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Clostridium difficile: A difficult diagnosis made possible by real-time PCR

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Introduction:

Clostridium difficile is considered one of the most important bacterial pathogens that cause diarrhea and enterocolitis in horses[3]. *C. difficile* is a short, rod shaped, anaerobic bacteria that is transmitted via spores in contaminated feces, and often flourishes after anti-microbial treatment[1]. The bacterium itself is not pathogenic, however it can produce toxins, and these toxins are what damage the host cells and cause disease. The *C. difficile* toxins of interest for this project include Toxin A and Toxin B[4]. A quick diagnosis of a *C. difficile* infection is difficult because its clinical signs are nearly identical to those of other bacterial infection such as *Clostridium perfringens* and *Salmonella spp*. infections[5]. *C. difficile* is also difficult and expensive to diagnose using bacterial culture due to its anaerobic requirements, toxin degradation and the possibility that the isolate cultivated in laboratory conditions does not necessarily produce Toxin A or Toxin B[2]. Thus, instead of detecting the toxins themselves, molecular methods such as PCR are utilized to detect the presence of the toxin genes.

PCR is an effective tool for diagnosing *C. difficile* infection because it doesn't require growth of bacterial colonies. The PCR assay also uses DNA primers that specifically detect the Toxin A and Toxin B genes, which identifies a potentially pathogenic *C. difficile* isolate in the sample[4]. The Wyoming State Veterinary Laboratory has been diagnosing *C. difficile* using anaerobic culture on specific *Clostridium* agar plates. This is a slow, labor-intensive process, and is not ideal for a rapid diagnosis. A more efficient method of diagnosis would be an end-point PCR assay that detects the genes for toxins A and B using gel electrophoresis, but this method has very similar issues that make it less ideal for rapid diagnosis. Modern quantitative PCR assays (qPCR) pose a solution to these problems. Because the thermocycler detects fluorescence emitted from the probe in the sample after each cycle of the polymerase-chain reaction, it not only detects the presence of *C. difficile* DNA twice as quickly (only taking about 1.5 hours for 40 cycles) as end-point PCR, but it more precisely quantifies the amount of DNA in the sample by reporting the number of cycles needed to pass a threshold DNA concentration (CT). Furthermore, by negating the need for gel electrophoresis, qPCR cuts down on material cost, active labor time and risk of contamination, making it an attractive alternative to veterinary diagnostic laboratories.

Here I report the optimization and ongoing validation of a qPCR assay that detects DNA coding for *Clostridium difficile* Toxin A and Toxin B.

Methods

Primers

We designed two sets of primers for each Toxin using IDTPrimerQuest and the gene sequences for Toxin A and Toxin B. (Table 1 and Table 2)

Table 1. *Toxin A primer and probe design*

Toxin A IDT PrimerQuest			
Primer Set	Forward (Sense)	Reverse (Antisense)	Probe (Sense)
Set 4	CAGCTACTGGATGGCAAACTAT 22 bases Position 7194-7216	ACCAGTTGAGGCTATGAAAGTG 22 bases Position 7309-7331	TGAAGCAGCTACTGGATGGCAAACT 25 bases Position 7252-7277
Set 5	CAGCTACTGGATGGCAAACTAT 22 bases Position 6450-6472	CCAGTTGAAGCTATAGCAGTGT 22 bases Position 6564-6586	TGAAGCAGCTACTGGATGGCAAACT 25 bases Position 6508-6533

Table 2*. Toxin B primer and probe design*

Toxin B IDT PrimerQuest			
Primer Set	Forward (Sense)	Reverse (Antisense)	Probe (Sense)
Set 2	GACTGTAGGCGATGATAAATACTACT 22 bases Position 1762-1788	CCTGTTTGTAACACTCCACTTTG 23 bases Position 1859-1882	TGGTGGAGCTGCTTCAATTGGAGA 24 bases Position 1801-1825
Set 3	ATTCCAATACAAGCCCTGTAGAA 23 bases Position 4901-4924	CTGCATTAATATCAGCCCATTGTT 24 bases Position 4985-5009	TGGATAGGTGGAGAAGTCAGTGA TATTGCT 30 bases Position 4942-4972

We chose which set to use for each Toxin during the first step of the optimization process, where we used a gradient of annealing temperatures using end-point PCR and gel electrophoresis. The primer sets that generated the clearest and brightest bands were selected. We also took into consideration the optimal annealing temperature of each set, as we wanted the annealing temperatures for both Toxins to be the same in the final assay. Of the sets listed above, we chose Set 5 for Toxin A and Set 2 for Toxin B.

To verify that the primers we designed matched genes coding for Toxin A and Toxin B in *C. difficile*, we sequenced the amplicons from the two chosen primer sets using Sanger sequencing. We identified the sequencing product using BLAST, and confirmed that the primers amplified their intended DNA targets for Toxins A and B.

Optimization

We optimized the *C. difficile* qPCR assay by testing key elements of the assay and procedure while drawing on previous knowledge of qPCR best practices[6] for the universal elements of the assay.

Annealing Temperature. We first optimized the annealing temperature of each primer set using a temperature gradient of 48-60°C with 12 samples per set. The positive *C. difficile* DNA that we used during the entire optimization process was the ATCC *C. difficile* (BAA-1870DQ

Quantitative Genomic DNA from *Clostridium difficile* Strain 4118) which had been previously verified to contain genes for Toxin A and Toxin B. The positive control was diluted with nuclease free water to 1:1,000 and was stored at -80°C. We amplified the DNA using end-point PCR in a Peltier Thermal Gradient Cycler followed by gel electrophoresis to visualize the relative effectiveness of each annealing temperature and to validate that our amplicon was the expected size. For the gel electrophoresis, we used a 2% gel agarose with 1X TAE. We ran the gel at 450 V for 45 minutes. There were four primer sets tested separately, two for each toxin. During these tests, the primer concentration was 10 µM, which is a concentration known to work reasonably well for most PCR assays. We also used best-practices temperatures and times for the other elements of the PCR protocol. Our reaction volume was 25 µL (20 µL mastermix and 5 µL sample DNA). The PCR protocol is below:

Table 3. *Thermal cycler conditions for annealing temperature gradient.*

Thermocycler Conditions	
95°C	10 min
95°C	30 sec
48-60°C gradient	60 sec
72°C	15 sec
40X cycles	
4 °C	Forever/Storage

Primer Concentration. We then optimized the primer concentration for each toxin, using the same thermal cycler and visualizing the DNA using gel electrophoresis. Each set was tested with four different primer concentrations: 2.5 µM, 5 µM, 10 µM, and 20 µM. Drawing from our results of the optimal annealing temperature, the PCR protocol was as follows:

Table 4. *Thermal cycler conditions for primer concentration.*

Thermocycler Conditions	
95°C	10 min
95°C	30 sec
57 °C	60 sec
72 °C	15 sec
40X cycles	
4 ºC	Forever/Storage

Annealing Time. Next, we tested three different annealing times for each toxin: 30 seconds, 45 seconds and 60 seconds. After that, we began optimizing the assay for qPCR and from this point forward tested and imaged samples using a Bio-RAD C1000 Touch Thermal Cycler. For fluorophores, we chose FAM for Toxin A and HEX for Toxin B because they have different wavelengths that can be detected through different filters by the thermal cycler. We tested each toxin for optimum probe concentration: 2.5 µM, 5 µM, 10 µM, and 20 µM. We adjusted the Mastermix formula to accommodate the new probe concentrations and to keep the final reaction

volume 25 µL. We also tested whether the Xeno-Liz internal control would work for this assay by adding it to the Mastermix and ATCC DNA, then comparing the fluorophore activity of the samples with Xeno to the samples without.

Multiplex. Once the internal control was added to the assays for each Toxin, we attempted to multiplex into one assay. To keep the final reaction volume at 25 µL, we adjusted the Mastermix water concentration to make room for the additional set of primers and probes. However, as shown in the results, the multiplex did not work due to interference between the two primers sets and was not used in the remainder of the optimization. The assay going forward continued to use two different reactions for each sample (one for each Toxin).

Assay Efficiency. We calculated the efficiency of the completed assay using a 12-sample serial dilution of ATCC positive control DNA ranging from 1:10- $1:10^{11}$. The efficiency of the assay was calculated to be 91%.

Validation

To validate the qPCR assay, we tested known positive and negative samples. We used 30 boiled colonies and 30 extracted DNA samples known of *Clostridium perfringens* and utilized these as negative samples. These samples were obtained from fecal samples submitted to the Wyoming State Veterinary Laboratory between 2018- 2019 that had been stored at -80°C. The Xeno LIZ internal control was added at the time of diagnosis. The internal control is especially necessary during validation of negative samples because it ensures that the PCR protocol itself is working and is able to amplify DNA. This provides confidence that the sample is truly negative and not negative due to inhibition. The samples were from different host origins including horses, cows, and goats.
For the positive validation, we have only tested one equine sample positive for *C. difficile.* because of limited access to positive veterinary samples. The positive validation is ongoing and will be completed over time.

Results

To determine the optimum annealing temperature and the efficacy of each primer set, we ran the ATCC positive control sample with each set through a temperature gradient PCR ranging from 48-60°C. For Toxin A, we found that despite Set 5 not producing bands as bright as Set 4, Set 5 was more specific to Toxin A DNA because of its solid bands at the expected product size of ~300 bp. Because the Set 4 produced a gradient of bands, this suggests that Set 4 is not as specific to the Toxin A DNA because it has multiple priming sites. For Toxin B, both primer sets produced strong bands at the expected size without extra bands further up the gel, suggesting that both primer sets are sufficiently specific to Toxin B DNA. However, the bands produced by Set 2 are noticeably brighter, suggesting that the Set 2 primers bind more readily to Toxin B DNA. Because of these results, we decided to use the Set 5 and Set 2 primers in the final assay. To determine the annealing temperature, we compared the Set 5 and Set 2 gradients to see which bands in the gradients were brightest, which shows the temperature at which the primers bind

most readily to the DNA. We found that 57°C was the temperature that produced bright bands for both primer sets, as shown in Figure 1, so we used 57°C as the annealing temperature for all following tests and in the final assay.

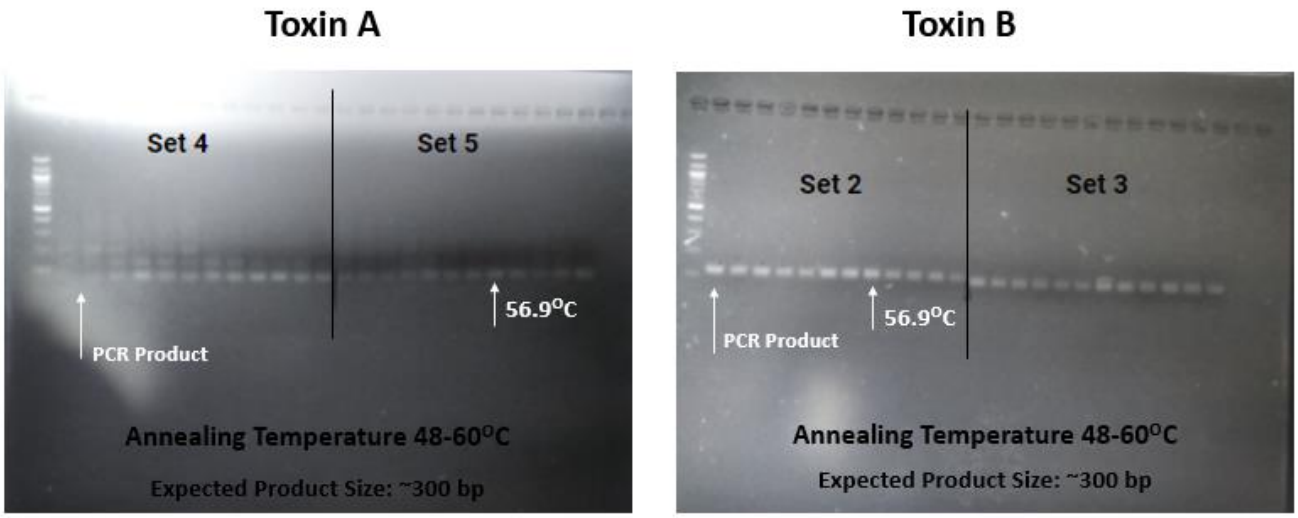

Figure 1. *Annealing temperature of Toxin A and Toxin B primer sets. Images are 2% agarose gels with PCR product following a reaction with annealing temperature gradient of 48-60°C. Lane 1 contains the DNA ladder for size reference. Each lane corresponds to a different annealing temperature, as shown in Table 5 (below). The expected PCR product was 300 bp. The optimal annealing temperature chosen was 56.9 °C, which was rounded up to 57°C for the final assay:*

Table 5. *Annealing Temperature Gradient*

Sample Number	***Temperature (°C)***	***Sample Number***	***Temperature (°C)***
1	*48*	***7***	*55.2*
2	*48.3*	***8***	*56.9*
3	*49.1*	***9***	*58.2*
4	*50*	***10***	*59.1*
5	*51.4*	***11***	*59.8*
6	*53.1*	***12***	*60*

Primer Concentration

Each primer set (Set 5 and Set 2) was tested using four different primer concentrations: 2.5 µM, 5 µM, 10 µM, and 20 µM. For both toxin genes, 10 µM primer concentrations yielded the brightest bands in gel electrophoresis imaging. Going forward, all tests and the final validation included primer concentrations of 10 µM.

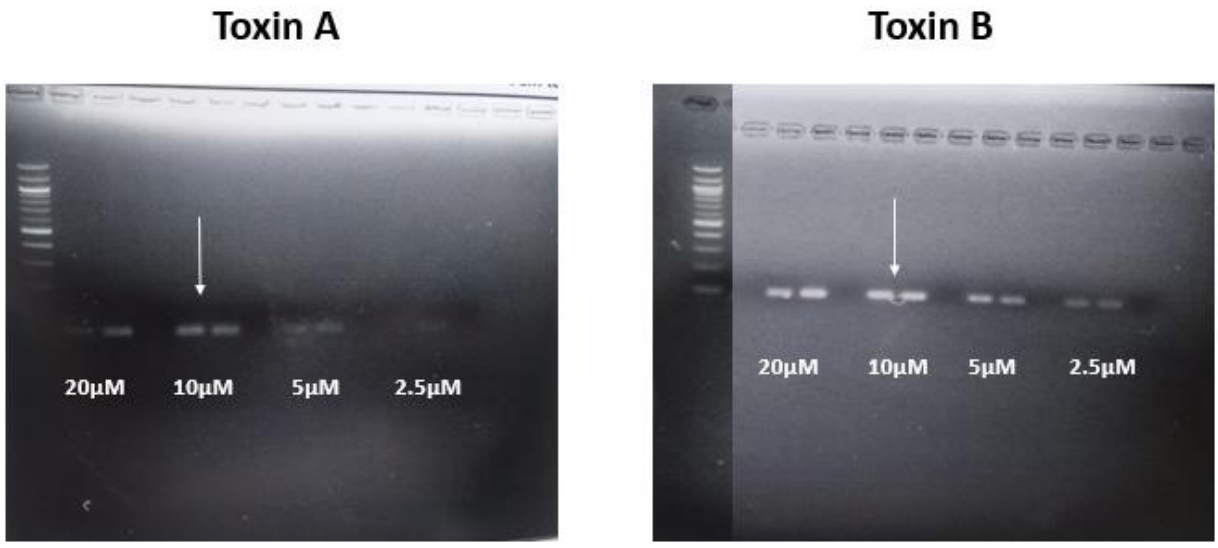

Figure 1. *Primer concentrations of Set 5 and Set 2.*

Annealing Time

Three annealing times were tested for the Toxin A gene: 30 seconds, 45 seconds and 60 seconds. Because the Toxin B assay consistently produced more amplicon than the Toxin A assay, we only tested Toxin A for shorter annealing times because it would be more sensitive to those changes. We chose 60 seconds for the annealing time of the final assay because the bands were sharper/more well-defined.

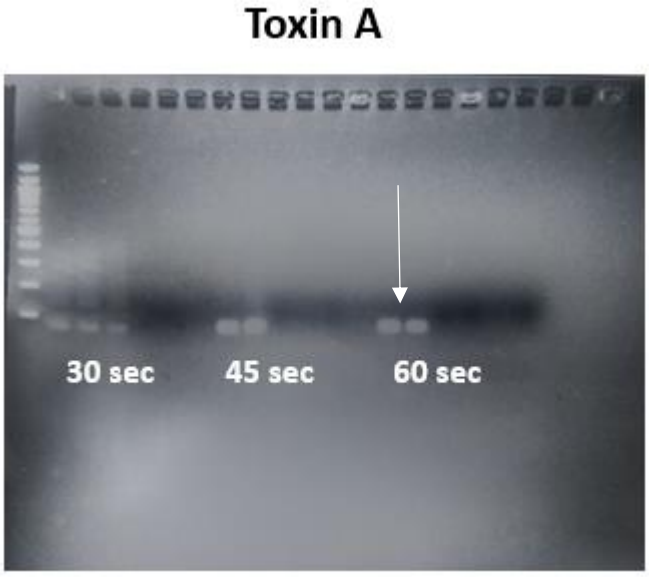

Figure 2. *Annealing Time Gradient for Toxin A Set 5.*

Probe Concentration

Probe concentration was optimized using qPCR. For fluorophores, we used FAM for the Toxin A gene and HEX for the Toxin B gene. Four probe concentrations were initially tested: 2.5 µM, 5 µM, 10 µM, and 20 µM. Toxin B had the lowest CT (29.34) at 5 µM with around 1,000 relative fluorescence units (RFU) after 40 cycles. While the 10 µM sample had higher RFU's, the CT's for that concentration were also slightly higher, and we wanted to use the minimum concentration for optimal results to maximize resources. Toxin A didn't have adequate RFU at these concentrations, and we hypothesized that the concentration of probe was too high and that it was interfering with the primers. After testing the Toxin A probe at the lower concentrations of 0.313 µM, 0.625 µM, 1.25 µM, and 2.5 µM, the highest RFU for this test came from the 1.25 µM concentration at about 300 RFU with a CT of 29.4, which confirmed our hypothesis. We chose to use 1.25 µM for Toxin A and 5 µM for Toxin B in the final assay.

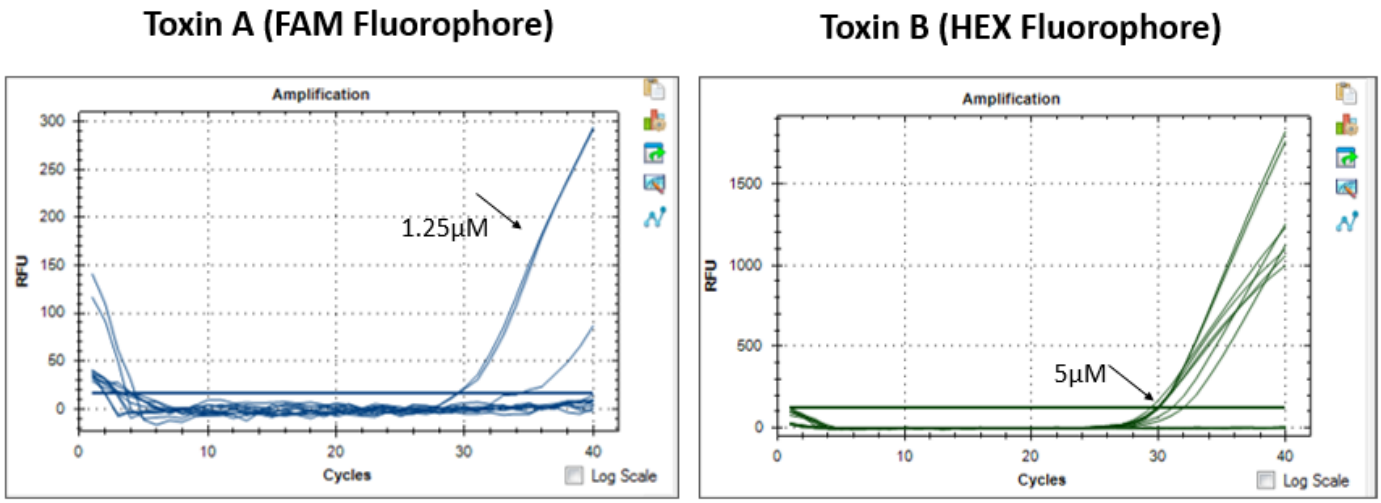

Figure 3. *Fluorescence at different probe concentrations.*

Failed Multiplex

Attempting to multiplex the Toxin A and Toxin B assays did not produce sufficient RFU's.

Addition of Internal Control

The addition of the Xeno-Liz internal control had no significant negative effects on the Toxin A gene or Toxin B gene assays. Xeno-Liz contains Cy5 fluorophores, which didn't interfere with the other fluorophores.

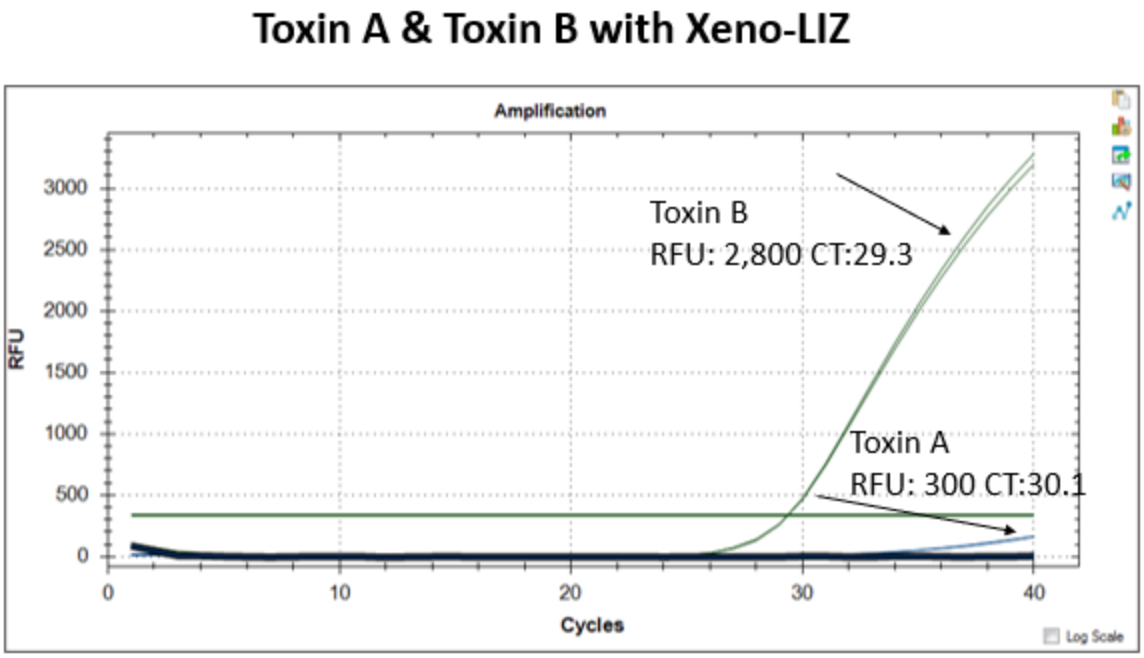

Figure 4. *Toxin A and Toxin B assay with Xeno-LIZ internal control added.*

Calculating Efficiency of the qPCR assay

We ran a 12-sample serial dilution of ATCC *C. difficile* DNA ranging from 1:10- 1:10^{11}. The efficiency of the assay was calculated to be 91.3% for Toxin A and 97.6% for Toxin B. This is based on the slope of the standard curve. For every 10-fold dilution, the cycles should increase by about 3 cycles. This will result in a standard curve slope of approximately -3. The slope of the Toxin A standard curve is -3.549, and the slope of the Toxin B standard curve is -3.382, which are both acceptably close to the ideal of a -3 slope.

Based on the data from the serial dilution assay, we calculated the detection limit of the protocol to be approximately 2,483 copies.

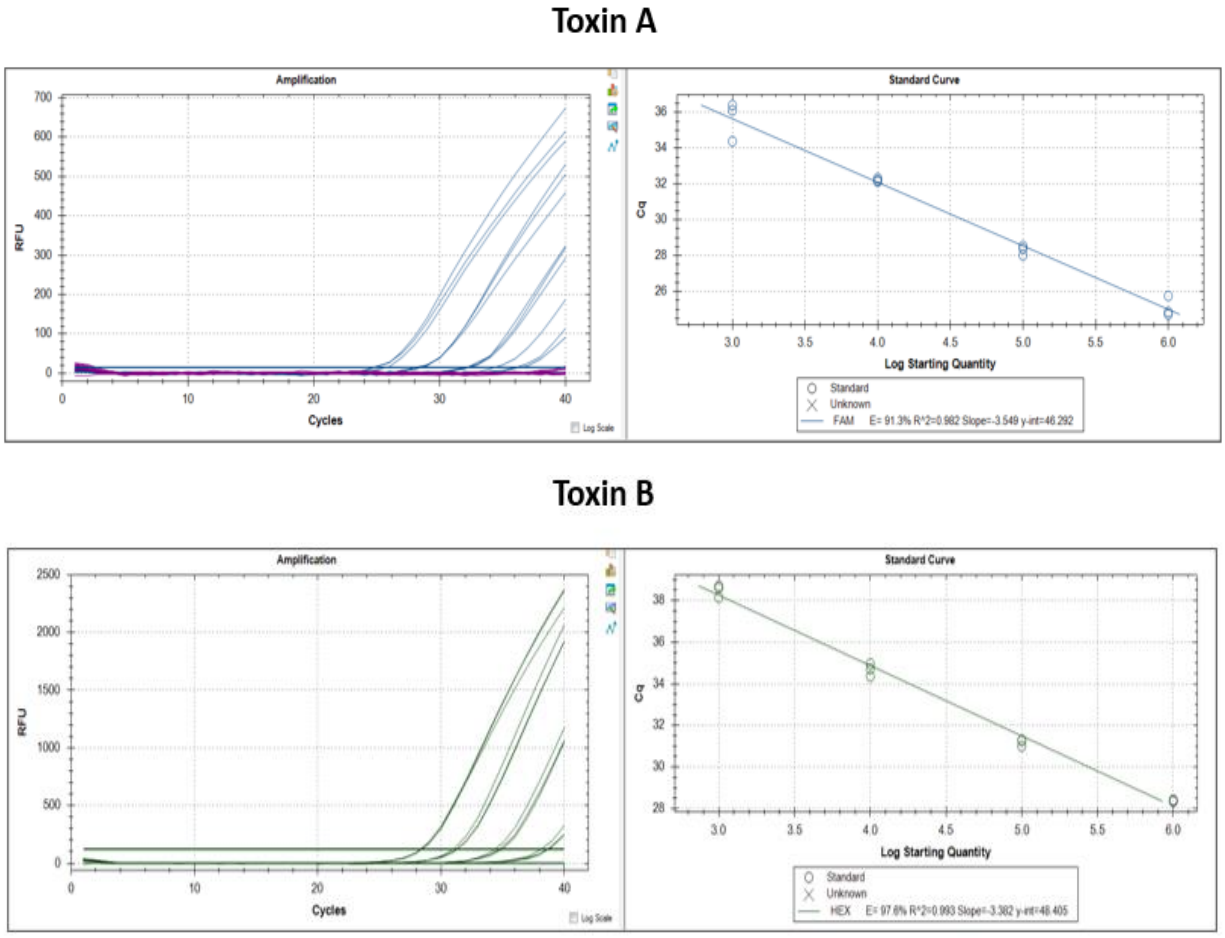

Figure 5. *Assay efficiency tests using dilution series. Samples were run in triplicate, and for every 1:10 dilution, the cycles should increase by 3 cycles. This will result in a slope of approximately -3 on the Standard Curve.*

Validation

For twenty-nine of the thirty negative samples that were tested, there was no detection of the Toxin A or Toxin B genes. We detected the gene for Toxin A in one of the negative samples, but not Toxin B. However, these Toxin genes may not be specific to *C. difficile*, and other Clostridial species may contain these genes. To make up for the unexpected positive result, we tested an extra negative sample, in which there was no detection of the Toxin A and Toxin B genes.
We have so far tested one known positive sample, and both Toxin A and Toxin B genes were detected in that sample.

Discussion

The goal of this project was to develop a multiplex PCR assay for detection of *C. difficile* Toxin A and Toxin B genes. We developed the PCR conditions separately for each toxin gene, however when multiplex was attempted; we were unsuccessful. This could be due to a number of reasons including interference between the primer sets and differences in target affinity between primer sets. Both primer sets for Toxin A gene amplified less DNA than the primer sets for Toxin B, thus knowing the reason for the reduction in affinity to the DNA may be a useful next step. There are likely other primer sequences for the Toxin A gene that may bind more efficiently to the target gene and result in more greater DNA amplification. While the assay can be performed in two different reactions, it would be useful to further explore multiplexing in order to increase laboratory efficiency.

Since this assay will be used in the diagnostic laboratory, it is important to include an internal amplification control. Failure to produce a PCR product can indicate a problem with the assay because the primers are designed to match the gene of interest. If a sample tests negative, it is difficult to determine whether the sample is truly negative for the gene of if the assay simply didn't work. An internal control allows assessment of the assay without affecting the results for the gene of interest. We used Xeno-LIZ as the internal control for this assay, which is most commonly used internal control for qPCR assays at the Wyoming State Veterinary Laboratory.

The most difficult aspect of this project was troubleshooting the source of unexpected problems that arose. For example, we began the project using positive *C. difficile* samples derived from horses as our positive control. However, when the assay suddenly failed to produce amplicons part-way through the project, it took a long time and lots of failed tests to determine the source of the issue. The problem was that the positive *C. difficile* sample didn't actually have the toxin A and toxin B genes, so it couldn't bind to the primers and produce an amplicon. This was very frustrating, but we solved the problem by re-starting the project with a *C. difficile* ATCC strain that contained the toxin A and toxin B genes. This both improved the potency of the produced amplicon during tests and the consistency of our positive control throughout the project.

In conclusion, we were able to successfully develop a qPCR assay for the detection of *C. difficile* Toxin A and Toxin B genes, with positive validation ongoing. The final PCR conditions for this assay are outlined in Table 6. This assay will be now utilized by the Wyoming State Veterinary Laboratory for the detection of *C. difficile* in fecal samples.

Table 6. *Final C. difficile qPCR conditions*

Toxin A Reaction Assay	
Reagent	Volume/Reaction µL
Sso Advanced Supermix	4.875
Nuclease Free Water	12.5
Toxin A Forward Primers Set 5	1
Toxin A Reverse Primers Set 5	1
Toxin A Probe FAM	0.125
Xeno-LIZ internal control	0.5
Subtotal	20
Template	5
Total Reaction Volume	25
Toxin B Reaction Assay	
Reagent	Volume/Reaction µL
Sso Advanced Supermix	4.5
Nuclease Free Water	12.5
Toxin B Forward Primers Set 2	1
Toxin B Reverse Primers Set 2	1
Toxin B Probe FAM	0.5
Xeno-LIZ internal control	0.5
Subtotal	20
Template	5
Total Reaction Volume	25

Thermocycler Conditions	
95ºC	10 min
95°C	30 sec
57 °C	60 sec
72 °C	15 sec
40X cycles	
4 °C	Forever/Storage

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