



Research paper

Homogeneous and digital proximity ligation assays for the detection of *Clostridium difficile* toxins A and B



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ABSTRACT

Background: The proximity ligation assay (PLA) detects proteins via their interaction with pairs of proximity probes, which are antibodies coupled to noncomplementary DNA oligonucleotides. The binding of both proximity probes to their epitopes on the target protein brings the oligonucleotides together, allowing them to be bridged by a third oligonucleotide with complementarity to the other two. This enables their ligation and the detection of the resulting amplicon by real-time quantitative PCR (qPCR), which 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 proximity probes targeting *Clostridium difficile* toxins A (TcdA) and B (TcdB) and used hydrolysis probe-based qPCR and digital PCR (dPCR) assays to detect antibody/antigen interactions.

Results: The performance of the PLA assays was antibody-dependent but both TcdA and TcdB assays were more sensitive than comparable ELISAs in either single- or dualplex formats. Both PLAs could be performed using single monoclonal antibodies coupled to different oligonucleotides. Finally, we used dPCR to demonstrate its potential for accurate and reliable quantification of TcdA.

Conclusions: PLA with either qPCR or dPCR readout have potential as new diagnostic applications for the detection of pathogens where nucleic acid based tests do not indicate viability or expression of toxins. Importantly, since it is not always necessary to use two different antibodies, the pool of potential antibodies useful for PLA diagnostic assays is usefully enhanced.

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1. Introduction

The proximity ligation assay (PLA) [1] combines the specificity of antibody-based detection of proteins with the exquisite sensitivity and dynamic range of real time quantitative PCR (qPCR) to detect antigens in body fluids and tissue samples [2]. Two antibodies coupled to non-complementary oligonucleotides are incubated with a sample and in the presence of target protein the oligonucleotides come into close proximity in solution. A third oligonucleotide complementary to the 3'-end of one and the 5'-end of the other antibody-linked oligonucleotides anneals to both and facilitates a ligation event between the two oligonucleotides. This generates a

ligation product that can be detected by various methods including qPCR (Fig. 1A). PLA offers several advantages over traditional ELISAs, including better sensitivity, a broader dynamic range, simpler workflow and faster time to results [3]. A proof of principle for the detection of bacterial proteins has shown PLA to be as sensitive as nucleic acid-based assays [4], although to-date there has been only one report describing its use to detect a pathogen [5].

The spore-forming gram-positive bacillus *Clostridium difficile* is the cause of *C. difficile*-associated infection (CDI) in hospital patients [6] and has become the most common health care-associated pathogen [7]. Elevated levels of two key virulence factors, toxins A (TcdA) and B (TcdB) [8] are associated with a sharp increase in fatalities [9,10]. There are non-pathogenic *C. difficile* strains that do not express either toxin [11] and remain asymptomatic in more than one-half of infected patients [12]. Such symptomless *C. difficile* colonisation is associated with a decreased risk of CDI [13].

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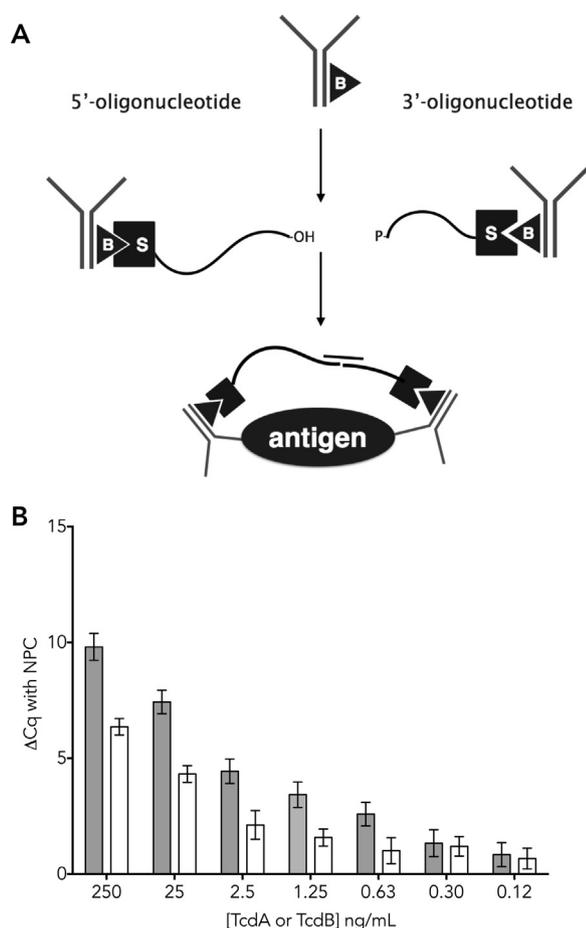


Fig. 1. PLAs targeting purified *C. difficile* toxins using single mAbs. (A) The PLA principle using a single mAb is shown schematically. Following biotinylation and a successful forced proximity test, the mAb is split into two pools and one is coupled to the 5'-, the other to the 3'-oligonucleotide. The pools are mixed with antigen, ligated and analysed by qPCR. (B, biotin, S, streptavidin). (B) The difference in quantification cycle (ΔCq) obtained at each concentration compared to the "no protein control" is plotted at each of seven antigen concentrations (250, 25, 2.5, 1.25, 0.625, 0.31 and 0.12 ng/mL). The dark boxes show the results from five independent PLAs with the α -TcdA mAb coupled to either 5'- or 3' oligonucleotide (Am5m3). The fluorescent signal was detected using either duplicate (one PLA) or five replicate qPCR reactions. The light boxes show the results from a PLA carried out in duplicate with the α -TcdB mAb (Bm5m3). Error bars show standard deviations.

Hence PCR detection of asymptomatic *C. difficile* colonisation may be contributing to rising CDI rates by reporting a significant number of false positives [14] and may result in unnecessary treatment for many patients.

We report the development of a hydrolysis probe-based PLA assay [15] to detect both *C. difficile* toxins. We have adapted the assay for accurate quantification of bacterial proteins by digital PLA. The assays can be performed using single mAbs, take 1.5 h to complete, are more sensitive than traditional ELISAs and have a dynamic range of five orders of magnitude.

2. Materials and methods

2.1. Equipment

All pipetting steps were carried out using Biohit micropipettes (Sartorius Ltd, Epsom, UK). ELISA results were read on an iMark™ Microplate Absorbance Reader (160-1130, BioRad, Hemel Hempstead, UK). Centrifugation steps were carried out using a Rotina 380R centrifuge (Hettich Zentrifuge, Germany) for 96 well or 48 well plates or a 5424 microfuge (Eppendorf, Stevenage UK) for

microfuge tubes. qPCR assays were carried out using either a Biorad CFX Connect (Biorad, Hemel Hempstead, UK) or a Eco48 (PCRMax, Stone, UK). PCR reactions for digital PCR were carried out on a TC9639 flatbed thermal cycler (Denville Scientific Inc. South Plainfield, USA) before detection of amplicons on a Constellation dPCR instrument (Formulatrix, Bedford, MA, USA).

2.2. Antigens and antibodies

Purified and lyophilised *C. difficile* TcdA and TcdB (CDA-TNL and CDB-TNL, The Native Antigen Company, Upper Heyford, UK) were reconstituted in 250 μ l of sterile distilled water (10245203, Thermo Scientific, Loughborough, UK), giving final concentrations of 0.4 μ g/ μ l and 0.2 μ g/ μ l of antigen, respectively, 0.05 M Hepes, 0.15 NaCl and 5% sucrose. Ten aliquots of antigen (25 μ l each) were stored at 4 °C.

Protein G-purified mouse monoclonal anti-*C. difficile* TcdA antibody (ab19953, Abcam, Cambridge, UK) was raised against full length protein and was supplied at a concentration of 1.16 μ g/ μ l in 0.1% Sodium azide with 10 mM of PBS, pH7.2. It does not cross react with TcdB.

Protein G-purified mouse monoclonal IgG1 anti-*C. difficile* TcdB antibody (ABIN234836, antibodies-online, Aachen, Germany) was raised against full length protein and was supplied at a concentration of 1.14 μ g/ μ l in 10 mM PBS, pH7.4. It does not crossreact with TcdA.

Chicken polyclonal IgY anti-*C. difficile* TcdB antibody (PAB29154, Abnova, Tapei, Taiwan) was raised against native purified *C. difficile* toxin B with Freund's adjuvant. It was supplied at a concentration of 2 μ g/ μ l in 0.075% Sodium azide with 10 mM of PBS, pH7.2.

2.3. ELISA

TcdA and TcdB antigen concentrations were assayed in the range of 1.25, 0.625 and 0.312 ng/mL and 2.5, 1.25, 0.625 ng/mL respectively to determine the sensitivity of using a commercial sandwich ELISA (TGC-E001-1, tgcBiomics, Bingen, Germany) as per manufacturer's instructions. The ELISA was performed using the commercial TcdA and TcdB antigens diluted in buffer supplied with the kit and also in a suspension of canine faeces. Canine faeces was chosen as a model as it simplified ethics considerations.

2.4. Biotinylation of antibodies

Biotinylation of the three antibodies was performed using EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation kit (21327, Thermo Scientific, Loughborough, UK). 50 μ g of antibody was added to 1 x PBS, pH7.4 (10051163, Thermo Scientific, Loughborough, UK) making a final volume of 200 μ l to which 0.67 μ l of 10 nM biotin was added. Tubes were centrifuged at 10,000g for 10 s and incubated at 20 °C for 1 h. Two \times 100 μ l of each antibody-biotin solution were transferred to two Slide A- Lyzer mini dialysis units (69562, Thermo Scientific, Loughborough, UK) per antibody and free biotin was removed by dialysis in 1 L of 1x PBS, pH 7.4 at 4 °C. The buffer was changed after 2 h, then twice after one hour intervals, with a final overnight dialysis against 2 L of buffer.

2.5. Forced proximity probe preparation and test

In a PLA, the term proximity probe refers to the antibody/oligonucleotide conjugate, ie there are two proximity probes per PLA, one with the 5'-oligonucleotide, the other with the 3'-oligonucleotide. The forced proximity test helps to assess the quality of biotinylated antibodies by determining whether or not they can bind to the two oligonucleotides, which are streptavidin-linked. Following biotinylation, individual antibodies are incubated

with both 5'- and 3'-oligonucleotides, resulting in both oligonucleotides binding to the same antibody. A ligation is carried out, which should be highly efficient since both oligonucleotides are close together and the resulting DNA templates are amplified by PCR. At the same time 5'- and 3'- oligonucleotides are incubated without antibodies, ligated and subjected to PCR. This should be an inefficient process and the Cqs recorded in these reactions are due to background ligation events. The kit's suppliers suggest that a difference in quantification cycle (ΔCq) of 8.5 or greater between the two treatments indicates efficient biotinylation of the antibodies. If they do not pass this test, i.e. the ΔCqs are less than 8.5, either the biotinylation reaction was not sufficiently efficient and antibodies are under-biotinylated or there still is free biotin in the solution because the dialysis of the biotin-antibody conjugates was inadequate. The forced proximity test was carried out exactly as per manufacturer's instructions using antibody dilution buffer (4453745, Life Technologies, Carlsbad, CA, USA), 3' and 5' Prox-Oligo (4448549, Life Technologies), assay dilution buffer (4448571, Life Technologies), ligation solution (4448592, Life Technologies), fast master mix (4448616, Life Technologies) and universal PCR assay buffer (4448592, Life Technologies). The qPCR was carried out on a Biorad CFX Connect with the following cycling conditions: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. Biotinylated proximity probes passing the quality threshold were diluted using an equal volume of antibody dilution buffer, aliquoted and stored at -80 °C at a final concentration of 0.125 $\mu\text{g}/\mu\text{l}$.

2.6. Proximity probe preparation

Two proximity probes for each antibody were prepared by combining 5 μl of 200 nM biotinylated antibody with 5 μl of 200 nM 3' (proximity probe A) or 5' (proximity probe B) Prox-Oligo. Following a 10 s spin at 10,000g, the mixture was incubated at 20 °C for 60 min. Following the addition of 90 μl of probe storage buffer, the mixture was incubated for a further 30 min at 20 °C. Ten aliquots of 10 μl of proximity probes A and B were made and stored at -20 °C.

2.7. qPCR-PLA

A proximity probe mix was prepared fresh each time by combining 2.5 μl each of proximity probes A and B with 125 μl probe dilution buffer and placing the mixture on ice. For each PLA, 2 μl of this proximity probe mix was placed in a single well of a standard white qPCR 96 well plate (Biorad, Hemel Hempstead, UK) followed by 2 μl of target antigen, which was appropriately diluted with 1x serum dilution buffer (SDB II, 4483013, Life Technologies). No protein controls (NPC) consisted of 2 μl of proximity probe mix and 2 μl of 1x SDB II. The plate was sealed using an optically clear heat seal with a PX1 PCR plate sealer (Biorad, Hemel Hempstead, UK), centrifuged at 780g for 2 min (Rotina 380R Hettich Zentrifuge, Germany) and incubated for 1 h at 20 °C. Following removal of the seal, 16 μl of ligation solution II (4483013 Life Technologies) 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 following conditions: 18 °C for 15 min (ligation) and 60 °C for 10 min (ligase deactivation). Since the ligation solution also contains proprietary primers and FAM-labelled hydrolysis probe with a non-fluorescent quencher, ROX as well as the *Taq* polymerase and dNTPs, qPCR was carried out immediately using the following conditions: 95 °C for 2 min and 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

Alternatively, the PLA assay was carried out as described, but using 48 well plates suitable for the Eco48 sealed with Eco-specific adhesive optically clear seals (PCRMax, Stone, UK). Since the instrument cannot be programmed to run at 18 °C, the plate was placed in a cooled water bath (Grant Instruments Ltd, Shepreth, UK) prior

to the qPCR reaction. Cycling conditions were the same as before, i.e. 95 °C for 2 min and 40 cycles of 95 °C for 5 s and 60 °C for 10 s.

For PLAs carried out on antigen suspended in canine faeces 50 mg of faeces were added to 450 μl of 1x SDB II, the suspension was vortexed for 1 min and centrifuged at top speed in a microfuge for 3 min. Supernatants were collected, spiked with 10 or 1 ng/mL of toxin A, transferred to Slide A- Lyzer mini dialysis units and dialysed against 1 L of 0.5 \times TE Buffer (T11493, Molecular Probe, Eugene, OR, USA) at 4 °C. The buffer was changed after two hours and dialysis was continued overnight against 1 L of buffer. A non-spiked sample was also dialysed for use with the no protein control assay. After dialysis samples were transferred to microfuge tubes, the 10 ng/mL sample was diluted to 1 and 0.5 ng/mL, the 1 ng/mL sample was diluted to 0.1 ng/mL in 1x SDB and used for PLAs.

2.8. qPCR analysis

qPCR data (Cq values) were recorded from both the Biorad CFX and the Eco qPCR instruments. Those from the Biorad CFX were acquired using the regression algorithm supplied with the instrument's software, those from the Eco48 were obtained using the threshold method. For the forced proximity test the ΔCq values were calculated for each biotinylated antibody: average Cq (negative controls)—average Cq (forced proximity probes). If the ΔCq was at least 8.5, the biotinylated antibody was considered suitable for use in the PLA. For the PLA, results were recorded as average Cqs \pm standard deviations. The NPC was used as a reference background and its Cq value determined the non-target ligation background noise of the assay. Three replicate PLAs were performed for each sample and NPC.

2.9. PLA with dPCR readout

The PLA was performed as described above, except that after the inactivation of the ligase the white 96 well plate was centrifuged at 780g for 2 min and placed on ice without carrying out the qPCR step. The Formulatrix Constellation dPCR system uses a special microplate that contains 96 input wells on the top surface and 496 microfluidic chambers per input well on the bottom surface. 10 μl of each assay were transferred from single wells on the white 96 well plate to single wells on the Formulatrix microplate. The dPCR 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 min and involves pins pushing from above on the plate seal over each well to force the liquid into the channels. A roller from below then forces a sealing 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 any ligated DNA templates using the following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s. Following endpoint PCR, the microplate was placed on the imaging station at the top of the Constellation instrument, which takes imaging profiles of each well.

2.10. dPCR analysis

An imaging profile is a collection of imaging settings that are used to image the microplate and typically defines ROX as a reference filter and FAM and HEX as the reporter dye filters. A suitable threshold is set for the reference filter to allow the analysis software to distinguish between partitions that contain an assay from those that have not been properly primed. Similarly, suitable thresholds are set for the reporter dye filters to determine which partitions have successfully amplified.

Thresholds for each filter are set using a separate threshold screen and delineate an average brightness value, below which a partition will be interpreted as empty if applied to the reference filter or negative if applied to a reporter dye filter. Above that threshold a partition is interpreted as containing an assay or a positive amplification result. The threshold is set using a histogram image, which shows the number of partitions with an average brightness value from 0 (completely dark) to 255 (completely bright). Typically, the histogram will show a bimodal distribution.

An initial assessment with the “raw images” view used the ROX in the master mix to confirm that all partitions were properly filled with reagents and provided a visual estimate of target concentration. The ROX histogram displays two peaks, a tiny one on the left representing empty partitions and a much bigger one on the right representing partitions that contain reagents. The threshold was placed between 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. Threshold settings were validated by using the Auto Threshold facility, which confirmed the manual placing of the thresholds between the various peaks.

Unlike some other dPCR instruments which typically have thousands of partitions, the Formulatrix microplate has only 496 compartments per 96 well. This means that the instrument has a relatively small dynamic range, and requires the user to determine sample concentrations by qPCR first to ensure that not all compartments contain templates. Typically, target concentrations that record Cqs above 28 are sufficiently dilute to generate reliable dPCR counts.

3. Results

3.1. ELISA

The lowest concentration tested that the ELISA was able to detect was 1.25 ng/mL for both TcdA and TcdB using neat dilutions of antigen. Suspension of antigens in canine faeces at the same concentrations did not substantially alter the results of the ELISA (Fig. S1).

3.2. Forced proximity test

This test compares the amplification results obtained with an antibody/oligonucleotide conjugate to those obtained with oligos only. A large ΔCq between the two suggests that the antibody has been efficiently biotinylated and the manufacturer’s stipulate a minimum ΔCq value of 8.5. Biotinylation of all antibodies was successful, as they significantly exceeded the forced proximity quality threshold, with the TcdA proximity probe recording a ΔCq of 13.14, the monoclonal TcdB 15.97 and the TcdB polyclonal 10.91 (Fig. S2 and Table S1).

3.3. PLA with qPCR readout

3.3.1. TcdA

PLAs using two pools of a single mAb targeting *C. difficile* TcdA conjugated separately to either the 5’ or 3’ oligonucleotides (Am5m3) resulted in a sensitive PLA, detecting purified TcdA down to 0.12 ng/mL of toxin. The repeatability of the assay was assessed by performing four additional independent repeats of the PLA assay, with five replicates of each concentration. When all the results are combined, the associated variability ($\pm SD$) shows that it is possible to detect as little 0.12 ng/mL of TcdA, which is five to ten times more sensitive than the ELISA (Fig. 1B, coloured boxes). When the PLA was

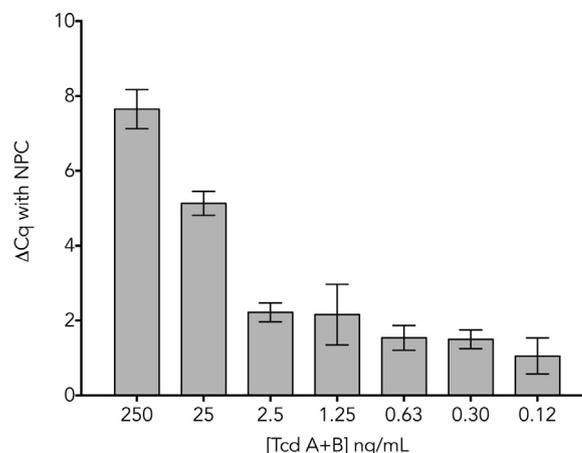


Fig. 2. Dualplex assay targeting TcdA and TcdB with Am5m3 and Bm5m3. The difference in quantification cycle (ΔCq) obtained at each concentration compared to the “no protein control” is plotted. PLAs were carried out in duplicate using two separate pools of 3’ oligonucleotide- and 5’ oligonucleotide-mAb targeting TcdA (Am5m3) and TcdB (Bm5m3). Error bars show standard deviations.

performed using antigens suspended in canine faeces, the assay was less sensitive, but sensitivity was partly restored by overnight dialysis (Table S2).

3.3.2. TcdB

PLAs using two pools of a single mAb targeting *C. difficile* TcdB conjugated separately to either the 5’ or 3’ oligonucleotides (Bm5m3) resulted in a PLA showing similar sensitivity to the assay targeting TcdA, with 0.12 ng/mL the lowest level of antigen detectable (Fig. 1B, clear boxes). The performance of the mAb-based PLA was compared to that of a conventional PLA utilising a combination of the same anti-TcdB monoclonal and polyclonal antiserum. An initial assessment showed that the 5’-oligonucleotide-monoclonal/3’-oligonucleotide-polyclonal combination (Bm5p3) gave a better result than the 3’-oligonucleotide-monoclonal/5’-oligonucleotide-polyclonal combination (Bm3p5) (Fig. S3). PLAs carried out using Bm5m3 or Bm5p3 performed very similarly, with Bm5m3 slightly more sensitive (Fig. S4).

3.4. Dualplex PLA targeting TcdB and TcdA

Next, the two mAb-based PLA assays were combined into a dualplex test with assay conditions the same as for the individual PLAs. Results were similar to the ones recorded with the singleplex assays, with quantification down to 1.25 ng/mL and detection at 0.12 ng/mL of TcdB, although the variability at each concentration was less (Fig. 2).

3.5. PLA with dPCR readout

Three independent PLAs targeting TcdA were analysed by dPCR in duplicate with two different concentrations of TcdA (0.6 and 0.3 ng/mL) and indicated that it is possible to obtain reliable quantification of the copy numbers of ligated proximity probes (Fig. 3). A comparison between PLA results obtained using either qPCR or dPCR at the lowest concentration of antigen tested showed that the results generated by the two readout methods are similar, with dPCR recording an average difference of 3.1 ± 1.4 copies compared with 0.8 ± 0.5 Cqs for qPCR (Fig. 4). Coefficients of variation were 44% and 70%, respectively.

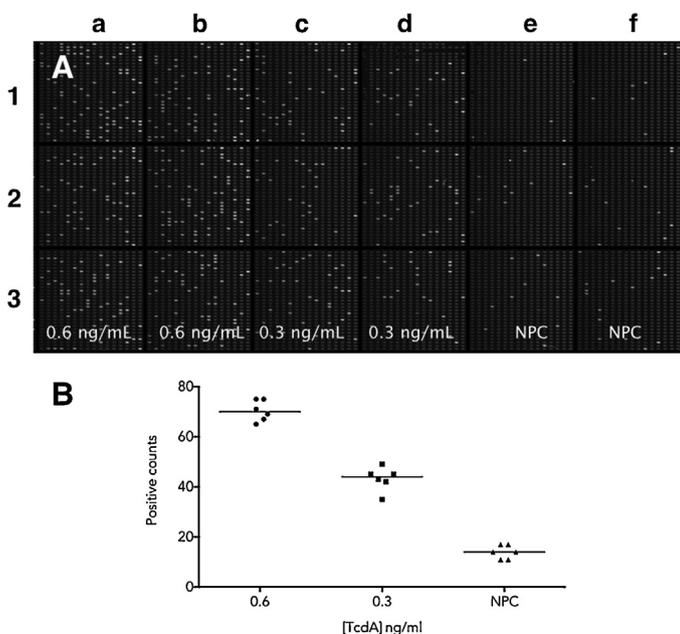


Fig. 3. PLA with dPCR readout targeting TcdA. (A) Each row of the screen image (labelled 1–3) corresponds to an independent PLA, carried out in duplicate at 0.6 (a,b) and 0.3 (c,d) ng/mL of antigen and with the no protein control (NPC) (e,f). (B) The positive counts calculated by the Constellation software are shown in the graph, with the horizontal bar showing the median count.

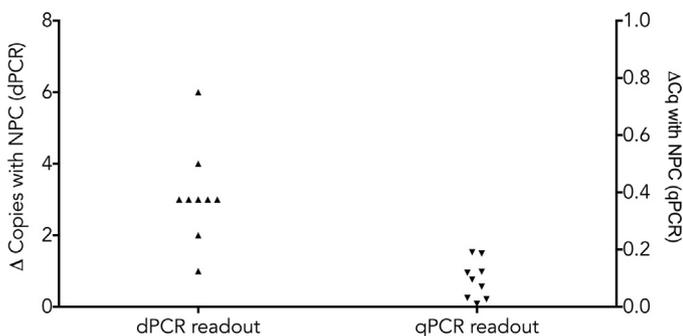


Fig. 4. Comparison of PLA results obtained with qPCR and dPCR at the limits of detection. The difference in copies (dPCR) or copy numbers (qPCR) obtained by diluting TcdA to 0.1 ng/mL were compared to the no protein controls (NPC).

4. Discussion

There are numerous molecular methods available for the molecular analysis and diagnosis of pathogens. Two key technologies in widespread use target nucleic acids using PCR-based methods and proteins using a range of antibody-based techniques. Both approaches have their distinct advantages and disadvantages: PCR assays are easier to develop, are generally more sensitive, but detection of DNA does not prove the presence of a viable, infectious pathogen and that of RNA provides no information about protein functionality. Antibody-based tests can give a result in less than five minutes (e.g. a lateral flow device) and have a low target purity requirement, but can be difficult to develop and the quality of the test depends on the quality of the antibody.

Immuno-PCR [16] and real-time immuno-qPCR (iqPCR) [17] represent the first attempts to combine the advantages of the two approaches whilst overcoming the limitations associated with traditional immunoassays. However, the sensitivity and specificity of iqPCR assays as originally developed are antibody-dependent and do not necessarily exceed that of the conventional ELISA because both are reduced by nonspecific background signals [18] probably

caused by cross-reactive binding of the single antibody to non-target proteins [19]. Nonetheless, appropriately designed sandwich formats can improve the sensitivity of iPCR to make them 1000-fold or more sensitive than ELISAs [20]. However, the most limiting aspect of iPCR are (i) that its workflow involves a multi-step process that results in significant hands-on time requirements and introduces the potential for contamination and (ii) that there is an enhanced consumption of reagents [21].

In contrast, PLA is a homogeneous assay that uses two interacting proximity probes to generate a signal and has significant potential to improve the sensitivity of detection [1]. Indeed, PLA has been used for a wide variety of applications that range from detection of proteins in single cells [22], cancer biomarkers [23] and prions [24] to proposals for its use with personalised medicine [25]. However, apart from a report detailing the proof of principle for the detection of bacteria, [4], there is only one other report of its use to detect pathogenic *Escherichia coli* [5].

The emergence of hyper virulent strains of *C. difficile* and the increased rate of morbidity and mortality associated with CDI world-wide [26] makes reliable local surveillance to detect and control endemic and epidemic CDI critical for patient management and infection control. However, enzyme immunoassays lack an adequate combination of sensitivity, specificity, and timeliness [27,28] and a recent comparison of *C. difficile*-specific in-house and commercial qPCR assays found that the former lacked specificity, whereas the latter lacked sensitivity [29].

Our report details the development of a PLA-based test that targets the two main bacterial toxins responsible for the pathogenicity associated with *C. difficile*. TcdA has multiple epitopes at its C-terminal end that are recognised by a single antibody [30]. Hence we reasoned that a single mAb, coupled differentially to oligonucleotides, might be able to perform a PLA whilst minimising background noise. Our results show that it is indeed possible to use this approach to develop a highly sensitive assay against this bacterial toxin. However, since TcdB is generally thought to be the key virulence determinant [31] and TcdA-negative, TcdB-positive isolates appear to be on the increase [32], we also developed a TcdB-specific PLA. Like TcdA, TcdB has several repeated motifs at its C-terminal end [33], hence we decided to see whether a single mAb might also work in a TcdB-specific PLA. The results show that it is likely that any protein with multiple identical epitopes can be targeted by single mAb-PLAs. This finding has important implications for the practical usefulness of the PLA, especially for pathogen detection, since proteins expressed by many pathogens have such repeated elements [34]. Dualplexing also worked, with the limitation that the TaqMan Protein Assay kit currently does not allow the use of two different DNA reporter sequences and fluorophores to distinguish two targets. However, Fig. 2 shows that dualplexing resulted in less variability and that having four sets of PLA proximity probes does not affect the performance of the assay.

One of the advantages of ELISAs is that samples do not need to be purified before analysis as they are generally not inhibited by biological fluids. This is confirmed by our own results with antigen-spiked faeces shown in Fig. S1, where the PLA did not work well. This was not unexpected since faeces are known to contain numerous inhibitors of the PCR reaction. However, inhibition was relieved by dialysis and dilution, making it likely that methods can be developed to overcome faeces-related inhibition.

One of the potential drawbacks of the PLA is that the negative (no protein) controls always generates a Cq due to background ligation events between the two proximity probes. Hence it is important to shift the background to as high a Cqs as possible, for example by diluting the PLA reaction after the proximity probe and antigen interaction step [22]. When we compared Cqs recorded by PLAs subjected to ligation and amplification with and without dilution we did indeed obtain an increase in Cq (Table S3). In con-

trast, changing the antibody concentrations used for generating the proximity probes did not affect any of the Cqs (not shown).

dPCR is increasingly seen as an alternative to qPCR as it provides more precision, may be less sensitive to inhibitors [35] and allows the determination of absolute copy numbers. PLA in combination with digital RT-PCR has been used to quantify proteins and mRNA from single mammalian cells and demonstrate that there is a low correlation between the two macromolecules [36]. The Formulatrix dPCR instrument provides a simple platform with physical partitions, rather than the alternative, more complex droplet-based dPCR system and results in an easy to understand readout. It is also not restricted to any one company's reagents, has a small footprint and software that is easy to use. Its main limitation is its relatively low dynamic range due to the small number of partitions (currently 496) available for each dPCR reaction. We decided to determine the usefulness of a dPCR approach and transferred the qPCR-based assay to the digital platform. The need to use a PCR instrument able to handle flat-bottomed plates resulted in slight modifications to the PCR protocol, but resulted in a very sensitive, rapid and reproducible PLA allowing us to count individual ligation events without the need for any standard curve. We suspect that the sensitivity of the PLA is dependent upon the efficiency of the ligation event that is required to generate a template that can be amplified by PCR. Hence improvements to the sensitivity of the PLA will require improvements to the ligation efficiency and there is a need to target that aspect of the PLA.

In summary, we report the first use of two single mAbs in a PLA targeting toxins produced by the clinically important bacterial pathogen *C. difficile*. Since the assay is as sensitive and possibly less variable if it contains four proximity probes, there is clear potential for a dual- or even multiplex application. Results can be obtained from faecal samples, with maximum sensitivity currently requiring a dialysis step. The inclusion of dialysis is of course not ideal for the simplicity of the assay and significantly increases the time required to complete the assay. However, the development of high-capacity antibody-binding magnetic beads together with a simple workflow [37] will streamline the process of selecting and enriching antigen/proximity probe complexes and minimize the co-purification of inhibitors of the PCR reaction. Finally, we have substituted qPCR with a dPCR and report that it works reliably, reproducibly and with high sensitivity.

Conflict of interest

There is no conflict of interest.

Authors' contributions

HSD carried out all steps associated with the PLA assays. CG designed, carried out and interpreted the ELISAs. GJ and MS instructed and advised on the development of the PLAs. DR instructed and advised on all aspects of the dPCR. SB conceived, planned and supervised the work. All authors contributed to the data analysis and writing of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bdq.2016.06.003>.

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