ELISA-Based Correlation of *Rathayibacter toxicus* Antigen With Corynetoxins in Pasture and Hay

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Abstract

Enzyme-linked immunosorbent assays (ELISAs) for a *Rathayibacter toxicus* antigen and for corynetoxins were developed in the 1990s and in 2000-2002, respectively, to assist in the management and control of annual ryegrass toxicity (ARGT). The relationship between the results of these two ELISAs has not previously been established. This relationship was examined in an inter-laboratory study and in samples collected from the field in Western Australia (WA). The inter-laboratory study compared the results obtained by three laboratories, each having performed both ELISAs on the same samples. There was good agreement between laboratories in results for both analytes. The correlations between pairs of laboratories for the *R. toxicus* ELISA were r = 0.86, 0.85, and 0.92; and for the corynetoxins ELISA the correlations between pairs of laboratories were r = 0.98, 0.97, and 0.98. The results of the corynetoxins ELISA were also compared with those of the *R. toxicus* antigen ELISA in infected annual ryegrass and hay samples collected from paddocks, windrows, and hay bales on four different properties in WA at various times throughout spring and summer. The inter-laboratory study and the analysis of field samples described in this paper show that *R. toxicus* antigen and corynetoxins are present simultaneously in the analyzed samples and that there is an overall positive correlation: r = 0.70 ($p<0.001$, n = 100) and 0.80 ($p<0.001$, n = 389) in the two studies respectively between *R. toxicus* antigen and corynetoxins concentrations in extracts of ryegrass and hay samples.

Keywords: ARGT, annual ryegrass toxicity, immunoassay

Introduction

Annual ryegrass toxicity (ARGT) occurs when livestock graze on pastures or ingest hay containing annual ryegrass (*Lolium rigidum*) infected by the bacterium *Rathayibacter toxicus* (Sasaki et al. 1998). This bacterium produces corynetoxins (Payne et al. 1983) that, if ingested in sufficient quantities, will cause neurological signs and subsequent death in livestock (McKay et al. 1993). A parasitic nematode (*Anguina funesta*) carries *R. toxicus* into the growing ryegrass plant. The nematodes induce the formation of galls, instead of seeds, inside the developing seedheads, and multiply within these structures. The gall and seedhead may be colonized by *R. toxicus* when sufficient numbers of the bacterium are present (figure 1). These bacterial galls contain a yellow mass of toxin-producing bacteria (Bird and Stynes 1977, Vogel et al. 1981). In vitro studies have

shown that corynetoxins are mainly produced when bacterial growth is in the stationary phase, although some corynetoxins are detectable at the end of log-phase growth (Payne and Cockrum 1988). In vivo, the quantity of corynetoxins in bacterial galls is implicitly expected to be proportional to the bacterial load and the weight of the galls. Indeed, analysis of Cockrum and Edgar's (1985) data shows a high correlation between gall weight and corynetoxins content (r = 0.91). The production of the corynetoxins is greatest when the plants senesce (Stynes and Bird 1983).

Figure 1. (top) Annual ryegrass (*L. rigidum*) seedheads and (bottom) from left: two dehusked *R. toxicus* bacterial galls, three bacterial galls, two nematode galls, two *L. rigidum* seeds. [Magnification 10x]. Courtesy of Leo den Hollander.

ARGT has been reported in South Australia (SA), Western Australia (WA), and South Africa (McIntosh et al. 1967, Gwynne and Hadlow 1971, Schneider 1981). *R. toxicus* has also been implicated in stock losses because of its presence in galls induced by *Anguina paludicola* (Bertozzi and Davies 2009) in blowngrass, *Lachnagrostis filiformis* (formerly *Agrostis avenacea*, Jacobs 2001) in northern New South Wales, and annual beardgrass, *Polypogon monspeliensis*, in southeastern SA (McKay and Ophel 1993). *R. toxicus* infection in these grasses is similar to that in annual ryegrass, and the same corynetoxins are produced (Edgar et al. 1994). In addition, corynetoxins have been implicated in stock poisoning related to nematode seed galls of *Festuca nigrescens* in the United States and New Zealand (Anderton et al. 2004a). In 1996, ARGT was diagnosed in cattle in Japan after they had eaten hay imported from WA. This incident resulted in the introduction of a voluntary hay testing protocol to minimize contamination of exported hay with *R. toxicus* (Elson 2002a,b). The protocol was made compulsory in 2005.

The hay-testing protocol requires laboratories to provide a sensitive, high-throughput assay for the presence of *R. toxicus*. The assay in use since 1996 has been an enzyme-linked immunosorbent assay (ELISA) for a water-soluble antigen of *R. toxicus* (Masters et al. 2006, 2011). Since this ELISA could only be regarded as providing an indirect measure of the toxicity of samples, the Commonwealth Scientific and Industrial Research Organisation (CSIRO) developed an ELISA that quantitates the amount of corynetoxins in samples (Than et al. 2002). An inter-laboratory study was performed in 2002-2003 to compare the robustness of the two ELISAs by testing the same samples in three different laboratories. Since the bacterial antigen ELISA protocol formed the backbone of the hay-testing protocol, and since the more complex corynetoxins ELISA was a direct measure of the potential for toxicity, the correlation of corynetoxins to the level of bacterial antigen—and hence the validation of the bacterial antigen ELISA—was investigated in the inter-laboratory study and in additional samples collected from the field in WA.

Materials and Methods

Bacterial Galls

R. toxicus bacterial galls were separated from ryegrass samples collected in 1999 from an infected location in the Wongan Hills-Ballidu district of WA. The ryegrass was threshed, and the galls were identified and sorted visually over a light box by two independent operators. One operator did the initial sorting, and a second checked the selected galls and removed any that were not bacterial galls (figure 2).

Corynetoxin Standards

The corynetoxins (figure 3) used as analytical standards and to develop and validate the corynetoxins ELISA were isolated from grain screenings collected during harvest in WA in the

Figure 2. Sorting galls over a light box. Courtesy of Leo den Hollander.

early 1990s. The extraction and purification process afforded the corynetoxins with a purity of >95% (Anderton et al. 2004b). Briefly, the grain screenings were sieved to concentrate the bacterium-colonized galls that were subsequently extracted with 80% methanol in water. After evaporation of the solvent, the crude extract was treated with aqueous sodium hydroxide to hydrolyse co-extracting glycerides (Frahn et al. 1984). Adapting the method of Vogel et al. (1981), acidification of the hydrolysis mixture resulted in a precipitate enriched in corynetoxins that was subsequently partitioned with ethyl acetate and water to produce an insoluble, more-enriched corynetoxin fraction at the ethyl acetate/water interface. Further solvent-solvent partitioning using a phase transfer reagent and final preparative HPLC, based on the analytical HPLC described by Cockrum and Edgar (1985), provided the pure corynetoxins in gram quantities (Anderton et al. 2004b). Similar to the confirmation of corynetoxins in bacterial "galls" from New Zealand and the United States (Anderton et al. 2004a), the identities of the individual corynetoxins were further confirmed by diverting the effluent from the HPLC column to a LCQ ion trap mass spectrometer (Thermo-Finnigan) operated in the positive ion, electrospray ionization mode. Prior to the analysis, the mass spectrometer was tuned as per operating guidelines by infusing a solution of a purified, major corynetoxin (figure 3).

Samples for Inter-Laboratory Study

One hundred oaten hay samples were obtained from hay export companies in WA (Gilmac Pty Ltd) and SA (Balco Australia Pty Ltd and Golden Plains Fodder Australia Pty Ltd). The samples were collected by farm staff on various farms early in 2001 from hay bales made in the year 2000 with a steel hay-bale core sampler attached to the front or rear of a 4-wheel-drive vehicle. The hay-bale core samplers were not commercially available but were custom made based on the design of those used for bale core sampling for nutritional testing. The hay-bale core samplers were cylindrical, 35±10 mm in diameter, and >800 mm long. There was a collection hole at the vehicle end of the sampler, and a plastic collection bag was attached under the hole so that the hay sample was forced into the bag when the core sampler was driven 800 mm into the bale (figure 4). Cores (~50 g) were collected from each end of 10 bales, all into the same collection bag, so that each final composite sample weighed 1000±200 g. Residual hay was cleaned out of the core sampler after collection of each composite sample.

Field Samples

Samples were collected from four farms: farm A (in the Brookton Shire, where ARGT had not been reported and *R. toxicus* had not been detected) and farms B, C, and D (in the Shires of Victoria Plains and Serpentine-Jarrahdale, respectively, where ARGT had occurred and a high prevalence of *R. toxicus* in extracts of pasture and hay samples had been detected by use of the *R. toxicus* antigen ELISA in the previous 2 years). Oaten hay (on farms A and B) or meadow hay (on farms C and D) and standing ryegrass samples were collected. Hay exporters (farms A and B) and dairy farmers (farms C and D) cut 5-10 g ryegrass samples or ryegrass heads from fence lines immediately adjacent to areas to be cut for hay at 10 meter intervals until 200 g in total was collected per fence line. Samples were collected every 14 days from July 2001 to January 2002 (except on farm D: July to end of November 2001) from a total of 21 fence lines on the 4 farms (2 on farm A, 10 on farm B, 4 on farm C, and 5 on farm D; most of the fence lines being in different paddocks). After cutting the pasture for hay, random grab samples were collected from the windrows until 1 kg of composite sample was collected per paddock (twice per week). Once the hay was baled, sampling was to be performed as for the inter-laboratory study except that at each collection time, the sample on each paddock or farm was a 200g core taken from one end of a single bale. This was done from mid-November 2001 to the middle of February 2002 on farms A, B, and D and to the middle of June 2002 on farm C. On farm B, the bales weighed 650-750 kg (dimensions 1.2 x 1.2 x 2.4 m), and on farm D the bales weighed 250 kg (1.5 x 1.2 m round bales). Information about the bales on the other two farms was not provided. Windrows and bales were sampled in two

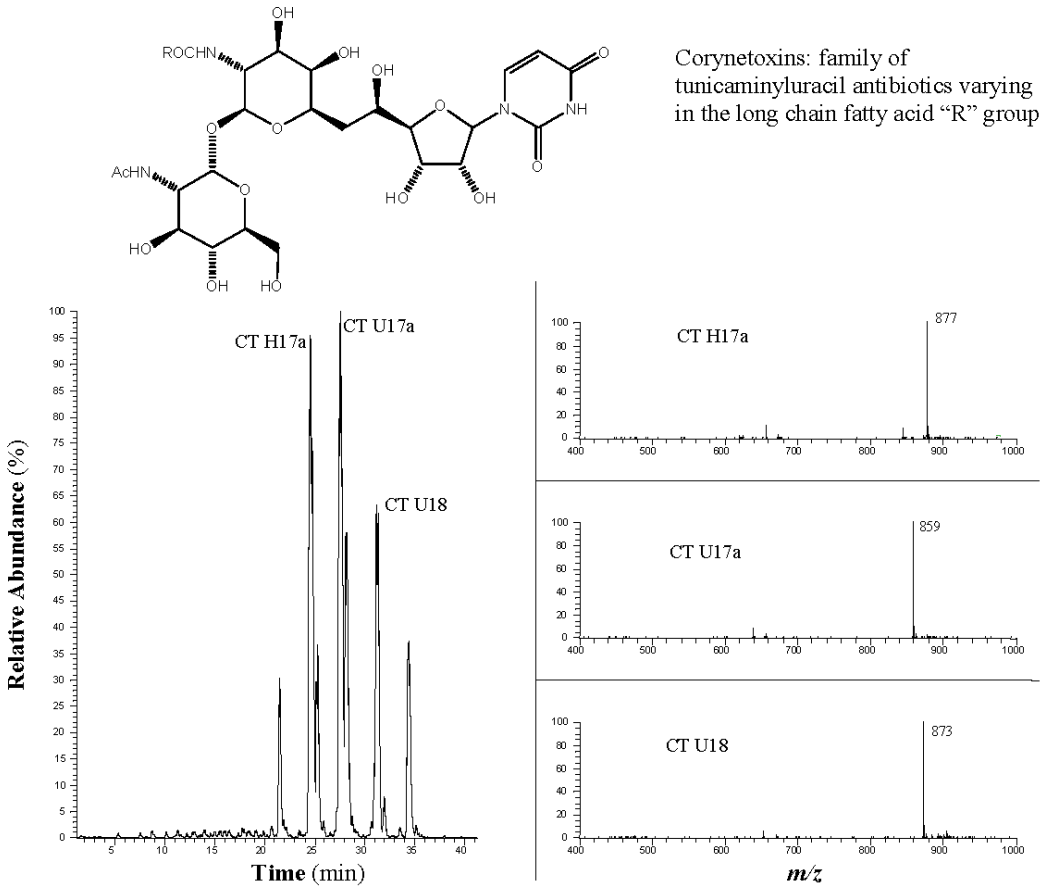

Figure 3. The general structure of the corynetoxins and a HPLC-esi(+)MS ion chromatogram of a corynetoxins sample showing, for example, the mass spectra for corynetoxins H17a, U17a, and U18.

paddocks on farm A, one paddock on farm B, four paddocks on farm C, and two paddocks on farm D. Samples of ryegrass heads from uninfected farm A for a spiking experiment were collected from along fence lines as for growing ryegrass at 14-day intervals from early September to mid-December 2001, providing a total of 8 samples (approximately 200 g each). On each farm, the repeat sample collections were taken from the same fence lines, the same windrows, or the same bales, as appropriate.

Ryegrass and hay samples were transferred to a cooler at 4 °C within an hour after collection and kept cool until delivery to the laboratory either in the afternoon the same day or early morning the next day. On arrival in the laboratory, the samples were kept at 4 °C until late afternoon (the same day or the next day), when they were soaked in 1% cyclodextrin to extract the *R. toxicus* antigen and the corynetoxins (*vide infra*). The soak extracts were stored at -15 °C ± 5 °C until the day of assay. All these samples were analyzed only in the Department of Agriculture and Food (DAFWA) laboratory using the described *R. toxicus* (Masters et al. 2006) and corynetoxins (Than et al. 2002) ELISAs.

Figure 4. (top) Driving corer into hay bale and (bottom) corer with hay sample in bag. Courtesy of Leo den Hollander.

Sample Extraction

All samples in this study were extracted by soaking overnight (approximately 17 h) in 5 volumes (v/w) of 1% methyl-β-cyclodextrin (Wacker Chemicals) in water in a tough plastic bag (figure 5). At the end of the soaking period, each sample was mixed thoroughly by kneading before cutting a corner of the bag to collect a mid-stream aliquot (1.5 mL) of the extract (to minimize the collection of soil or debris in addition to the clear extract). The soak extract samples were stored at −20 °C until tested.

Figure 5. Soaking hay samples (each plastic bag contains 1 kg hay plus 5 L of 1% methyl-β-cyclodextrin in water). Courtesy of Leo den Hollander.

The R. toxicus *Antigen ELISA*

The *R. toxicus* antigen ELISA (*R. toxicus* ELISA) used in these studies was performed as described by Masters et al. (2006) except that samples were extracted as above. The presence of 1% methyl-β-cyclodextrin does not affect the *R. toxicus* ELISA results (Masters, unpublished 2001). Semi-quantitative estimations of the amount of antigen present were performed as follows: Samples with an absorbance at 450 nm (Abs_{450}) reading higher than 2.0 when tested undiluted were tested in serial 10-fold dilutions (from 1 in 10 to 1 in 10,000, or higher if required) in 0.02 M phosphate buffered saline (pH 7.2) with 0.05% Tween 20 (Sigma P-1379) (PBST). The positive reference sample for this assay was an aqueous extract of contaminated oaten hay from the Wongan-Ballidu district of WA, containing bacterial galls as determined by identifying galls over a light box. The negative reference sample was an aqueous extract from ryegrass hay collected from Bedfordale (32.172°S, 116.047°E), an area where ARGT has not been recorded and that contained no bacterial galls. Both reference samples had been prepared when the ELISA was first developed and stored in1 mL aliquots at <−10 °C until used in assays. The positive reference was diluted as described (Masters et al. 2006). The ELISA results for each plate were accepted if the Abs_{450} for the positive reference was between 1.8 and 2.6 and the Abs_{450} for the negative reference was less than 0.25. For each sample dilution series from 1 in 10 to 1 in 10,000, the result chosen for calculating ELISA Units (EU) was that which fell within the linear range of the response curve (between 0.2 and 2.0); and if more than one result fell in this range, the result closest to the middle of the linear response curve was used (Masters et al. 2014). The EU calculation was simplified: [(Test Abs_{450} – Neg. reference Abs_{450}) x 100] x (reciprocal of the dilution for the sample). Experiments involving spiking of hay samples with bacterial galls have shown that using this simplified EU calculation, the relationship between EU and galls/kg is such that 10, 100, 1,000, 10,000, and 90,000 EU are equivalent to about 0.3, 1.7, 9.5, 52, and 300 galls/kg, respectively (Gregory, unpublished). The limit of sensitivity for this ELISA is 20 EU. The measurement uncertainty of the ELISA as determined at the DAFWA laboratory by repeated analysis of the same sample on different days and by different operators over a period of several months, during which different reagent batches were used, is 13% (coefficient of variation) at a mean value of 20 EU (n = 223) and 8.1% (n = 100) at a mean value of 5,943 EU.

The Corynetoxins ELISA

A quantitative indirect competitive ELISA for corynetoxins was developed by Than et al. (2002). The corynetoxins ELISA reagents were developed at the CSIRO and are not commercially available. Briefly, 96-well flat-bottom microtiter plates (Nunc 439454 Maxisorp) were pretreated with 0.2% glutaraldehyde in pH 9 carbonate buffer for 2 h at 56 °C. Plates were washed with pure water and then coated with chemically modified tunicamycins (4 ng/well in 100 µL pure water) for 2 h at 56 °C. The latter were prepared by treating commercially acquired tunicamycins (Fluka Analytical) with trifluoroacetic acid anhydride in trifluoroacetic acid, followed by hydrolysis to afford the diaminotunicaminyluracil derivative used for directly coating the glutaraldehyde-treated microtiter plates (Than et al. 1998). After washing 4 times with normal saline containing 0.5% Tween 20, dilutions of sample extracts in assay buffer (phosphate buffered saline pH 7.3 containing 0.5% protease-free bovine serum albumin and 0.05% Tween 20) and corynetoxin standards in 0.1% methyl-β-cyclodextrin in assay buffer were added, 100 µL/well in quadruplicate, followed by 50 µL sheep anti-corynetoxin antibodies (diluted 1 in

12,000 in assay buffer). The plate was mechanically shaken so that the contents of each well were mixed and then incubated at room temperature for 2 h. The wells were again washed 4 times with normal saline containing 0.5% Tween 20 and then incubated with anti-sheep IgG conjugated to horseradish peroxidase in assay buffer for 1 h at 37 °C. After washing again in the same way, the wells were finally incubated with 3,3'5,5'-tetramethyl benzidine substrate (K Blue, Cat. No. ESKE 1000, from ELISA Systems) for 30 min at room temperature. The color reaction was stopped by adding 50 µL/well of 0.5 M sulfuric acid, and the Abs_{450} was read. A standard curve was constructed by plotting the average Abs_{450} against the amount of standard corynetoxins added, and the resulting curve was used to determine the amount of corynetoxins in the diluted samples. Test samples were initially diluted 1 in 10; then samples with results that fell above the highest point on the standard curve were tested again at 1 in 100 in 0.1% methyl-β-cyclodextrin. The limit of quantitation for corynetoxins in hay with this ELISA was determined by Than et al. (2002) to be 40 µg/kg. The measurement uncertainty of this ELISA, determined at the DAFWA laboratory by repeated analysis of the same sample on different days and by different operators over a period of several months, during which different reagent batches were used, is 12% (coefficient of variation) at a mean value of 136.4 µg/kg (n = 172). Throughout this study, values below the level of quantitation were entered into data analysis spreadsheets as the values recorded (not as zero).

Inter-Laboratory Study

In 2001, an inter-laboratory study was conducted to ensure that the *R. toxicus* and corynetoxin ELISAs met acceptable standards for the quality assurance (QA) of hay in terms of reproducibility within and between laboratories and the robustness of the assays. The participating laboratories were the Animal Health Laboratories at DAFWA, the CSIRO Plant Toxins Research Group at the Australian Animal Health Laboratories, and the Plant Research Centre at the South Australian Research and Development Institute (SARDI). Soak extracts from 100 hay samples were made at SARDI (samples 1-10) and DAFWA (samples 11-100), each divided into 1.2 mL aliquots and stored frozen until 1 aliquot of each extract was dispatched in a polystyrene insulated container with ice packs to each of the 3 participating laboratories. The laboratories performed the *R. toxicus* ELISA with reagents and procedures supplied by DAFWA (Masters et al. 2006), and the corynetoxins ELISA with reagents and procedures supplied by CSIRO (Than et al. 2002) on the same aliquot, stored frozen between assays.

Comparison Methods and Statistical Analysis for Inter-Laboratory Study

The data obtained by the three laboratories were analyzed at DAFWA in five ways.

(A) *R. toxicus* ELISA versus corynetoxins ELISA in each laboratory. First, it was determined how many samples each laboratory categorized as "higher" (>90,000 EU) and "lower" (<90,000 EU) by the *R. toxicus* ELISA. The value 90,000 EU was chosen because this level is just below the mean (but above the median) found in 14 samples spiked with 100 bacterial galls/kg in a previous study (Masters et al. 2014). The two categories of *R. toxicus* ELISA results were each further divided into four corynetoxins result categories, based on the average corynetoxins ELISA result from the three laboratories, such that each category contained roughly one quarter (20-30) of the samples. The first category, 0 – <40 µg/kg, was all results below the limit of quantitation. The other three categories were 40 – <100 µg/kg, 100 – <400 µg/kg, and ≥400 µg/kg. The number of "higher" or "lower" *R. toxicus* results in each corynetoxins result category was then determined for each laboratory.

(B) Reproducibility of each assay. The coefficient of variation (CV) of the difference in results between the three laboratories was calculated as the between laboratory standard deviation expressed as a percentage of the mean for each assay.

(C) Inter-laboratory agreement. The results were divided into three categories: high, medium, and low, each category containing roughly one-third of the results. Each pair of laboratories (DAFWA and SARDI, DAFWA and CSIRO, and CSIRO and SARDI) was then compared on the basis of the number of samples falling into the categories high (*R. toxicus* antigen >183,000 EU; corynetoxins >180 µg/kg), medium (*R. toxicus* antigen 38,000 to 183,000 EU; corynetoxins 60 to 180 µg/kg), and low (*R. toxicus* antigen <38,000 EU; corynetoxins <60 µg/kg).

(D) Inter-laboratory correlation. The correlation coefficients between results from pairs of laboratories were calculated for each ELISA.

(E) Correlation between *R. toxicus* ELISA and corynetoxins ELISA results. The correlation between results for the two ELISAs obtained within each laboratory was calculated, and an overall correlation between the average *R. toxicus* EU and the average corynetoxins concentrations for each sample obtained by the three laboratories

was calculated. These correlations were also calculated after natural logarithm transformation to achieve a closer to normal distribution. A constant of 10,000 was added to *R. toxicus* before the log transformation was applied.

Statistical Analysis for Samples Collected From the Field

Samples were collected from the field to examine the association between the *R. toxicus* antigen and corynetoxins as assessed by the ELISAs when testing (i) infected, standing ryegrass over its normal growth and maturation cycle; (ii) ryegrass pasture after cutting, baling, and storage in sheds; and (iii) an experiment with spiked ryegrass samples. The correlation between *R. toxicus* antigen and corynetoxin concentrations was calculated for each of the three datasets. The spiked samples experiment was performed with samples collected on uninfected farm A. The samples were split equally into two with one subsample being kept as a reference sample and the other having five bacterial galls added to it to create a spiked sample. The bacterial galls were randomly picked from bacterial galls identified over a light box in 1999. An overall correlation between the *R. toxicus* EU and the corynetoxins concentrations in all the samples from the field was calculated.

After collection of all the data for the above studies, it was found that DAFWA used the ProSciTech brand of glutaraldehyde to prepare wells of the corynetoxins ELISA plates for coating with modified tunicamycin, whereas CSIRO used the AJAX brand. The two brands were compared on the same type of ELISA plates on the same day with the same reagents and the same batch of samples (that were negative in the *R. toxicus* ELISA) at DAFWA. The significance of the difference between the two sets of corynetoxins results was calculated using a paired t-test.

Results

Inter-Laboratory Study

Table 1 shows the results obtained by the three laboratories after analyzing 100 hay sample extracts for the bacterial antigen and the corynetoxins.

Table 1. *R. toxicus* antigen and corynetoxins ELISA results as determined at three laboratories: DAFWA (D), CSIRO (C), and SARDI (S)

Sample No.	*R. toxicus* antigen (ELISA Units x 10^{-2})					Corynetoxins (µg/kg)				
	D	C	S	3-Lab Mean	CV (%)	D	C	S	3-Lab Mean	CV (%)
1	50	49	70	56	21	63	41	15	40	61
2	18,805	10,602	14,690	14,699	28	989	1,335	916	1,080	21
3	5,935	1,782	1,379	3,032	83	384	499	606	497	22
4	0.2	0.1	0.1	0.1	16	47	50	17	38	48
5	0.1	0.1	0.0	0	42	68	65	12	48	65
6	0.1	0.1	0.0	0	58	77	75	41	64	31
7	10,495	12,377	11,460	11,444	8	323	402	527	417	25
8	1,467	802	790	1,019	38	423	350	146	306	47
9	0.8	0.5	0.6	1	30	105	58	42	68	48
10	19,670	12,887	18,400	16,986	21	1,496	1,921	1,271	1,563	21
11	15,920	7,762	6,520	10,067	51	3,890	3,201	2,405	3,165	23
12	12,835	8,512	7,360	9,569	30	1,561	1,920	1,746	1,743	10
13	16,650	7,182	6,240	10,024	57	2,531	2,497	2,157	2,395	9
14	4,405	1,755	2,820	2,993	45	894	938	1,179	1,003	15
15	12,600	1,510	4,060	6,057	96	2,466	1,965	2,482	2,304	13
16	74	77	90	80	10	91	81	26	66	53
17	37	17	17	24	48	58	43	24	42	41
18	8,780	1,698	3,560	4,679	78	395	422	248	355	26
19	0.5	0.2	0.4	0.4	45	64	56	11	44	65
20	4,800	1,710	3,140	3,217	48	514	542	403	486	15
21	1,647	892	884	1,141	38	146	171	119	145	18
22	744	472	518	578	25	104	86	71	87	19
23	172	151	153	159	7	99	83	42	75	39
24	10,135	1,308	0.5	3,814	145	2,436	2,807	2,286	2,510	11

25	118	106	148	124	17	40	35	37	37	7
26	16,395	8,217	11,750	12,121	34	318	385	403	369	12
27	18,500	18,417	15,040	17,319	11	908	933	1,128	989	12
28	1,204	1,607	1,749	1,520	19	164	127	97	129	26
29	3,005	1,875	2,104	2,328	26	106	64	67	79	30
30	14,825	9,162	6,140	10,042	44	524	560	512	532	5
31	935	1,043	859	945	10	194	153	154	167	14
32	16,095	12,772	10,370	13,079	22	381	211	249	280	32
33	107	141	155	134	18	31	15	15	20	44
34	1,692	1,546	1,485	1,574	7	193	143	155	163	16
35	5,395	5,327	3,100	4,607	28	1,731	1,840	1,607	1,726	7
36	18,695	16,737	12,610	16,014	19	2,274	2,789	2,193	2,418	13
37	4,495	4,037	2,960	3,831	21	531	602	562	565	6
38	18,295	15,477	13,210	15,661	16	3,364	4,546	3,060	3,656	21
39	4,005	6,287	154	3,482	89	653	812	598	688	16
40	0.9	5.0	6.7	4	70	27	23	12	21	37
41	380	354	273	335	17	120	67	56	81	42
42	0.5	0.3	0.3	0	24	36	26	18	26	35
43	6.0	0.9	81.1	29	153	38	30	18	29	36
44	0.8	0.5	0.6	1	22	40	25	14	26	50
45	0.2	0.1	0.1	0.1	50	34	35	19	29	30
46	0.7	0.4	0.6	1	27	69	50	36	52	32
47	38	800	376	404	94	37	35	16	30	39
48	3,025	2,022	1,373	2,140	39	188	154	159	167	11
49	6	6	8	7	19	36	39	19	31	33
50	0.1	0.1	0.0	0	39	42	47	34	41	16
51	0.1	0.0	0.0	0	60	37	40	19	32	35
52	13	14	27	18	42	32	2	9	15	107
53	0.3	0.1	0.2	0.2	33	30	20	16	22	33
54	30	49	25	35	36	40	21	20	27	41
55	1,085	877	436	799	41	64	48	37	50	27
56	7,065	1,959	1,547	3,524	87	211	120	143	158	30
57	1,138	1,544	974	1,218	24	93	56	69	73	26
58	1,097	1,247	608	984	34	90	51	48	63	37
59	1,553	1,593	899	1,348	29	193	120	115	143	31
60	7,885	2,040	1,645	3,857	91	302	193	192	229	28
61	3,715	1,483	689	1,962	80	112	75	73	87	25
62	8,485	11,177	6,120	8,594	29	483	708	494	561	23
63	1,708	1,333	838	1,293	34	132	110	138	126	11
64	65	151	49	88	62	36	32	42	36	14
65	9,715	7,417	3,460	6,864	46	580	449	516	515	13
66	1,864	1,617	1,020	1,500	29	173	120	143	145	18
67	1,833	1,592	924	1,449	32	166	111	149	142	20
68	158	756	482	465	64	89	54	64	69	27
69	2,545	11,827	5,760	6,711	70	846	1,046	778	890	16
70	269	895	471	545	59	100	54	43	66	45
71	917	4,677	3,130	2,908	65	398	319	324	347	13
72	2,585	6,987	3,940	4,504	50	700	528	493	574	19
73	4	17	32	17	82	49	11	19	27	76
74	431	1,605	1,193	1,076	55	170	179	181	176	3
75	178	1,128	873	726	68	117	97	91	102	14
76	369	195	275	280	31	67	33	39	46	38
77	497	574	346	472	25	59	54	57	56	4
78	1,602	1,375	878	1,285	29	149	112	122	128	15
79	126	103	315	181	64	44	40	38	40	8
80	1,609	5,500	4,620	3,910	52	122	76	100	99	23

81	1,391	1,809	4,300	2,500	63	121	96	100	106	13
82	521	558	852	643	28	40	46	21	36	36
83	1,072	1,575	1,700	1,449	23	98	104	81	94	13
84	1,371	1,250	1,389	1,336	6	139	157	128	142	10
85	6,375	5,595	7,020	6,330	11	590	508	493	530	10
86	14,660	16,745	21,720	17,708	20	1,275	1,573	1,043	1,297	20
87	1,727	3,525	3,460	2,904	35	143	143	124	137	8
88	3,810	5,205	6,690	5,235	28	308	341	291	314	8
89	286	440	663	463	41	64	50	34	49	31
90	1,038	1,395	1,831	1,421	28	128	118	106	118	9
91	949	1,239	1,693	1,294	29	86	75	69	77	11
92	469	591	1,019	693	42	64	84	73	73	13
93	533	733	975	747	30	55	40	28	41	34
94	1,687	1,791	3,290	2,256	40	186	151	136	158	16
95	302	343	549	398	33	66	54	42	54	22
96	377	484	736	532	35	90	64	55	70	26
97	242	168	440	283	50	55	35	28	39	36
98	38	49	76	54	37	36	43	33	37	14
99	2,220	3,845	3,950	3,338	29	144	135	116	132	11
100	1,510	1,886	3,570	2,322	47	97	121	109	109	11

(A) *R. toxicus* ELISA vs corynetoxins ELISA in each laboratory (table 2). There were 24 extracts for which the mean of the three laboratories showed a high corynetoxins concentration (>400 µg/kg), and two of the laboratories identified all these extracts as "higher" according to the *R. toxicus* ELISA. The third laboratory identified 22 of the 24 extracts as "higher" according to the *R. toxicus* ELISA. All laboratories identified the 21 extracts with the lowest corynetoxins concentrations (0–<40 µg/kg) as "lower" according to the *R. toxicus* ELISA. Most of the 27 extracts with corynetoxins concentrations between 100 and <400 µg/kg were identified as "higher," while most of the 28 extracts with corynetoxins concentrations between 40 and <100 µg/kg were identified as "lower" in the *R. toxicus* ELISA by each laboratory.

(B) Reproducibility of each assay. The average between laboratory CV for the *R. toxicus* ELISA was 41.6% (SD 26.4, range 6-153), while for the corynetoxins ELISA it was 25.4% (SD 17.0, range 3-107). Analysis of variance showed a significant difference in CV between the assays ($p<0.001$) indicating better reproducibility for the corynetoxins ELISA.

(C) Inter-laboratory agreement (tables 3a-e). For both the *R. toxicus* ELISA and the corynetoxins ELISA, there was high consistency between laboratories in terms of the broad categorization of high/medium/low. For the corynetoxins ELISA, there were no instances where one laboratory gave a high result (top third) and another laboratory gave a low result (bottom third), while for *R. toxicus* ELISA, this happened three times (1% of comparisons).

(D) Inter-laboratory correlation. For the *R. toxicus* ELISA, the correlations between laboratories were DAFWA and SARDI r = 0.86, DAFWA and CSIRO r = 0.85, and SARDI and CSIRO r = 0.92. For the corynetoxins ELISA, the correlations between laboratories were DAFWA and SARDI r = 0.98, DAFWA and CSIRO r = 0.97, SARDI and CSIRO r = 0.98. All correlations for both analytes were highly significant ($p<0.001$). An observation, however, was that the corynetoxins ELISA results obtained at DAFWA were higher than the results of the other two laboratories for 59 samples out of 100, and this tendency was even more pronounced when results below 100 µg/kg were compared: DAFWA result was higher in 33 out of 41 (80%).

(E) Correlation between *R. toxicus* ELISA and corynetoxins ELISA results. The correlations found between *R. toxicus* ELISA results and corynetoxins ELISA results within each laboratory were DAFWA r = 0.75, CSIRO r = 0.64, and SARDI r = 0.57. The overall correlation between the average *R. toxicus* EUs and the average corynetoxins concentrations for each sample obtained by the three laboratories was r = 0.70. The correlations on the log transformed data were DAFWA r=0.88, CSIRO r=0.81, SARDI r=0.80, and Average r=0.87. Again, all correlations were highly significant ($p<0.001$).

At the low end of the scale for both analytes, taking the average of the results from the three laboratories for each analyte, there were detectable quantitation in 16 samples (table 1 numbers 25, 33,

Table 2. Number of extracts classified as higher according to the *R. toxicus* ELISA, in each of four categories based on corynetoxin concentrations, for each of the three laboratories

		Number of samples			
		Corynetoxins, µg/kg (average of three labs)			
		0-<40	40-<100	100-<400	≥400
DAFWA	higher (*R. toxicus* > 90,000 EU)	0	8	25	24
	lower (*R. toxicus* < 90,000 EU)	21	20	2	0
CSIRO	higher (R. toxicus >90,000EU)	0	7	25	24
	lower (R. toxicus < 90,000 EU)	21	21	2	0
SARDI	higher (*R. toxicus* > 90,000 EU)	0	7	20	22
	lower (*R. toxicus* < 90,000 EU)	21	21	7	2

Table 3. Between laboratory agreement for *R. toxicus* and corynetoxins ELISAs

(a) CSIRO v DAFWA

R. toxicus	DAFWA low[1]	DAFWA medium[1]	DAFWA high[1]
CSIRO low	30	1	0
CSIRO medium	6	23	9
CSIRO high	0	4	27

(80% correct match, 0% high-low)

(b) CSIRO v DAFWA

Corynetoxin	DAFWA low[2]	DAFWA medium[2]	DAFWA high[2]
CSIRO low	24	12	0
CSIRO medium	0	26	7
CSIRO high	0	0	31

(81% correct match, 0% high-low)

(c) CSIRO v SARDI

R. toxicus	SARDI low	SARDI medium	SARDI high
CSIRO low	29	2	0
CSIRO medium	3	29	6
CSIRO high	1	3	27

(85% correct match, 1% high-low)

(d) CSIRO v SARDI

Corynetoxin	SARDI low	SARDI medium	SARDI high
CSIRO low	34	2	0
CSIRO medium	6	26	1
CSIRO high	0	1	30

(90% correct match, 0% high-low)

(e) SARDI v DAFWA

R. toxicus	DAFWA low	DAFWA medium	DAFWA high
SARDI low	29	2	2
SARDI medium	7	20	7
SARDI high	0	6	27

(76% correct match, 2% high-low)

(f) SARDI v DAFWA

Corynetoxin	DAFWA low	DAFWA medium	DAFWA high
SARDI low	24	16	0
SARDI medium	0	21	8
SARDI high	0	1	30

(75% correct match, 0% high-low)

[1]The categories for *R. toxicus* ELISA are <38,000 EU (low), 38,000 to 183,200 EU (medium), and >183,200 EU (high).
[2]The categories for Corynetoxin ELISA are <60 µg/kg (low), 60 to 180 µg/kg (medium), and >180 µg/kg (high).
The categories for both assays were chosen on the basis of one-third of the samples falling in each category and do not necessarily reflect risk (of ARGT) levels.

40, 42, 43, 44, 47, 49, 52, 53, 54, 64, 73, 82, 97, and 98), *R. toxicus* levels but corynetoxins concentrations below the limit of but still all in the lower half of the dataset with *R. toxicus* levels below 65,000. There were detectable corynetoxins but *R. toxicus* levels below the limit of quantitation in three samples (table 1 numbers 5, 6, and 50); however, for these three samples, the level of corynetoxins was still very low (<100). There were also three samples with both *R. toxicus* levels and corynetoxins concentrations below the limit of quantitation (table 1 numbers 4, 45, and 51).

Samples Collected From the Field in WA

(i) Infected ryegrass over its normal growth and maturation cycle. Compliance with sample collection instructions for this section was generally poor: sample weights ranged from 1 g to 225 g. The time between consecutive sample

collections became longer in the 2 months after cutting (late October) and baling (first week of November). The correlation between *R. toxicus* antigen and corynetoxins concentrations in ryegrass heads in these samples was r = 0.79 ($p<0.001$, n = 252).

(ii) Correlation of the bacterial and toxin antigens in pasture after cutting for hay. Compliance with the sample collection instructions for this part of the field study was good on farms A and B, where staff followed the export industry protocol, except that sampling from windrows was infrequent on farm A. The number of days between cutting and baling was approximately 24, 7, 26, and 12 on farms A, B, C, and D, respectively. Samples from the windrows on farm A varied from 800 g to 1,000 g (mean 967 g, n = 6), and samples from the bales ranged from 106 g to 296 g (mean 204 g). The correlation between *R. toxicus* antigen and corynetoxins concentrations for this experiment on cut pasture before and after baling was r = 0.77 ($p<0.0001$, n = 121).

(iii) Spiked ryegrass samples. Results for the eight reference samples were all below the respective limits of quantitation of the two ELISAs. The *R. toxicus* ELISA results for the 8 spiked samples ranged from 8125 to 78,800 EU (average 48,622 EU), and the corresponding corynetoxins results ranged from 18 to 65 µg/kg (average 49.6 µg/kg, only one result being below 40, the limit of quantitation). The correlation between the *R. toxicus* and corynetoxins results in this experiment was r = 0.87 ($p<0.001$, n = 16).

In the samples collected from the field, including the spiked samples, the overall correlation between results obtained with the *R. toxicus* ELISA and the corynetoxins ELISA (figure 6) was r = 0.80 ($p<0.001$, n = 389). At the low end of the scale for both analytes, there were detectable *R. toxicus* levels but corynetoxins concentrations below the limit of quantitation in 92 samples, although none of these had *R. toxicus* greater than 100,000 EU. There were also 23 samples with detectable corynetoxins but *R. toxicus* levels below the limit of quantitation, but these all had very low corynetoxin concentration (<150). There were 152 samples with both *R. toxicus* levels and corynetoxins concentrations below the limit of quantitation. The relationship at the lower end of the scale can be seen better when the data are plotted on the log scale (figure 7).

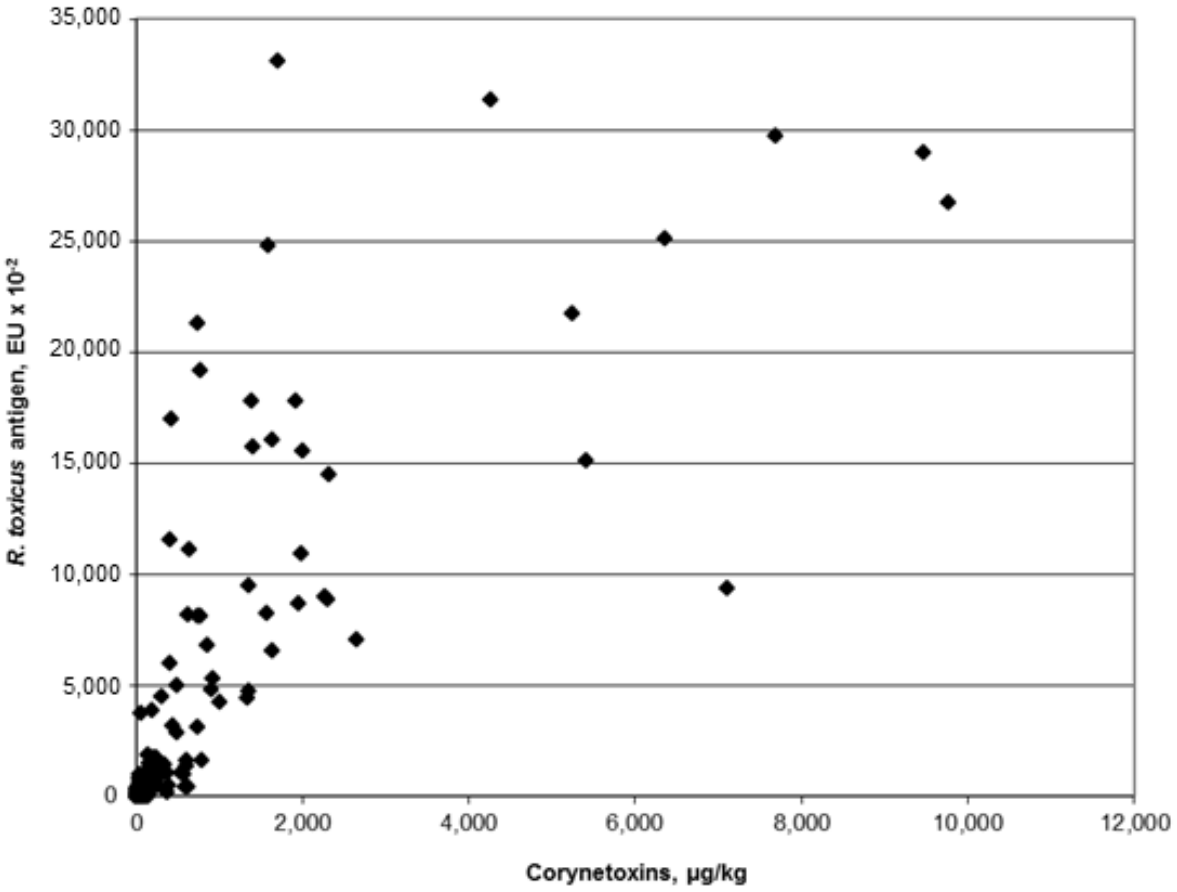

Figure 6. Scatter diagram showing the relationship between *R. toxicus* antigen and corynetoxins observed in all samples (n = 389) collected from the field in Western Australia.

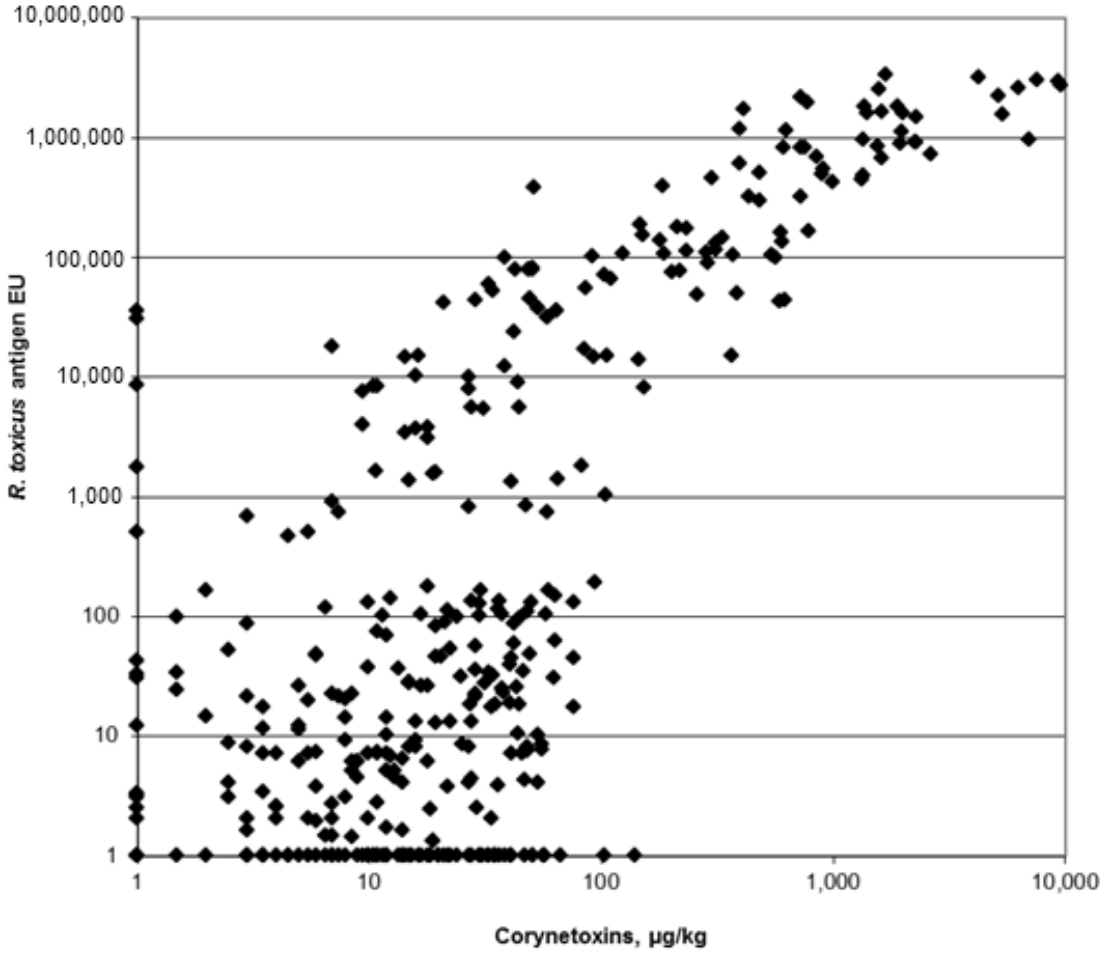

Figure 7. Scatter diagram showing results for *R. toxicus* antigen and corynetoxins on a log scale, in samples collected from the field in Western Australia (n = 389). So that the many zero results were included on the log scale, all values less than 1 were plotted as 1.

When tested on plates coated after pre-treatment with ProSciTech glutaraldehyde, the mean corynetoxins result for a set of samples with negative results for *R. toxicus* was 57 µg/kg (SD = 14.7, n = 19), compared with a mean of 32 µg/kg (SD = 8, n = 19) on plates pre-treated using AJAX glutaraldehyde. This difference was significant by a paired t-test ($p<0.001$) and was unexpected (see discussion).

Discussion

Throughout both studies, samples were stored for up to 2 days before extraction by soaking in 1% cyclodextrin. It is possible that during storage, toxin production may continue inside the galls, particularly when moisture is still present; and this may possibly occur in the early stages of soaking when galls take up water. It is not known how much additional toxin would be elaborated during 2 days of storage or during soaking (if this occurs). Further studies may be required.

In the inter-laboratory study, the results of comparison method A indicated that all three laboratories generally found that when the *R. toxicus* ELISA result was high, the corynetoxins result was also high, and when the *R. toxicus* ELISA result was low, the corynetoxins result was also low. The corynetoxins ELISA was more reproducible than the semi-quantitative *R. toxicus* ELISA (comparison method B). The features that potentially reduce variability of the corynetoxins ELISA between laboratories are (1) there is a standard curve on every plate, (2) all samples and standards are assayed in quadruplicate, and (3) there is only a single dilution of each sample. Probable explanations for the greater variation in the *R. toxicus* ELISA would include the performance of the serial 10-fold dilutions performed in the ELISA plate (differences between operators and between pipettes) because any error is compounded in successive dilutions. Additionally, when for some samples, the absorbance result for the calculation falls in a non-linear section of the antigen/absorbance response curve, the variation is greater than when it falls in the linear range. The above explanations, however, do not account for the disparity in results seen for 12 samples (numbers 3, 15, 18, 24, 39, 43, 47, 56,

60, 61, 73, and 75 in table 1). It is possible that for these samples, there were errors such as inadvertent sample substitution or calculation error (such as entering the wrong dilution or failing to multiply by the reciprocal of the dilution) or failing to notice that precipitation of the substrate had occurred before reading the ELISA plate. Precipitation can occur in wells with high levels of antigen and will reduce the measured OD by variable amounts depending on the degree of precipitation at the time of reading the plate. Comparison method C indicated good agreement between the laboratories with 81% of comparisons giving the same ranking of high/medium/low, and no high-vs-low discrepancies for the corynetoxin ELISA. Comparison method D showed highly significant positive correlations between laboratories for both ELISAs, the correlations being higher for the corynetoxins ELISA. Comparison method E demonstrated that within each laboratory, a highly significant positive correlation between *R. toxicus* ELISA results and corynetoxins ELISA results was found. Also, when the results of each analyte from all three laboratories were averaged for each sample, there was a highly significant positive correlation between the average *R. toxicus* antigen ELISA results and the average corynetoxins ELISA results. Overall, the inter-laboratory study showed that both ELISAs are robust enough to be performed reliably in different laboratories, as well as showing a significant positive correlation between results of the two ELISAs.

The results of the spiking experiment in samples collected from the field showed that both the *R. toxicus* antigen and the corynetoxins concentrations, when determined by the ELISAs described in this paper, are indeed associated with bacterial galls. Other possible sources of toxin in ryegrass and hay samples are bacterial slime and fines. There was a highly significant correlation between the *R. toxicus* and corynetoxins ELISA results in this experiment. Bacterial galls can vary with respect to weight and the quantity of corynetoxins (Cockrum and Edgar 1985). The range of results seen in the spiked samples reflects variation in the amounts of both the bacterial antigen and corynetoxins between galls and possibly variation in extraction efficiency between samples.

There were 3 samples in the inter-laboratory study and 23 among the field samples for which low levels of corynetoxins were detected and *R. toxicus* antigen was not detected. It is quite possible that most of these are false positives. As mentioned in the Results section, the DAFWA corynetoxins results were generally higher than those of the other two laboratories for the samples at the low end of the range. The coating step of the corynetoxins ELISA requires pre-treatment of the wells with glutaraldehyde, and it was found after testing of the samples from the field in WA was completed that the brand of glutaraldehyde makes a difference to the results at the low end of the range. The DAFWA laboratory used a different brand (Pro Sci Tech) from CSIRO (Ajax), and this apparently raised the limit of quantitation in the DAFWA laboratory. This result was not expected because the Pro Sci Tech brand is of higher purity. In hindsight, glutaraldehyde of higher purity may cause greater “activation” of sites on the polystyrene surface of the wells and thereby promote binding of a higher proportion of the modified tunicamycin to the plate at the first step of the ELISA. Since it is a competitive ELISA, the amount of anti-corynetoxin antibody bound to the modified tunicamycin will be affected very little for the high corynetoxin standards, but it will be increased for the low corynetoxin standards. This would change the slope of the standard curve and thereby potentially cause false positive results.

There was a significant positive correlation between the results of the two ELISAs when they were performed on the same soak extracts of ryegrass or hay samples. Distribution and abundance of bacterial galls within a paddock can be highly variable. When assessing the suitability of pasture or hay for stock to graze, sampling strategies must ensure that the samples taken represent the whole paddock or bale. Before baling, this requires collecting at least 20 subsamples while walking in zigzag fashion across the paddock, then pooling the subsamples for testing. After baling, the procedures to follow have been described in detail (Elson 2002b). Even when the *R. toxicus* ELISA result is low, stock still need to be observed frequently for signs of ARGT, because there have been cases where only part of a paddock or just one bale was highly infected and caused stock deaths. In these situations, it is hard to track down the source of the corynetoxins.

In both the inter-laboratory study and the samples collected from the field, the correlation between *R. toxicus* antigen and corynetoxins was positive and highly significant. At the low end of the range of both analytes and in both studies, more samples were positive for *R. toxicus* and negative for corynetoxins than vice versa. The reasons may be that the *R. toxicus* ELISA is more sensitive and that log-phase bacterial multiplication precedes toxin production (Payne and Cockrum 1988). The measurement uncertainty of the assays may also be an important factor at the low end of the range. At the medium and high end of the range of results, we found that if *R. toxicus*

antigen was detected in hay or pasture samples, corynetoxins were also detected.

Implications/Recommendations

The *R. toxicus* ELISA can therefore be used as an indirect measure of corynetoxins in hay and pasture samples.

The *R. toxicus* ELISA has remained the method for export testing because it is better suited for high-throughput testing (42 samples per ELISA plate vs 10), it requires only water for extracting the antigen in soak extracts of the hay samples, and it is very sensitive. In addition, the hay export *R. toxicus* ELISA is on undiluted extracts and for this reason is very robust (no chance of dilution errors or calculation errors).

If no *R. toxicus* antigen is detected by the *R. toxicus* ELISA in a representative sample of hay, then there should be little or no risk of poisoning.

The corynetoxins ELISA is a potentially useful research tool but is not practical for routine testing. When the corynetoxins ELISA is performed, the limit of quantitation should be re-evaluated for the brand/batch of glutaraldehyde in use.

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Gastrolobium spp. Poisoning in Sheep—A Case Report

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Abstract

This report describes the history and investigation of a suspected plant poisoning event in Western Australia where 15 sheep died. One of the poisoned sheep was necropsied, and gross and microscopic pathology of the poisoned sheep is described. Monofluoroacetate was detected in rumen contents from the necropsied sheep. The case history, pathological changes, and detection of monofluoroacetate in the rumen contents support a diagnosis of monofluoroacetate intoxication. A review of the literature suggests this is the first example of detection of monofluoroacetate in the rumen contents of an animal poisoned by a plant containing monofluoroacetate.

Keywords: *Gastrolobium* spp., monofluoroacetate, poisoning, sheep

Introduction

Many plants found primarily in the southern continents of Africa, Australia, and South America, belonging to the Fabaceae, Rubiaceae, Bignoniaceae, Malpighiaceae, and Dichapetalaceae families, contain monofluoroacetate and adversely affect livestock production on these continents (Lee et al. 2014). Australian species of *Gastrolobium* and *Acacia* produce monofluoroacetate and have had a significant impact in the settlement of Australia (McKenzie 2012). Early colonists of Western Australia (WA) experienced heavy livestock losses due to animal consumption of *Gastrolobium* spp., which resulted in the colloquial term for the plants as "poison peas" (Marchant 1994). There are over 100 *Gastrolobium* spp., primarily in WA, and many continue to affect modern-day farming (Chandler et al. 2002, 2003).

Aplin (1971) reported large variations in monofluoroacetate concentrations between different *Gastrolobium* spp. and between plants in the same species even at the same location.

Monofluoroacetate concentrations tend to be highest in reproductive tissues such as pods, flowers, and young leaves and much lower in mature leaves and wood (Aplin 1971, Hall 1972, Twigg et al. 1996b, Twigg et al. 1999). There are varying degrees of evidence for toxicity in 39 species of *Gastrolobium* (Bennetts 1935, Gardner and Bennetts 1956, Gardner 1964, Aplin 1971, Twigg et al. 1996a,b, Twigg et al. 1999, Chandler et al. 2002) and an additional 7 species are suspected or presumed to be toxic (Chandler et al. 2002).

This report describes the history and investigation of a suspected plant poisoning event in WA. The gross and microscopic pathology of one of the poisoned sheep is described. Analysis of the rumen contents suggested that the ingestion of a toxic monofluoroacetate-containing plant (*Gastrolobium* spp.) was the probable cause of the poisonings. This appears to be the first report of detection of monofluoroacetate in the rumen contents of a poisoned animal being used to support a diagnosis that they have consumed a *Gastrolobium* sp.

History

In February 2013, 1,200 young mated Merino ewes were introduced into a pasture near Merredin, WA. On or about April 18, 2013, there was a rainfall event. The next day, 10 ewes were found dead. The owner decided to remove the remaining ewes by walking them out of the pasture. Several animals walked slowly, kept stopping, and became recumbent. One of the ewes that became recumbent subsequently died, for a total of 11 dead sheep in this incident. The owner left about 20 sheep in the pasture. The following May, there was another rainfall event, and the owner revisited the pasture the next day to find four dead sheep. A total of 15 sheep died in the two episodes.

Gross Pathology

A sheep in good post-mortem condition from the second episode was necropsied. The ewe was in good body condition and not pregnant. Petechiae were in the myocardium, kidneys were soft, and rumen contents were green and fluid in consistency. Most of the rumen contents were accidentally discarded, with only a few plant pieces saved for identification and analysis.

Suspected Cause of Death

Findings at necropsy suggested enterotoxemia, but the sheep had received two vaccinations against this disease. Also, two episodes of synchronous deaths immediately after rainfall are inconsistent with enterotoxemia, being more consistent with fluoroacetate poisoning, since after a rainfall sheep nibble on toxin-containing bushes. The attending veterinarian suspected that *Gastrolobium stenophyllum* (narrow-leaved poison) was available to these sheep.

Histopathology

Tissues collected at necropsy were submitted to the Animal Health Laboratories of the Department and Agriculture and Food Western Australia for microscopic examination. In heart sections, rare myofibers were swollen, hypereosinophilic, fragmented, and had pyknotic nuclei (necrosis) (figure 1). There was also hypertrophy of interstitial cells, diffuse congestion, and multiple hemorrhages. Lung sections were congested, and proteinaceous fluid filled airways. A light lymphocytic and plasmacytic, periportal infiltrate was in liver sections. No significant findings were observed in sections of brain.

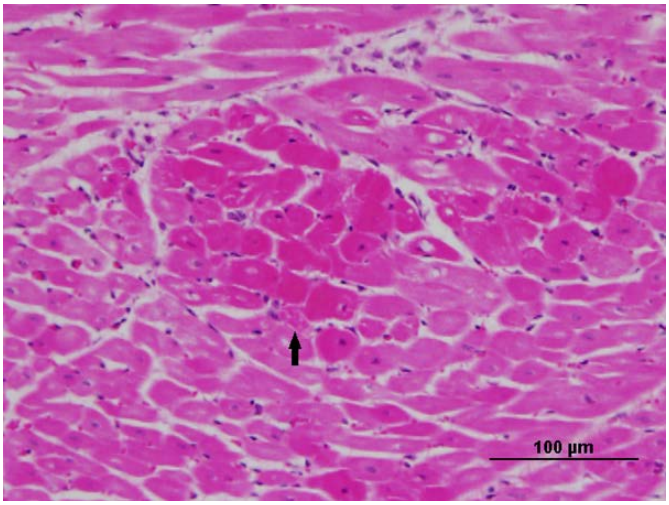

Figure 1. Heart (myocardium) with a focal area of necrosis. The necrotic myofibres are swollen, hypereosinophilic, and fragmented with pyknotic nuclei (arrow).

Mild, multifocal, acute, myocardial necrosis, together with moderate, diffuse pulmonary edema, are suggestive of cardiac toxicosis. In WA, the ingestion of plants containing either monofluoroacetate or cardiac glycosides is usually suspected as the cause of such changes in ruminants that have died unexpectedly.

Plant Identification

Plant fragments recovered during necropsy were examined and could not be positively identified as from a *Gastrolobium* spp.

Chemistry

Using a HPLC-APCI-MS method developed by Lee et al. (2012), monofluoroacetate was detected in the plant material collected from the rumen during necropsy at a concentration of 63mg/kg (figure 2). The identification of monofluoroacetate-containing plant material in the rumen at the time of death supported a diagnosis of monofluoroacetate poisoning, and the veterinarian's suspicion that the

plant fragments collected were from a *Gastrolobium* spp.

Summary

In conclusion, the case history, pathological changes, and detection of monofluoroacetate in rumen contents supported a diagnosis of monofluoroacetate intoxication. A review of the literature suggests that this is the first example of detection of monofluoroacetate in rumen contents of an animal poisoned by a plant containing monofluoroacetate. Methods have been developed to detect monofluoroacetate in rumen contents and liver samples. Monofluoroacetate was spiked into the liver or gastric contents, and it was determined how much could be recovered (Minnaar et al. 2000). In addition, monofluoroacetate has been detected in the kidneys of a lamb and a ewe diagnosed with 1080 (monofluoroacetate) poisoning (Giannitti et al. 2013), and monofluoroacetate has been detected in the blood, heart, skeletal muscle, and liver of sheep that died due to experimental poisoning with 1080 (monofluoroacetate). Significantly, in the last situation, monofluoroacetate was not detected in any of the organs of animals that survived (Gooneratne et al. 2008). In none of these examples was plant material containing monofluoroacetate consumed by the animals. This case demonstrates the diagnostic value of using modern chemical instrumentation to detect toxins in gastrointestinal contents from animals intoxicated by poisonous plants.

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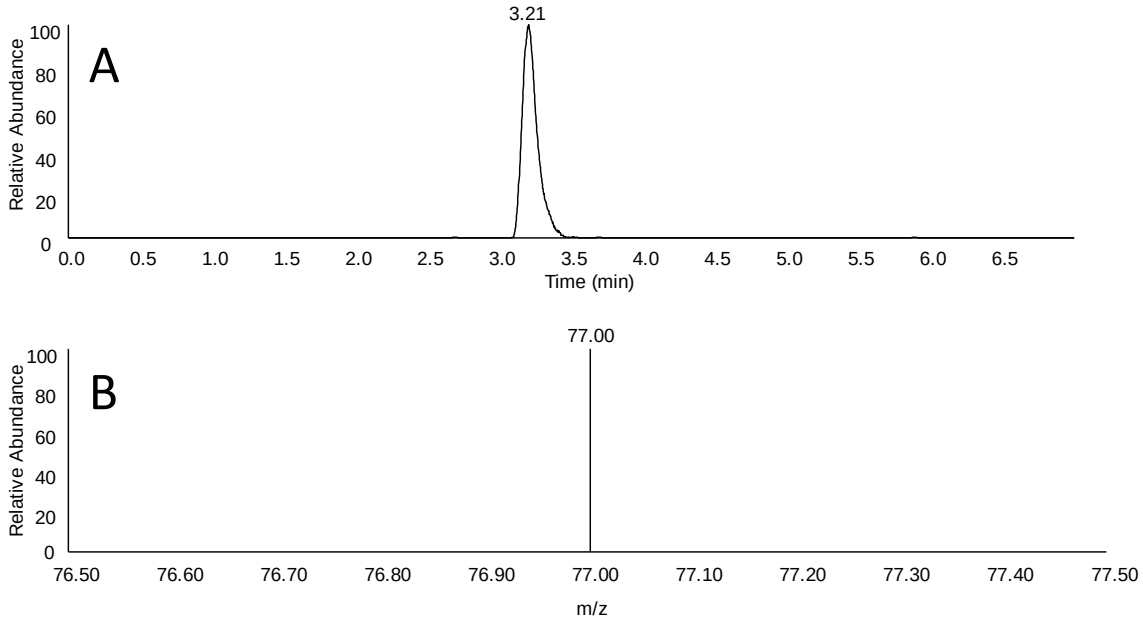

Figure 2. Selected negative ion monitoring HPLC chromatogram at *m/z* 77; of (A) water extract of sheep rumen plant material and (B) the mass spectrum of the peak. *m/z*, mass to charge ratio.

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