable for the Illumina Eco48 instrument. Since the instrument cannot be programmed to run at 25°C, the PLA was done in 2 steps as shown in table 2.14, step 1; the plate was placed in a water bath prior to the qPCR reaction at 25°C for 5 minutes. Step 2; qPCR Cycling and thermal conditions were 95 °C for 2 minutes and 40 cycles of 95°C for 5 seconds and 60°C for 10 seconds.

Temperature (°C)	Time	Function
25	5 minutes in Water Bath	Ligation

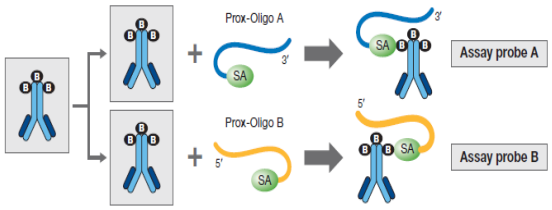
Temperature (°C)	Time	Cycles	Function
95	2 minutes	1	Ligation deactivation and Denaturation
95	5 seconds	40	Denaturation
60	30 seconds	40	Amplification

Table 2.14 PLA cycling conditions for Illumina Eco48 qPCR instrument

5.4.3 qPCR analysis

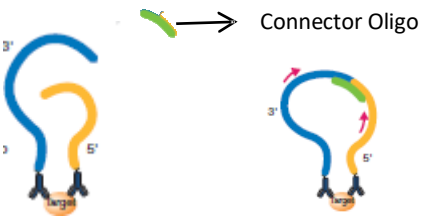
qPCR data obtained from the Biorad CFX and Eco48 were analysed using the using the threshold setting at 10^3 and an automatic baseline. For the PLA, results were recorded as average Cqs ± standard deviations. The NPC was used as a reference background and its Cq value determined the non-target ligation background noise of the assay. Two replicate PLAs were performed for each sample and control. The ΔC_q is also calculated as the difference in the average of the C_q values obtained for the positive sample and no protein control (NPC).

Step 1
Making Proximity Probes

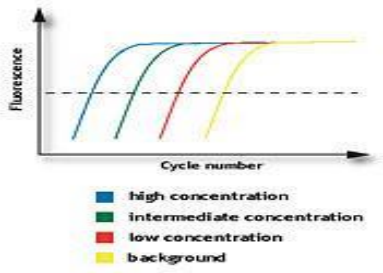


Step 2
Binding reaction
Binding of proximity probes
With target antigen

Step 3
Ligation
Templated ligation of the oligos in proximity using a connector oligo and DNA ligase



Step 4
PCR Amplification
qPCR amplification & detection



Step 5
qPCR Data Analysis

Flowchart F5 Performing Proximity Ligation Assay

5.5. Duplex PLA for *C. difficile* TcdA and TcdB

5.5.1 Preparing Duplex 3' and 5' probes

The two PLA assays were combined into a duplex assay. In a duplex assay the 3' and 5' probes were prepared by combining an equal volume of 3' probes of C*. difficile* TcdA and *Tcd*B. Similarly, equal volume of 5' probes of TcdA and TcdB were also combined (Table 2.15).

Probes	3' (µL)	5' (µL)
***C. difficile* TcdA**	1	1
***C. difficile* TcdB**	1	1
***C. difficile* TcdA+TcdB**	2	2

Table 2.15 Components for Duplex 3' and 5' probes

5.5.2 Antigen for Duplex PLA

An equal volume of *C. difficile* toxin TcdA and TcdB (CDA-TNL and CDB-TNL, The Native Antigen Company, Upper Heyford, UK) were combined for the duplex assay Table 2.16. Both the antigens were diluted to the same concentration from 400,000ng/mL to 20,000ng/mL.

Antigen (20,000ng/mL)	Volume (µL)
***C. difficile* TcdA**	5
***C. difficile* TcdB**	5
Total	10

Table 2.16 Antigen preparation for duplex PLA

C. difficile TcdA and TcdB positive control supplied with commercial ELISA TGC-E001-1 by tgcBiomics, Bingen, Germany was also used for performing the duplex PLA assay.

5.5.3 Performing Duplex PLA assay

A probe mix was prepared by combining both proximity probes (3' and 5') to the assay probe dilution buffer II as shown in the Table 2.17.

Reagents for probe mix	1X (µL)
Assay probe dilution buffer II	1.92
3' proximity probe (TcdA+TcdB)	0.04
5' proximity probe (TcdA+TcdB)	0.04
Total volume	2

Table 2.17 Reagents and volume for duplex probe mix

For each duplex PLA 2µL of this probe mix was placed in a single well of a 96 well plate, followed by combining 2µL of target antigen which is a mixture of both TcdA and TcdB pure antigen and was diluted appropriately in a 1X Serum Dilution Buffer II. No protein controls (NPC) consisted of 2µL of proximity probe mix and 2µL of 1x Serum Dilution Buffer II. The plate was sealed, centrifuged at 780 g for 2 min (Rotina 380R Hettich Zentrifuge, Germany) and incubated for 1 hour at 20°C. Following removal of the seal, 16µL of ligation solution II (Table 2.12) was added to each well, the plate was sealed again and spun. The ligation was performed on a CFX Connect qPCR instrument with the conditions as used in the section 5.4.2 which are 25°C for 5 minutes (ligation step), 95°C for 2 minutes (denaturation step) followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds (amplification step).

Function	Temperature (°C)	Time	Cycles
Ligation Step	25	5 minutes	1
Ligation deactivation and Initial Denaturation	95	2 minutes	1
Denaturation	95	10 seconds	40
Amplification (Extension/Amplification)	60	30 seconds	40

Table 2.19 PLA cycling conditions for Perfecta Toughmix on CFX connect qPCR instrument

5.6 qPCR – PLA using Canine faeces

The canine faeces sample for performing PLA was prepared in two different methods and reagent used for performing the dialysis of faeces sample and PLA are as follows (Table 2.18)

Reagent	Name of Reagent	Company
Faeces sample	Canine faeces stored at 4˚C	-------
Dilution Buffer	1X Serum Dilution Buffer II	#4483013 Life Technologies, USA
Dialysis Buffer	0.5 X TE Buffer	T11493 by Molecular Probe, Eugene, Oregon, USA
Dialysis Chamber	Slide A- Lyzer mini dialysis units	69562,Thermo Scientific, Loughborough, UK
***C. difficile* toxin**	TcdA and TcdB	The Native Antigen Company, Upper Heyford, UK

Table 2.18 Reagents for of dialysis canine faeces

5.6.1 Preparing canine faeces sample for PLA without dialysis

The compact stool sample (50mg) was added to 450µL of the 1X Serum Dilution Buffer II and the suspension was homogenised by vortexing. The sample was then centrifuged at 2500X g in a 5424 microfuge for 2-5 minutes and the supernatant was then spiked with 10ng/mL of TcdA which was further diluted to 1ng/mL and 0.5ng/mL in 1X SDB. This TcdA spiked samples were used for carrying out qPCR-PLA.

5.6.2 Preparing canine faeces sample for PLA with dialysis

A 100µL aliquot of the TcdA antigen spiked faecal sample (10ng/mL and 1 ng/mL) was transferred to two Slide A- Lyzer mini dialysis units and dialysis was performed in 1 litre of 0.5 X TE Buffer at 4°C. The buffer was changed 2 times after 2 hours and then overnight dialysis against 1 litre of the buffer. After dialysis, the spiked samples were transferred to Eppendorf tubes, further 1:10 and 1:20 dilution of both samples were carried out with 1X SDB to perform qPCR-PLA.

5.6.3 qPCR-PLA canine faeces samples using ABI mastermix

The PLA was performed as shown in section 5.4.2. The NPC was also included which consisted of 2µL of faeces supernatant with no antigen and 2µL of probe mix. The same conditions for the PLA as shown in Table 2.11 were used on the CFX connect qPCR instrument.

5.6.4 qPCR-PLA TcdA/TcdB spiked canine faeces samples using Perfecta qPCR Toughmix

The compact stool sample (50mg) was added to 450µL of the 1X Serum Dilution Buffer II and the suspension was homogenised by vortexing. The sample was then centrifuged at 2500X g in a 5424 microfuge for 2-5 minutes and the supernatant was then spiked with the concentration of 250ng/mL of TcdA and 250ng/mL of TcdB in separate tubes. The spiked toxins in the canine faeces were further diluted to the 25ng/mL and 2.5ng/mL and 0.625ng/mL in 1X SDB. The PLA was performed as shown in section 5.4.2 and NPC was also included which consisted of 2µL of faeces supernatant with no antigen and 2µL of probe mix. The thermal condition used on CFX connect qPCR instrument for performing the PLA with Perfecta qPCR Toughmix are shown in Table 2.19.

5.7 qPCR-PLA clinical faeces and swab samples using Perfecta qPCR Toughmix

5.7.1 Preparation of clinical faeces samples

The six clinical faeces samples stored at -80°C were defrosted by keeping them at room temperature. Once defrosted the 50µL of semi-solid stool sample was added to 200µL of the 1X Serum Dilution Buffer II and the suspension was homogenised by vortexing. The sample was then centrifuged at 2500X g in a 5424 microfuge for 2-5 minutes. The supernatant was

pipette out into a new microfuge tube and is considered as a neat sample. The neat supernatant was than diluted to 1:10 and 1:100 dilutions in 1X SDB.

5.7.2 Preparation of clinical swab samples

The thirteen clinical swab samples stored at -80°C were defrosted by placing them for 30 minutes at room temperature. The swabs were tipped down and transferred to the sterile falcon tubes. 500µL of the 1X SDB was added to each falcon tube followed by vortexing of each tube so that the stool materials on the swab tip are mixed into the buffer. This suspension was considered as a neat swab suspension. This neat suspension was diluted to 1:10 dilution in 1X SDB. The swab sample was then ready to be used for testing with qPCR-PLA.

5.7.3 Performing qPCR-PLA for clinical faeces/swab samples using Perfecta qPCR Toughmix

The PLA was performed as shown in section 5.4.2 using 1:10 and 1:100 dilution of the clinical faecal samples and neat and 1:10 dilutions of the clinical swab samples. The thermal conditions for Perfecta qPCR Toughmix were used as shown in the Table 2.19. The clinical swab and faeces sample were tested for both TcdA and TcdB. Purified TcdA/TcdB and their respective NPCs in 1XSDB were used as a control in order to test the working of the PLA.

5.7.4 Ethics Statement

The work was ethically approved by the East London & the City Local Research Ethics Committee. Participants were recruited from Barts and the London. Study title: Novel biomarkers to predict outcome in clostridium difficile – infection.

REC reference number: 10/H0709/91.

Ethics amendment dated: 25/11/2010.

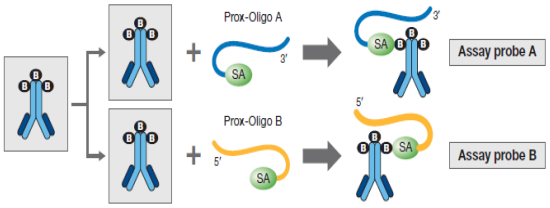
5.7.5 Statistical Analysis

All statistical analysis was done using computer assisted statistical analysis software, GraphPad Prism, version 6. The Mann Whitney U test was used for the statistical assessment . The p-value of less than 0.5 was considered statistically significant.

5.8 Digital PLA

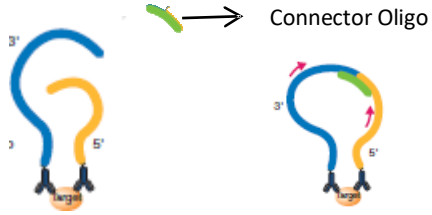
The PLA was performed as in section 5.4.2 except that the qPCR amplification step was performed separately from the ligation reaction (see Flowchart F6).

Step 1
Making Proximity Probes

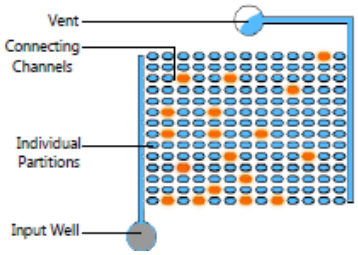


Step 2
Binding reaction
Binding of proximity probes with target antigen

Step 3
Ligation
Templated ligation of the oligos in proximity using a connector oligo and DNA ligase in CFX Connect qPCR machine

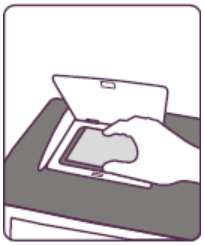


Step 4
Priming
Pin pushing on plate seal over each well and thus isolating individual partitions from one another and diving each sample into 496 identical portions



Step 5
PCR Amplification

Step 6
Imaging



Step 7
Result Analysis

Flowchart F6: Steps for performing digital PLA

The Ligation step was performed on a CFX Connect qPCR instrument with the conditions as shown in Table 2.20.

Temperature (°C)	Time (minutes)	Number of Cycles	Function
18	15	1	Ligation
60	10	1	Ligation Deactivation

Table 2.20 Cycling condition for Ligation for dPLA

Following ligation, the 96 well plate was centrifuged at 780 g for 2min before 10µL of each assay were loaded into a single well on a "Constellation dPCR 96 Well Microplate" (Formulatrix). The dPLA plate was sealed with a rubber seal (3M 300LSE, Formulatrix, Bedford, MA, USA) and placed in the priming drawer of the dPCR machine. Priming takes 15 minutes and involves pins pushing on the plate seal over each well to force the liquid into the channels and a roller forcing the tape into the connecting channels, thus isolating the individual partitions from one another and dividing each sample into 496 identical partitions. The microplate was then placed on a flat block thermal cycler to amplify the DNA using the following conditions: 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds (Table 2.21). Following endpoint PCR, the microplate was placed on the imaging station at the top of the Constellation instruments, which takes images of each well.

Function	Temperature (°C)	Time	Number of Cycles
Initial denaturation	95	3 minutes	1
Denaturation	95	10 seconds	40
Annealing and Amplification	60	30 seconds	40

Table 2.21 Cycling condition for PCR amplification for dPLA

5.8.1 dPCR analysis

Data were analysed using a threshold method to separate positive from negative partitions. An initial assessment with the “raw images” view used the ROX which is a passive reference dye in the master mix to confirm that all partitions were properly filled with reagents and provided a visual estimate of target concentration. The analysis was performed by setting a threshold for both the ROX and FAM filters. The ROX histogram displays two peaks, a small one on the left representing empty partitions and a larger peak on the right representing partitions that contain reagents. A threshold was set manually just to the left of the two peaks. The FAM histogram also displays two peaks: one represents partitions without target DNA, the other those containing PCR amplicons. The threshold was placed halfway between the two peaks and the software then counted the number of positive partitions and calculated the amount of target DNA.

5.9 Enzyme Linked Immuno-sorbent assay (ELISA) for *C. difficile* TcdA and TcdB

A diagnostic/research Elisa kit to detect *C. difficile* TcdA and TcdB was purchased from tgc Biomics, Bingen, Germany and the components of the kit are shown in table 2.22

Component	Name of Component	Company
ELISA Kit	C. *difficile* toxin A and toxin B ELISA kit	TGC-E001-1) supplied by tgcBiomics, Bingen, Germany
Antigen	TcdA and TcdB	The Native Antigen Company, Upper Heyford, UK)
ELISA Reader	iMark™ Microplate Absorbance Reader	#160-1130, Bio-Rad, Hertfordshire, UK

Table 2.22 Components for ELISA

5.9.1 Dilution of the *C. difficile* TcdA and TcdB

TcdA and TcdB antigen concentrations were assayed in the range of 1.25, 0.625 and 0.312ng/mL and 2.5, 1.25, 0.625ng/mL respectively to determine the sensitivity of using this commercial ELISA kit. The sample antigen was diluted in dilution buffer supplied in ELISA kit for C. *difficile* TcdA and TcdB.

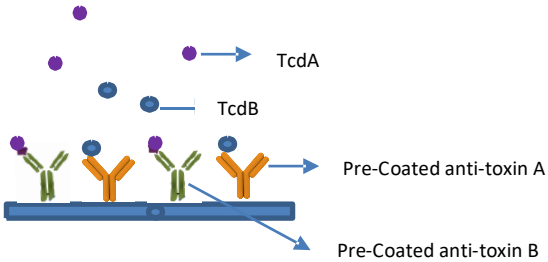
All reagents were brought to room temperature before use. The kit is supplied with the 96 well microtitre ELISA plate already coated with anti-toxin A and anti-toxin B antibodies. The workflow of the ELISA can be seen in flowchart F6. 100µL of the TcdA, TcdB and 100 µL of the positive control (*C. difficile* TcdA & TcdB) supplied with the ELISA kit were added to individual wells. The negative control wells were also included in which 100µL of dilution buffer (Supplied in ELISA kit) was added into the wells. Now 50µL of the conjugate anti-toxin

A & B –HRP was added to each well to detect TcdA & TcdB and the sample and conjugate mixture was then incubated at 37°C for 60 minutes. Wells were washed 3 times with the 1X washing buffer in order to remove the unbound components and thereafter 100µL of the substrate was added to each well followed by incubation at 20°C for 15 minutes. The development of colour was seen after incubation which was stopped by adding 50µL of stop reagent (Supplied in ELISA kit) into each well. The optical density was measured at 450nm and 620nm with iMark™ Microplate Absorbance Reader.

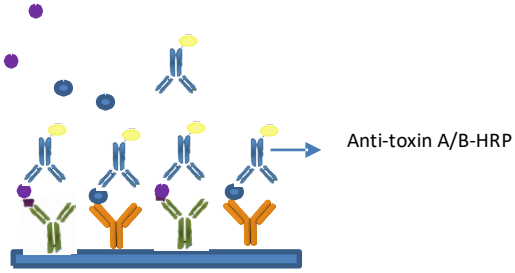
ELISA Flowchart F6

Step 1
Preparation of Sample
TcdA and TcdB is diluted to appropriate concentration

Step 2
Addition of Sample
Diluted sample is added to the ELISA plate pre-coated with anti-toxin A and anti-toxin B antibodies

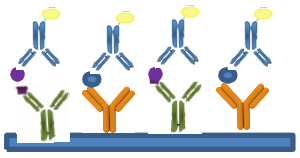


Step 3
Addition of HRP conjugated Ab
Anti-toxin A/B-HRP conjugate is added to the sample and pre-coated antibody complex

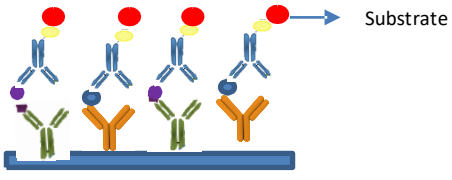


Step 4
Incubation
The sample and conjugate mixture was incubated at 37˚C for 1 hour

Step 5
Washing
Wells containing mixture were washed 3X in order to remove unbound components



Step 6
Addition of Substrate
Substrate was added to each well followed by incubation at 20˚C for 15 minutes



Step 7
Addition of Stop reagent & Reading the Absorbance
Stop reagent was added to stop the reaction followed by reading the absorbance.

5.10 Western Blot

5.10.1 Gel preparation and electrophoresis

For western blotting, resolving layer (8%) and stacking layer (4%) were prepared using protogel from National Diagnostics, Hull, UK and loaded on the Hoefer Mini VE vertical electrophoreses system. Initially, the gel cast was assembled with 1.5 mm spacers and tested with distilled H_2O for leaks. Once the leak was tested, 8% of the stacking layer was prepared by combining the following components in the W/V:

- sterile water
- Tris (1.5 M pH 8.8) (BDH)
- SDS (10%) (BDH)
- acrylamide:bisacrylamide (30%) 19:1 ratio w/w) (Sigma)
- ammonium persulphate (APS) (10%) (Sigma)
- N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma).

The tube containing the above components was mixed thoroughly and 5mL was added in the space between the glass plates, covered with 1mL of the 70% ethanol and was left to set for 30 minutes. Once the resolving layer was set, the 70% ethanol was poured off and 4% stacking layer was prepared by combining the following components:

- sterile water
- Tris (0.5M pH6.8)
- SDS (10%)
- acrylamide:bisacrylamide (30%) 19:1 ratio w/w) (Sigma)
- APS (10%)
- TEMED

All these components were mixed and 2mL of the stacking gel was poured above the set resolving layer and the comb was put into it. Once the gel was set, comb was taken out, wells were formed which were cleaned by sterile water in order to remove all acrylamide.

5.10.2 Sample preparation

20µL of the sample was prepared by combining 10µL of *C. difficile* toxin A antigen (0.5µg/10µL) with 10µL of 2X loading buffer (10% SDS (w/v) , Glycerol, 1M Tris H 6.8, 1M DTT and water). Similarly, 20µL of TcdB sample was also prepared by combining 10µL of *C. difficile* toxin B antigen (0.5µg/10µL) with 10µL of 2X loading buffer. Breast cancer cell line MCF10 cell was used as a negative control. Samples were boiled for 10 minutes at 100°C on a heat block (#DB2A, Techne DRI-BLOCK, Staffordshire, UK.)

5.10.3 Gel Electrophoresis:

A 6µL of protein stained marker HiMark (#LC5699 from Novex life technologies, Paisley UK) was added to the first well and samples were added to the other wells. The amount of samples added to each well was made up to the same volume by adding the appropriate amount of sample reducing buffer plus sample. The gel was run in 1X running buffer (pH 8.56) that contains the following components:

- 250mM Tris
- 1.92M glycine (VWR, East Grinstead, UK)
- 1% w/v SDS

The gel was run at 180V, 50mA, 25W for 2 hours. The run was stopped once the dye reached the bottom of the glass plate.

5.10.4 Transfer of protein to nitrocellulose

Once the gel had finished running it was removed from the electrophoresis set up and proteins in the gel were transferred to a nitrocellulose membrane (Watman, Schleicher & Schuell, and Dassel, Germany). The nitrocellulose membrane, filter papers and sponges were soaked in 1X transfer buffer containing (250mM Tris, 1.92M glycine, pH8.48 and methanol). The gel was then sandwiched between the blotting paper and sponges. The order of the layers in the transfer cassette is as follows:

- black cassette
- sponge
- filter paper
- gel

- nitrocellulose membrane
- filter paper
- sponge
- red cassette

This sandwich blot was kept and soaked in the 1X running buffer in the electro blotting tank and transfer of proteins from the gel to the membrane were conducted at 50V, 180mA, and 22W for 3 hours.

5.10.5 Western blot analysis

The transfer of the protein was checked with Ponceau S red stain (0.5% Ponceau S in 1% acetic acid) (Sigma). The membrane was blocked overnight at 4°C in 3 % (w/v) milk powder in 1X PBS with gentle shaking. The blocked membrane was washed in TBST (1X TBS-Tween20) for 1 minute and probed for 3 hours at room temperature with 1:1000, 1:2000 and 1:5000 primary antibody for *C. difficile* toxin A diluted in 3% MPBS (3% Marvel (w/v) + 1X PBS) and 1:1000 and 1:2000 primary antibody for *C. difficile* toxin B diluted in 3% MPBS (3% marvel (w/v) + 1X PBS). After being washed twice with 10mL PBS/Tween for 15 minutes, the membranes were incubated in an anti-mouse antibody which was conjugated with horse-radish peroxidase- (Pierce Biotechnology, Rockford, USA) at 1:1000 and incubated at room temperature for 1 hour. The membranes were then washed twice as before. Enhanced chemiluminescent (ECL) (#98490B from Interchim, Montlucon, France) reagents were used for 1 minute for visualisation (Millipore, Watford, UK). The dark room was set up and under red light, 1 piece of X-Ray film was placed over the membrane and cassette was closed. The X-Ray was exposed to the membrane and incubated for 30 seconds. The film was then removed and placed in the developer (Sigma) for 1 minute in the agitated movement so that bands are visible on the film. The exposed film was then washed in sterile water to remove the developer and then placed in fixer (Sigma) for 1 minute followed by washing with sterile water. Once the film was dried it was scanned in the image scanner and analysed.

Chapter 3

Results

6.0 Results

6.1 Validation of *C. difficile* TcdA and TcdB antigens and specific antibodies with SDS-PAGE and Western blotting

In order to perform successful PLAs, high-quality target antigens of C. *difficile,* as well as antibodies specific to the toxins TcdA and TcdB are required for the assay. There is concern about the specificity and efficacy of many commercial antibodies (Voskuil, 2014).Therefore, validation of the target antigens (quality/molecular weight) and antibody specificity prior to PLA is required. Full-length TcdA and TcdB were purchased from The Native Antigen Company, Upper Heyford UK and 10µL of both at the concentration of 0.5µg/10µL were added to 10 µL loading buffer which was resolved on 8% of resolving layer and 4% of stacking gels followed by western blotting. A cell lysate of the breast cancer cell line MCF10 was used as a negative control. Primary antibodies to anti-*C. difficile* toxin A mAb (#Ab19953, Abcam, Cambridge,UK) and anti-*C. difficile* toxin B mAb (#ABIN234836, Antibodies-online Aachen, Germany) were used to probe the blot.

Figure 11(A) and Figure 11(B) shows the presence of a protein band of molecular weight around ~290kD for TcdA and ~270kD for TcdB respectively and matches the expected size from the manufactures data sheet. This shows that the *C. difficile* TcdA and TcdB which will be used for the development of proximity ligation assay are both full-length proteins. No bands were seen in the negative control which shows that *C. difficile* toxins do not cross react.

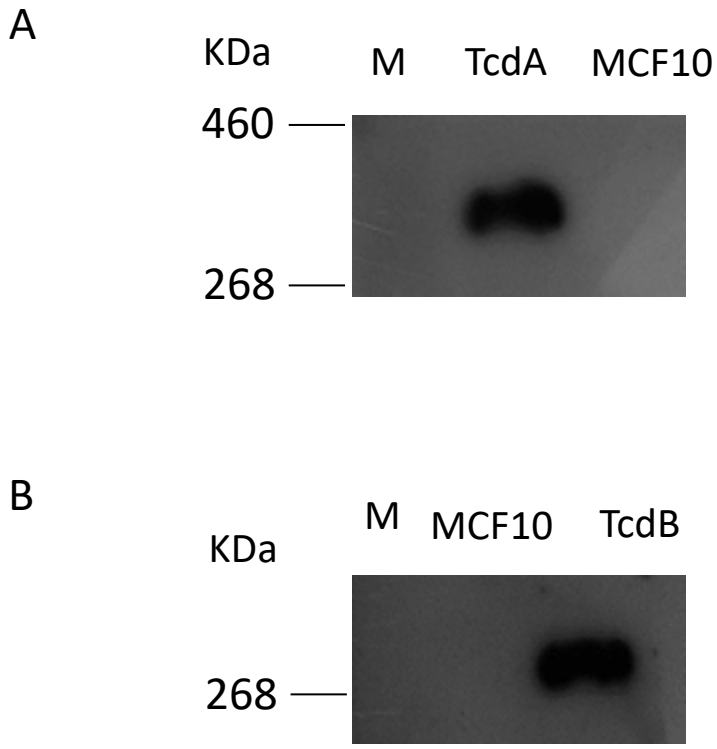


Figure 11: (A) Western blot analysis of *C. difficile* TcdA. Lane 1 shows the molecular weight marker (M), HiMark (#LC5699 from Novex life technologies, Paisley UK). Lane 2 shows the *C. difficile* TcdA band at a molecular weight of approx. 300kDa. The 0.5µg of TcdA was blotted with 1:1000 dilution of primary antibody and 1:1000 dilution of secondary antibody (Anti-mouse HRP). The membrane was exposed to ECL for 1 minute followed by exposure to X-ray film for 30 seconds in a dark room. No bands were seen in the negative control lane.

(B) Western blot analysis of *C. difficile* TcdB. lane 1 shows the molecular weight marker, HiMark (#LC5699 from Novex life technologies, Paisley UK). Lane 2 shows the *C. difficile* TcdB band at a molecular weight of approx. 270kDa. The 0.5µg of TcdB was blotted with 1:1000 dilution of primary antibody and 1:1000 dilution of secondary antibody (Anti-mouse HRP). The membrane was exposed to ECL for 1 minute followed by exposure to X-ray film for 30 seconds in a dark room. No bands were seen in the negative control lane.

6.2 Determining the sensitivity of C. *difficile* TcdA and TcdB with Enzyme Linked Immuno-Sorbent Assay

ELISAs are the most common clinical laboratory test for the detection of *C. difficile* toxins and give results within 3 hours as compared to several days for anaerobic culture and cell cytotoxin assays. Several *C. difficile* TcdA/TcdB ELISA kits are commercially available such as Premier toxin A and B (Meridian Bioscience, Inc.) and manufacturers suggest that these kits have a limit of detection (LOD) of 2.5ng/mL for TcdA and 1.25ng/mL for TcdB. Similarly, *C. difficile* TOX A/B II (TechLab, Inc., Blacksburg, VA) shows LOD of 1.25ng/mL for TcdA and 2.5ng/mL for TcdB (Novak 2008). Finally, we selected an ELISA kit sold by tgcBiomics, GmbH because it apparently has the highest sensitivity of all the ELISA kits compared with a LOD of 0.5ng/mL for TcdA and 1ng/mL for TcdB, moreover, it was less expensive than other ELISA kits available.

C.difficile TcdA and TcdB quantification was determined twice following to the manufacturer's instruction (section 5.9). The quantification and LOD of the kit were analysed using purified TcdA and TcdB (The Native Antigen Company, Upper Heyford, UK) at the concentration range of 1.25, 0.625 and 0.312ng/mL and 2.5, 1.25, 0.625ng/mL respectively. This particular concentration range was selected in order to test the minimum LOD of the ELISA. The sensitivity of the kit when the antigen was suspended in canine faeces over the same concentration range (methods section 5.9) was also determined. The canine faeces sample was used as the substitute to the human sample as there was no human clinical sample available as this stage of optimization of the assay and moreover, no ethics permission was required to work on canine faeces.

The readout of the assay is based on the measurements of the optical density at 450 and 620 nm and is calculated as OD_{450}-OD_{620}. The average $OD_{450\text{-}655}$ of the negative control of the neat and faecal suspension was below 0.05 and according to the kit specifications; the cut-off for the positive result was 0.2 if the background was below 0.1. The positive control did not have any OD as it was highly concentrated in both TcdA and TcdB.

Figure 12 (A) shows the LOD for the ELISA for neat purified TcdA was 1.25ng/mL with the average $OD_{450-655}$ of 0.275. Antigens suspended in canine faeces sample, suspended with the purified TcdA at the concentration of 1.25ng/mL, 0.625ng/mL and 0.312ng/mL, did not alter the results of the ELISA and gave the same LOD of 1.25ng/mL with the average $OD_{450-655}$ of 0.297 for TcdA.

Figure F12 (B) shows that the LOD for neat purified TcdB was 1.25ng/mL with the average $OD_{450-655}$ of 0.280. Purified TcdB with the concentration range of 2.5ng/mL, 1.25ng/mL and 0.625ng/mL spiked into canine faeces sample gave the same LOD of 1.25ng/mL showing the average $OD_{450-655}$ of 0.244.

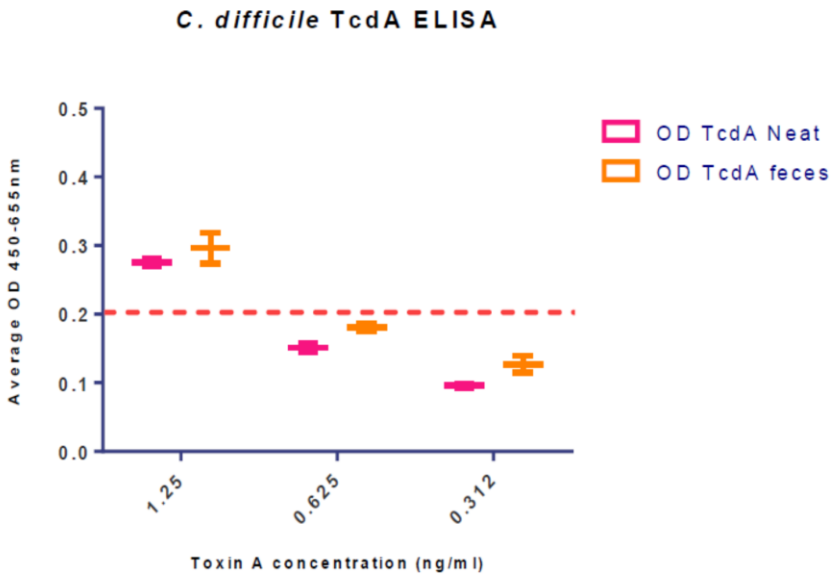


Figure 12: (A) Sensitivity of a commercial ELISA used to detect TcdA using neat antigen (Pink) and antigens suspended in canine faeces (orange). According to kit specification, the LOD for the assay had a cut off of 0.2 optical density (OD) $_{450\text{-}655nm}$ (pink dashed line) when the negative control was less than 0.05 $OD_{450\text{-}655nm}$ (the negative control for the assay was 0.04 $OD_{450\text{-}655nm}$). Error bars show standard deviations.

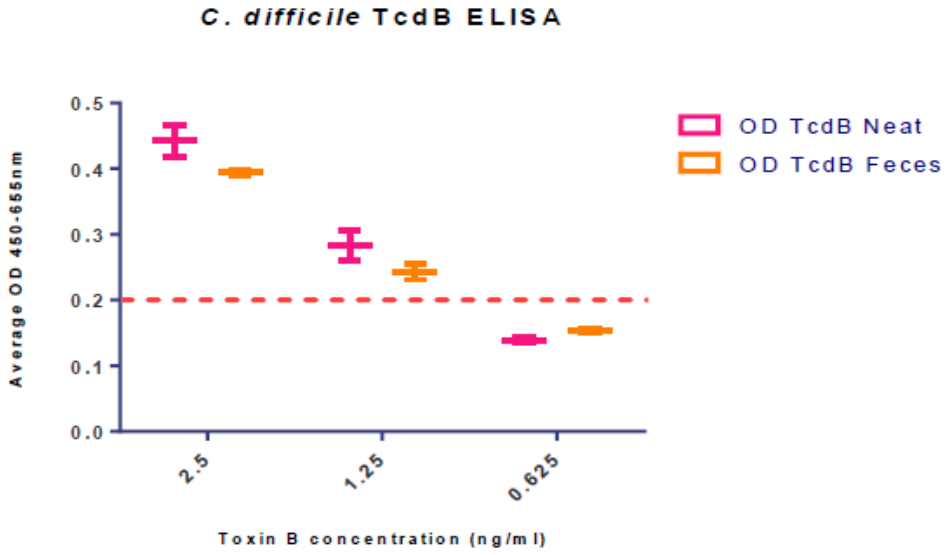


Figure 12: (B) Sensitivity of a commercial ELISA used to detect TcdB using neat antigen (Pink) and antigens suspended in canine faeces (orange). The LOD (ng/mL) to detect purified *C. difficile* TcdB (Pink) with antigen concentration (2.5,1.25,0.625) and *C. difficile* TcdB fecal suspension (Orange) with antigen concentration (2.5,1.25,0.312). The cut off with the negative control <0.05 is OD 0.2 OD $_{450\text{-}655nm}$ (according to kit specification) and is shown by the dotted horizontal pink line.

6.3 Development of Proximity ligation assay (PLA) to detect *C. difficile* TcdA and TcdB

The PLA used in the project is commercially available known as TaqMan Protein Assay II (TPA II) (Applied Biosystems, Life Technologies). To perform the TPA II (methods 5.4.2), biotinylation of the antibodies specific to the *C. difficile* TcdA and TcdB is first required and this was performed using two commercial kits. Initially, biotinylation of the selected Abs was performed using the APEX Biotin-XX Ab labelling kit (Invitrogen) (methods section 5.1.2) and then EZ-Link Sulfo-NHS-LC-Biotin; No-Weigh Format Biotinylation kit (Thermo Scientific) (section 5.1.1) was used.

6.3.1 Results for forced proximity probe test (FPPT)

The forced proximity probe test was performed to determine whether or not the biotinylated antibodies can bind to the oligonucleotides in order to perform the PLA. The result of the FPPT for the antibody that was biotinylated using the APEX Biotin-XX Ab labelling kit (Invitrogen) can be seen in Figure 3. The ΔCq was calculated which is a difference between the Cqs of the negative control containing oligonucleotides only and Cqs of the forced proximity probes containing both biotinylated antibodies and oligonucleotides. The ΔCq was calculated for each biotinylated antibody. The arbitrary cut-off value of ΔCq≥8.5 was mentioned in the TaqMan assay protocol from Life Technology for the antibody to pass the FPPT. Figure 13 shows the FPPT of the biotinylated TcdA mAb (Abcam) and ΔCq is <8.5, therefore the TcdA mAb failed the test.

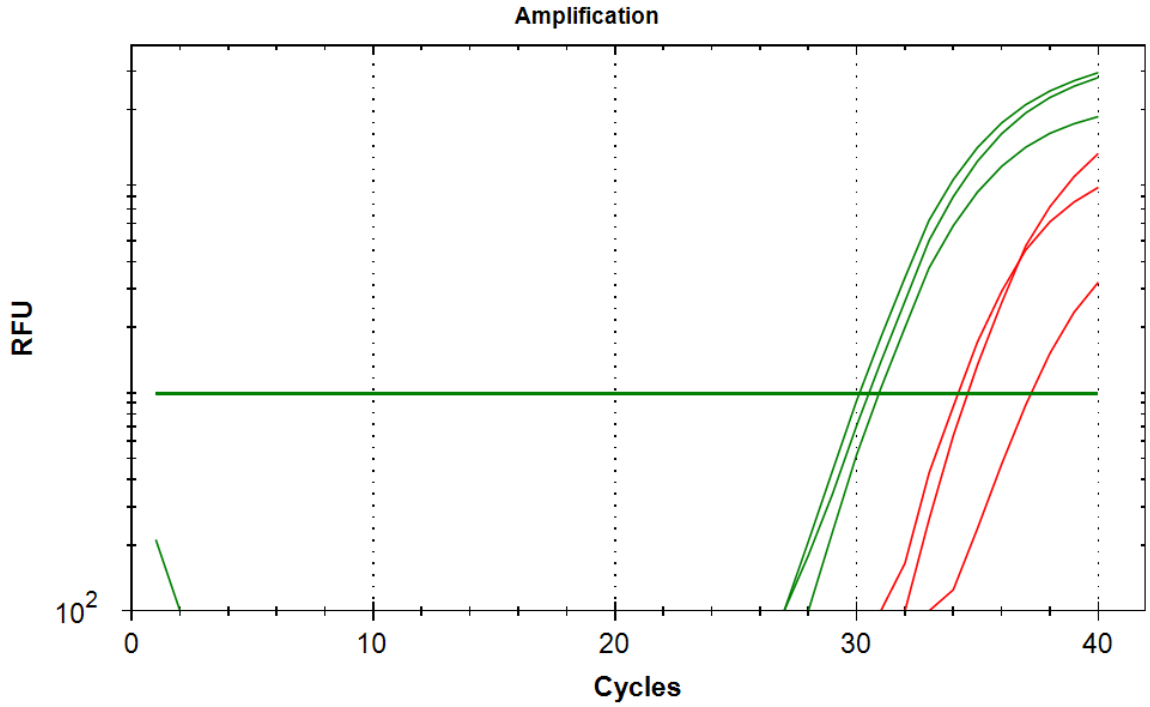


Figure 13 Forced proximity test amplification curve for TcdA mAb forced proximity probe, containing 3'and 5' prox oligo mix and biotinylated antibodies (Green) and the negative control containing 3' and 5' prox-oligo mix and Antibody Dilution Buffer II (Red). Three replicate were performed for both TcdA mAb forced proximity probe and negative control. The forced proximity probe amplifies at the average Cq (quantification cycle) of 26.5 and since the average Cq for the negative control is 31.5, the ΔCq is 5 which is <8.5. (RFU : Relative Fluorescence Unit)

After the biotinylation of TcdA mAb (Abcam) was unsuccessful, the decision was made to use EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation kit (Thermo Scientific) for biotinylation of the antibodies. The results in Figures 14 show an amplification plots for FPPT for mAb for TcdA, mAb for TcdB and pAb for TcdB with an average Cq of for the forced proximity probe and average Cq for the negative control.

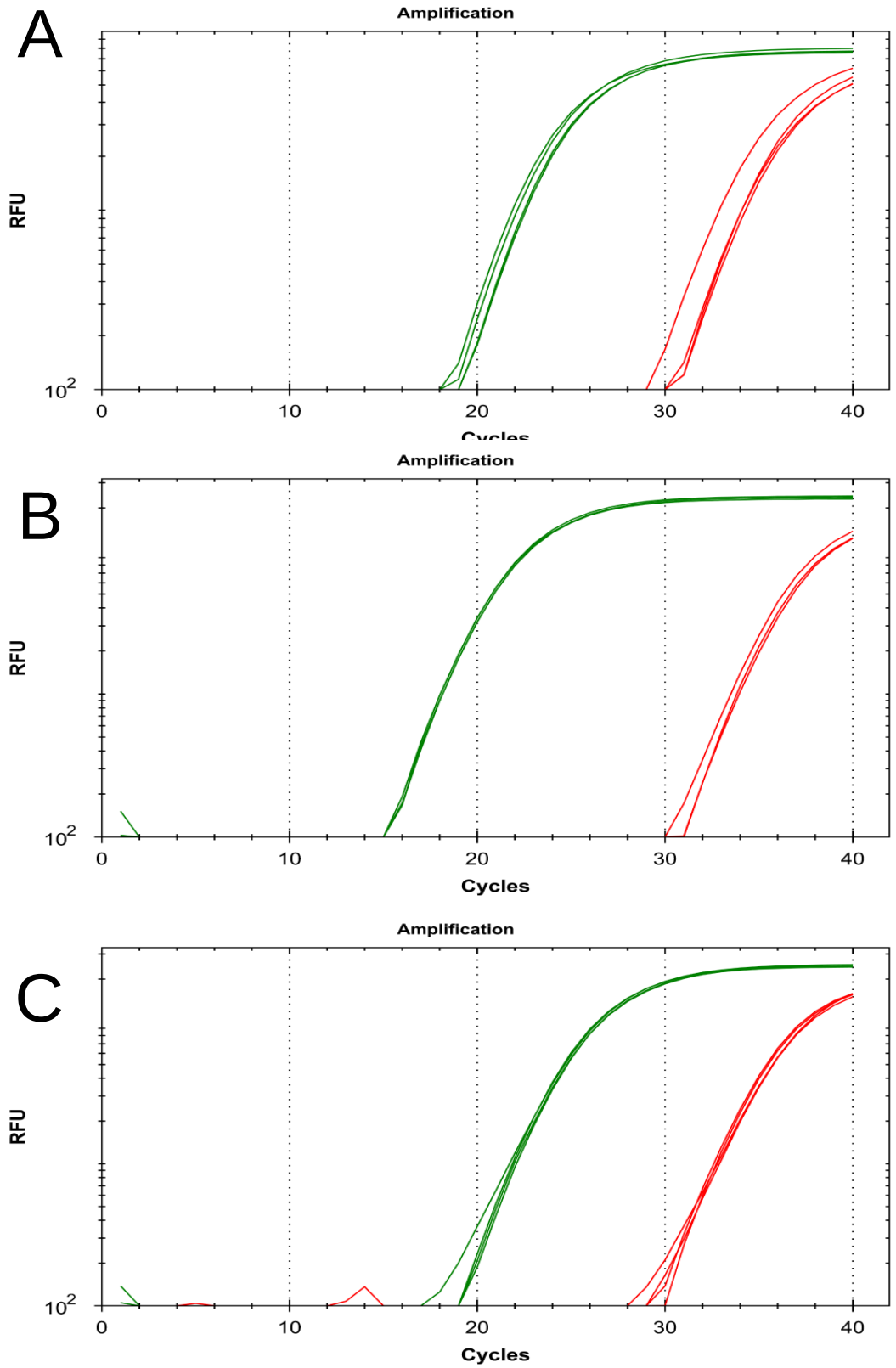

A
Amplification
RFU
10^2
0
10
20
30
40
Cycles
B
Amplification
RFU
10^2
0
10
20
30
40
Cycles
C
Amplification
RFU
10^2
0
10
20
30
40
Cycles

Figure 14 Result of the forced proximity tests (A) mAb for TcdA with ΔCq 12.96, (B) mAb for TcdB with ΔCq 15.97 and (C) pAb for TcdB with ΔCq 10.92 using use EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation kit (Thermo Scientific) kit for biotinylation of antibodies. The green amplification plots were obtained in the presence of biotinylated antibodies with oligonucleotides and the red ones in the absence of biotinylated antibodies, with only prox-oligonucleotides present. (RFU : Relative Fluorescence Unit)

Table 3.1 below shows, all three antibodies tested (TcdA mAb, TcdB mAb and TcdB polyclonal (pAB) passed the forced proximity probe test as they significantly exceeded the forced proximity probe quality threshold of a ΔCq ≥ 8.5.

Sample Name	Cq	Mean Cq	ΔCq = (Mean Cq NPC – Mean Cq antibody)
Abcam TcdA mAb negative control	30.47	30.24	12.96
	29.92		
	30.18		
	30.41		
Abcam TcdA mAb Forced proximity probes	17.37	17.28	
	17.32		
	17.32		
	17.12		
TcdB mAb negative control (antibodies-online)	33.82	33.89	15.97
	33.89		
	34.45		
	33.38		
TcdB mAb Forced proximity probes (antibodies-online)	17.83	17.92	
	17.98		
	17.88		
	17.98		
TcdB pAb negative control (Abnova)	32.80	32.66	10.92
	32.63		
	32.50		
	32.68		
TcdB pAb Forced proximity probes (Abnova)	21.62	21.74	
	21.93		
	21.72		
	21.71		

Table 3.1: The Cq value of negative control (containing prox-oligonucleotides and Ab dilution buffer) and Forced proximity probes (containing biotinylated antibodies and prox oligonucleotides) of three different biotinylated antibodies are seen. The average of the Cq values is calculated followed by the ΔCq values for antibodies tested using forced proximity probe test. All three antibodies tested (TcdA mab, TcdB mAb and TcdB polyclonal (pAB) passed the forced proximity probe test as they significantly exceeded the forced proximity probe quality threshold of a $\Delta Cq \geq 8.5$.

mAb for TcdA recorded a ΔCq of 12.96, mAb for TcdB with ΔCq of 15.97 and the pAb for TcdB with ΔCq of 10.92 as seen in the table 3A above. These results indicated that all three antibodies do not contain an excess of free biotin and are sufficiently biotinylated therefore they are suitable for use in the homogenous PLA.

6.3.2 Detection of *C. difficile* TcdA and TcdB with proximity ligation assay

6.3.2.1 Development of TcdB specific PLA

The biotinylated stock antibodies were used to prepare the 3' probe ("probe A") and 5' probe ("probe B") (methods 5.4.1) and further used to perform PLA for detection of TcdA and TcdB (methods 5.4.1). Since TcdB is generally thought to be the key virulence determinant (Lyras et al., 2009) and TcdA-negative, TcdB-positive isolates appear to be on the increase (Kim et al., 2008), we initially targeted the TcdB antigen for the development of the first proximity ligation assay. No information on the epitope recognition of the selected antibodies for TcdB was available from the manufacturer, therefore we performed a single repeat of the TcdB specific PLA using a conventional combination of monoclonal (mAb) and polyclonal (pAb) probes and compared the 3'-oligonucleotide polyclonal /5'-oligonucleotide-monoclonal combination (Bp3m5) with the combination of 3'-oligonucleotide-monoclonal /5'-oligonucleotide-polyclonal (Bm3p5) probes specific to TcdB to determine the best pair of probes suited for the detection of TcdB. PLAs were carried out in duplicate over the following TcdB antigen concentrations (250, 25, 2.5, 1.25, 0.625, 0.3 and 0.12ng/mL). The result for Bp3m5 probes and Bm3p5 probes are shown in Figure 5. The result was determined as the difference in quantification cycle (ΔCq) obtained at each concentration compared to the "no protein control" (NPC). The result in figure 15 shows that the combination of 3' pAb - 5'mAb (Bp3m5) probes were more sensitive than 3'mAb -5'pAb and Bp3m5 is an ideal set of probes for the TcdB-specific PLA as it can detect TcdB at the concentration of 0.125ng/mL but 3'mAb and 5' pAB combination (Bm3p5 probes) can detect 1.25ng/mL. Therefore, the PLA run performed in duplicate showed that we can use the Bp3m5 combination of the probes for the optimization of the TcdB specific PLA.

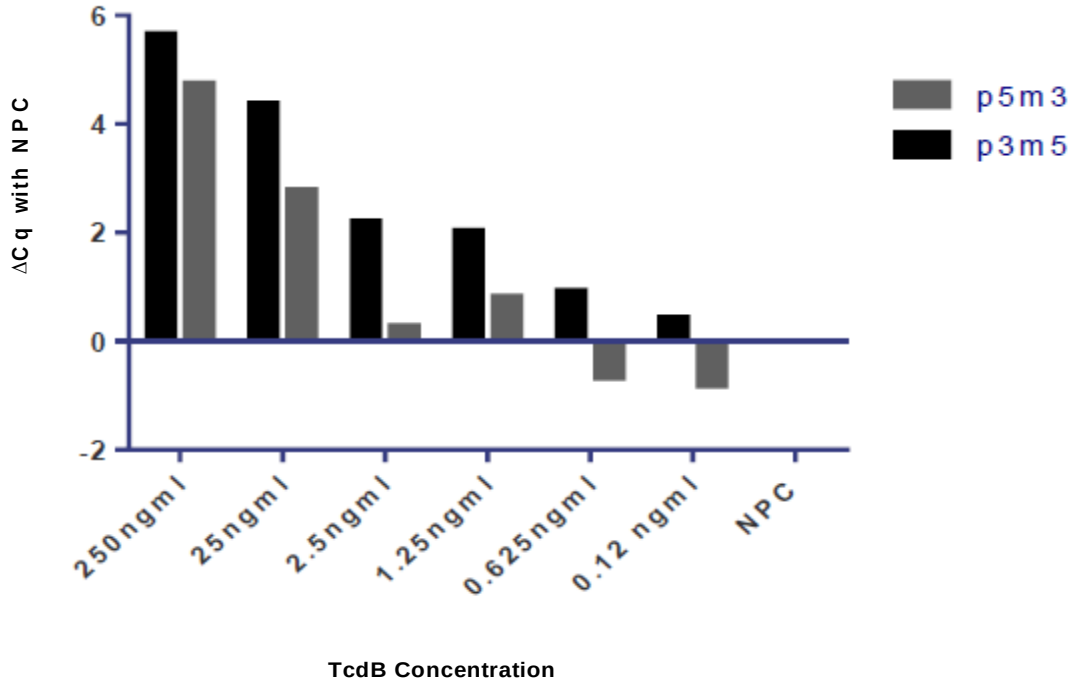


Figure 15 Result for TcdB PLA: A single PLA run was carried out in duplicate reactions on purified TcdB antigen using either 3'-oligonucleotide polyclonal /5'-oligonucleotide-monoclonal combination (grey). ((Bp3m5 probes) and 3'-oligonucleotide-monoclonal /5'-oligonucleotide-polyclonal combination (black) (Bm3p5 probes). ΔCq obtained at each concentration in plotted on the Y-axis and concentration of TcdB is seen on the X-axis of the graph. NPC is referred to as negative control or no protein control.

Since the C-terminal end of the TcdB gene is characterised by several repeat motifs (Pruitt and Lacy, 2012b), we surmised that it might be possible to target TcdB using a single mAb. Hence, we also tested a PLA using only the single mAb and used the combination of 3'-oligonucleotide-monoclonal /5'-oligonucleotide-monoclonal (Bm3m5) probe for the PLA and compared this with the of 3'-oligonucleotide-polyclonal/5'-oligonucleotide-monoclonal Bp3m5 probes. In order to check the repeatability of an assay, two replicate PLAs were carried out in duplicate at each of antigen concentrations (250, 25, 2.5, 1.25, 0.625, and 0.12ng/mL). The difference in quantification (ΔCq) obtained at each concentration compared to the NPC was calculated. The statistical analysis of the data was done using multiple t-tests for each concentration of the TcdB used for both sets of probes and p-value for each concentration was calculated. Figure 16 shows no statistically significant difference in sensitivity in the ΔCqs for PLA at difference concentrations using either combinations of the probes (p=0.754 (250ng/mL), p=0.502 (25ng/mL), p= 0.119 (2.5ng/mL), p= 0.270 (1.25ng/mL), p=0.754 (0.625ng/mL) and p= 0.953 (0.125ng/mL), n=2) and allowed the detection of 0.12ng/mL of TcdB which is more sensitive than the corresponding ELISA.

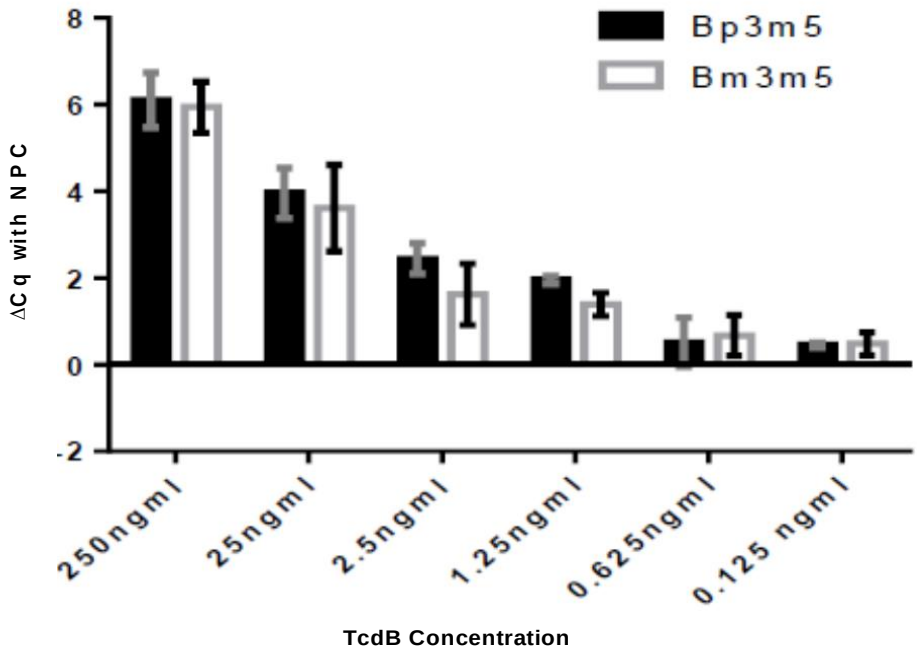


Figure 16 Repeatability of TcdB PLA: Two replicates of the PLAs carried out in duplicates on purified TcdB antigen using either 5'-oligonucleotide-monoclonal/3'-oligonucleotide-polyclonal combination ((Bp3m5 -dark boxes) or 5'-oligonucleotide-monoclonal /3'-oligonucleotide-monoclonal combination (Bm3m5- (light boxes). The difference in quantification cycle (ΔCq) obtained at each concentration compared to the "no protein control" is plotted (Y-axis). PLAs were carried out in duplicate at each of seven antigen concentrations (250, 25, 2.5, 1.25, 0.625, and 0.12ng/mL). A., B., Error bars show standard deviations. The p-value for each concentration is more than 0.05 which shows that there is no statistically significant difference in sensitivity in the ΔCqs for PLA using either of the probes.

6.3.2.2 Development of TcdA specific PLA

Since TcdA also has multiple repeat epitopes at its C-terminal end, we hypothesised that it might also be possible to develop a sensitive PLA using a single mAb, (Frey and Wilkins, 1992). Therefore, the combination of single mAb probes 3'-oligonucleotide-monoclonal /5'-oligonucleotide-monoclonal (Am3m5) were used and PLA was carried out with purified TcdA at the concentration range of 250, 25, 2.5, 1.25, 0.625 and 0.125ng/mL. Figure 17 (A and B) shows that this approach of using single mAb to perform PLA was successful for TcdA as well and resulted in a sensitive assay, detecting purified TcdA down to 0.125ng/mL.

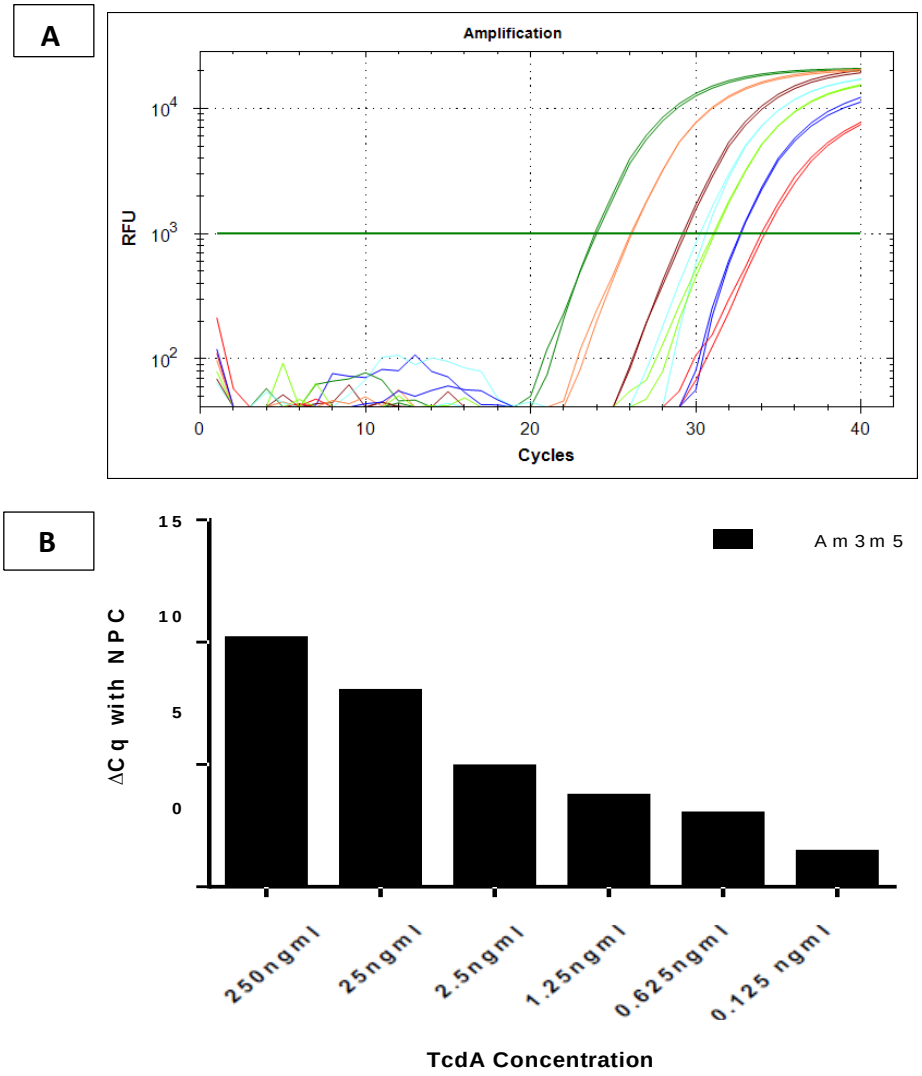


Figure 17: Single TcdA PLA using Am3m5 probe

(A) PLA amplification curve for TcdA using Am3m5 probes. The green colour trace denotes the highest TcdA concentration of 250ng/mL. The amplification plot for other concentrations are 25ng/mL (orange), 2.5ng/mL (purple), 1.25ng/mL (light blue), 0.625ng/mL (light green), 0.125ng/mL (dark blue) and Red Trace denotes the NPC (no protein control). The data was analysed using a threshold setting of 10^3 with automatic baseline. NPC is referred as no protein control

(B) Single PLA carried out on purified TcdA antigen, with 3'-oligonucleotide-monoclonal /5'-oligonucleotide-monoclonal Am3m5 probes. The difference in quantification cycle (ΔCq) obtained at each concentration compared to the NPC is plotted. The Δ Cq calculated between 250ng/mL of purified toxin and NPC was 10.13 and with the minimum concentration of 0.125ng/mL was 1.41.

The repeatability of the assay was assessed by performing four additional independent repeats of the PLA assay with two replicates of each concentration. Figure 18 shows the average results and suggest that it is possible to quantify reproducibly as little as 0.625ng/mL and detect 0.125ng/mL of purified TcdA which is five to ten times more sensitive than the ELISA.

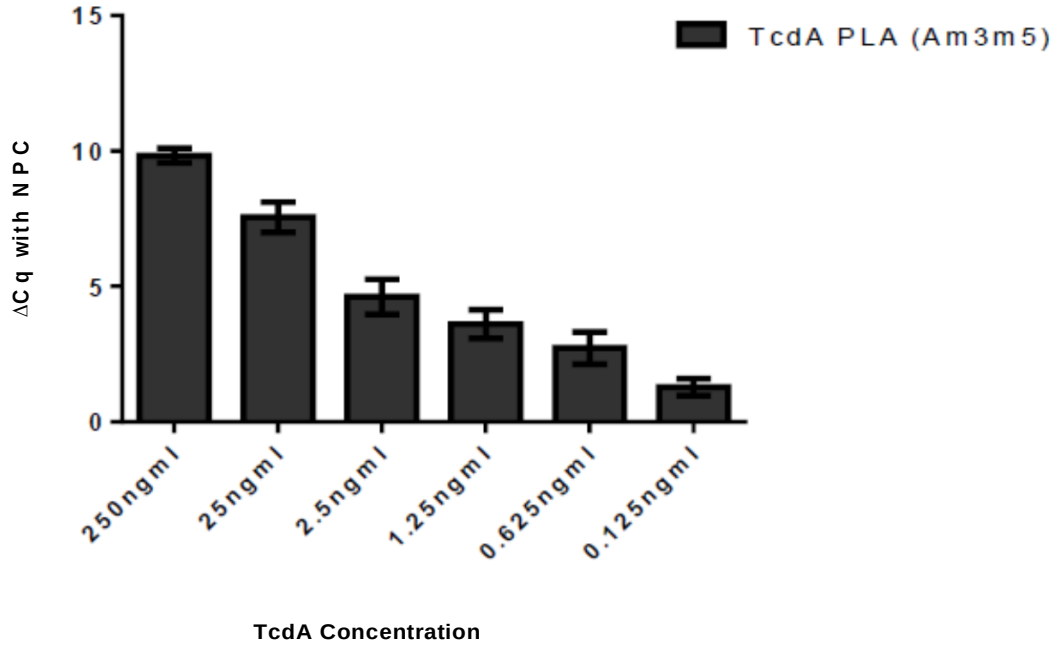


Figure 18: Repeatability of the TcdA PLA: The difference in quantification cycle (ΔCq) obtained at each concentration compared to the NPC is plotted at each of seven antigen concentrations (250, 25, 2.5, 1.25, 0.625 and 0.125ng/mL). The black bars show the results from four independent PLAs with the TcdA mAb coupled to either 5'- or 3' oligonucleotide (Am5m3). Error bars show standard deviations of ΔCqs of four different PLAs. The p –value calculated from the t-test with n=4 is more than 0.05 (p=0.851), which show no statistical difference in the ΔCqs of the 4 different PLAs performed for each concentration.

Along with sensitivity and specificity of an assay, early detection of an antigen is also an important factor in order to develop an assay for a clinical diagnosis. Therefore in order to reduce the time of an assay, Eco48 instrument was used which has a superior temperature control which helps in maintaining the thermal uniformity across the sample plate, therefore, performs the 40 PCR cycles in approximately 40 minutes. Therefore, PLA was also carried out using the Eco48 qPCR instrument for TcdA at the same concentration range as shown in figure 8 to reduce the time taken to perform the qPCR stage of the PLA. Since the instrument cannot be programmed to run at 25°C, the plate was placed in a water bath prior to the qPCR reaction (method section 5.4.2) which makes the assay tedious. Figure 19 shows the PLA assay performed with a similar sensitivity with the detection limit of 0.125ng/mL with faster qPCR reaction and reducing the assay time by 20 minutes. The statistical comparison for the PLAs for TcdA performed with Illumina Eco and CFX was done using Mann-Whitney U test. The t-test calculated the p-value of 0.571 which show no significant difference in the results irrespective of the instrument used for the PLA thus shows that PLA can be performed in the Illumina Eco 48 without affecting the PLA result and also reduces the assay time from 1 hour to 45 minutes.

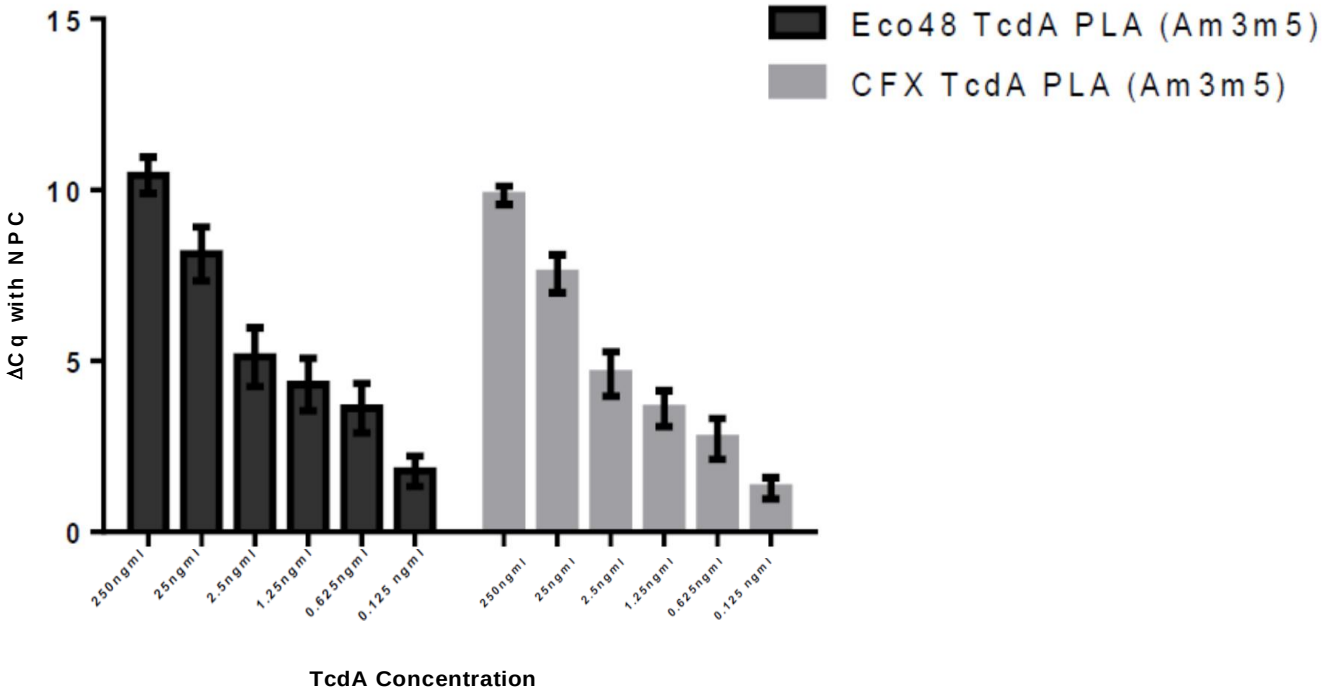


Figure 19: PLA performed on the Illumina Eco48 instrument vs PLA performed on CFX: The difference in quantification cycle (ΔCq) obtained at each concentration compared to the NPC is plotted at each of seven antigen concentrations (250, 25, 2.5, 1.25, 0.625 and0.12ng/mL). Results from four independent PLAs with the TcdA mAb coupled to either 5'- or 3' oligonucleotide (Am5m3). Error bars show standard deviations from 4 replicates of PLA in duplicate for each concentration. No Statistical difference was seen between PLA results from both the instruments using Mann-Whitney U test (P value=0.571 and n= 4)

6.3.3 Development of a Duplex PLA targeting TcdA and TcdB

C. difficile toxins, TcdA and TcdB have similar structure see Figure 1 in introduction chapter. More than half of the *C. difficile* strains do not express toxins (A^-B^-) and therefore, they colonise the patient without showing any symptoms. But there are strains of the *C. difficile* which express either TcdA or TcdB ($TcdA^+/TcdB^-$ or $TcdA^-/TcdB^+$) but most pathogenic strain produces both TcdA and TcdB (Drudy, Fanning and Kyne, 2007). Therefore, after the development of TcdA and TcdB specific single PLA, we focused on developing a duplex PLA targeting both toxins (TcdA and TcdB) into a single assay. In a duplex assay, the 3' and 5' probes were prepared by combining equal volume of 3' mAb probes of C*. difficile* TcdA and 3' pAb probes of *C. difficile* TcdB. Similarly, equal volume of 5' mAb probes of TcdA and 5' mAb probes of TcdB were also combined followed by assay conditions the same as for individual PLAs (methods 5.5). Two independent duplex PLA were performed targeting combined TcdA/TcdB toxins. The analysis of the results was done by determining the ΔCq same as in singleplex assays. Figure 20 shows that the assay was able to quantify target toxin to 1.25ng/mL and LOD was 0.125ng/mL. These results were similar to the singleplex assay for which LOD of TcdA and TcdB was 0.125ng/mL. Since, the TaqMan mastermix (supplied by Life Technologies) uses single hydrolysis probe with FAM labelled as the marker for PCR detection, therefore, the duaplex PLA cannot distinguish between the two toxins TcdA and TcdB.

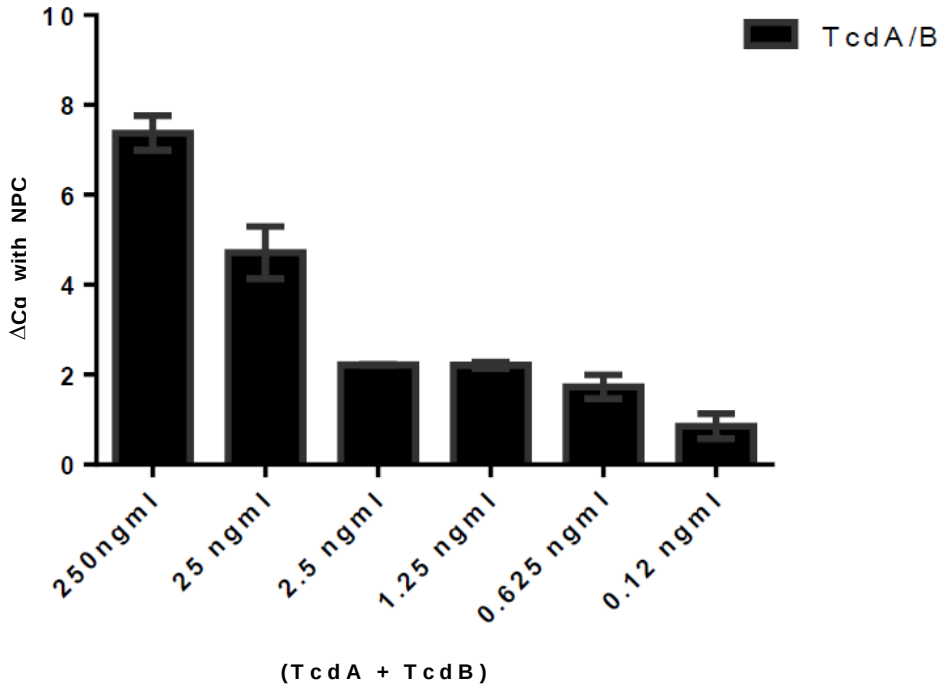


Figure 20: Duplex assay targeting TcdA and TcdB with Am5m3 and Bm5p3. The difference in quantification cycle Δq obtained at each concentration compared to the "NPC" is plotted. Two independent PLAs were carried out in duplicate using two separate pools of 3' oligonucleotide- and 5' oligonucleotide-mAb targeting TcdA (Am5m3) and TcdB (Bm5p3). Error bars show standard deviations from two replicates of PLA performed in duplicate for each concentration.

6.3.4 Validation and optimization of *C. difficile* TcdA and TcdB specific PLA using Canine faeces

Development of a PLA using pure antigen is not representative of the clinical setting so TcdA and TcdB specific PLAs were performed with the antigen spiked into canine faeces sample as a model of human faecal samples. Previous studies have shown the isolation of pathogenic *C. difficile* toxins from the diarrhoeic and non-pathogenic *C. difficile* from non-diarrhoeic dogs, therefore, the canine faecal sample was used (Chouicha and Marks, 2006). Initially, the canine faeces sample was prepared by spiking 10ng/mL of TcdA toxin (methods section 5.6) into 50mg of canine faeces and further diluted to 1ng/mL and 0.5ng/mL in 1X Serum Dilution Buffer II. The non-spiked faecal sample was also used as the negative control or no protein control assay. Two independent PLA s were carried out in duplicates using the standard PLA conditions (methods 5.4.2). The results in table 3.1 show that the assay did not work as no amplification was seen in the neat TcdA spiked faecal sample. But when the neat TcdA spiked faecal sample was diluted in Serum Dilution Buffer II the working of the assay was restored.

	PLA 1				PLA 2			
TcdA Concentation	10ng/mL(NEAT)	1ng/mL 1:10 of neat	0.5ng/mL 1:20 of neat	NPC (neat)	10ng/mL(NEAT)	1ng/mL 1:10 of neat	0.5ng/mL 1:20 of neat	NPC (neat)
Replicate 1 (ΔCq)	NA	NA	37.43	NA	NA	NA	37.58	NA
Replicate 2 (ΔCq)	NA	NA	38.31	NA	NA	NA	38.02	NA

Table 3.2: TcdA spiked Canine faecal PLAs. Two separate PLAs were performed in duplicate and ΔCq of the values were calculated. The neat sample showed no amplification (NA) but the dilution of the sample with 1XSDB restored the working of the assay in both PLAs.

Even with this dilution the neat sample still did not amplify which was most likely due to the presence of PCR inhibitors such as bile salts and other complex polysaccharides in the faeces sample (Oikarinen et al., 2009). Therefore in order to remove any potential inhibitors of the qPCR, canine faeces sample for PLA were dialysed in the 1X TE buffer and further diluted in order to remove any leftover PCR inhibitors (methods 5.6.2). PLAs were performed using five replicates of each concentration. The results in Table 3.3 show that the dialysis and dilution of the spiked canine faeces helped to partially restore the sensitivity of the assay to the levels of the ELISA assay which has the detection of 0.5ng/mL.

TcdA Concentation	10ng/mL(Neat)	1ng/mL 1:10 of Neat	0.5ng/mL 1:20 of Neat	NPC (Neat)
Replicate 1	38.04	34.26	33.02	NA
Replicate 2	38.14	36.63	33.20	NA
Replicate 3	NA	NA	33.45	NA
Replicate 4	NA	NA	33.38	NA
Replicate 5	NA	NA	33.28	NA

Table 3.3: Result for TcdA spiked canine faecal PLA with dialysis and dilution of the sample. Five replicates of each concentration (10ng/mL (Neat: no dilution), 1ng/mL(1:10 dilution) and 0.5ng/mL (1:20 dilution)) were used. Dialysis of faecal samples resulted in restoring the sensitivity of PLA with the spiked faecal sample.

Although dialysis and dilution of the spiked canine faeces helped to partially restore the sensitivity of the assay this additional step increased both the time of the assay by 24 hours as well as its complexity (with additional dialysis), which would limit its use as a diagnostic test. Therefore, to overcome the inhibitory elements in the spiked faecal sample the Perfecta qPCR Toughmix (Quanta) was used to perform the qPCR step of the PLA instead of the ABI mastermix (Life Technologies, USA). The Perfecta qPCR Toughmix (Quanta) contains additives which prevent inhibition of PCR by common PCR inhibitors.

Initially, PLAs were performed using purified TcdA toxin. Figure 21 shows that replacing the ABI mastermix with Perfecta qPCR master mix did not change the LOD of the assay and gave the similar results as the ABI mastermix when performed with the purified TcdA. Interestingly, Perfecta qPCR Toughmix did not allow amplification for the NPCs which shows that Toughmix inhibits the non-specific ligation of the free oligonucleotides in the NPC whereas background ligation was seen in the case of PLA using ABI mastermix.

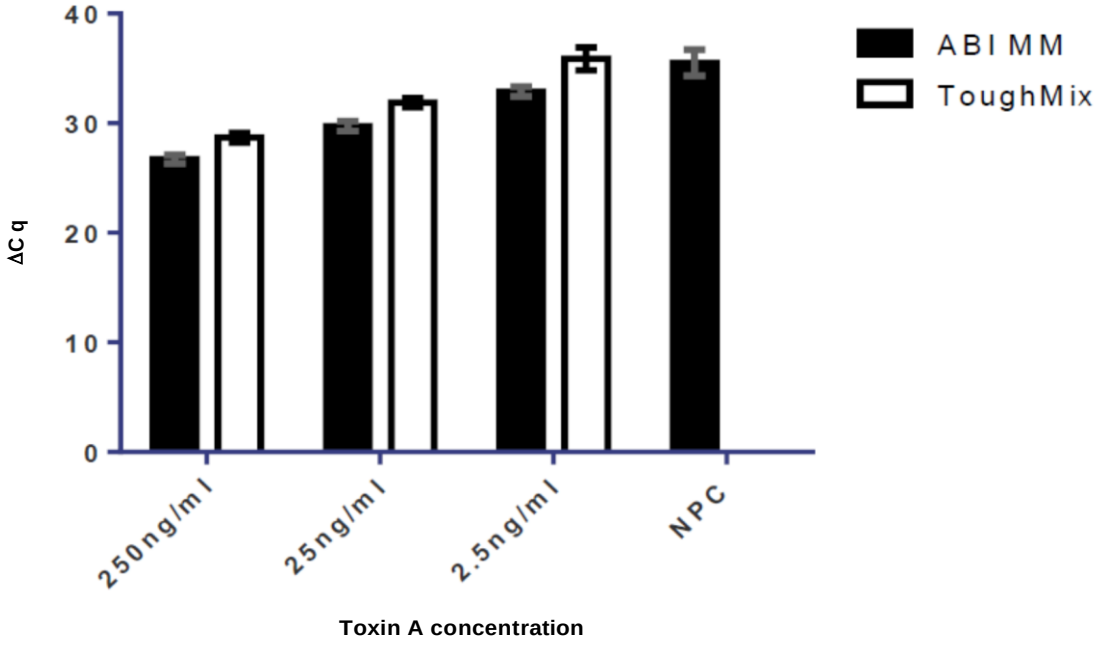


Figure 21: Purified TcdA specific PLA using ABI mastermix and Perfecta qPCR Toughmix. Quantification cycle (Cq) obtained at each concentration of purified TcdA is plotted. PLA was carried out in duplicate. The error bars shows the standard deviation of the three PLA replicates. The graph shows no bar for toughmix NPC because toughmix inhibits the non-specific binding of oligonucleotides. Statistical analysis shows no significant difference between the Cqs of the PLA using ABI mastermix and Perfecta ToughMix (p-value = 0.8571 and n=3. T-test (Mann-Whitney U)).

These results showed that Perfecta qPCR Toughmix worked successfully in the PLA using purified toxin. Therefore, further PLAs was carried out using canine faeces sample spiked with the purified TcdA and TcdB giving a final concentration of 250ng/mL. The spiked sample was further diluted to 25 and 2.5ng/mL and 0.625ng/mL in 1X SDB. The non-spiked faecal sample was also used with the NPC (method section 5.6.3). Two replicate of PLAs were carried out in duplicate using the above-said concentration for both TcdA and TcdB spiked sample. The results in Table 3.4 show that the dilution of the sample in 1X SDB and performing PLA with Toughmix overcame the inhibitors in the faecal sample. The 250ng/mL (neat) for both TcdA and TcdB did not show any amplification, but the further dilution of the sample showed the amplification of the samples giving the LOD of 0.625ng/mL for both TcdA and TcdB spiked canine faeces. Finally, the use of Toughmix partially restores the partial sensitivity of the assay without requiring dialysis thus reduces the time and complexity of the assay.

Canine Faeces	ΔCq 250ng/mL	ΔCq 25ng/mL	ΔCq 2.5ng/mL	0.625ng/mL
TcdB Concentration	250ng/mL(NEAT)	25ng/mL 1:10 of neat	2.5ng/mL 1:10 of 25ng/mL	0.625ng/mL 1:5 of 25ng/mL
PLA Replicate 1	NA	4.97	2.67	1.09
PLA Replicate 2	NA	4.2	1.27	0.62

Canine Faeces	ΔCq 250ng/mL	ΔCq 25ng/mL	ΔCq 2.5ng/mL	0.625ng/mL
TcdA Concentration	250ng/mL(NEAT)	25ng/mL 1:10 of neat	2.5ng/mL 1:10 of 25ng/mL	0.625ng/mL 1:5 of 25ng/mL
PLA Replicate 1	NA	4.73	1.25	1.65
PLA Replicate 2	NA	4.7	3.06	1.04

Table 3.4: Two independent PLAs were carried out in duplicate with Perfecta qPCR Toughmix using canine faeces sample spiked with TcdA and TcdB antigen at the concentration of 250ng/mL. The spiked sample was further diluted in 1X SDB to the concentration of 25ng/mL, 2.5ng/mL and 0.625ng/mL along with their NPCs. The neat 250ng/mL of the sample showed no amplification (NA) for both TcdA and TcdB. But dilution of the faecal sample in 1xSDB shows the ΔCq range of concentration samples used in the PLA for TcdA spiked canine faeces and TcdB spiked canine faeces.

6.4 Validation of *C. difficile* TcdA and TcdB specific PLA using clinical faeces and swab sample

After the successful validation of the PLA using the TcdA and TcdB spiked canine faeces sample using the perfecta Toughmix, the validation of the assay was done on the *C. difficile* positive human faecal and swab samples. The *C. difficile* human faecal and swab sample was used as a negative control which was tested negative by PCR detection method. The control qPCR-PLA was also performed along with the clinical faeces and swab samples using purified toxins and NPCs with 1XSDB in order to test the working of PLA using standard purified toxins.

6.4.1 PLA using clinical faecal samples

In total, six *C. difficile* positive human faecal samples were tested for the presence of both TcdA and TcdB using PLA. The faecal samples were prepared as shown in methods section 5.7.1 and PLA was performed in duplicates for each faecal sample using perfecta qPCR Toughmix as shown in methods section 5.7.3. The results in Figure 22 showed that the PLA performed to detect the presence of TcdA in the human faecal sample diluted to 1:10 and 1:100 had amplified later than the negative human faecal sample (background ligation) diluted to 1:10 and 1:100 respectively. Therefore, higher background ligation gave the lower Cq values as compared to the positive sample. The ΔCqs ($Cq_{NPC} – Cq_{Pos}$) for each sample was calculated against the NPC faecal sample. The majority of the samples gave negative ΔCq values which suggested the low sensitivity of the assay. The positive sample 1, 2 and 6 amplified earlier then the NPCs (background ligation) giving the ΔCq value less than 1, but this was not significantly different to prove the sample to be positive. The ΔCq for the control PLA was 9.1 which showed that the qPCR-PLA using purified TcdA (250ng/mL) and NPC with 1X SDB has worked with the same sensitivity as before. The statistical analysis gave no significant difference between the mean Cqs of the each TcdA positive faecal sample (1:10) and NPC (1:10) dilution (p=0.569, n=6, Mann-Whitney U) and TcdA positive faecal sample 1:100 and NPC (1:100) dilution (p= >0.999, n=6, Mann-Whitney U).

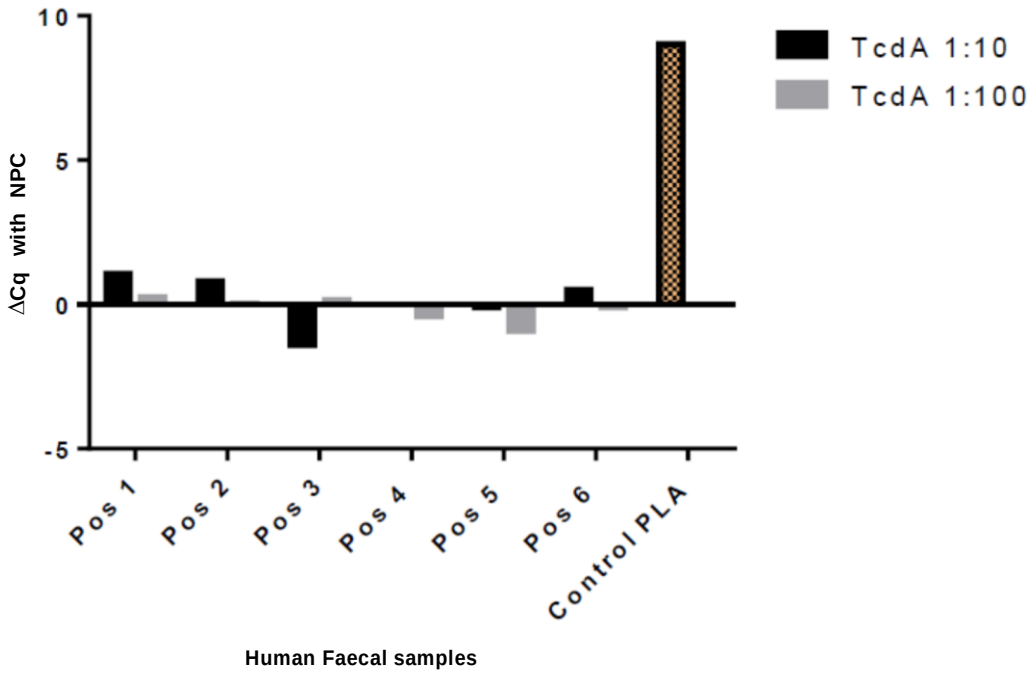


Figure 22: TcdA specific PLA using clinical faecal samples: The ΔCqs for TcdA PLA of 1:10 dilution of positive human faecal sample (dark boxes) calculated against the 1:10 of NPC (p=0.569, n=6, Mann-Whitney U). The ΔCqs of 1:100 dilution of positive human faecal sample (light boxes) calculated against the 1:100 of NPC (p= >0.999, n=6, Mann-Whitney U). The orange bar shows the control PLA performed to test the working of PLA using purified TcdA (250ng/mL) and NPC using 1XSDB with ΔCq 7.8. Pos 1 -6 are referred to as C. *difficile* positive sample.

Figure 23 showed that the results for the detection of TcdB in the positive human faecal samples were similar to the TcdA PLA. The ΔCqs for the all the positive faecal samples diluted to 1:10 and 1:100 dilutions were calculated against the negative faecal sample 1:10 and 1:100 respectively. The NPCs (background ligation) amplified earlier than the positive faecal samples thus gave negative ΔCqs, again suggesting the poor sensitivity of the PLA assay with TcdB. The statistical analysis showed no significant difference between the mean Cqs of the each TcdB positive faecal sample (1:10) and NPC (1:10) dilution ($p=0.156$, $n=6$, Mann-Whitney U) and TcdB positive faecal sample 1:100 and NPC (1:100) dilution ($p= 0.081$, $n=6$, Mann-Whitney U).

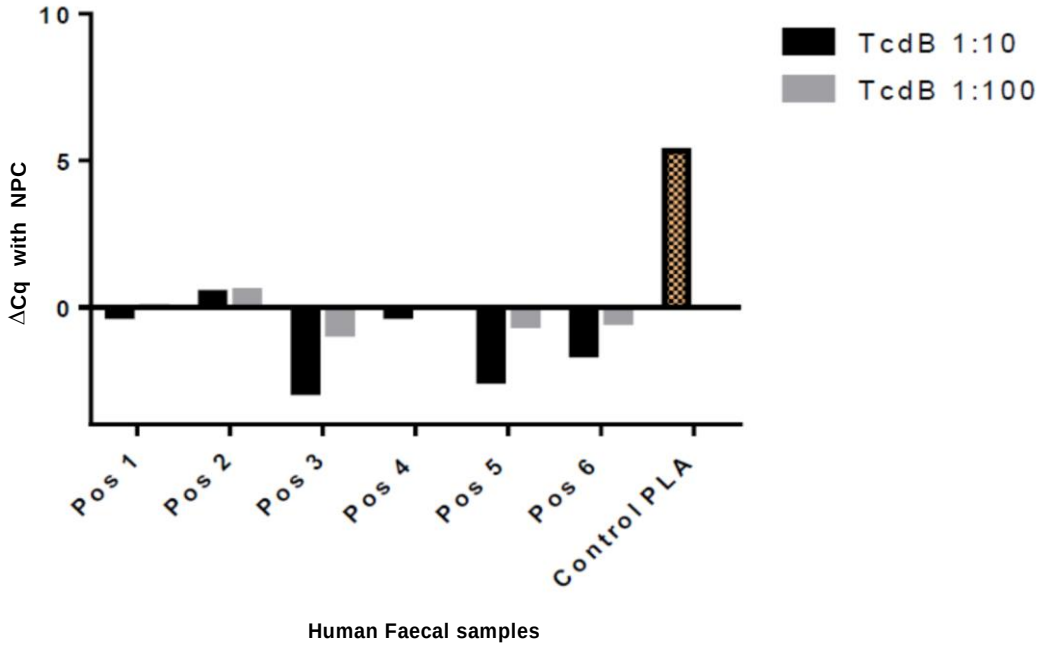


Figure 23: TcdB specific PLA using clinical faecal sample: The ΔCqs for TcdB PLA of 1:10 dilution of positive human faecal sample (black bar) calculated against the 1:10 of NPC (p=0.156, n=6, Mann-Whitney U). The ΔCqs of 1:100 dilution of positive human faecal sample (black bar) calculated against the 1:100 of NPC (p= 0.081, n=6, Mann-Whitney U). The orange bar shows the control PLA performed to test the working of PLA using purified TcdB (250ng/mL) and NPC using 1XSDB with ΔCq of 5.03. Pos 1 -6 are referred to as C. *difficile* positive sample

6.4.2 PLA using clinical swab samples

The *C. difficile* positive human swab samples were also tested using PLA for TcdA and TcdB. In total, there were 13 swab samples which were prepared as shown in the method section 5.7.1 and PLA was performed in duplicates with Perfecta qPCR Toughmix using thermal conditions as shown in the methods section 5.7.4.

Figure 24 shows the PLA results for the detection of TcdA in a neat human swab sample and 1:10 dilution of the human swab sample. The ΔCqs for the all the positive swab samples neat and 1:10 dilutions were calculated against the negative swab sample neat and 1:10 dilutions respectively. As the majority of the swabs samples, neat or 1:10 dilution had amplified later than their respective negative swab sample, this shows that the assay has worked with poor sensitivity giving negative ΔCq values due to higher background ligation. Although neat swab samples 4 and 10 gave the ΔCq values of 1 and 1.02 respectively but this was not significantly different to prove the sample to be positive. Cq values of the positive samples and NPCs were significantly different when compared statistically ($p=0.0216$ (neat), $p= 0.0001$ (1:10), $n=13$) due to higher background ligation and early amplification of the NPCs as compared to the positive swab samples which amplified very late due to inhibition in the samples.

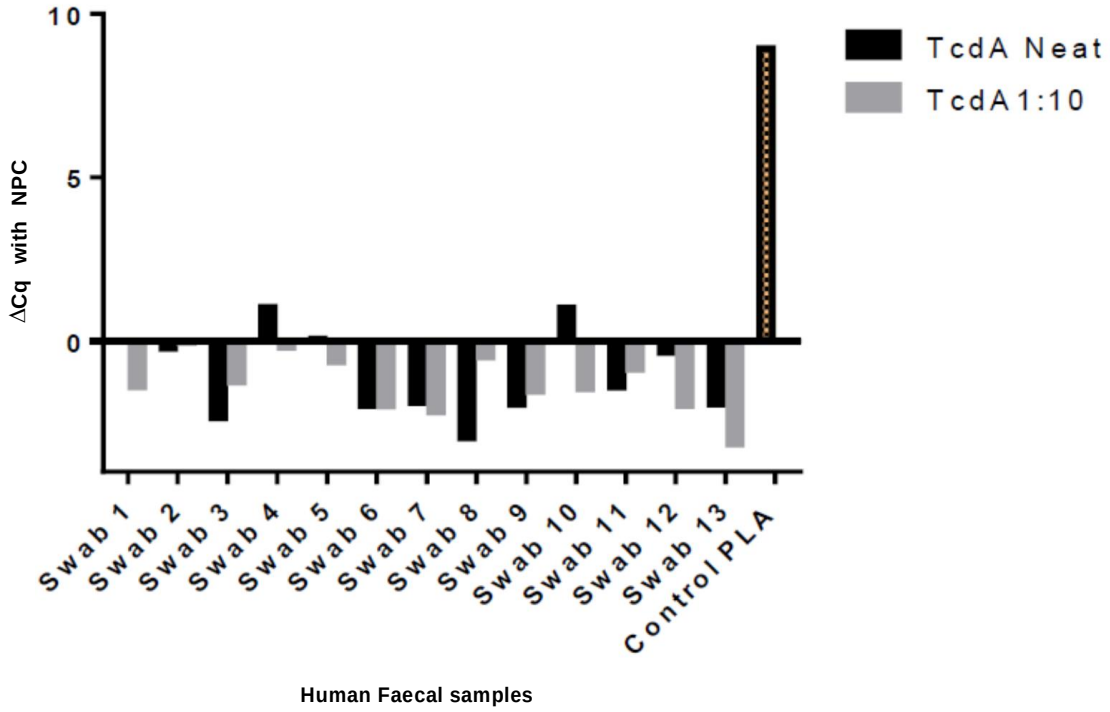


Figure 24: TcdA specific PLA using clinical swab samples : The ΔCqs for TcdA PLA of neat positive human swab sample (black bar) calculated against the neat NPC or negative swab sample (p=0.0216, n=13 , Mann-Whitney U). The ΔCqs of 1:10 dilution of positive human swab sample (black bar) calculated against the 1:10 of NPC (p=.0001, n=13, Mann-Whitney U). The orange bar shows the control PLA performed to test the working of PLA using purified TcdA (250ng/mL) and NPC using 1XSDB with ΔCq 8.9.

Figure 25 shows the ΔCqs of TcdB specific PLA for 13 swab samples, neat and 1:10 dilution against the NPC neat and NPCs 1:10 respectively. The results showed that 6 out of the 13 neat swab samples have ΔCq value as negative showing the poor sensitivity of the assay due to higher background ligation in NPCs and late amplification of the positive sample due to the inhibition of the PCR reaction. The remaining 7 samples gave the ΔCq values less than 1, thus showing no significant difference to prove the samples to be positive. Moreover, when Mann-Whitney U test was performed on the data, no significant difference was seen between the neat positive swab samples and Neat negative swab sample ($p=0.0791$, $n=13$).

On the other hand, ΔCq values for the 1:10 dilution of the positive swab samples against negative sample were calculated. The results in figure 25 showed ΔCq value for entire 13 samples to be negative due to early amplification of the NPCs as compared to the positive sample($p=0.0001$, $n=13$, Mann-Whitney U). This result shows that inhibition in the positive samples and high background ligation had led to the poor sensitivity of the assay.

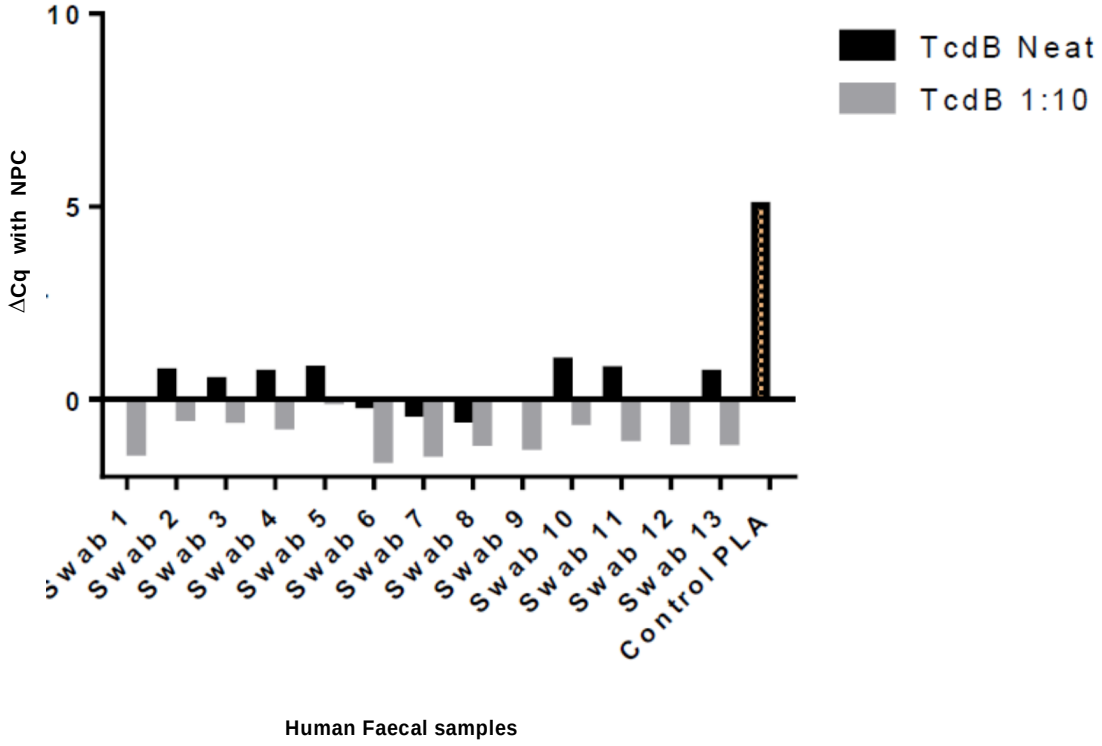


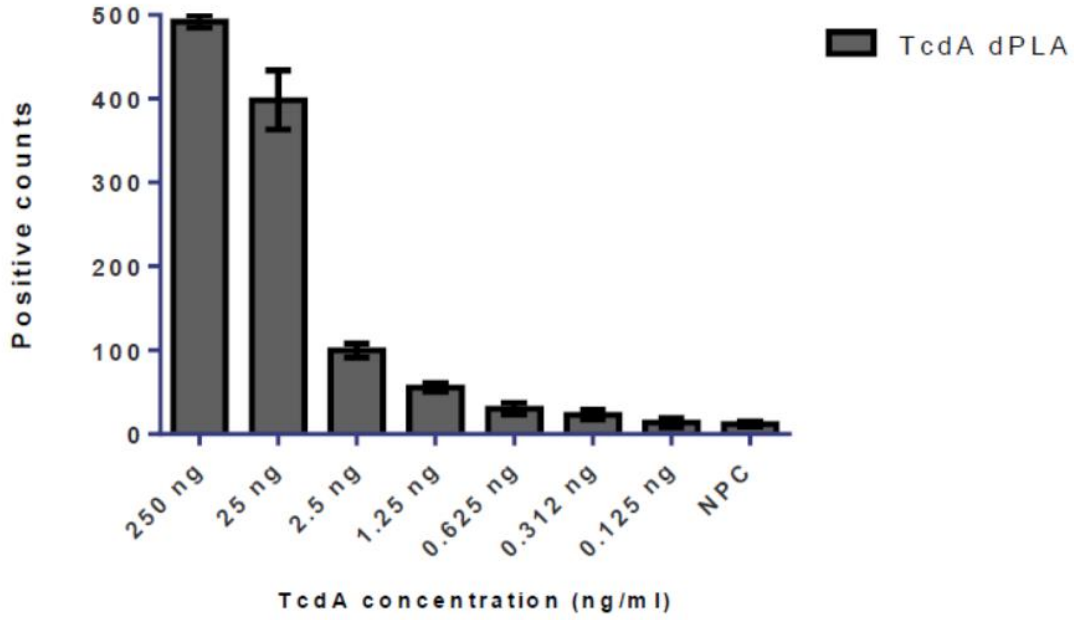
Figure 25: TcdB specific PLA using clinical swab sample: The ΔCqs for TcdB PLA of neat positive human swab sample (dark boxes) calculated against the neat NPC or negative swab sample (p=0.0791, n=3, Mann-Whitney U). The ΔCqs of 1:10 dilution of positive human swab sample (light boxes) calculated against the 1:10 of NPC ((p=0.0001, n=13, Mann-Whitney U). The orange bar shows the control PLA performed to test the working of PLA using purified TcdB (250ng/mL) and NPC using 1XSDB with ΔCq 5.03.

6.5 Digital PLA specific to *C. difficile* TcdA and TcdB

Digital PCR (dPLA) is a precise readout method alternate to qPCR. The dPCR helps in determining absolute copy numbers, it is highly tolerant to complex inhibitors and the results do not reply on standards or references (Pohl and Shih, 2004). PLA was performed using the Formulatrix dPCR instrument as an alternative readout method to the qPCR-based PLA.

Initially, dPLA was carried out using purified TcdA in five replicates with range of concentration of 250, 25, 2.5, 1.25, 0.625, 0.312, and 0.125 (ng/mL). At the same time, the same set of reagents were used to perform the same PLA on the qPCR machine (CFX qPCR), in order to check and compare the LOD of the experiments. Figure 26 shows that the dPLA produced the same LOD of 0.125ng/mL as the qPCR PLA targeting purified TcdA. Once working of dPLA was confirmed with TcdA, the repeatability of a PLA assay was tested. Three independent PLAs targeting TcdA were analysed in duplicate by dPCR and the results in figure 27 indicated that it is possible to obtain the precise quantification of the copy numbers of ligated PLA probes. Average copy numbers at 0.6ng were 70 (range 65-75), at 0.3ng 49 (range 35-49) and NPCs 14 (range 11-17). Coefficients of variation were 5.9%, 10.8% and 19.2%, respectively; suggesting that quantification by dPLA has the potential to be more precise and robust than qPCR-based PLA. Figure 28 shows that when a comparison between dPLA and qPCR-based PLA at the lowest concentration of antigen tested (0.1 ng/mL) was performed the lowest levels of detection were similar, with the lowest limits probably determined by ligation efficiencies.

A



B

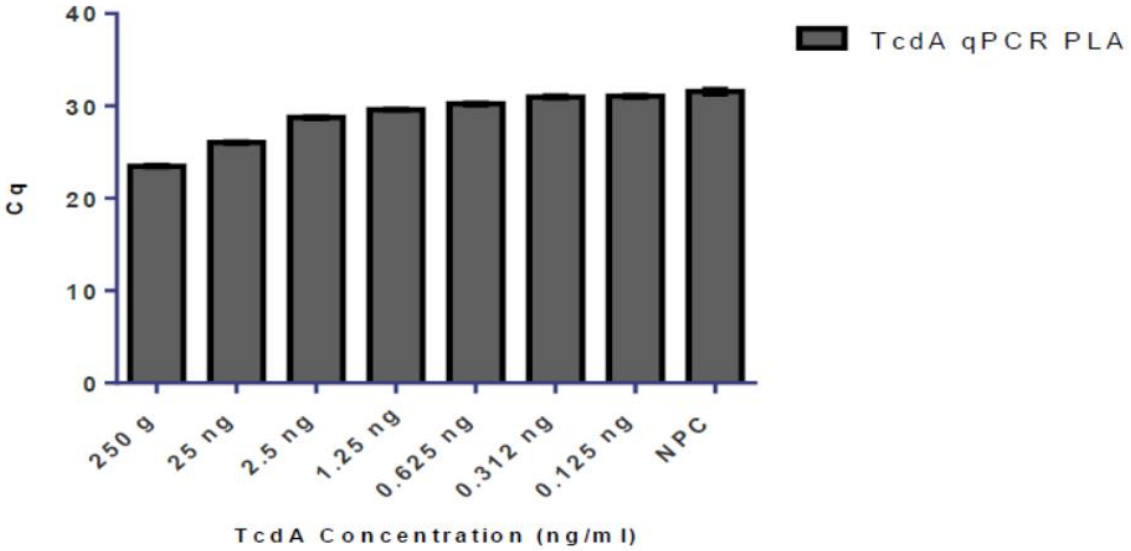


Figure 26: TcdA qPCR PLA vs TcdA dPLA: A: dPLA was carried out using purified TcdA in five replicates with the range of concentration of 250, 25, 2.5, 1.25, 0.625, 0.312, and 0.125 (ng/mL) NPC (as no protein control or negative control) and positive count shows the number of ligation events in each PLA. **B:** qPCR PLA was also carried out using purified TcdA using the same reagent mix and the same range of concentration in order test the working of PLA using dPCR setup. Error bar demonstrates the standard deviation.

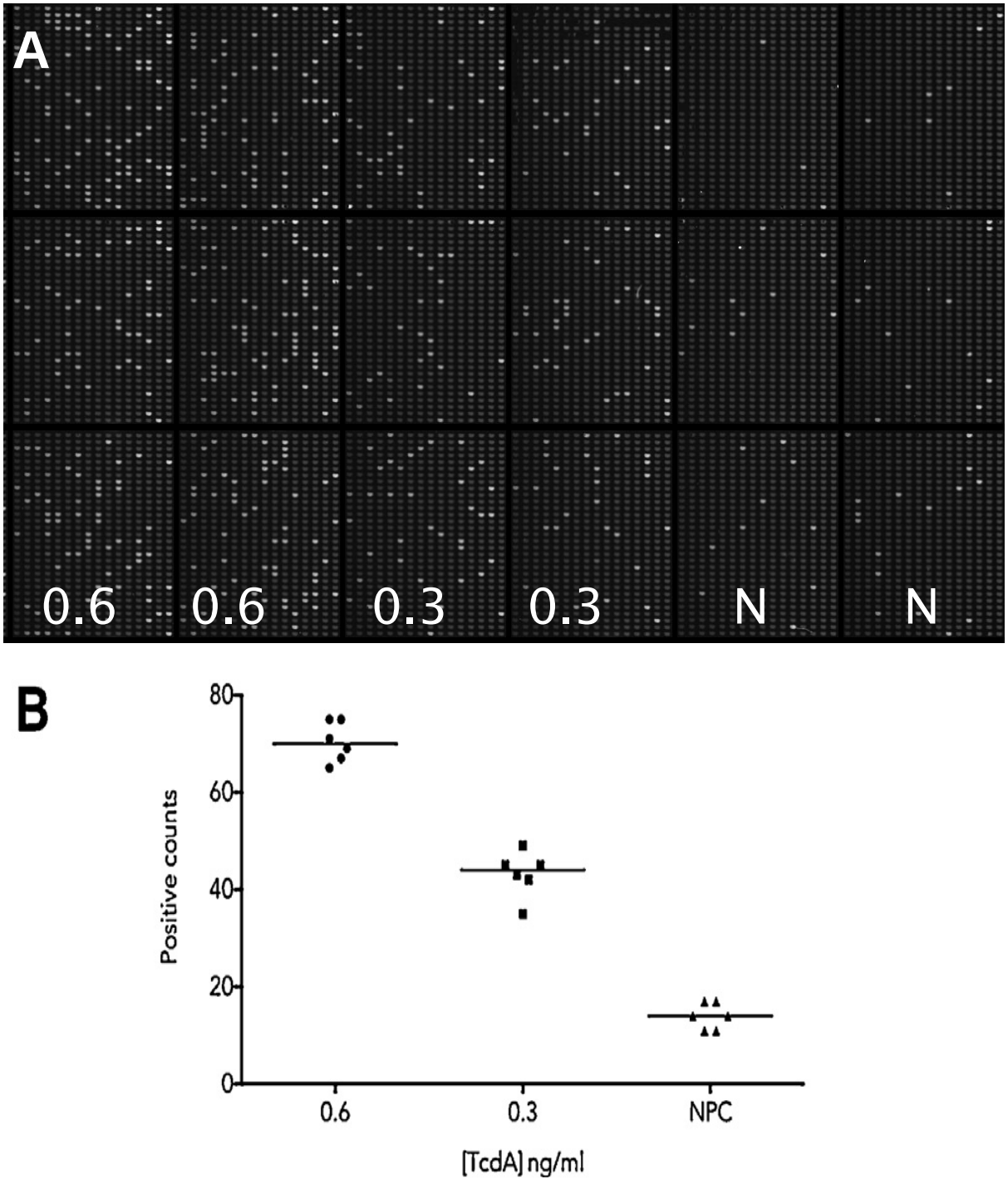


Figure 27: dPLA assay targeting TcdA pure toxin: (A) Each row of the screen image corresponds to an independent PLA, carried out in duplicate at each concentration of antigen and no protein control (NPC). **(B)** The counts are shown in the graph, indicating the median counts.

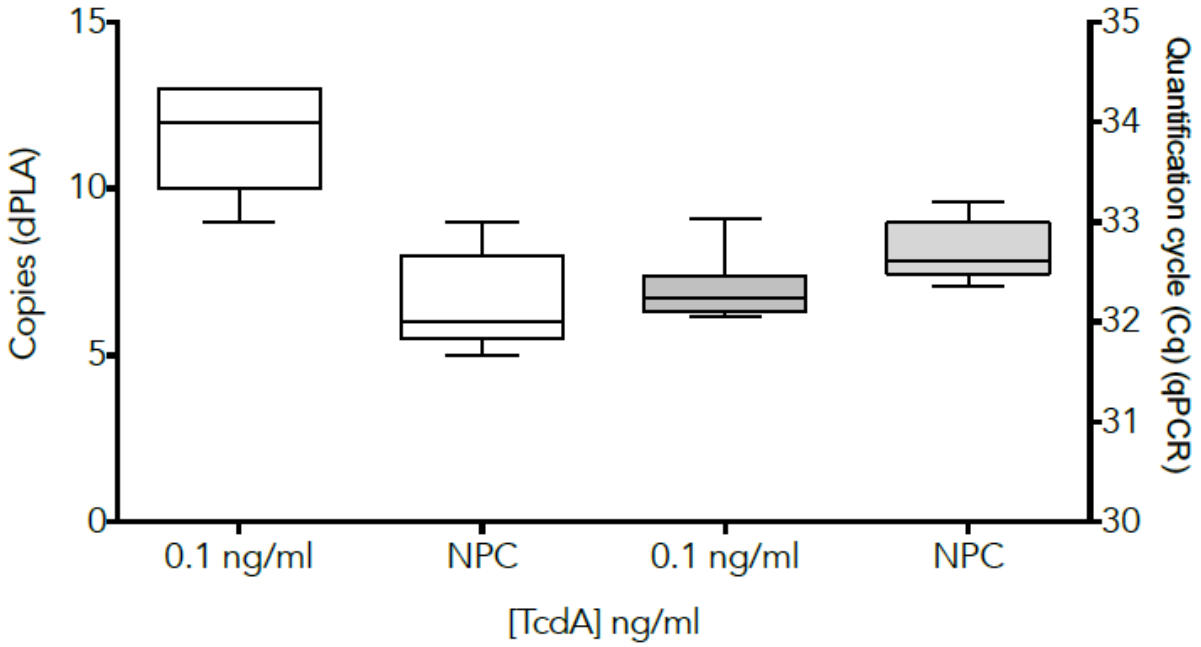


Figure 28: Comparison of dPLA and PLA at the LOD: Copies (PLA) or Cqs (PLA) obtained by diluting TcD to 0.1ng/mL was compared to the no protein controls (NPC). Nine independent PLA reactions were amplified in duplicate either using dPCR (indicated by white bars) or qPCR (indicated by grey bars) and the resulting average differences in copy numbers (dPLA) or Cqs (qPCR) for each PLA are shown.

Similarly, four independent dPLAs targeting using purified TcdB were also performed in five replicates with range of concentration of 2.5, 1.25, 0.625, 0.312 (ng/mL) using the combination of Bp3m5 probes (as used for qPCR PLA for TcdB). The result in Figure 19 shows that TcdB PLA assay did not work with the dPLA set up. Average copy numbers at 2.5 ng/mL were 8 (range 4-13), at 1.25ng/mL were 6 (range 1-12), at 0.625ng/mL were 5 (range 2-9), 0.312ng/mL were 3 (range 2-7) and NPCs were 5 (range 0-13). Due to the uneven positive counts, large error bars can be seen and which makes it difficult to differentiate the LOD of the assay. Finally, the combined results for the TcdA and TcdB dPLA shows that TCdA specific dPLA shows similar sensitivity as the standard qPCR PLA but TcdB specific PLA does not work with the dPLA set up.

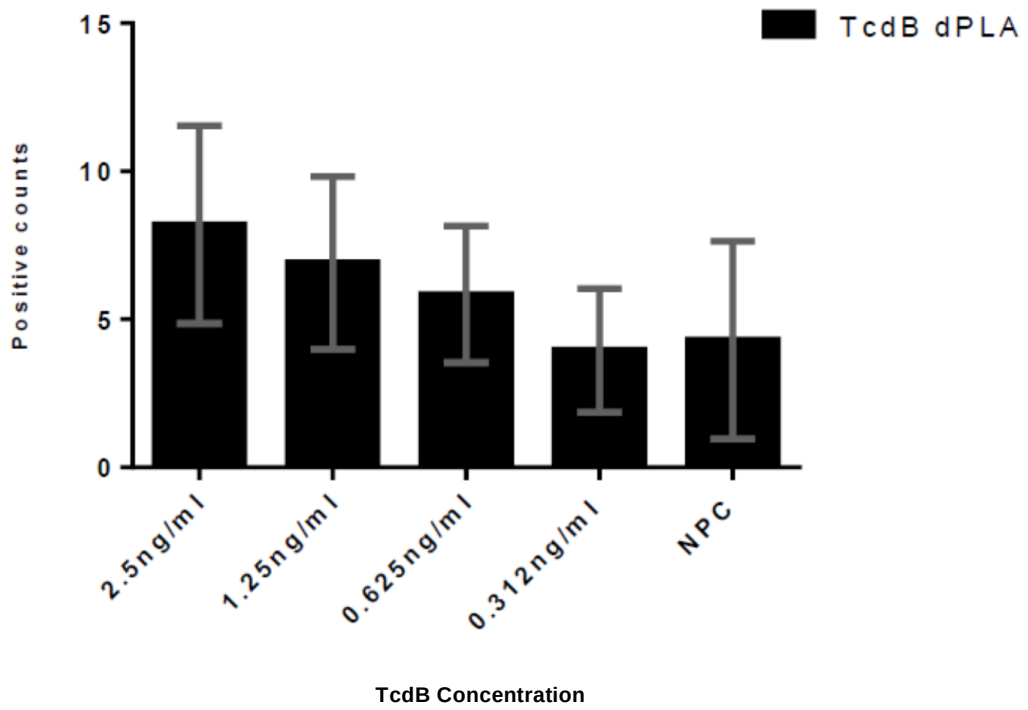


Figure 29: TcdB specific dPLAFour independent: dPLAs targeting purified TcdB were also performed in five replicates with the range of concentration of 2.5, 1.25, 0.625, and 0.312 (ng/mL) using the combination of Bp3m5 probes. The counts are shown in the graph, with the vertical bar indicating the median counts.

Chapter 4

General Discussion

7.0 Discussion & Conclusion

C. difficile-associated infection (CDI) particularly in hospital patients (Shah et al., 2010) has led to increase in mortality and morbidity rate in US, UK and Europe mainly due to elevated level of two main virulence factors TcdA and TcdB and also due to the limitations of the current CDI diagnostic in terms of sensitivity, specificity or sometime time required for diagnosis. PCR and antibody-based methodologies used in CDI diagnostic assay have their distinct advantages and disadvantages: PCR assays are sensitive and easy to develop but detection of DNA does not prove the presence of the viable and infectious pathogen (Platts-Mills, Liu and Houpt, 2013). For instance, there are asymptomatic strains of *C. difficile* that do not produce either of the toxins but they are colonised in the patient, thus, PCR detection of the asymptomatic *C. difficile* colonisation can lead to unnecessary treatment of many patients. On the other hand, antibody-based diagnostic methods such as ELISA is specific but they are relatively insensitive as compared to the nucleic acid based tests (Planche et al., 2008) (Sajid, Kawde and Daud, 2014). Therefore, both of these molecular tools, qPCR and ELISA has been combined together to develop PLA which uses the sensitivity of the qPCR assay and specificity of the ELISA method. PLA has been used for wide variety of applications ranging from detection of cancer biomarkers (Zhu et al., 2006), proteins in the single cells (Stahlberg et al., 2012) and prions (Hammond et al., 2014). The use of PLA in detecting bacterial pathogens is somewhat limited. To date, there are only two studies showing the use of PLA for detection of bacterial proteins. The very first study was performed by (Gustafsdottir et al., 2006), detailing the proof of principle for the detection of bacterium *Lawsonia intracellularis* using PLA. The second publication demonstrated homogenous PLA for pathogenic detection of human pathogenic E.coli (O157:H7) (Leslie et al., 2010).

So this MPhil study shows the development of the first ever PLA for the detection of *C. difficile* bacterial toxins TcdA and TcdB. As both TcdA and TcdB are encoded by the tcdA and tcdB gene respectively showing major similarity in their structures, the criterion for the development of the PLA was the selection of purified full-length target antigen TcdA and TcdB (The Native Antigen company) and specific antibodies raised against these whole *C. difficile* toxins so that they don't cross react with each other. The validation of *C. difficile*

TcdA/ TcdB antigens and specific antibodies were performed by SDS-PAGE and western blotting, showing clear single protein bands for both commercial antigens and with expected molecular weights of ~308KDa (TcdA) and ~270KDa (TcdB). Following this validation step, an initial attempt at biotinylating the TcdA specific mAb using the APEX Biotin-XX Ab labelling kit (Invitrogen) resulted in a failed FPPT with ΔCq less than 8.5 which is an arbitrary cut-off set by Life technologies, USA. There could be three reasons for this FPPT failure: 1) the Inadequate biotinylation of the antibody, 2) longer dialysis may be required and insufficient dialysis could have also led excess or free biotin in the solution 3) low recovery of biotinylated antibody (the Apex kit indicates the recovery of biotinylated antibody is between 40- 80%). Due to the failure of FPPT using the APEX method for biotiinylation, a new biotinylation method was tried known as EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation kit (Thermo Scientific). This kit involved simple and fewer steps as compared to the Apex biotinylation kit, thus reducing the chances of manual errors during biotinylation and helps in better recovery of the biotinylated antibodies as compared to the apex method. Therefore, the use of this method led to successful biotinylation of TcdA and TcdB specific antibodies with all biotinylation exceeding the forced proximity probe quality threshold of a $\Delta Cq \geq 8.5$.

We initially targeted TcdB for PLA, since there are some TcdA-ve pathogenic strains of *C. difficile* and developed a TcdB-specific PLA using a combination of two different antibodies (anti-TcdB monoclonal antibodies (mAb) and polyclonal (pAb) antibodies). This PLA had a LOD of 2.5ng/mL; three times lower than that of an equivalent ELISA (company) which was 1.25ng/mL. Since the C-terminal end of the tcdB gene is characterised by several repeated motifs (Pruitt and Lacy, 2012b), we surmised that it might be possible to target these using a single mAb tagged with two different oligonucleotides. Hence, we tested a PLA with the purified TcdB using only the single mAb probes and were able to detect toxin LOD 0.125ng/mL. Similarly, PLA targeting TcdA was also developed and since TcdA also has multiple repeat epitopes at its C-terminal end, we hypothesised that it might also be possible to develop a sensitive PLA using a combination of single mAb (Frey and Wilkins, 1992). This approach was also successful and resulted in the highly reproducible detection of purified TcdA at 0.125ng/mL. In both the cases detection of purified TcdA and TcdB using

PLA resulted in 10 times more sensitivity than the ELISA performed in the lab with the purified toxin which has LOD of 1.25ng/mL for TcdA and TcdB.

Since there were few regulatory complications with ethics regarding the use of the human clinical sample in our lab, therefore, TcdA and TcdB specific PLAs were performed with the antigen spiked in a canine faeces sample as a model of human faecal samples. The canine faecal sample was used because there have been previous studies which showed the isolation of pathogenic *C. difficile* toxins from the diarrhoeic and non-pathogenic *C. difficile* from non-diarrhoeic dogs (Chouicha and Marks, 2006). The initial PLA was performed by spiking known concentration of purified TcdA (as mentioned in section 5.6) resulting no amplification. The most likely reason for this result could be the presence of PCR inhibitors such as bile salts and other complex polysaccharides in the faeces sample which have been shown to inhibit PCR reactions (Oikarinen et al., 2009), (Chouicha and Marks, 2006). To remove these potential inhibitors we performed dialysis and dilution of the 10ng/mL of TcdA spiked canine faeces which helped in the amplification of the spiked samples and partially restore the sensitivity of the assay giving LOD of 1ng/mL. However, this additional step increased the time and complexity of the assay, which would limit its use as a diagnostic test. Therefore, in order to eliminate the additional steps of dialysis and dilutions, an alternative mastermix was used called Perfecta qPCR toughmix (Quanta Bioscience). This Toughmix contains highly processive thermostable DNA polymerase combined with the high avidity monoclonal antibodies and this combination is highly resistant to the PCR inhibitors. Therefore, the use of this Toughmix instead of Taqman mastermix (recommended by Life Technologies) helped in reducing the inhibition caused by the PCR inhibitors in the spiked faeces sample thus restoring the sensitivity of the assay with LOD of 0.625ng/mL for both TcdA and TcdB.

The LOD of PLA compared to ELISA performed in the lab showed the 10 times more sensitivity when using purified TcdA and TcdB, however, the sensitivity of the PLA reduced to 2 times using spiked canine faeces sample for PLA and ELISA. Therefore, this suggests that sensitivity of the PLA was still inhibited by the presence of faecal inhibitors even after the dilution and use of Toughmix for the qPCR part of the PLA.

Another detection method such as *C. DIFF QUIK CHEK COMPLETE* test which is a rapid membrane test has the detection level of TcdA at 0.63ng/mL and TcdB at 0.16ng/mL in the faecal sample. When the sensitivity of this test was compared to PLA, this shows that PLA has the similar LOD for TcdA specific PLA but was 4 more times less sensitive for TcdB PLA when using the spiked canine faecal sample. Although the *C. DIFF QUIK CHEK COMPLETE* test is better than PLA in term of sensitivity but this method is not specific as it cross-reacts with isolates of *C. sordelli* (according to manufacture specification) which is not the case in *C. difficile* TcdA and TcdB specific PLAs making PLA better than this particular test.

After the development of TcdA and TcdB specific PLA, we developed a duplex PLA targeting both purified toxins in a single PLA assay. The results of the duplex assay were similar to the singleplex assay giving a LOD of 0.12ng/mL with good repeatability. However, one of the limitation in this PLA assay was that using a TaqMan protein kit uses only single fluorophore (FAM) as the marker for PCR detection, therefore, it is incapable of distinguishing between two targets (TcdA and TcdB) in real time amplification but more importantly this result shows that we can see more reliable and robust detection of the toxins at the very low concentration of 0.12ng/mL. The next stage of the research could be to design own 3' and 5' oligonucleotides attached with two different fluorophore that are capable of distinguishing between TcdA and TcdB in real time. As TcdB is approx. 1000 fold more toxic than TcdA (Sun, Savidge and Feng, 2010) and TcdB positive isolates appear to be on increase development of this duplex assay will help in providing the diagnostic information which will help in treating the CDI patients accordingly.

Along with sensitivity and specificity of an assay, early detection of an antigen is also an important factor in a good diagnostic assay. Currently, proximity assay cannot be completed in less than ~2.5 hours, mainly because binding of proximity probes and antigen requires an hour of incubation for maximum sensitivity, the ligation step takes 10 minutes and the use of hydrolysis probes requires minimum extension time for the PCR reaction (~1 hour in CFX). Hence, we focused on reducing the time taken to complete the PCR step of PLA from 65 minutes in CFX qPCR machine to 45 minutes which was achieved using Illumina PCRMax Eco48 using same thermal conditions. The reduction of 20 minutes in the qPCR reaction of the PLA was seen which is very important and useful for this type of assay. Hence, the

overall time taken to complete the PLA has now reduced from 2.5 hours to less than 2 hours without compromising the sensitivity and specificity of an assay which is very important. The PLA now give results in approximately less than half the time of the *C. difficile* TcdA/TcdB ELISA which takes ~3.5 hours to give the results. Although there is a rapid membrane test known as *C. DIFF QUIK CHEK COMPLETE* test which can give results in 30 minutes but it cross reacts with other strains of *clostridium* test making it less specific than PLA. The speed of the PLA can be further increased by many potential methods such as by reducing hybridisation time with molecular crowding, use of faster chemistries such as scorpions and developing the mastermix which can be designed to work with minimal activation and annealing/polymerisation.

Once the validation of the PLA was successful in the TcdA and TcdB spiked canine faeces sample using perfecta Toughmix, the assay was then validated with the CDI infected human faeces and swab samples in Public Health England, London, UK. Initially, the CDI positive human faecal samples were tested and in order to remove the PCR inhibitors from the faecal samples, we diluted the neat sample and performed the PLA-specific to TcdA and TcdB on the diluted samples. The assay worked with poor sensitivity for both TcdA and TcdB specific PLA because of early amplification of negative control than the positive faecal samples. Similar results with poor sensitivity were also seen when TcdA and TcdB specific PLAs were performed on the CDI positive human swab samples. The possible reason for these results may be presence of high PCR inhibitors in the human faecal samples which inhibited the assay, even after pre-treatment of the faecal sample by dilution and use of Toughmix. But, the dilution of the faecal sample as the pre-treatment method had been successful before in the case of detection of *L. interacellularis* in the pig faeces using homogenous PLA with high sensitivity (Gustafsdottir et al., 2006). Moreover, homogenous PLA for the detection of invasive aspergillosis had also been successfully developed in our lab which used broncho-alveolar lavage (BAL) fluid samples from the patients. The dilution of BAL fluid sample in 1XPBS overcame the inhibition for the BAL sample for detection of aspergillosis and showed 1000X greater sensitivity than the current lateral flow device for the detection of invasive aspergillosis. On the other hand, the dilution of the BAL fluid did not largely affected the fungal load in the sample as there was enough target antigen to detect with high sensitivity but this was not in the case of *C. difficile* PLA. Therefore, the

other probable reason for poor sensitivity of the assay could be due to the presence of fewer toxins in the sample due to degradation of toxin because of protease (Chouicha and Marks, 2006) (Corthier et al., 1989), which were undetectable by PLA setup or possibly the target was lost when the samples were diluted in the 1X SDB to remove the inhibitors in the faeces and swabs. Although, the dilution of the sample as these pre-treatment methods had been successful in the case of detection of invasive aspergillosis in our lab but this pre-treatment methods did not help in minimising the PCR inhibitors for *C. difficile.* Therefore, alternative pre-treatment methods should be performed such as heat treatment of the faecal sample, treatment of the faeces with bovine serum albumin (BSA) and use of single stranded DNA binding T4 gene 32 proteins (gp32) for reduction of the PCR inhibitors in the faeces sample (Schrader et al., 2012). The use of the pre-treatment method other than dilution method might increase the time of the assay but hands on time for the assay will still be less than the current diagnostic methods for detection of *C. difficile* toxins such as ELISA which involved high hands on time due to multiple wash steps. Finally, the successful treatment of the faecal sample could make *C. difficile* specific PLA a promising assay to be used in the clinical setup with high sensitivity and specificity.

The stability of the *C. difficile* specific antibodies used in the PLA could be another important factor which affects the sensitivity of the PLA when using human faecal samples. The antibodies used in this assay works fine when used with purified toxins and canine faecal samples (as seen in the results chapter), but might not be stable and ideal for the *C. difficile* PLA when using human faecal and swab samples. In order to develop the sensitive and specific *C. difficile* PLA for the clinical samples different set of mAbs can be used or new antibodies can be bought from the different manufacturer. Since, the generation of own new *C. difficile* antibodies is difficult, as it requires biological system, moreover, the activity of the antibody varies from batch to batch; therefore, use of aptamers are the suitable alternative for developing the PLA-specific to *C. difficile* toxins. Despite having similar functions to antibodies, the aptamers have many advantages over antibodies which can help in developing highly sensitive and specific *C. difficile* PLA. The advantages of aptamers includes, uniform activity regardless of the batch synthesis, they bind there target with specificity and affinity comparable to the monoclonal antibodies, they are non-immunogenic although they are difficult to synthesise but once prepared they have

unlimited shelf life unlike the antibodies who have limited shelf-life (Toh et al., 2015). Moreover, as aptamers are nucleic acid, they can be easily labelled and linked to the linkers, reporter molecules and other functional groups which is beneficial for the development of probes for the PLA (Luzi et al., 2003). Finally, the other main issue remains the detection of the background caused by ligation of non-interacting antibodies in the NPCs, which impedes achieving the sensitivity of an assay with clinical samples.

We have also developed a first digital PLA (dPLA) protocol using digital PCR (dPCR) as a readout method for TcdA and TcdB as an alternative to qPCR. The advantages of dPCR are that it provides more precision by determining absolute copy numbers, it is highly tolerant to complex inhibitors and the results do not reply on standards or references (Pohl and Shih, 2004). Therefore, we used, the Formulatrix dPCR instrument for the PLA. It uses a simple platform with physical partitions in order to count actual ligation events for each PLA and results in an easy to understand readout of the copy numbers. The quantification of the PLA using dPCR did give similar sensitivity and LOD for TcdA with a quantification limit of 0.312ng/mL with canine faeces sample and using Taqman mastermix (recommended by Life Technologies). This showed that although the dPLA had a similar LOD as qPCR-PLA for TcdB, the use of dPLA overcame the inhibition caused by the bile salt and other complex inhibitors in the toxin spiked canine sample as the superior master mix was not used.

But when the similar set up was used for performing TcdB specific dPLA, the assay did not work similar to the sensitivity and LOD as of TcdB qPCR PLA. This may be due to the nature of the TcdB assay or the probe set up, but further steps need to be taken to optimise TcdB dPLA assay. Moreover, as the dPLA counts the actual ligation events therefore, the problem of background ligation was still seen in the dPLA in both TcdA and TcdB dPLA assay thus affecting the sensitivity of the assay.

In summary, we have developed the first PLA-specific to *C. difficile* TcdA and TcdB combining the specificity of antibody-based assay with sensitivity and dynamic range of the qPCR. As the results for *C. difficile* PLA above suggests, PLA is more specific than commercial immunoassay and has an ability to be more sensitive than nucleic-acid based tests. The potential for development of duplex assay adds further advantage to the specificity of the

assay. Finally, we also established the first digital PLA for *C. difficile* diagnosis, with initial results suggesting that it reliable, reproducible and show similar sensitivity for detection of TcdA antigen when using qPCR. However, in order gain the maximum sensitivity further optimisation is required to overcome the inhibition caused by the faecal inhibitors in the clinical sample and also decreasing the detection of background caused by ligation of non-interacting antibodies in the NPC, which hampers achieving maximum sensitivity.

8.0 Future Directions

This study has led to the development of a PLA-based diagnostic test for the detection of *C. difficile* TcdA and TcdB. However, future studies should be performed in order to optimise and improve several factors to create the diagnostic kit that can be used in the clinical laboratories. Most importantly, unlike a nucleic acid-based test, non-specific ligation of the oligonucleotides in the absence of antigen always results in the detection of a background signal which is the main drawback of PLA. The background signal can be minimised by optimising certain components in the PLA which may include an optimising choice of probe and ligase concentrations, reaction times and PCR conditions which may help in increasing the performance of PLA. The concentration of antibody reagents can be reduced which keeps the assay background very low, thus reducing the chance of proximity in the absence of target.

Increasing the concentration of the connector oligonucleotides in the PLA reaction can also help in reducing the background signal by hybridising the unbound probe which is not in close proximity to one connector each thus stop the to undergo ligation (Gustafsdottir et al., 2006).

The use of asymmetric connector hybridization model in PLA can also help in reducing the background ligation thus increasing the sensitivity and dynamic range of the assay. In this model, the affinity of one side of the connector or splint towards the antibody or aptamer is weakened which ultimately reduce the non-target specific ligation (background noise) without affecting the target specific ligation (Kim et al., 2010). Therefore adopting the similar model in the *C. difficile* TcdA and TcdB may help in solving the issue of background ligation.

The use of alternative PLA methods such as *in situ* and solid phase PLA may also minimise the problem of background signal. The extensive washing steps involved in the solid phase PLA can also help in solving the problem of background ligation by removing the free and unbound probes thus enhance sensitivity owing to the reduce background ligation.

Although the PCR inhibition in the TcdA/TcdB spiked canine faeces was reduced by dilution and use of perfecta Toughmix the CDI positive clinical samples performed very poorly. Therefore, the alternate method for removing the PCR inhibitors in the human faecal sample is the development of solid phase PLA, which shares the properties of the classic sandwich immunoassay. In this method, the target specific biotinylated antibody linked to the magnetic bead is captured on the solid support to which the target antigen binds followed by washing steps which allegedly remove any unbound antigen and other inhibitors in the faeces sample. This complex of the target antigen and the antibody is then detected by 3' and 5' probes during incubation followed by washing to remove the unbound probes. Finally, the ligation step following qPCR assay is done. Despite the fact that this assay is more complex and involves more step but it can be a promising assay for the detection of the TcdA and TcdB in the faecal sample full of PCR inhibitors.

The limitation of the duplex PLA assay we have developed can be solved by designing new 3' and 5' oligonucleotide which will be attached with the different fluorophores replacing the oligonucleotide supplied in the Taqman kit from life technologies. Using two different fluorophores can help in the development of the duplex PLA for *C. difficile* TcdA and TcdB capable of distinguishing the two individual targets on the qPCR.

The duplex assay also has the potential of further developing it into a multiplex PLA, which will be capable of detecting TcdA, TcdB and glutamate dehydrogenase (GDH) enzyme in a single PLA assay. As mentioned previously, GDH is a metabolic enzyme which is produced by both toxigenic and non-toxigenic *C. difficile* strains. This enzyme is used as a marker for the presence of *C. difficile* in clinical specimens. Therefore, a new PLA could be developed using a probe containing the antibody specific to the GDH enzymes, which will show the presence of either pathogenic or non-pathogenic *C. difficile* in the sample. Once the GDH specific PLA is developed, it can be incorporated into the duplex assay, ultimately it will result in development of highly specific multiplex PLA capable of detecting the presence of colonised

C. difficile either capable of producing toxin of not and also detection of the released TcdA and TcdB toxins or other hypervirulent strains of *C. difficile* in a single PLA test, which may have the positive impact on the health of at-risk patients of CDI.

Once this assay is developed into a diagnostic kit for *C. difficile* TcdA and TcdB, PLA can be used for further development of an assay for other pathogenic organisms resulting in more targeted clinical decision-making, helping reduce the mortality rate for high-risk individuals. Finally, in the future, the combined testing of DNA and protein targets from the same sample on the same analytical platform (i.e. qPCR) may further improve the sensitivity and specificity of disease diagnosis leading to improved clinical outcomes, patient satisfaction and reduced associated costs.

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