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Improvements to the immunoassay for detection of *Rathayibacter toxicus* in hay

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Abstract. An improved protocol for the previously described enzyme-linked immunosorbent assay for *Rathayibacter toxicus* in hay is described. The improvements were driven mainly by the export hay industry requirement of same-day turnaround for testing of hay extracts. The preparation of hay extracts was shortened by 8 h. The time for adding samples to the enzyme-linked immunosorbent assay plates was shortened by the use of sample tubes with penetrable stoppers combined with specially designed racks. The monoclonal antibody used in the original protocol was purified and conjugated to horseradish peroxidase. This eliminated the need for a secondary step with an anti-mouse horseradish peroxidase conjugate and thereby shortened the assay by over 1 h. Results with the improved assay protocol showed a very high correlation with results obtained with the original protocol ($r = 0.98$). The assay is still sensitive enough to detect antigen equivalent to less than 1 average gall per kg of hay. These cost-effective changes have streamlined the testing of large numbers of samples for the presence of *R. toxicus*, in support of the hay export industry.

Additional keywords: ARGT, conjugation, corynetoxins, ELISA, IgM.

Introduction

Annual ryegrass toxicity (ARGT) is a poisoning of livestock caused by the ingestion of hay or pasture containing annual ryegrass (*Lolium rigidum*) infected by a toxic bacterium, *Rathayibacter toxicus* (Sasaki *et al*. 1998), earlier thought to be similar to *Corynebacterium rathayi* (Bird and Stynes 1977) and then given the basonym *Clavibacter toxicus* (Riley and Ophel 1992) before the current classification. *R. toxicus* poisoning causes mainly neurological signs and can be lethal if sufficient toxin is consumed (McKay and Ophel 1993). *R. toxicus* is carried into the growing ryegrass plant by a parasitic nematode (*Anguina funesta*). The nematode induces the formation of a gall inside the developing seed head, within which it multiplies. When sufficient numbers of *R. toxicus* are carried into the gall, they may multiply to displace the nematodes and form a bacterial gall. Such a gall contains a yellow mass of toxin-producing bacteria in place of the normal seed (Bird and Stynes 1977).

ARGT has been reported in Western Australia (Gwynn and Hadlow 1971), South Australia (McIntosh *et al*. 1967) and South Africa (Schneider 1981). *R. toxicus* has also been implicated in stock losses because of its presence in *Anguina paludicola* galls (Bertozzi and Davies 2009) in common blown-grass, *Lachnagrostis filiformis* [syn. *Agrostis avenacea*, (Jacobs 2001)], in northern New South Wales and annual beard-grass, *Polypogon monspeliensis*, in south-eastern South Australia (McKay *et al*. 1993). *R. toxicus* infection in these grasses is similar to that in annual ryegrass and the same toxins (corynetoxins) are produced (Edgar *et al*. 1994). In 1996, ARGT was diagnosed in cattle in Japan after they had eaten hay imported from Western Australia. This incident resulted in the introduction of a voluntary hay testing protocol for all hay exported from Australia, to minimise the risk of significant contamination of hay with *R. toxicus* (Elson 2002*a*). Hay containing 1 average bacterial gall or more per kg of hay is rejected for export. The protocol was made compulsory in 2005.

The hay testing protocol required laboratories to provide a sensitive, high throughput assay for the presence of *R. toxicus*. The assay developed and used since 1996 is an enzyme-linked immunosorbent assay (ELISA) that detects a water-soluble antigen of *R. toxicus* (Masters *et al*. 2006). The lengthy extraction time and the turnaround time within the laboratory presented problems for the hay producers. With this assay, the extraction time for the hay samples was 17 h (overnight) and as a result it took at least 24 h to collect hay samples, extract them and transport the extracts to the laboratory for testing. When there were large numbers of bales on the property, the collection, extraction and transport often took 2 days or more. To ensure same-day turnaround of the laboratory testing, hay industry staff were required to deliver the extracts to the laboratory by 10 a.m. To achieve this, they were required to start collecting extracts at 3–4 a.m. at some sites. In turn, it was essential to put hay samples to soak before midday the previous day, which limited the time available for actually collecting the hay samples. In the laboratory, while robust and sensitive, this ELISA involved three 60-min incubation steps as well as four plate washing steps and 15 min of incubation with substrate. In addition, the sample identification numbers were checked manually before samples were added to the test plates. Each sample was pipetted

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individually in duplicate and the lid of each sample vial was unscrewed and replaced with one hand during the sample addition step. As a result, the results were frequently delayed at the end of the day, when hay producers were waiting to unload their (non-contaminated) bales at the export hay processing plant. The late results were a major issue for several hay producers. The overall turnaround time for testing the hay was also an issue when hay was dry and ready for storage, and there was a threat of rain. Shortened extraction and testing times would reduce the time from sampling to transfer of the hay to covered storage by at least several hours and up to 1 day. There were Occupational Health and Safety considerations for the hay industry staff preparing hay extracts – long hours and very early mornings to be ready for early transport of extracts to the laboratory. Occupational Health and Safety concerns for the laboratory staff performing repetitive pipetting every day and frequent late finishes were an additional incentive to improve the assay. This paper describes modifications to the procedure for the same assay in order to achieve high sample throughput in a shorter same-day turnaround time as required by the hay export industry, as well as reducing the volume of repetitive tasks in the laboratory. This was achieved by validating a shorter time for sample extraction, by changes to the method of sample addition to the ELISA plates and by conjugating the monoclonal antibody with horseradish peroxidase to shorten the laboratory assay time. Furthermore, the cut-off for the original laboratory assay (Masters *et al*. 2006) was set very conservatively and this resulted in the rejection of some hay that gave false positive results. The experiments performed in this study enabled us to safely reset the cut-off, for both the original and the shortened assay now described, to detect 1 average bacterial gall/kg to optimise the amount of hay suitable for export. The development, performance and validation of the new assay are described.

Materials and methods

Determination of the optimal time for hay extraction

Experiments were conducted to find the optimal extraction time in terms of efficiency of detecting contaminated hay and efficiency of time management. In other words the experiments were to determine if a shorter extraction time than the standard 17 h is possible without compromising the sensitivity to detect 1 average bacterial gall/kg of hay. Oaten hay free of *R. toxicus* was obtained from Queensland, where ARGT has never been recorded. Hereafter, this will be referred to as 'negative hay'. Bacterial galls were sorted from highly positive pasture samples collected from four locations in Western Australia during the previous 2 years. The galls were separated from the pasture samples using sieving and aspiration methods followed by visual sorting over a light box and confirmed as bacterial galls by two independent operators.

Fifty 1-kg negative hay samples were placed in large plastic bags and each spiked with 10 bacterial galls before the addition of 5 L of water. Air was squeezed out as much as possible by applying pressure on the bag, which was then tied with a reusable garden tie. Before collection of extracts each bag was mixed by stepping on each side at least 5 times. Extracts were collected at 1, 3, 6, 9, 12, 15 and 17 h for testing in the *R. toxicus* ELISA (both the Standard ELISA and the Improved ELISA described below). Samples were tested diluted 1/10 in 0.02 M phosphate-buffered saline pH 7.2 containing 0.05% Tween 20 (Sigma P-1379, Castle Hill, NSW, Australia) (PBST) to obtain results for 1 average gall/kg. All samples were tested in duplicate and the mean of the duplicates used for analysis. The ELISA absorbance values of the positive and negative controls (Masters *et al*. 2006) were assigned 100 ELISA units (EU) and 0 EU, respectively, for the purpose of calculating the EU values for samples. Samples with results outside the linear range of the ELISA (absorbance >2.6) were further diluted (1/100). The cut-offs for the Standard ELISA were: 10 EU between negative and weak positive (called the 'negative cut-off') and 100 EU between weak positive and positive (Masters *et al*. 2006). Any hay with a result above the negative cut-off is rejected for export. Weak positive hay cannot be exported as it contains $\geq$1 bacterial gall/kg.

In a second experiment we determined if extraction times shorter than the standard 17 h are possible in routine hay samples without affecting the end result of the test. A total of 202 Western Australian hay samples were collected (between 0.75 and 1.25 kg) from bales in paddocks where sampling in the previous season had shown weak positive results (low level contamination). Extracts from these samples were collected at the same time intervals as described above for the spiked samples and tested in the Standard *R. toxicus* ELISA.

Modifications to the Standard ELISA to shorten the turnaround time in the laboratory

Several changes were made to shorten the Standard ELISA: The specific monoclonal antibody was purified and modified by direct conjugation with horseradish peroxidase thus making the detection step with horseradish peroxidase-conjugated sheep anti-mouse antibody unnecessary. The conjugated monoclonal antibody needs to be stored at a higher concentration for longevity, so the dilution for use in the test is higher. Plate sealing film was used for all the 37°C incubation steps. The sample addition step was streamlined to reduce the time taken to add samples to the plates (*vide infra*).

Preparation and purification of monoclonal antibody

The cell culture for the production of the monoclonal antibody was as described in detail previously (Masters *et al*. 2006) except that the specific monoclonal antibody (IgM, hybridoma 2A1C7D8) was produced in serum-free medium (GIBCO Hybridoma SFM, Invitrogen Australia, Mulgrave, Victoria) without the addition of monoclonal antibody production enhancer (GIBCO OPTIMAb, Invitrogen Australia, Mulgrave, Victoria). Culture supernatant containing a high concentration of monoclonal antibody was clarified by centrifugation at 4000*g* at 10°C for 8 min to remove the cells and this clarified supernatant was used for the Standard ELISA procedure. For the Improved ELISA the IgM was purified from the clarified supernatant by ammonium sulfate precipitation. Saturated ammonium sulfate (BDH) adjusted to pH 7 was added slowly, while mixing, to the clarified culture supernatant to a final concentration of 40% (v/v). The suspension was centrifuged at 1500*g* at 10°C for 60 min and additional saturated ammonium sulfate was added slowly to the resulting supernatant to a final concentration of 50% (v/v). The precipitate was collected by centrifugation at 1500*g* for 60 min. The IgM-containing precipitate was dissolved in a minimal

volume of water and then dialysed extensively against 0.02 M phosphate-buffered saline pH 7.5.

The protein concentration of the monoclonal antibody (IgM) was estimated before conjugation by using a modified Bradford protein assay (BioRad, Hercules, CA, USA). The purification of the monoclonal antibody was checked by polyacrylamide gel electrophoresis. The titre of the dialysed purified monoclonal antibody was checked in the Standard *R. toxicus* ELISA.

Conjugation of the monoclonal antibody with horseradish peroxidase

The purified monoclonal antibody was conjugated with activated horseradish peroxidase using a 'Peroxidase labelling kit' (Roche Applied Science, Indianapolis, IN, USA, Cat. No. 1 829 696) following the manufacturer's instructions. After conjugation, the antibody was dialysed against the buffer provided in the kit. The conjugated monoclonal antibody was stabilised and protected from freezing by adding two parts of Super Freeze Buffer (Pierce, Rockford, IL, USA, Cat. No. 31503) to one part of conjugated monoclonal antibody and stored below −15°C. The required dilution of the conjugated monoclonal antibody for the Improved ELISA (to obtain an absorbance at 450 nm of between 1.8 and 2.6 for the positive control) was determined by chequer-board titrations.

The Improved ELISA procedure

The Improved ELISA was a modification of the Standard ELISA as follows: the sample addition step was streamlined (*vide infra*). The ELISA plate was covered with sealing film (Axygen PCR-SP Seal Plate, Union City, CA, USA) for all incubations except the last incubation with substrate, in order to avoid evaporation of the sample and to keep the temperature even in all the wells. After the 60-min incubation of the hay extracts and controls in the ELISA wells, the wells were washed 5 times with PBST, then incubated with the conjugated monoclonal antibody diluted 1 in 800 in high salt diluent [0.45 M NaCl, 10% fetal bovine serum (Sigma F9423), 1% Tween 20 in deionised water] for 60 min at 37°C. The plate was washed 5 times with PBST and 100 µL per well of the substrate 3,3,5,5-tetramethylbenzidene (K Blue, ELISA Systems) was added. Colour development, spectrophotometric reading and the calculation of EU proceeded as described previously. To optimise the dilution of the conjugated monoclonal antibody and the positive control, various dilutions of the former were tested against various dilutions of the latter (data not shown). The Improved ELISA was calibrated so as to get the value of the positive control in the same range (absorbance at 450 nm between 1.8 and 2.6) as that of the Standard ELISA.

Semi-automating and streamlining the sample addition step

The availability of 'MiniCollect tubes' (Greiner, Frickenhausen, Germany, Cat. No. 450 448) introduced the possibility of pipetting samples out of tubes without unscrewing the caps. The stoppers on the MiniCollect tubes have a soft centre, allowing a pipette tip to be easily pushed through to take a sample. To ensure that the stoppers stay on the tubes when pipette tips are withdrawn and to enable the pipetting of four samples simultaneously, custom-made metal racks with snap fit metal covers were designed (Fig. 1). The covers had a small hole over each MiniCollect tube, so that the stoppers are held on to the tubes, while allowing pipette tips to be pushed through the stoppers to draw up 100 µL of extract for transfer to an ELISA well. The spacing of the holes in the metal racks and covers was such that samples of four hay extracts could be drawn up simultaneously and transferred to four ELISA wells by using an 8-channel pipette with a tip on every second channel. This significantly shortened the time for the addition of samples to ELISA plates when processing large numbers (several hundred) of samples.

Validation of the Improved R. toxicus *ELISA*

In order to validate the Improved ELISA, a total of 374 hay samples from Western Australia were tested (in duplicate) in both the Standard and Improved ELISA formats. The results were compared graphically and by calculating the correlation coefficient (r) and by counting the number of negatives, weak

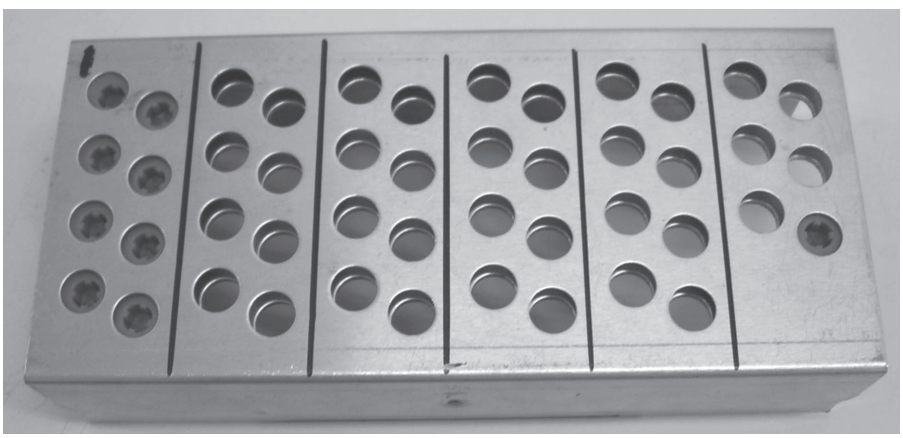

Fig. 1. Metal rack specially designed to hold 'MiniCollect tubes' so that stoppers are held in place while sampling contents of four tubes at a time. Stoppers of nine tubes are visible.

positives and positives determined by each test. The Standard ELISA used for comparison with the Improved ELISA was performed as described previously under ARGT export ELISA procedure (Masters *et al*. 2006).

Revision and re-validation of Improved ELISA negative cut-off

The Improved ELISA cut-off was revised using the results from 50 negative hay samples, each spiked with 10 bacterial galls/kg and extracted for 9 h. The extracts were tested at 1 : 10 dilution (1 average bacterial gall/kg) and the negative/weak positive cut-off set to give 95% confidence (*vide infra*) that a sample containing 1 gall/kg can be detected but to minimise false positive results by not setting the cut-off too low.

Determination of measurement uncertainty for the Standard and Improved ELISA

Two pools of hay extracts were prepared. Previously tested extracts with results in the range of 7–20 EU were thawed, pooled and mixed well. The pool ('low pool') was divided into 1-mL aliquots and stored below −15°C. A pool of extracts in the range 80–120 EU ('high pool') was prepared similarly. The measurement uncertainty was determined using reproducibility data obtained by testing aliquots of each pool on different ELISA plates (≥5 aliquots per plate), on different days, by different operators, using different batches of reagents over a period of 3 months. The measurement uncertainty of the Improved ELISA was determined about 1 year after that of the Standard ELISA, so freshly made low and high pools were used for the Improved ELISA.

Results

Determination of the optimal time for hay extraction

The results for 1 average bacterial gall/kg from 50 spiked hay samples (Table 1) showed that after 1 and 3 h of extraction, only 29 and 36 samples (respectively) were above the negative cut-off in the Standard ELISA and 24 and 36 samples in the Improved ELISA (*vide infra*). By 6 h of extraction time, 47 were above the negative cut-off in both ELISAs and by 9 h, the number was 49 (98%) of the 50 samples in the Standard ELISA and 48 (96%) in the Improved ELISA. At 17 h, 49 (98%) of the 50 samples were above the negative cut-off in both ELISAs. The one sample that would be missed by soaking for 9 instead of 17 h, had a weak positive result at 17 h, well below the median result for 1 gall/kg.

The results for the 202 field samples from bales in paddocks where low level contamination had previously been detected were as follows: 75 were weak positive, 64 were negative and 63 were positive, based on the results after 17 h of extraction (Table 2). Of the 75 weak positive Western Australian hay samples, seven (10%) were classified negative after 3 h of soaking (with results of 7, 6, 8, 6, 9, 9 and 9 EU) and five (7%) were classified negative after 9 h of soaking (4, 9, 9, 8 and 7 EU at 9 h compared with weak positive results of 10, 10, 12, 10 and 60 EU at 17 h, respectively). The results after 17 h extraction for the first four of the latter five samples were therefore either right on the negative cut-off or 2 EU above, indicating a very low level of *R. toxicus* contamination. The difference between the 9 h and 17 h results was within the measurement uncertainty of the ELISA for these four samples (*vide infra*). The result of 60 EU for the fifth sample is still well below the median result for 1 average gall/kg (see Revision and re-validation of the

Table 1. ELISA units (EU) after 1, 3, 6, 9 and 17 h extraction for 1 average gall/kg in spiked oaten hay samples

Result (EU) in the Standard ELISA is stated first, followed by result in the Improved ELISA (after comma). The samples were sorted according to the result in the Improved ELISA at 17 h. Negative results are in bold (<10 EU for Standard ELISA and <20 EU for Improved ELISA)

Sample #	1 h	3 h	6 h	9 h	17 h
1	**1, 1**	**1, 2**	**2, 3**	**3, 4**	**4, 5**
2	**1, 1**	**1, 1**	**3, 4**	**8, 10**	32, 34
3	**8, 9**	**9, 15**	20, 26	36, 45	38, 49
4	**2, 2**	**6, 8**	19, 22	21, 25	78, 84
5	**8, 8**	22, 23	37, 40	48, 59	70, 88
6	**5, 5**	**7, 9**	26, 26	44, 61	89, 105
7	**5, 5**	**7, 9**	31, 34	58, 70	94, 125
8	**1, 1**	**3, 4**	46, 42	65, 63	145, 129
9	**6, 9**	15, **14**	56, 62	58, 67	128, 141
10	18, **15**	29, 25	50, 44	64, 59	177, 142
11	**7, 8**	12, **14**	25, 25	40, 42	150, 143
12	55, 47	49, 62	53, 71	75, 103	123, 146
13	**4, 5**	**6, 8**	21, 28	27, 36	82, 155
14	**4, 5**	**8, 9**	12, **12**	28, 32	127, 169
15	**4, 6**	**8, 11**	24, 24	52, 52	141, 170
16	39, 47	39, 51	63, 82	80, 108	129, 176
17	17, **18**	23, 22	28, 37	71, 81	163, 180
18	15, **15**	51, 43	73, 77	120, 108	161, 190
19	22, 25	26, 30	44, 56	66, 81	139, 202
20	**7, 10**	**7, 10**	18, **19**	38, 44	172, 250
21	**6, 9**	17, **16**	42, 51	137, 184	215, 255
22	21, 21	33, 34	53, 56	84, 94	262, 255
23	29, 32	41, 50	68, 88	87, 148	174, 256
24	28, 27	41, 44	80, 94	172, 171	262, 260
25	23, 24	34, 42	54, 63	91, 123	230, 264
26	31, 28	49, 51	54, 63	109, 102	245, 269
27	41, 47	40, 47	87, 131	142, 199	198, 277
28	24, 29	67, 89	117, 182	202, 277	215, 286
29	19, 24	33, 40	46, 57	66, 89	258, 307
30	**9, 12**	22, 21	40, 40	78, 86	265, 309
31	18, 20	37, 41	56, 57	67, 72	284, 311
32	29, 28	65, 81	175, 178	223, 239	301, 314
33	**7, 10**	21, 22	74, 85	183, 200	256, 322
34	21, 22	52, 62	76, 92	86, 136	260, 340
35	17, **19**	37, 41	58, 67	150, 171	472, 369
36	16, **15**	56, 59	197, 207	229, 255	322, 375
37	14, **13**	61, 74	153, 166	198, 194	326, 386
38	35, 36	42, 46	69, 74	171, 178	350, 408
39	**6, 8**	28, 32	74, 89	210, 216	403, 428
40	18, 20	68, 77	128, 158	220, 270	465, 551
41	**7, 11**	44, 50	131, 147	280, 306	505, 556
42	26, 27	45, 50	185, 200	255, 255	551, 596
43	21, 22	50, 37	134, 161	484, 631	630, 805
44	21, 23	81, 104	213, 248	351, 457	686, 840
45	31, 35	199, 218	327, 401	450, 538	716, 853
46	18, **12**	72, 91	320, 413	521, 674	748, 1580
47	21, 23	86, 94	593, 677	711, 730	892, 1930
48	26, 28	92, 122	199, 217	517, 637	792, 2920
49	28, 31	95, 124	334, 418	705, 874	1240, 3030
50	18, 20	41, 46	186, 200	433, 538	886, 3610

Table 2. Determination of optimal extraction time: results of 202 field samples

Core samples from bales were extracted with water and 2-mL samples were collected at 3, 6, 9, 12, 15 and 17 h and these sample extracts were tested in the Standard ELISA. After testing all the extracts, the samples were grouped according to whether they were negative [<10 ELISA units (EU)], weak positive ($10 \leq 100$ EU) or positive (100 EU) at 17 h. The ranges of results at the earlier extraction times were then compared with the range of results at 17 h for each group. Results under the extraction times are the ranges of EU* for each group of samples, and (in brackets)** the number of samples having results that agreed qualitatively with the result at 17 h

Sample group	Extraction time (h)					
	3	6	9	12	15	17
Negative at 17 h (64 samples)	0–16* (62)**	0–14 (60)	0–10 (60)	0–10 (63)	0–14 (63)	0–9 (64)
Weak positive at 17 h (75 samples)	6–114 (66)	5–88 (70)	4–88 (70)	2–291 (71)	5–239 (71)	10–95 (75)
Positive at 17 h (63 samples)	16–5640 (45)	34–5230 (51)	65–6390 (53)	92–8195 (60)	105–9370 (63)	171–9590 (63)

Improved ELISA negative cut-off). Of the 63 positives, none were negative after 3 h (18 weak positive and 45 positive), nor after 9 h (10 weak positive and 53 positive) of soaking.

Modifications to the Standard ELISA to shorten the turnaround time in the laboratory

Preparation and purification of monoclonal antibody

Polyacrylamide gel electrophoresis showed that protein consistent in molecular weight with immunoglobulins predominated in the redissolved 50% ammonium sulfate precipitate by comparison with the redissolved 40% ammonium sulfate precipitate. The titre of the dialysed purified monoclonal antibody in the Standard *R. toxicus* ELISA was 1 in 500 000.

Conjugation of the monoclonal antibody with horseradish peroxidase

The conjugation of the monoclonal antibody (IgM) was successful. The 'Peroxidase labelling kit' was designed for the conjugation of IgG, so its applicability to IgM was not guaranteed. The required dilution of the conjugated monoclonal antibody for the Improved ELISA, determined by chequer-board titrations, was 1 in 800.

The Improved ELISA procedure

The time for performing the ELISA was reduced by over 1 h. The reduction was mainly achieved by the introduction of the conjugated monoclonal antibody, which obviated the need for the fourth 1-h incubation with sheep anti-mouse horseradish peroxidase conjugate. The reduction in time for addition of samples to plates (*vide infra*) was partly offset by the use of plate sealing film and the need for five washes instead of four after each incubation. The latter two innovations, however, reduced the number of samples and/or plates that required re-testing due to high background and the edge effect (higher results in wells at the edge of the plate) respectively, improving overall efficiency on a daily basis.

Semi-automating and streamlining the sample addition step

The time for addition of samples and controls to one full ELISA plate was reduced from 7 min ± 2 min (depending on the operator) to 3–4 min. Since the number of pipettings per plate was reduced to one-quarter of the number previously required, and there was no need to unscrew and replace sample tube caps, the level of operator fatigue was also substantially reduced.

Validation of the Improved R. toxicus *ELISA*

Ten pairs of ELISA plates were used when comparing the Improved ELISA with the Standard ELISA (374 samples). The correlation between results (expressed in EU) obtained with the Improved ELISA and the Standard ELISA was high ($r = 0.98$, $n = 374$) as illustrated in Fig. 2. There were 170 samples that were negative by both assays (using the 10 EU negative cut-off) and 29 samples that were negative by the Standard ELISA and weak positive by the Improved ELISA. There were no samples that were weak positive in the Standard ELISA and negative in the Improved ELISA (i.e. no false negatives in the Improved ELISA). There were 122 samples that were weak positive by both assays, 13 samples that were weak positive by the Standard ELISA and positive by the Improved ELISA (the difference being <28 EU in all these samples). This difference of <28 EU seen between samples near the weak positive/positive cut-off is within the measurement uncertainties of the two ELISA (*vide infra*). There were 40 samples that were positive by both assays.

Revision and re-validation of Improved ELISA negative cut-off

The median result for 1 average gall/kg in the Improved ELISA after 9 h was 116 EU with a 95% confidence interval of 14 EU to 1093 EU. The negative/weak positive cut-off was therefore set at 20 EU to ensure that the measurement uncertainty covers the 95% confidence interval (*vide infra*) at the lower end of the spectrum and to minimise the inclusion of negative samples. The range of results was very large, so if we set the cut-off to give 100% confidence of detecting 1 gall/kg, it would be so low that some negative hay would be rejected for export.

With a cut-off of 20 EU, two of the results were false negatives. The distribution of the results was not normal, so to check for outliers, the results were transformed to logarithms as the distribution of the $\log_{10}$ results was approximately normal. The lowest result of 4 EU at 9 h was an outlier by Chauvenet's criterion, when applied to the log-transformed results.

Determination of measurement uncertainty for the Standard and Improved ELISA

The measurement uncertainty of the Standard ELISA at 13 EU is 9.6–16.3 EU with 95% confidence ($n = 223$) and at 105 EU is

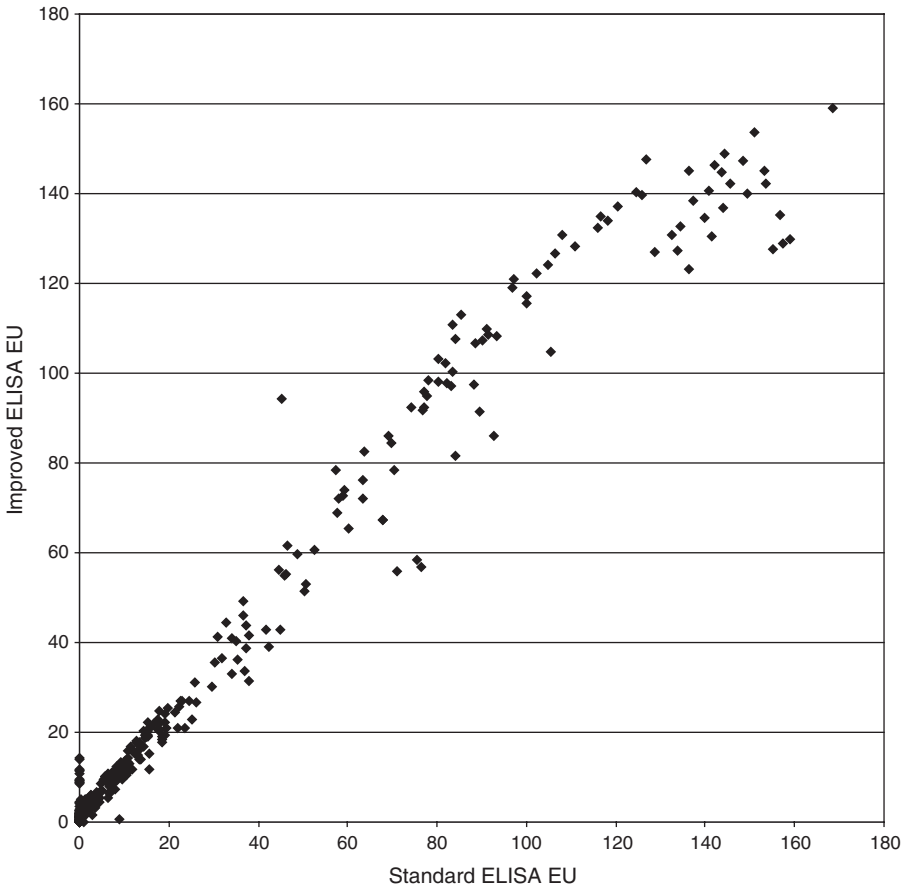

Fig. 2. ELISA units (EU) measured by the Improved *Rathayibacter toxicus* ELISA compared with EU measured by the Standard *R. toxicus* ELISA.

91.5–119.5 ($n = 223$) with the same confidence level. The measurement uncertainty of the Improved ELISA at 20 EU is 13.3–23.6 EU with 95% confidence ($n = 96$) and at 115 EU is 88–142 EU ($n = 89$) with the same confidence level. During everyday procedures, if duplicate results differ by more than 5 EU across a cut-off, the extract is routinely re-tested.

Discussion

This study showed that a level of contamination of 1 average bacterial gall/kg of hay can be detected after 9 h soaking of hay samples in 5 volumes of water, with thorough wetting at the start and adequate mixing at the end of the soak. Very little increase in sensitivity was seen when the extraction time was increased to 17 h. One hay sample was negative at 9 h but with further soaking and mixing became weak positive by 17 h. The result for this sample at 17 h was at the lower end of the range for 1 average gall/kg. Even considering the fact that most samples submitted for testing are composite samples, a result at the low end of the range for 1 gall/kg does not represent hay that contains enough corynetoxin to cause ARGT. This is because the composite samples represent a maximum of 40 bales and if all the *R. toxicus* came from one bale, that bale would contain 40–160 galls/kg (given that research work has demonstrated a 3–4-fold difference in the results for different core samples collected from the same bale). Clinical ARGT has never been encountered with hay containing less than 300 bacterial galls/kg. It is possible, however, for the disease to occur with lower levels of contamination if the hay is fed for months (J. Allen, 2003, pers. comm.). The hay soaking and mixing procedure requires thorough wetting of all the hay, including any bacterial galls that are present, to ensure solubilisation of the *R. toxicus* antigen and sufficient mixing to ensure homogeneity of the extract before a small sample is collected. Both of these requirements are difficult to achieve due to the nature of hay being a tangle of fibrous matter that can trap air bubbles and impede mixing of the water. Even with the use of a vacuum pump to remove the air from the bag of hay and water, small bubbles of air may remain and if a bubble happens to be adjacent to the only gall in 1 kg of hay, the antigen will not dissolve efficiently. Soaking and mixing efficiencies are therefore probably the reasons why the results changed (generally increased) between 9 and 17 h, and may also account for the outlier result at 1 average gall/kg. The shorter extraction procedure is more convenient for hay exporters as it facilitates a 24-h turnaround time from sampling of the hay to receiving a result. While soaking and mixing efficiencies may

partly account for the large variation in results for 1 average gall/kg, it is also likely that there is a huge variation in antigen content between individual galls (N. Lowry, D. Palmer, L. den Hollander, M. Kalkoven, A. Masters, unpubl. obs.). The *R. toxicus* bacterial count per gall is also likely to vary between galls. When sorting galls, operators see that there is a variation in size of the galls. Strain variation in antigen content is also possible and this needs investigation.

While the validation of the improved procedure was done with oaten hay, it is expected that this test would also be suitable for meadow hay and pasture samples. The results after re-sampling bales in paddocks with previous low level contamination illustrated that the distribution of bacterial galls in paddocks and bales is frequently not uniform. There are procedures, however, to minimise the risk of failing to detect *R. toxicus* when present (Elson 2002*b*).

In the experiments on 1 average gall/kg, PBST was chosen as the diluent rather than an extract from the unspiked negative hay because it had been shown previously in our laboratory that extracts from negative hay frequently suppress the result – that is, when a positive sample is diluted in unspiked negative hay extract, the result is generally lower than the result obtained if the positive sample is diluted in water or PBST (by ~10–30%). Different negative hay extracts suppress the result by different degrees, so using a negative hay extract as a diluent would not only produce lower results, but would be hard to interpret. This effect was investigated during specificity assessment of the standard *R. toxicus* ELISA and it was found that a secreted product of a bacterium commonly found in hay (*Bacillus subtilis* or a very closely related bacterium) inhibits the ELISA (Masters *et al*. 2006).

The adaptation and production of the monoclonal antibody in 100% serum-free medium and purification with simple ammonium sulfate precipitation resulted in sufficiently pure monoclonal antibody for direct conjugation with horseradish peroxidase without further purification.

There was a high correlation between results with the Improved and the Standard *R. toxicus* ELISA but the graphical comparison showed more scatter above 120 EU. This is because the relationship between absorbance and the amount of antigen is linear up to just below an absorbance of 2.6, (which corresponds to 100–120 EU). Above this level, the increase in absorbance readings becomes progressively less as the amount of antigen in the ELISA well increases. For this reason serial 10-fold dilutions of positive samples are tested when a quantitative estimate of the concentration of bacterial antigen is required, such as assessing the safety of hay for domestic use.

The Improved ELISA protocol allowed rapid export testing for *R. toxicus* in the laboratory facilitating the earlier release of results. This is useful for the hay industry to streamline processing of hay for export, especially when rain is expected and also when a consignment of hay is waiting at the exporter's gate, only to be accepted if negative for *R. toxicus*.

The revised cut-off of 20 EU is still a conservative cut-off between negative and weak positive samples, when compared with the median result of 116 EU and 95% confidence interval of results for 1 average bacterial gall/kg. A conservative cut-off is necessary because most of the hay samples tested for export are composite samples collected from 15 to 100% of the bales in a paddock (Elson 2002*a*). The tested bales are grouped into lots of up to 40, so the composite samples may represent as many as 40 bales. The effect of the revised cut-off is that more hay can be exported from Western Australia and South Australia, while still maintaining a sufficient safety margin.

A request was sent to AQIS to increase the negative–weak positive cut-off from 10 EU to 20 EU and to reduce the hay soaking time from 17 to 9 h, based on the above results of spiked samples and weak positive field samples. The request was made on 15 December 2005. Interim approval was granted on 8 September 2006 and final approval was given on 20 February 2007.

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