Development of a PCR-Based Method for Detection of Delphinium species In Poisoned Cattle

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Abstract

Toxic plants such as Delphinium spp. (i.e., larkspur) are a significant cause of livestock losses worldwide. Correctly determining the cause of death is crucial in developing strategies to prevent future losses. The objective of this study was to develop an alternative diagnostic tool using Polymerase Chain Reaction (PCR) to detect the presence of a particular poisonous plant within the rumen contents. A pair of oligonucleotide primers specific to Delphinium spp. were developed and larkspur was detected in samples from an in vivo, in vitro, and in vivo / in vitro coupled digestion of Delphinium occidentale. Typical larkspur poisoning occurs when larkspur comprises 5-10% of the rumen content. This PCR method was able to detect larkspur in an experimental dilution containing 2% larkspur. The PCR-based molecular detection technique holds potential to diagnose larkspur and perhaps other toxic plant-caused losses.

Keywords: Delphinium, Larkspur, PCR, cattle poisoning, genetic identification

Introduction

Larkspurs (Delphinium spp.) are poisonous plants found on rangelands in the western United States. They are responsible for significant financial losses to the cattle industry and have been the subject of extensive research over the past century (Marsh et al. 1916; Nielsen et al. 1994; Pfister et al. 1999; Pfister et al. 2002). Seven Delphinium species are responsible for most of the cattle losses in North America, D. andersonii, D. barbeyi, D. geyeri, D. glaucum, D. glaucescens, D. nuttallianum, and D. occidentale (Pfister et al. 1999; Green et al. 2011).

Larkspur-induced poisoning in cattle is caused by norditerpene alkaloids that can represent up to three percent of the plant dry weight. When sufficient larkspur is ingested by cattle, these alkaloids block nicotinic acetylcholine receptors causing neuromuscular paralysis which leads to labored breathing, increased heart rate, fatigue, collapse, and ultimately death (Pfister et al. 1999; Green et al. 2011).

Determining the agent responsible for the death of an animal is crucial in developing strategies to prevent future losses (Stegelmeier et al. 2009). Each year cattle die on foothill and
mountain ranges leaving little evidence to the cause of death other than the presence of larkspur in the pasture. Providing evidence of ingestion of a particular plant is an important step in determining the cause of the livestock loss. Microscopy has been used to examine the rumen contents of animals for the presence of a particular plant (Sparks and Malachek 1968; Holochek et al. 1982). Norditerpene alkaloids can be detected in the rumen contents of poisoned animals by liquid chromatography-mass-spectrometry (LC-MS), but may not be available in all veterinary labs. Polymerase Chain Reaction (PCR) is a molecular tool that may allow detection of genetic material from a plant species within the rumen contents. Studies conducted with cattle have shown that plant-derived DNA is degraded throughout the digestive tract, but fragmented DNA was detectable in the gastrointestinal tract (Chowdhury et al. 2004; Einspanier et al. 2004). The objective of this study was to evaluate the use of PCR as an alternative diagnostic tool to determine if larkspur could be detected within rumen contents in vitro and in vivo.

METHODS AND MATERIALS

**Primer Design.** The internal transcribed spacer of the ribosomal DNA sequences (ITS) from several *Delphinium* species and *Aconitum columbianum* from the Intermountain West were aligned using Sequencher. The ITS sequence representing each respective species was previously published (Koontz et al. 2004) and available on GenBank: *D. andersonii* (AF258773), *D. barbeyi* (AF258709), *D. bicolor* (AF258711), *D. geyeri* (AF258762), *D. glaucescens* (AF258754), *D. glaucum* (AF258739), *D. novomexicanum* (AF258718), *D. nuttallianum* (AF258688), *D. ramosum* (AF258687), *D. scaposum* (AF258732), *A. columbianum* (AF258683), and *A. delphiniifolium* (AF258681). An oligonucleotide forward primer Delph1F (5’ GTGAAAAYAAACCGRGACGG 3’) and reverse primer Delph1R (5’
GGGGATGATGAGCACACAACCA 3’) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

**Ruminally-cannulated animals.** Three gentle ruminally-cannulated Angus cows (510 kg) were maintained on a diet of alfalfa hay at maintenance levels (circa 10 kg/day). The diet was offered twice daily at 08:30 and 16:00 hours. All procedures were approved by the Utah State University IACUC committee and were conducted under veterinary supervision.

**Plant Material.** Plant material from previous collections representing the following species: *D. occidentale* (Logan, UT), *D. nuttallianum* (Colbrun, CO), *D. andersonii* (Garrison, UT), *D. glaucesens* (Dillon, MT), *D. scaposum* (Tuweep, AZ), *D. ramosum* (Cuchara, CO), *D. barbeyi* (Crested Butte, CO), *D. occidentale* (Wilsal, MT), *A. columbianum* (Elko, NV), and *Medicago sativa* (Logan, UT) were used to validate the specificity of the primers. Samples were placed on dry ice at the time of collection, frozen and freeze-dried upon return to the laboratory, and ground for subsequent DNA extraction.

**In Vivo Rumen Digestion.** *Delphinium occidentale* leaves were cut into pieces with a diameter ranging from 0.5-1.5 cm². Five grams of cut leaves were placed in each of 27 nylon bags (10 cm x 20 cm and 50 µm pore size, Ankom Technology, Macedon, NY), respectively (Ørskov et al. 1980). Three bags of material were freeze-dried as control samples. Eight bags each were incubated in the rumen of three ruminally-cannulated cows. All eight bags were placed in a larger nylon bag with a weight enclosed so the samples would be continually submersed in rumen fluid in the ventral sac. A 50 cm nylon string was attached to allow movement within the rumen and for the retrieval of the bags. One bag was removed from each animal (n = 3) at the following time points: 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 36 h, and 48 h.
Samples were immediately placed in a -20°C freezer upon removal and subsequently freeze-dried and ground (1 mm screen).

**In Vitro Rumen Digestion.** *Delphinium occidentale* leaves were cut into pieces with a diameter ranging from 0.5 - 1.5 cm². Five grams of cut leaves were placed in each of 21 nylon bags (5 cm x 5 cm, 25 um pore size, Ankom Technology). Three nylon bags were freeze-dried as control samples. Six bags were placed in each of three non-rotating digestion vessels containing freshly-collected, anaerobic rumen fluid (Daisy II incubator, Ankom Technology) at 39°C from the three cannulated cows. One bag was removed from each digestion vessel (n = 3) at the following time points: 8 h, 16 h, 24 h, 32 h, 40 h, and 48 h. Samples were immediately placed in a -20°C freezer upon removal and subsequently freeze-dried and ground.

**In Vivo and In Vitro Rumen Digestion.** *Delphinium occidentale* leaves, previously frozen, were cut into pieces with a diameter ranging from 0.5-1.5 cm². Five grams of cut leaves were placed in each of 21 nylon bags as noted above. Three nylon bags were freeze-dried as control samples. Eighteen bags were placed in a larger bag with a weight enclosed in the rumen of a cannulated cow. A 50 cm string was attached to allow movement and for the retrieval of the bags. All the bags were retrieved at 8 hrs and three were immediately frozen. The remaining 15 bags were immediately placed into three non-rotating digestion vessels (Daisy II Incubator, Ankom Technology) containing freshly-collected, anaerobic rumen fluid maintained at 39°C from the three cannulated cows. One bag was removed from each digestion vessel (n = 3) at the following time points: 16 h (24 h total; 8 h in vivo + 16 h in vitro), 24 h (32 h total), 40 h (48 h total), 48 h (56 h total), and 64 h (72 h total). Samples were immediately placed in a -20°C freezer upon removal and subsequently freeze-dried and ground.
**In Vivo Rumen Digestion Sensitivity.** Different ratios of *D. occidentale* leaves and alfalfa hay mixture were prepared. Four replicate nylon bags of the following ratios were prepared (g larkspur: g alfalfa): 5:0, 1:2.13, 1:5.25, 1:11.5, 1:24, 1:49. One bag of each ratio was freeze-dried as a control sample. One sample of each ratio was placed in the rumen of each of the three cannulated cows. All bags were placed in a larger nylon bag with a weight. A 50 cm string was attached to allow movement within the ventral sac and for the retrieval of the bags. After 8 h all the samples were retrieved from the cannulated cows. Samples were immediately placed in a -20°C freezer upon removal and subsequently freeze-dried and ground.

**DNA extraction.** DNA was extracted from ~150 mg of freeze-dried ground tissue using the ZR plant/seed DNA MiniPrep kit (Zymo Research Corp., Irvine, CA, USA). Extractions were preformed according to the manufacturer’s instructions with one exception, the volume of lysis solution used was increased to 1.2 mL to obtain a higher concentration of DNA. DNA was quantitated with the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

**Polymerase Chain Reaction (PCR).** All PCR was performed with a Bio-Rad Dyad PCR Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA). Thermal cycling conditions for all material was as follows: 1) initial denaturation at 94 °C for 3 min, 2) followed by 10 cycles of 30 s at 94 °C, 45 s at 60 °C, and 30 s at 72 °C, 3) followed by 25 cycles of 30 s at 94 °C, 45 s at 54 °C, and 30 s at 72 °C, and 4) a final extension of 5 min at 72 °C. Each reaction had a total volume of 50 µL containing 500 ng DNA. GoTaq DNA polymerase (Promega Corporation, Madison, WI) was used following the reaction conditions recommended by the manufacturer. PCR results were visualized on a 1% agarose gel containing ethidium bromide at 118 volts for 20
RESULTS

Primer Specificity. Candidate specific oligonucleotide DNA primers were developed by aligning and comparing the ITS sequence from several Delphinium spp. as well as representative Aconitum spp. (Figure 1). A 500 base pair amplicon was obtained from each of the seven Delphinium species but not from the Aconitum or Medicago sativa (alfalfa) sample (Figure 2). The Delphinium primers, therefore, successfully amplified a product with all of the Delphinium species tested including five of the more important Delphinium species in the Western United States.

Figure 1. Alignment of the internal transcribed spacer (ITS) of the ribosomal DNA from several Delphinium and Aconitum species. Oligonucleotide primers Delph1F and Delph1R are shown.
**Figure 2.** Agarose gel electrophoresis showing specificity of DNA primers. 10000bp marker (LD) shown as reference. Lanes: 1, *D. occidentale* (Logan, UT); 2, *D. nuttallianum*; 3, *D. andersonii*; 4, *D. glaucescens*; 5, *D. scaposum*; 6, *D. ramosum*; 7, *D. barbeyi*; 8, *D. occidentale*; 9, *A. columbia*; and 10, *M. sativa*.

**In Vivo Rumen Digestion.** Amplicons were obtained from *D. occidentale* samples that had been incubated in the rumen of the three cannulated cows after 4 h, 8 h, 12 h, and 16 h (Figure 3). At the 20 h and 24 h time point, amplicons were observed from two of the replicate samples (Figure 3). Amplicons were not observed from the 36 h and 48 h time points. Amplicons were obtained from the three control samples but not from the *Aconitum* or alfalfa sample (Figure 3).

**Figure 3.** Agarose gel electrophoresis with amplicons from the in vivo rumen digestion. 10000 bp marker (LD) shown as a reference. Triplicates of undigested positive control (+ ctrl) and digested (4 - 48 h) *D. occidentale*. Negative control (- ctrl) representing *A. columbia* and *M. sativa*. 
**In Vitro Rumen Digestion.** Amplicons were obtained from *D. occidentale* samples that had been incubated in digestion vessels of rumen fluid in each of the three replicates up to 40h (Figure 4). At the 48h time point, larkspur was detected from one of the replicate samples (Figure 4). Amplicons from the three control samples were obtained but not from the *Aconitum* or alfalfa sample (Figure 4).

![Agarose gel electrophoresis with amplicons from the in vitro rumen digestion.](image)

**Figure 4.** Agarose gel electrophoresis with amplicons from the *in vitro* rumen digestion. 10000 bp marker (LD) shown as a reference. Triplicates of undigested positive control (+ctrl) and digested (8 – 48 h) *D. occidentale*. Negative control (-ctrl) representing *A. columbianum* and *M. sativa*.

**In Vivo and In Vitro Rumen Digestion.** An *in vivo* digestion was coupled with an *in vitro* digestion to determine the length one may expect to detect larkspur in a bovine that consumed larkspur and died eight hours later. Amplicons were obtained from *D. occidentale* samples that had been incubated *in vivo* and *in vitro* from 8 h to 72 h in all three replicates.
Amplicons were detected from the three control samples but not from the *Aconitum* or alfalfa sample (Figure 5).

![Figure 5. Agarose gel electrophoresis with amplicons from the *in vivo* and *in vitro* coupled rumen digestion. 10000 bp marker (LD) shown as a reference. Triplicates of undigested positive control (+ctrl) and digested (8-72h) *D. occidentale*. Negative control (-ctrl) representing *A. columbianum* and *M. sativa.*](image)

**In Vivo Rumen Digestion Sensitivity.** Larkspur was detected from each of the triplicate digested ratios ranging from the *D. occidentale* only sample to 1 gram *D. occidentale* to 49 grams alfalfa (Figure 6). Amplicons were not detected from the *Aconitum* or alfalfa sample (Figure 6).

![Figure 6. Agarose gel electrophoresis with amplicons from undigested positive control (+ctrl) and the *in vivo* rumen digestion from different ratios of larkspur to alfalfa. 10000bp maker (LD) shown as a reference. Ratios represent grams of larkspur:grams of alfalfa. Negative control (-ctrl) representing *A. columbianum* and *M. sativa.*](image)
DISCUSSION

*In vivo* digestion study suggests that *D. occidentale* DNA is degraded over time and that by 36 h post incubation, larkspur is no longer detectable. However, the DNA degradation observed here may have occurred faster than in natural grazing, as the starting plant material was initially cut into smaller fragments compared to the large bites consumed by grazing cattle (Pfister et al. 1988). Additionally, much of the ingested larkspur remains in the upper layer in the ruminal dorsal sac. This is comprised primarily of rapidly fermenting, buoyant particles which could protect it from digestion and DNA degradation. Plant DNA may be rapidly degraded by the microbial activity in rumen fluid (Duggan et al. 2000, Alexander et al. 2004).

Higher quality amplicons were obtained from the *in vitro* digestion process and detected up to 48h. This may be due to lack of activity of the microflora, motility, and fermentation in the *in vitro* rumen compared to the *in vivo* rumen. Plant material in the nylon bags was buoyant, and moved to the top of the digestion vessel with the other plant material equivalent to the rumen mat. However, the expansion of the nylon bag provides evidence of digestion.

One concern when using PCR as a diagnostic tool is determining how much material would need to be sampled to provide confidence it is representative of the overall rumen contents. The data reported shows that larkspur was detected at a ratio of 1:49, while larkspur is likely to comprise 5-10% (1:20-1:10) or higher of the rumen contents of a poisoned animal (Pfister et al. 1999). However, in the cases of plants that are more acutely toxic such as water hemlock (*Cicuta* spp.), it may be even lower. So while larkspur may be detectable by the PCR assay, its relative abundance within the rumen is not yet quantifiable by the assay. Future studies should therefore pursue quantitative PCR (qPCR). A second concern when using PCR is the
integrity of the template DNA. Often there is a lapse in time between the death of an animal and its discovery and the subsequent sampling of rumen contents. The data reported here suggests that up to 48 h after the death of animal, larkspur can still be detected by PCR. This supports the idea that PCR could be used as a diagnostic tool if the dead animals are found and samples collected within two days.

In summary, a pair of oligonucleotide primers specific to Delphinium spp. were developed. Using these primers, larkspur was detected in samples from an in vivo, in vitro, and in vivo / in vitro coupled digestion of D. occidentale. Lastly, larkspur was detected at far lower concentrations than would be expected in a poisoned animal. These data demonstrate that this method and the primers are both specific and sensitive, a characteristic of a good diagnostic tool. This research suggests that PCR-based methods could be used as a diagnostic tool to detect larkspur in the rumen contents of a poisoned animal. Rumen samples need to be collected and analyzed from actual field cases of poisoned cattle to validate this method. A PCR-based method could also be developed for diagnosis of poisoning by any other plant species that is acutely toxic such water hemlock, death camas (Zigadenus spp.), and oleander (Nerium oleander).
References


