Proceedings of The National Conference
On Undergraduate Research (NCUR) 2014
University of Kentucky, Lexington, KY
April 3-5, 2014

Variance of Toxin Producing *Clostridium botulinum* in Utah Honey

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Abstract

Clostridium botulinum has been implicated in cases of infant botulism across the United States. It is recommended that infants under the age of one year not be fed honey because of the presence of *C. botulinum* spores. This study had two aims: to determine whether these spores can be detected through a multiplex polymerase chain reaction (PCR) without first culturing the organism and to determine whether honey produced in small and large apiaries in Utah contain varying amounts of toxin producing *C. botulinum*. *Clostridium perfringens* was used as a control organism in the proof of concept experiments. *C. perfringens* were isolated from the control honey sample, lysed through superheating, DNA was extracted, multiplex PCR and gel electrophoresis were performed proving that detection without culturing is possible. A multiplex PCR using primers specific for Clostridia species, and toxins A, B, E, and F was performed on honey samples that were collected from hives maintained in Utah. No DNA was detected in the honey samples.

Keywords: Honey, Botulism, PCR

1. Introduction

The current method of detection for *C. botulinum* in honey involves culturing the organism and then testing for the toxins it produces either through PCR or mouse lethality assay.[1] Because *C. botulinum* as a CDC Category A bioterrorism agent, a lab must have a high biohazard level and federal permission to culture this organism. In honey *C. botulinum* remains in spore form producing no toxins and would be safe to test in the clinical lab if a method could be used that did not require culturing of the organism. It has been estimated that up to 25% of honey samples contain *C. botulinum spores.*[2] *C. botulinum* poses minimal risk to adults and children over one year of age, yet in children under the age of one *C. botulinum* will produce a potent neurotoxin resulting in infant botulism.[3,4] *Botulinum* toxin can have various deleterious effects on the host including; mild hypotonia, paralysis, and sudden death.[4] The toxins most likely to be the cause of infant botulism include: A, B, E, and F.[1] While a few nationwide surveys have been conducted on this issue none have been focused in the state of Utah and none have tested between large and small hive operations. We hypothesized that larger apiaries would have a higher levels of contamination than backyard hives because of the greater potential for interaction between bees whose hives are within close proximity to one another as well as the increase in bee population. This study compared *C. botulinum* spores present in honey from large and small apiaries in Utah using a multiple PCR for the detection of specific botulism toxin genes.

2. Methods

2.1 Honey Sample Processing

At least eight ounces of honey was donated by 31 beekeepers from around the state of Utah during the 2013 harvest season. All participants were required to answer a short survey that discussed the type of hive, number of hives, species of bee and location of the hive from which the sample was harvested.

2.2 Multiplex PCR

Primers to detect *Clostridium* toxins A, B, E and F were designed from a previous study shown in Table 1.[5]

Table 1. primers used for multiplex PCR amplification of *Clostridium* toxins

Toxin Type	Sequence (5'-3')	Product size (bp)
Af	GGG CCT AGA GGT AGC GTA RTG[a]	101
Ar	TCT TYA TTT CCA GGA GCA TAT TTT[b]	
Bf	CAG GAG AAG TGG AGC GAA AA	205
Br	CTT GCG CCT TTG TTT TCT TG	
Ef	CCA AGA TTT TCA TCC GCC TA	389
Er	GCT ATT GAT CCA AAA CGG TGA	
Ff	CGG CTT CAT TAG AGA ACG GA	554
Fr	TAA CTC CCC TAG CCC CGT AT	
Closf	CTC AAC TTG GGT GCT GCA TTT	619
Closr	ATT GTA GTA CGT GTG TAG CCC	

f = Forward [a]R= C or T
r = Reverse [b]Y = A or G

Genus-specific primers were designed for *Clostridium* 16S rRNA also shown in Table 1. Genomic DNA samples for *C. botulinum* toxins 62A, Clovis (A, B), 17B, Beluga E, and PCF were provided by Kristin M. Marshall, Ph.D. of the US Food and Drug Administration and were used as positive controls. A temperature [53° - 63° Celsius (C)] and magnesium chloride gradient [1.0 mM – 3.0 mM] was performed to optimize the multiplex PCR assay, shown in Figure 1. The template extension was set at 72° C for 60 seconds (s) for a total of 39 cycles.

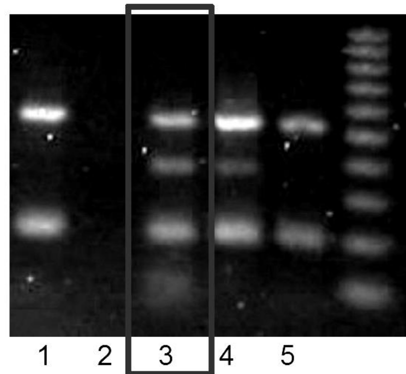

Figure 1. Multiplex PCR optimization for toxin detection

A $MgCl^{2+}$ (1.0 – 3.0 mM) and annealing temperature gradient (53.0 – 63.0° C) were performed simultaneously to find optimal PCR conditions. (Box) Optimal amplification determine to be 2.0 mM $MgCl^{2+}$ and 61.0° C.

A second temperature gradient was performed with alternate cycle conditions and varying sample concentration. All other PCR conditions remained constant with previous gradient. The extension temperature was reduced to 68° C for 37 s. Primers concentrations for toxins A & E were increased from 0.1 mM to 0.5 mM and two sets of samples were run with alternate DNA concentration, 4 ng/uL and 10 ng/uL. (Figure 2)

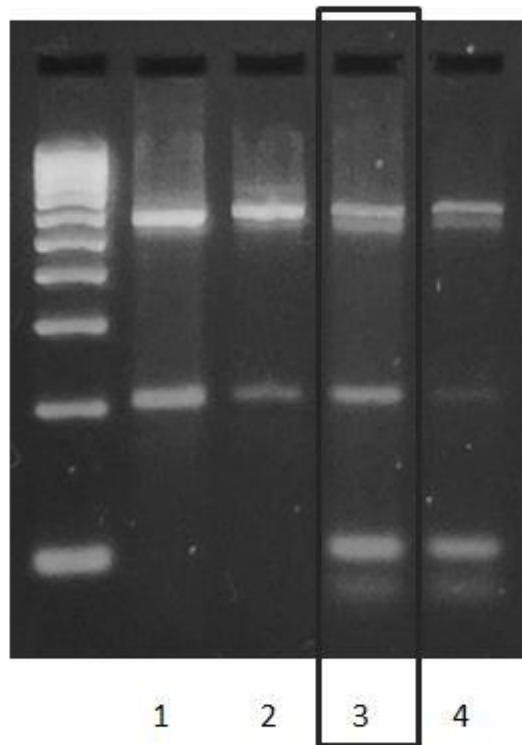

Figure 2: Second Multiplex PCR optimization

Lane 3 shows optimal amplification with 10 ng/uL of DNA and a annealing temperature of 56.9° C.

2.3 Spore Isolation In Saline Suspension

SBA plates were inoculated with lyophilized *C. perfringens* in CAMPY jars at 37° C for 24 hours (hr) then refrigerated at 2 – 8°C for 24 hr. Sporulation was then verified using malachite green spore stain.[6] (Figure 3.)

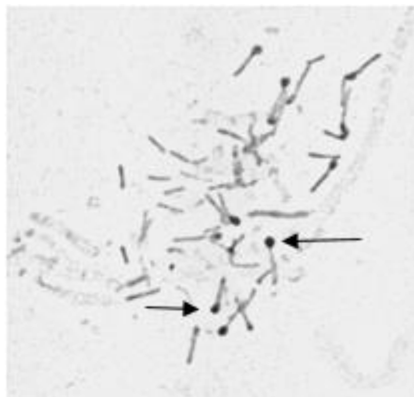

Figure 3. Malachite Green spore stain

Arrows represent *C. perfringens* spores

In order to verify spores could be lysed, two tubes were filled with saline and inoculated with organism until reaching a 0.5 & 1.0 McFarland turbidity standard. To obtain pure spore samples a Qiagen DNA extraction kit was used. The washes were saved instead of discarded as per standard protocol. (Figure 4)

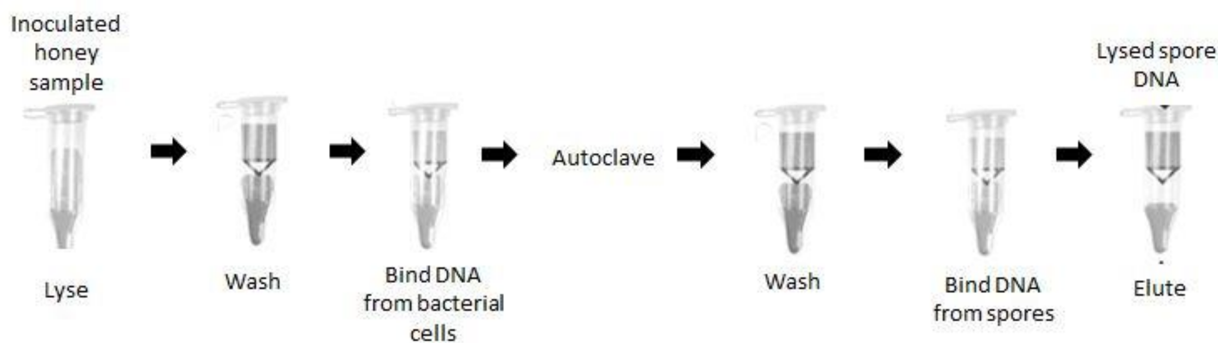

Figure 4: Spore isolation and lysing

The washes were centrifuged for five minutes (min) at 8,000 rpms and decanted before being placed in an autoclave for 30 min at 126° C to lyse the spores and release the DNA. This DNA was then reconstituted with 0.9% sterile saline and run through a second DNA extraction. PCR for 16s rRNA was then performed on the pre-autoclaved washes and the DNA extracted from the autoclaved washes. Results were visualized through gel electrophoresis. (Figure 5.A)

2.4 Spore Isolation In Honey Suspension

To determine if DNA can be detected from a honey sample inoculated with *C. perfringens* spores. Raw honey was dissolved at a 1:1 ratio with distilled water. Then 100 uL of a McFarland 3 spore solution was added to 125 uL of dissolved honey. Quantification could not be accurately performed on the spores so the McFarland standard of bacterial suspensions was used as a measurement tool for spore concentration. This mixture was then centrifuged for

30 min at 12,000 rpms. The supernatant was discarded and the pellet re-suspended with 200 uL of saline. DNA extraction was performed as previously described on the spore saline mixture discussed above. 16s rRNA PCR was performed on extraction and washes. (Figure 5.B)

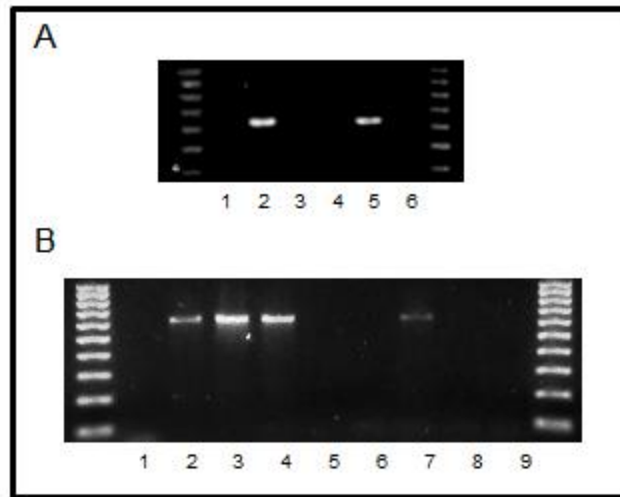

Figure 5: Confirmation of lysed spores

(A) 16s rRNA Clostridium specific PCR products from extracted spore lysate suspended in saline. Lanes: 1. negative primer control (*E. coli),* 2. positive primer control (*C. perfringens),* 3. first wash, 4. second wash, 5. autoclaved wash DNA extract, 6. negative PCR control (H_20). (B) 16s rRNA *Clostridium* specific PCR products from extracted spore lysate suspended in honey. Lanes: 1. negative primer control (*E. coli),* 2. positive primer control (*C. perfringens),* 3. pellet DNA extraction, 4. supernatant DNA extraction, 5. pellet DNA extraction – wash, 6. supernatant DNA extraction – wash, 7. autoclaved pellet DNA extraction – wash, 8. autoclaved supernatant DNA extraction – wash, 9. negative PCR control (H_2O).

2.5 Large Scale Honey Sample Processing

To determine if spore isolation could be performed from a large scale (~200g) sample, 224 g of honey was dissolved in 240 mL of DI water and mixed with 1.0 mL of a McFarland 3 *C. perfringens* spore mixture. This was aliquoted into two 200 mL tubes and centrifuged at 10,000 rpms for 30 min in an industrial Sorvall centrifuge. The supernatant was poured off and the pellets were reconstituted with 2 mL of sterile saline. This suspension was extracted for DNA using the standard Qiagen blood/tissue protocol and tested using multiplex PCR described above.

2.6 Utah Honey Sample Testing

224 g of honey was dissolved in 240 mL of DI water. This was aliquoted into two 200 mL tubes and centrifuged at 10,000 rpms (15,000 G) for 30 min in an industrial Sorvall centrifuge. The supernatant was poured off and the tubes were autoclaved. The pellets were reconstituted with 2 mL of sterile saline. The suspension was then combined into 15 mL tubes and vortexed. 200 uL of each honey sample were processed through the DNA Qiagen kit. Multiplex PCR was performed on *C. perfringens*, toxins A, B, E, and F, multiplex positive control, *E. coli* as the negative and the DNA extractions from the honey samples. This was all followed by a water blank. The amplified DNA was visualized through gel electrophoresis using SYBR green fluorescent dye.

A second multiplex PCR was performed using the second set of conditions on *C. perfringens*, toxins A, B, E, and F, multiplex positive control, *E. coli* as the negative, the DNA extractions from the honey samples and a final water blank. The amplified DNA was visualized through gel electrophoresis using ethidium bromide. (Figure 6)

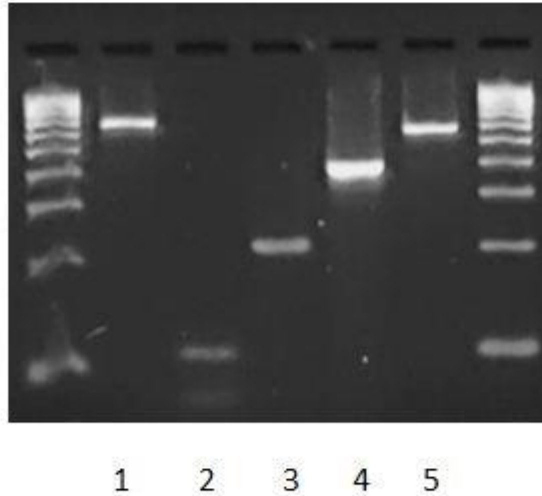

Figure 6: Multiplex PCR for detection of *Clostridium* species and toxin genes in Utah honey samples

Lanes: 1. Positive primer control (*C. perfringens),* 2. Toxin A, 3. Toxin B, 4. Toxin E, 5. Toxin F

3.Results

Initial multiplex PCR optimization resulted in an annealing temperature of 61° C and MgCl concentration of 2.0 mM. (Figure 1) Due to inconsistent amplification during the processing of honey samples, another gradient was performed to vary primer and DNA concentrations, as well as shortening the extension temperature and time. The initial annealing temperature was shockingly high and upon re-optimization a new annealing temperature of 56.9° C was found to be ideal using the new primer concentrations. (Figure 2)

C.perfringens spores were isolated from a homogenous mixture of bacteria and spores using a modified Qiagen DNA extraction procedure shown in Figure 4. The washes were negative for DNA confirming that the column retained the entire DNA from the lysed bacterial cells. Spores were lysed using a standard autoclave. The autoclaved sample was positive for Clostridium showing that the spores passed through the columns into the wash solution and that autoclaving it released the DNA from the spore without denaturing the sample. (Figure 5A) This process was repeated after adding the *C. perfringens* spores to dissolved honey and confirmed the ability to obtain DNA amplification from spores suspended in honey samples. (Figure 5B) This same method was used but with a larger volume of honey (224 g) to replicate actual sample conditions. Surprisingly, this large scale sample failed to amplify the 16s rRNA band.

Despite the lack of amplification in our large scale honey experiment the Utah honey samples were processed and tested for the presence of *Clostridium* toxin genes. While the positive and negative controls for the multiplex PCR assay passed there was no amplification in any of the 31 honey samples.

4. Discussion

This study is one of the first to research the prevalence of *C. botulinum* in Utah honey and to isolate and detect the spores directly from the honey without culturing. A multiplex PCR was optimized for the detection of *C. botulinum* toxins A, B, E, and F. Bacterial spores were successfully isolated and lysed using superheating by autoclave which could have application in the clinical laboratory setting for the direct detection of *C. botulinum* toxins.

The lack of amplification from the large volume experiments may suggest that something present in the honey is interfering with amplification or that the DNA concentration is below the limit of detection for the multiplex assay. It is also possible that Utah honey has a lower level of contamination of *C. botulinum* and that the small numbers of samples collected were not contaminated. The Qiagen DNA extraction kits have a limit of detection of 1 pg.

The limitations of this study include a small sample size and a lack of a defined limit of detection for this assay. An increased number of samples and sample volume could increase the power of the assay to detect a possibly lower prevalence of *C. botulinum* contamination. Establishing the limit of detection for this assay would add confidence to defining the possible level of contamination in Utah honey samples. Further studies with larger sample size and sample volumes would validate whether or not there is a lower prevalence of *C. boutilnum* in Utah honey.

5. Acknoweledgements

The authors wish to express their appreciation to Dr. Edward Walker of Weber State University Chemistry Department and Kristin M. Marshall, Ph.D., Research Microbiologist, United States Food and Drug Administration for their support of our project as well as the Nye Foundation and Weber State University Department of Undergraduate Research for funding this research.

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