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An enzyme-linked immunosorbent assay for the detection of *Rathayibacter toxicus*, the bacterium involved in annual ryegrass toxicity, in hay

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Abstract. An enzyme-linked immunosorbent assay (ELISA) for *Rathayibacter toxicus* is described. The development of a monoclonal antibody for a specific antigen from *R. toxicus* and a polyclonal antibody raised against the same *R. toxicus* preparation enabled a capture assay format. The assay is specific for a soluble polysaccharide produced by the bacterium and was found to be sensitive enough to detect antigen equivalent to less than one gall per kilogram of hay. The applicability of the assay to samples of pasture or hay is demonstrated. Cost-effective testing of large numbers of samples for the presence of *R. toxicus* is possible with the ELISA. This will assist stockowners, hay producers, and hay exporters in the management of the risk of annual ryegrass toxicity.

Additional keywords: ELISA, immunoassay.

Introduction

Annual ryegrass toxicity (ARGT) is a poisoning of livestock caused by the ingestion of annual ryegrass (*Lolium rigidum*) infected by a toxic bacterium (*Rathayibacter toxicus*), resulting in neurological signs and if severe, death (McKay and Ophel 1993; Sasaki *et al*. 1998). The bacterium is carried into the ryegrass plant by a parasitic nematode (*Anguina funesta*). Within the developing seedhead the nematodes induce the formation of a gall, within which they multiply. When sufficient bacteria are present, they colonise the gall, which then contains a yellow mass of toxin-producing bacteria in place of the normal seed (Bird and Stynes 1977). The toxins, known as corynetoxins, are glycolipids that are members of the tunicamycin group of antibiotics produced by some species of *Streptomyces* (Vogel *et al*. 1981; Edgar *et al*. 1982).

ARGT has been reported in South Australia (McIntosh *et al*. 1967), Western Australia (Gwynn and Hadlow 1971), and South Africa (Schneider 1981). *R. toxicus* has also been implicated in stock losses because of its presence in galls in blowngrass, *Lachnagrostis filiformis* (formerly *Agrostis avenacea*, Jacobs 2001) in northern New South Wales, and annual beardgrass, *Polypogon monspeliensis*, in south-eastern South Australia (McKay *et al*. 1993). *R. toxicus* infection in these hosts is analogous to that in annual ryegrass and the same toxins are produced. In 1996 in Japan, ARGT was diagnosed in cattle after they had eaten hay purchased from a Western Australian hay exporter. This resulted in the introduction of a voluntary hay-testing protocol for all exported hay, that was designed to minimise the risk of significant contamination with *R. toxicus* (Elson 2002*a*). Since 2005, the protocol is compulsory.

Pasture and grazing management programs for the prevention of ARGT have been developed for stockowners (Riley 1992; McKay and Ophel 1993). Knowledge of the risk of ARGT that a paddock may or does present is useful in these management programs. Two tests existed prior to 1994 for the detection of *R. toxicus* in the seedheads of green and mature ryegrass to assist stockowners establish this risk. These were a pre-flowering test and a mature ryegrass test (MRT) (Riley and McKay 1991; McKay and Riley 1993).

The pre-flowering test detects *R. toxicus* only in green ryegrass samples collected mid-season. Seedheads are collected soon after they emerge from the boot, when bacterial infection is advanced (McKay 1985), but toxin has not been produced (Stynes and Bird 1983). Timely sampling is important with this test (Riley 1992). The test procedure involves crushing the samples under high pressure to extract juice and any bacteria. The exudate is diluted and tested by an ELISA that uses polyclonal antibodies to capture the antigen/s and the same polyclonal antiserum conjugated to alkaline phosphatase for detection (Riley and McKay 1991). If the test is positive the paddock can immediately be spraytopped to minimise or stop toxin production.

The MRT detects and quantifies the number of bacterial and nematode galls in the seedheads of mature ryegrass. Seeds and galls are dislodged by threshing the plants, collecting and cleaning the seed fraction, and examining this over a light box. The concentration of galls is determined for a subsample of up to 10 g of seed. The sample is then

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classified into one of four risk categories: no risk (no galls), low risk (nematode galls only), medium risk (low numbers of bacterial galls), and high risk (medium to high numbers of bacterial galls) (McKay and Riley 1993).

This paper describes another test for the detection of the bacterium *R. toxicus*. It was developed when it was expected that a high-throughput test for pastures and hay would be required to cope with the rapidly increasing problem of ARGT in livestock in Western Australia. The test was optimised for the hay export industry when the need arose. The test is an ELISA and uses a monoclonal antibody (Mab) specific to a water-soluble polysaccharide of the bacterium *R. toxicus*. The test is simple and cost-effective and is able to handle large numbers of samples. The development and standardisation of the test, its performance, and application are described.

Materials and methods

Bacterial isolates

R. toxicus isolates CS14 and CS29, *Rathayibacter tritici* (isolate CS16), *Rathayibacter rathayi* (isolate CS18), *Arthrobacter ilicus* (isolate CS10), *Rathayibacter michiganense* subsp. *insidiosum* (isolate CS20), *Rhodococcus fascians* (isolate CS27) (Riley 1987) and another coryneform species, 'Corynebacterium agropyri' (isolate CS35), were used in this study. Isolate CS29 was from the Department of Agriculture of Western Australia Plant Pathology Unit's culture collection. All the other isolates were obtained by courtesy of the Plant Pathology Unit of the South Australian Department of Agriculture and their isolate codes were used for this paper.

Preparation of polyclonal antiserum

Cultures of the type strain *R. toxicus* (isolate CS14) were grown on 523M media (0.2% casein hydrolysate, 0.2% yeast extract, 0.2% K_2HPO_4, 0.03% $MgSO_4.7H_2O$, 1% sucrose, 1.5% agar in distilled water) for 7–14 days at 26°C (Riley and Ophel 1992). For immunisations, bacterial cells were suspended in phosphate buffered saline (PBS: 0.02 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) and heat killed (1 h at 58°C) then adjusted to an optical density of 1.0 (at 600 nm). Sheep immunisations were approved by the Animal Ethics Committee (AEC) of the Department of Agriculture of Western Australia (DAWA) and were performed in 1991–92. Suitable alternatives to the use of Freund's adjuvants were not available in the Department at that time. Polyclonal antisera to *R. toxicus* were raised in sheep by intramuscular immunisation with heat-killed bacterial suspensions (5×10^7 cells/mL PBS) of *R. toxicus* in an equal volume of Freund's complete adjuvant for the first immunisation and Freund's incomplete adjuvant for subsequent immunisations. The polyclonal antiserum collected was tested in an indirect ELISA to determine the antibody levels to *R. toxicus*. The IgG fraction was extracted from the serum by affinity chromatography using Sepharose 4B-protein G (Amersham Pharmacia Biotech, NSW, Australia) and stored in 20% glycerol and 0.02% sodium azide at 4°C.

Mouse immunisation and Rathayibacter toxicus *hybridoma development*

Mouse immunisations and fusions were approved by the AEC of the DAWA and were performed in 1991–92. Suitable alternatives to the use of Freund's adjuvants and the production of Mabs in ascites were not available in the Department at that time. BALB/c mice were each immunised by intraperitoneal (i.p.) injection with 0.2 mL (10^7 cells) of heat-killed CS14 in Freund's complete adjuvant according to previously described procedures (Galfrè and Milstein 1981; Harlow and Lane 1988). The same dose of bacteria was repeated monthly for 2 months using Freund's incomplete adjuvant. Booster immunisations of the same dose in PBS were given once a month until the mice were required for fusions. On each of the 4 days prior to a fusion, each mouse was inoculated with 4×10^7 cells of heat-killed CS14 in PBS. Fusion of splenic lymphocytes from the immunised mouse with mouse myeloma cells (P3-NS/1-Ag4-,1 NS-1) in the ratio of 5 : 1 was carried out as described by Galfrè *et al.* (1977). Hybridomas positive for antibody to *R. toxicus* (CS14) were detected by an indirect ELISA using CS14-coated ELISA plates (Tjissen 1985). The hybridomas were cloned twice by limiting dilution.

Cloned hybridoma cultures were evaluated in the CS14 capture ELISA described below, and isotypes of monoclonal antibodies were determined with a mouse monoclonal antibody isotyping kit (Isostrip, Roche, Mannheim, Germany). The selected hybridoma (IgM hybridoma 2A1C7D8) was used for ascites production according to previously described procedures (Galfrè and Milstein 1981; Campbell 1991). The ascitic fluid was centrifuged at 1000*G* for 10 min to remove any cells and filtered (pore size 0.2 µm). The supernatant was stored at 4°C with 0.05% sodium azide as preservative. Hybridoma cells were stored in liquid nitrogen. When monoclonal antibody (Mab) was required later, cells were thawed, and cultured in Gibco Hybridoma SFM complete DPM (Cat. No. 12300-067) with the addition of bicarbonate to adjust pH to within the range 7.2–7.4. The cells were then grown up *in vitro* (Integra BioSciences CL 1000) in the same culture medium, supplemented after 7 days with OPTIMAb Monoclonal Antibody Production Enhancer (GIBCO Cat. No. 11910) to produce a high concentration of Mab. The culture medium containing Mab was clarified by centrifugation at 16 000*G*, mixed with an equal volume of glycerol, then preserved by the addition of 0.05% sodium azide (final concentration) and stored at -15 ± 5°C.

Antigen characterisation

To characterise the antigen captured by the Mab, *R. toxicus* antigen was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis using a gradient sodium dodecyl sulfate gel with acrylamide concentrations of 8–25% in a Phast (Amersham Pharmacia Biotech, NSW, Australia) system (150 V, 4.0 mA, 1.5 W, 15°C, 105 Vh). The gel was stained for proteins using the Bio-Rad silver staining method (Bio-Rad Laboratories, Richmond, CA, USA). A second gel was run under the same conditions, blotted onto nitrocellulose, then probed with the Mab.

Further studies involved gel filtration of bacterial washings on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech, NSW, Australia). After culture on 523 M medium for 7–14 days at 26°C, colonies of CS14 were collected into 4 mL of normal saline, mixed by vortex for several minutes, centrifuged in a microfuge for 10 min (15 000*G* max.), then 0.22-µm filtered to remove all cell debris. A 100-µL aliquot of this suspension was injected onto the column and run for 60 min at a flow rate of 0.4 mL/min and fractions collected. These fractions were tested for protein concentration (absorbance at 280 nm and Pierce protein assay kit), antigenicity in the CS14 capture ELISA, and carbohydrate concentration by the anthrone reaction (Brink *et al*. 1960).

Hay and ryegrass samples

Samples of *R. toxicus*-infected oaten hay, ryegrass, and mature pasture containing ryegrass were collected from 3 areas (designated north, central and south) in Western Australia where ARGT had been recorded in 1991–92 (Roberts and Bucat 1992). For initial test development, in total, 264 samples of ryegrass or mature pasture containing ryegrass were used. During further development, 33 oaten hay samples were used.

Samples of oaten hay and ryegrass seedheads were obtained from areas where ARGT had never been recorded, for use in validating the assay. The hay included a bale of hay from Queensland, obtained by courtesy of the Queensland Department of Primary Industries and Fisheries. These samples will be referred to hereafter as 'uninfected hay' and 'uninfected ryegrass'.

Samples of dry mature ryegrass were obtained from 4 ARGT locations in South Australia (courtesy of the South Australian Research and Development Institute [SARDI]) and 2 ARGT locations in Western Australia in 1992 and 1999 respectively, and bacterial galls were sorted from the seeds as described by McKay and Riley (1993). The bacterial galls were used to spike samples of uninfected hay or uninfected ryegrass seedheads for determination of sensitivity, reproducibility, and the limit of detection.

Extraction procedures

The 264 samples from Western Australian ARGT areas were each mixed and divided into 2 portions, one of which was tested by the MRT at SARDI to determine the number of bacterial and nematode galls in a standard weight of grass or seed. The second portion of each sample was extracted for ELISA by the following procedure used during early test development. Sample extracts were prepared by soaking approximately 30 mL of the pre-mixed sample in an equal volume of tap water, and subsamples of each extract were collected after mixing at 10 min, 60 min, and overnight (16–18 h).

For the ARGT export ELISA, oaten hay samples were weighed and soaked overnight (16–18 h) in 5 times their weight of tap water in a tough plastic bag. After thorough mixing by kneading and tilting the bag from side to side, a 2-mL aliquot of the supernatant was taken from each sample.

Specificity assessment of the monoclonal antibody and the ELISA

The ascites Mab was tested for reactivity and cross-reactivity by indirect ELISA (Tjissen 1985). Wells were coated with 100 µL of bacterial suspension (5×10^7 cells/mL) diluted 1 in 100 in 50 mM carbonate buffer pH 9.6 (containing 0.02% sodium azide) for 60 min at 37°C. In addition to *R. toxicus* (isolates CS14 and CS29), the species tested were *Rathayibacter tritici*, *Rathayibacter rathayi*, *Arthrobacter ilicus*, *Rathayibacter michiganense* subsp. *insidiosum*, *Rhodococcus fascians* (Riley 1987), and another coryneform species, 'Corynebacterium agropyri' (see *Bacterial isolates*). After coating (and after all subsequent incubations) the wells were washed 3 times with PBS containing 0.05% Tween 20 (PBST). The wells were coated with Mab dilutions (1/2, 1/8, 1/32, and 1/128 in PBST, 100 µL per well) by incubation for 60 min at 37°C. After washing, the wells were incubated under the same conditions with 100 µL of sheep anti-mouse Ig conjugated with horseradish peroxidase (Silenus Laboratories) at 1/4000 in peroxidase protective buffer (EPA buffer, Silenus Laboratories, Melbourne, Australia). The wells were washed and incubated for 15 min at room temperature (RT) with 100 µL of the substrate 3,3,5,5-tetramethylbenzidine (TMB, Chemicon, USA). Colour development was stopped with 100 µL of 1 M H_2SO_4 and the plates were read spectrophotometrically in a Multiskan MS at 450 nm using air as a blank.

The polyclonal sheep antiserum to *R. toxicus* was tested at 1/100, 1/400, 1/1600, and 1/6400, following a similar ELISA protocol to that for the Mab. Donkey anti-sheep Ig conjugated with horseradish peroxidase (Silenus Laboratories) at 1/4000 in EPA buffer was used to detect ovine IgG.

The possibility of cross-reactivity, in the ARGT export ELISA (see below), to bacteria commonly found in hay samples was investigated as follows. Five samples (2 hay samples from farms in ARGT areas and a hay sample, a ryegrass sample, and a chaff sample from non-infected areas) were each extracted as described for the ARGT export ELISA. After mixing, a sample of each extract was collected into a sterile container. A 1/1000 dilution of each extract in sterile pure water was plated out in duplicate (150 µL per plate) on nutrient dextrose agar, sucrose peptone agar, and King's B medium. The duplicate plates were incubated overnight at RT and 37°C, respectively. Single colonies were re-streaked onto fresh media and grown at the same temperatures to obtain pure cultures. Colony colour and morphology, Gram stain and microscopic appearance of the bacteria were recorded. The original plates and the pure cultures were stored at 4°C. A suspension of each bacterial isolate was made by mixing one loopful in 5 mL of sterile PBS. The turbidity of the suspensions was compared with McFarland's standards. To test for cross-reactivity, each suspension was mixed 3 : 1 with PBST and tested in the ELISA. To test for inhibition of the reactivity of samples containing *R. toxicus* antigen, each suspension was mixed 3 : 1 with a dilution of a sample that was highly positive for *R. toxicus* antigen and compared with a control comprising PBS mixed 3 : 1 with the same positive sample. It was decided to limit speciation of the bacteria isolated to any that cross-reacted or inhibited reactivity of positive samples. Identification was attempted with 16S rRNA gene sequencing. Prior to sequencing, DNA was extracted from a bacterial colony with a PrepMan kit (PE Applied Biosystems). Polymerase chain reaction (PCR) was performed with primers 530F and 1100r for 16S rRNA (Lane 1991). The PCR incubation cycles began with 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with the exception of the final cycle, for which extension was at 72°C for 10 min. The PCR product was purified for sequencing with an Ultra Clean PCR Clean-up DNA Purification Kit (MoBio Labs Inc.). The sequencing reaction was performed with 4 µL of Big Dye terminator version 3.1 mix (Applied Biosystems), 20 pmoles of primer, and 4 µL of purified PCR product as template in a total volume of 10 µL. The thermocycling conditions were initial denaturation for 2 min at 96°C, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The DNA in the sequencing reaction was separated from excess dye terminators by ethanol precipitation in the presence of 81 mM sodium acetate. Sequencing gel electrophoresis (Applied Biosystems) and bacterial identification by comparison of the 16S rRNA sequence with those in the Genebank database were performed by staff at Murdoch University, WA.

ELISA development: comparison with bacterial gall counts

For the initial rapid assessment of the test for its potential, 3 configurations of the ELISA were evaluated for the 264 samples of ryegrass and mature pasture containing ryegrass collected from the field. The test configurations evaluated were: T1, overnight extraction of sample followed by 60 min incubation of subsample in the test plate; T2, 60 min extraction and 60 min incubation of sub-sample; and T3, 10 min extraction and 10 min incubation of subsample. Optimal dilutions of the antigen capture antibody (sheep anti-*R. toxicus* IgG), Mab, and conjugate were determined by performing checkerboard titrations (Crowther 2001). All incubations were performed with 100 µL of reagent per well, in Immulon 4 plates (Dynatech). After each incubation, wells were emptied and washed 3 times with PBST, then tapped dry. Wells were coated with an appropriate dilution of sheep anti-*R. toxicus* in 0.1 M glycine buffer (pH 9.6) and incubated at 37°C for 60 min. The test samples were added and incubated at RT for the times as above, according to the test configuration. Finally, wells were incubated with pre-mixed and pre-incubated (30 min at 37°C) Mab and horseradish peroxidase-conjugated sheep anti-mouse antibody (Silenus Laboratories Pty Ltd, Boronia, Vic.), each at a final dilution of 1/800 in EPA buffer, at RT for 10 min. The substrate TMB was added and colour development and detection proceeded as described under *Specificity assessment*.

The correlation coefficient was calculated to compare ELISA Abs_{450} values with gall counts by the MRT for each of the test configurations T1, T2, and T3.

ELISA development: repeatability and sensitivity

Two sets of 15 samples of uninfected ryegrass seedheads (30 mL) were spiked by the addition of 3 bacterial galls or 12 bacterial galls, respectively. The galls were collected in 1992. Each spiked sample was tested in triplicate, undiluted and at 1/10, 1/100, and 1/1000 in PBST, in test configurations T1, T2, and T3, for the estimation of the repeatability and sensitivity. The results used were from the lowest dilution at which the spectrophotometric absorbance at 450 nm (Abs_{450}) was below 2.0 (this being within the linear segment of the response curve of Abs_{450} to increasing antigen concentration).

Analysis of variance was used to compare Abs_{450} values for the 3 test development configurations when testing the same spiked samples to compare test sensitivity. Residual plots were used to check that the assumptions underlying the analysis of variance were valid. The data were transformed by conversion to natural logarithms prior to analysis as required, to stabilise the variance.

Analysis of variance was used to calculate the variance between triplicate Abs_{450} values of the same sample on the same plate as an estimate of repeatability for test configurations T1, T2 and T3.

ELISA development: incubation times

Incubation times for the 2nd, 3rd, and 4th incubations of the ELISA were investigated. Incubations of 10 or 60 min at RT and 60 min at 37°C were compared, using 25 samples of uninfected hay spiked with bacterial galls collected in 1992 (3 and 10 galls/100 g hay) and extracted as for the ARGT export ELISA. In addition, 6 hay samples from an ARGT area were tested when comparing 60 min at RT with 60 min at 37°C. The samples were tested undiluted.

The ARGT export ELISA procedure

The hay export ARGT bacterial ELISA was performed as follows. Hay samples were extracted as described under *Extraction procedures*. All incubations were performed with 100 µL of reagent per well, except as indicated. Wells of an Immulon 4 micro flat-bottomed ELISA plate (Dynatech) were coated with a 1/2000 dilution of sheep anti-*R. toxicus* in 0.1 M glycine buffer (pH 9.6) and incubated at 37°C for 60 min or plates were sealed and incubated at 4°C overnight or up to 30 days. After coating the wells (and after all subsequent incubation steps) the plate was washed 4 times with PBST. The next 3 incubations were performed at 37°C for 60 min. Sample buffer (0.5 M phosphate buffer, pH 7.8, 0.02% sodium azide), 10 µL per well, was mixed with the test sample or control sample (100 µL) and incubated, then washed. The next incubation was with Mab at an appropriate dilution in high-salt diluent (HSD: 0.45 M NaCl, 10% foetal bovine serum Sigma F9423, 1% Tween 20 Sigma P-1379 in deionised water). After washing, the wells were incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody at an optimal dilution in HSD. The plate was washed, substrate TMB was added, and colour development and detection proceeded as described under *Specificity assessment*. Optimal dilutions of the antigen capture antibody (sheep anti-*R. toxicus* IgG), Mab, and conjugate under the incubation conditions for the hay export ARGT bacterial ELISA were determined by 'checkerboard titrations' as described for the developmental ELISA.

Extraction time

The time required to extract antigen from hay samples was investigated initially using 8 samples, then in a second experiment 12 samples, each weighing 500 ± 0.5 g and all from an ARGT area. Samples were soaked in 5 times their weight of water. In the first experiment, 2-mL extracts were removed after mixing at 1, 17, and 25 h. In the second experiment, extracts of the same volume were removed after mixing at 0 min and at 0.5, 1, 2, 3, 17, 20, 24, 43, and 50 h. Each extract was tested in the ARGT export ELISA in serial 10-fold dilutions from neat to 1/10000. ELISA units were calculated as described below.

Sample pH

During and after soaking of the hay samples in water, the pH of the aqueous extracts was found to drop over time. To determine the effect of sample pH on the test result, 16 hay extracts from ARGT areas (extracted before submission to the laboratory) were tested in the ELISA with and without buffering with 1/10 volume of sample buffer to raise the pH above 6.0. To determine whether any antigen was destroyed by fermentation in hay extracts, 10 hay samples were extracted with water as described above for the ARGT export ELISA, then 10 mL of each extract was collected. Subsamples of the 10 mL were taken for pH and ELISA immediately after collection and after 1 and 2 days of incubation at 37°C, to simulate extreme conditions for storage and transport of the extracts. The subsamples were tested in the ELISA neat (either buffered with 1/10 volume of sample buffer or mixed with 1/10 volume of PBS) and at 1/10 in PBST. Further 10-fold dilutions in PBST were tested if necessary, to obtain absorbance values in the linear section of the response curve. ELISA units were calculated as described below. The results for the buffered subsamples before Day 1 and after Day 3 incubation were compared by using a *t*-test. To find the pH range for effective binding of antigen to the antibody-coated wells in the ELISA, 3 hay extracts with different antigen concentrations and starting pH values were used. Eight 1-mL subsamples of each extract were buffered with increasing volumes of sample buffer. The volumes of the subsamples were equalised by the addition of water, then the pH of each subsample was measured. Each extract was therefore represented by a series of subsamples with increasing pH and the same antigen concentration. The subsamples were then tested in the ELISA.

Standardisation of the ARGT export ELISA

Uninfected hay was used to prepare a negative control hay extract. A hay sample with a high bacterial gall count was selected for preparation of a positive control extract. To prepare each control, the hay was extracted as described under extraction procedures for the ARGT export ELISA. The negative control was stored frozen in aliquots and assayed neat, in duplicate on each ELISA plate. The positive control extract was titred in the ARGT export ELISA to find the dilution at which the Abs_{450} reached a value between 1.8 and 2.6. The stock extract was frozen in aliquots. A secondary stock of 100 times the concentration required for the ELISA was prepared by diluting the stock in PBS containing 50% glycerol and 0.05% sodium azide and stored at −20°C. Just before each assay, a portion of the secondary stock was diluted 1 in 100 in PBST and assayed in duplicate on each ELISA plate.

The positive and negative control values were 100 ELISA units (EU) and 0 EU, respectively, provided that the average Abs_{450} for the positive controls on the plate was between 1.8 and 2.6, and that for the negative control was below 0.25. If the Abs_{450} was outside the limits for either control, the test plate was repeated.

Calculation of ELISA units

EU were calculated for the ARGT export ELISA as follows:

$$EU = [(\text{Test } Abs_{450} - \text{Neg. contr. } Abs_{450})/(\text{Pos. contr. } Abs_{450} - \text{Neg. contr. } Abs_{450})] \times 100$$

When samples needed dilution in order to obtain a result on the linear section of the response curve (from Abs_{450} ~0.2 to ~2.0 after subtraction of Neg. contr. Abs_{450}), the EU value was multiplied by the reciprocal of the dilution.

Cut-off values between negative and low positive and positive

Conservative cut-off values to distinguish between negative, low positive, and positive samples were determined as follows. One hundred extracts of uninfected hay were assayed, to obtain a mean ± 3 standard deviations for negative hay samples. Twenty test samples consisting of one bacterial gall added to 1 kg of uninfected hay were extracted and tested in the ELISA. The galls were collected in 1992. Although 20 is a relatively small number, the galls were randomly selected from a large pool of galls, so it was expected that there would be galls with a range of different antigen contents. The cut-off was chosen so that the EU results for 19 of the 20 samples containing 1 gall/kg would be above the cut-off. This cut-off separated negative and low positive samples. A second cut-off, separating low positive and positive samples, was set below the results of extracts from 15 samples spiked with 10 galls/kg. While single galls are known to vary widely in their antigen content (see below), it was expected that there would be less variation between sets of 10 randomly selected galls than between single galls (Lumsden 1969); therefore, it was considered that a smaller number of spiked samples would be required to determine this cut-off. Low positive hay cannot be exported but is acceptable for domestic use.

Limit of detection of the ARGT export ELISA and variation in gall antigen content

The limit of detection of the ARGT export ELISA and the variation in gall antigen content were determined in 2 ways as follows.

(*i*) Twenty-eight bacterial galls from WA (collected in 1992) were each crushed in 1 mL of water and tested in 10-fold dilutions in PBST, from undiluted to 1 in 100 000. The experiment was repeated on 30 bacterial galls from SA (collected in the early 1990s), making a total of 58 test samples of 1 gall/mL.

(*ii*) Uninfected hay from Queensland was tested in the ELISA and found not to differ from the negative control value. Ten 1-kg samples of the hay were each spiked with 10 bacterial galls (collected in 1999) and extracted by the method described for the ARGT export ELISA. The extracts were tested at a dilution of 1 in 10 to obtain results representing 1 average gall/kg. ELISA units were calculated as described above.

Variation in weight of galls

One hundred bacterial galls (each with its husk) were weighed individually on a Sartorius BP210S balance. Summary statistics were calculated.

Results

Antigen detected by monoclonal antibody

The results of electrophoresis and Western blotting showed that reactivity with the Mab was not concentrated in a discrete band, but extended as a smear along part of the membrane. The ELISA antibody reactivity to the Mab (OD_{450}) coincided with a broad peak in fractions 6–17 from the Superose 6 column, which were shown to contain carbohydrate when tested by the anthrone reaction but no or little protein as measured by absorbance at 280 nm and protein assay (Fig. 1). No ELISA antibody reactivity was present in fractions 21–24, which also contained carbohydrate, and fractions 21–34, which contained protein when tested by UV absorbance at 280 nm (Fig. 1). It was concluded that the antigen has a polysaccharide moiety with repeating units, all of which are antigenic.

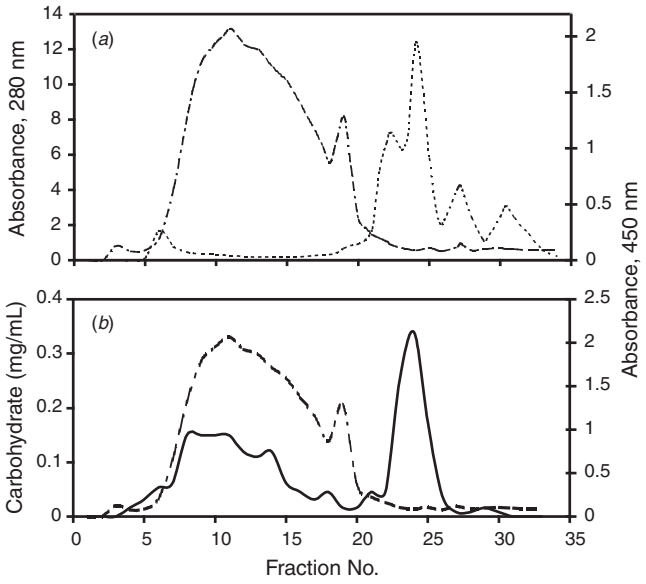

Fig. 1. The absorbance readings in each fraction collected from a 'Superose® 6 HR 10/30' column applied with *Rathayibacter toxicus* washings when tested for (*a*) .... protein (Absorbance at 280 nm) and (*b*) — carbohydrate (mg/mL), compared with results of antigen capture ELISA (·-·-·-·-·-, Absorbance at 450 nm) using the monoclonal antibody as the capture antibody. Fractions were diluted 1/50 000 in PBST for the ELISA.

Specificity assessment of the monoclonal antibody and capture ELISA

No cross-reactivity was found between the ascites Mab and *Arthrobacter ilicus* (CS10), *Rathayibacter tritici* (CS16), *Rathayibacter rathayi* (CS18), *Rathayibacter michiganense* subsp. *insidiosum* (CS20), *Rhodococcus fascians* (CS27), and 'Corynebacterium agropyri' (CS35) when tested by indirect ELISA. Reactivity occurred between the ascites Mab and *R. toxicus* isolates CS14 and CS29 (Fig. 2*a*).

The polyclonal antibody reacted with all the above species and isolates but higher Abs_{450} readings were seen for CS14 and CS29 at the highest dilution, 1/6400. The next highest Abs_{450} readings at 1/6400 were for CS18 and CS35 (Fig. 2*b*).

Twenty-nine bacterial isolates that differed with respect to colony size, morphology, and/or colour, and/or Gram stain and/or microscopic appearance, were found in the hay extracts in this experiment (Table 1). Twenty-eight isolates were Gram negative and one was Gram positive. Several isolates were excluded from Table 1 as they had identical characteristics (including ELISA non-reactivity) to another isolate from a different hay extract when grown under the same conditions. A number of isolates continued to grow when the plates were stored at 4°C. None of the bacteria isolated from hay samples gave false positive results. One isolate (# 3A in Table 1) reduced the EU result of the *R. toxicus* positive sample (when mixed with it) by about

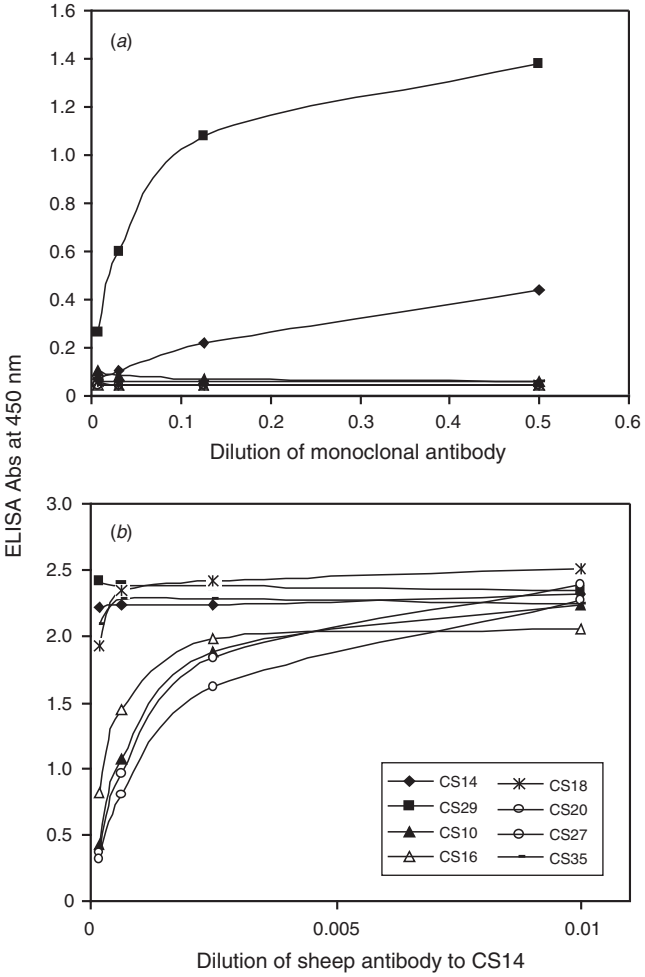

Fig. 2. Specificity and cross-reactivity of (*a*) the monoclonal antibody to *R. toxicus* and (*b*) the sheep anti-*R. toxicus* polyclonal antibody. CS14 and CS29, *R. toxicus*; CS10, *Arthrobacter ilicus*; CS16, *R. tritici*; CS18, *R. rathayi*; CS20, *R. michiganense* subsp. *insidiosum*; CS27, *Rhodococcus fascians*; 'Corynebacterium agropyri', CS35.

25%, in comparison with the same positive sample mixed with PBS. This was confirmed in a further experiment that showed reductions of 25 and 20% on different ELISA plates and also that the effect was reduced by diluting the suspension 1 in 10 (0 and 10% reduction in EU on different plates). This isolate was the only Gram positive bacterium isolated from hay in these experiments. It grew in sterile filtered hay extract both at 25 and 37°C. More growth was evident at the latter temperature. The supernatant of the culture of # 3A in sterile filtered hay extract at 37°C reduced the mean result of the positive sample from 75 to 21 EU (72% reduction). A sterile filtrate of the same culture, diluted 1 in 10 in uninfected hay extract, reduced the mean result of the positive sample from 70 to 46 EU (34% reduction) when compared with the same positive sample mixed with sterile filtered uninfected hay extract. This indicated that when this isolate was grown at 37°C in hay extract, a soluble metabolite of the bacteria interfered in the ELISA. The supernatant of a culture of # 3A in hay extract at 25°C reduced the mean result of the positive sample from 75 to 74 EU (<2%), which is not a significant reduction. The isolate # 3A also grew on blood agar. The 16S rRNA sequence amplified from this isolate was 99% identical (2 nucleotides unidentified) with that of *Bacillus subtilis*.

ELISA development: comparison between bacterial gall counts and ELISA results

Graphs of ELISA results for the 264 samples collected from various locations in 3 ARGT areas *v.* corresponding bacterial gall counts by the MRT are presented in Fig. 3. It is evident that high bacterial gall counts are associated with high ELISA readings ($P < 0.0001$; correlation coefficient $r = 0.343$ for T1, 0.364 for T2, and 0.420 for T3).

ELISA development: repeatability

The 95% confidence limits as a percentage of mean Abs_{450} for ELISA tests on the same plate when tested neat were 2.2% (3 galls/30-mL sample) and 2.1% (12 galls/30-mL sample) for T2, and 6.5% (3 galls/30-mL sample) and 4.6% (12 galls/30-mL) for T3. The ELISA results for T1 were based on diluted solutions and 95% confidence limits were ~12% of mean Abs_{450} value.

ELISA development: sensitivity

A plot of residual values *v.* fitted values (Abs_{450} values) for the analysis of variance for Abs_{450} indicated that residual variance was low for low Abs_{450} values, then increased for Abs_{450} values up to about 1.5. Residual variance stayed approximately constant for Abs_{450} greater than 2. This pattern of residual variance is probably related to the sigmoid pattern of response of Abs_{450} to increasing antigen concentration in the ELISA. An analysis of variance of Abs_{450} values for all samples would be invalid as residual variance was not constant. Instead, 2 analyses of variance were carried out, one for $\log_e(Abs_{450})$ values for all samples with an average Abs_{450} value less than 2, and another for Abs_{450} values for the remaining samples. There was a significant ($P < 0.001$) difference between Abs_{450} levels for the 3 test configurations. Average Abs_{450} values for each of the 3 tests (Table 2) indicate decreasing sensitivity from T1 to T3. Tests T1 and T2 were considerably more sensitive than T3. For example, for the samples containing 3 galls/30 mL, the dilution required to obtain an Abs_{450} in the linear range of the ELISA response curve for T1 was 1/100–1/1000, for T2 was neat −1/10, and for T3 was neat. For samples containing 12 galls/30 mL, the dilution required for T1 was 1/1000, for T2 was 1/10–1/100, and for T3 was neat −1/10. The sensitivity of the ELISA increased with the sample extraction time and the time of incubation of sample in the test well.

Table 1. Characteristics and ELISA results of bacteria isolated from 5 hay extracts

#	Media	Inc. temp	Colony diam. (mm), morphology, colour[A]	Gram stain	Microscopic appearance	McF[B] turbidity for ELISA	ELISA units with PBST[C]	ELISA units with +ve[D]
C[E]	–	n.a.	n.a.	n.a.	n.a.	0	0	114
1A	NDA	37°C	~1, mucoid, p.y.	–ve	Oval	5	0	117
1B	NDA	37°C	3–4, mucoid, cr.	–ve	Rods	2	0	113
1C	SPA	37°C	1–1.5, mucoid, y.	–ve	Rods	4	0	116
1D	SPA	37°C	1, mucoid, y.	–ve	Rods (diff. size)	4	0	116
1E	NDA	RT	1.5–2, mucoid, y.	–ve	Rods	3	0	116
1F	NDA	RT	1.5, mucoid, y.	–ve	Rods	1	0	114
1G	SPA	RT	1, mucoid, y.	–ve	Short rods	1	0	114
1H	KB	RT	<1, moist, smooth, fl.y[F]	–ve	Rods	3	0	115
2A	NDA	37°C	5–6, mucoid, p. y.	–ve	Rods	2	0	116
2B	SPA	37°C	8, mucoid, wh.	–ve	Rods	1	0	114
2C	SPA	37°C	1.5, mucoid, p. y.	–ve	Rods	4	0	113
2D	SPA	RT	4, mucoid, tr.wh.	–ve	Rods	1	0	118
2E	KB	RT	1.5, mucoid, y.	–ve	Rods	2	0	118
3A	NDA	37°C	5–6, dry wrinkled, cr.	+ve	Rods	1	0	85*
3B	SPA	37°C	1, mucoid, cr.	–ve	Short rods	4	0	114
3C	KB	37°C	~1.5, mucoid, y.	–ve	Rods	5	0	115
3D	KB	37°C	~1.5, flat moist, p. y.	–ve	Rods	4	0	121
3E	SPA	RT	3, mucoid, wh.	–ve	Rods	2	0	117
3F	SPA	RT	1, dry, cr.	–ve	Small rods	2	0	118
3G	KB	RT	2, mucoid, y.	–ve	Rods	3	0	114
4A	NDA	37°C	1, moist, y.	–ve	Short rods	5	0	113
4B	SPA	37°C	7–8, mucoid, tr. wh.	–ve	Narrow rods	1	0	112
4C	SPA	37°C	3, mucoid, p. y.	–ve	Rods	2	0	114
4D	NDA	RT	dry & mucoid cols, p.	–ve	Rods	3	0	112
5A	NDA	37°C	3–4, mucoid, cr.	–ve	Oval	4	0	112
5B	KB	37°C	~1, flat moist, cr.	–ve	Short Rods/oval	5	0	115
5C	SPA	RT	2–3, mucoid, cr.	–ve	Rods	2	0	114
5D	SPA	RT	<1, dry, wh.	–ve	Cocci	2	0	114
5E	KB	RT	1, moist, cr.	–ve	Short rods	3	0	113

*Reduction of absorbance of positive sample relative to control. n.a., Not applicable.
[A]p.y., Pale yellow; y., yellow; fl.y., fluoro yellow; cr., cream; wh., white; tr.wh., translucent white; p., pink.
[B]McFarland's turbidity.
[C]ELISA well contained 75 µL of bacterial suspension + 25 µL of PBST.
[D]ELISA well contained 75 µL of bacterial suspension + 25 µL of positive sample.
[E]Control (PBS).
[F]Fluorescent (green) when exposed to UV light.

ELISA development: incubation times

When incubations were at RT for 10 min, the results for all 25 spiked samples (Abs_{450} range 0.08–0.52) were considerably lower than at RT for 60 min (0.96–3.29) and 37°C for 60 min (0.91–3.02). When the 6 additional samples from an ARGT area were compared, 4 samples showed a higher Abs_{450} when incubations were performed at 37°C for 60 min (1.60, 0.70, 2.23, and 0.65) than at RT for 60 min (0.82, 0.44, 2.14, and 0.46, respectively); one showed no difference (0.51 *v.* 0.48) and one was lower (0.36 *v.* 0.49). Incubating for 60 min at 37°C was therefore chosen for the assay, being generally more sensitive.

Extraction time

In Expt 1, the EU values for 4 samples appeared to rise linearly from 1 to 25 h and for 4 samples the increase in EU was apparently more rapid between 17 and 25 h than before 17 h (Fig. 4*a*). In Expt 2, the EU values rose rapidly during the first 2–3 h, then at a slower rate between 3 and 20 h, and at an even slower rate from 20 to 47 h (Fig. 4*b*). The negative samples (<10 EU) were distinguishable from the high positive samples (>100 EU) before half an hour of extraction, and from all the low positive samples (11–95 EU) by 17 h. Overnight (17 h) soaking was considered acceptable and convenient.

Sample pH

The pH of the tap water in the laboratory was 6.1. The pH of the water immediately after wetting the hay was 5.8–6.0. The sample pH of 16 extracts after soaking the hay for 17 h ranged from 3.8 to 5.1. Buffering with 1/10 volume of 0.5 M phosphate, pH 7.8, raised the pH above 6.0. The ELISA results and pH values without and with pH correction are depicted in Fig. 5*a*.

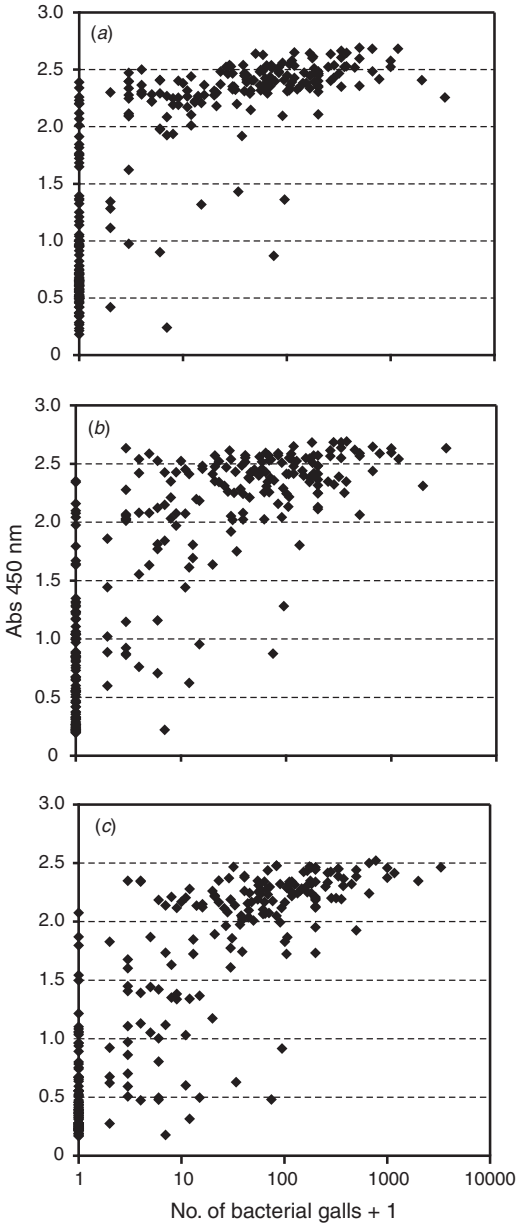

Fig. 3. ELISA absorbance results at 450 nm and their corresponding mature ryegrass test results for test configurations (*a*) T1, (*b*) T2, (*c*) T3, for 264 samples from ARGT areas.

The results of 10 hay extracts that were incubated at 37°C for 2 days after extraction are shown in Table 3. Correction of the pH with sample buffer resulted in an increase in the ELISA result in all samples. In samples from 2 extracts, after 2 days at 37°C, pH was not raised above 6.0 by sample buffer. These 2 samples had lower ELISA results after 2 days than after 1 day or before incubation at 37°C, but the effect of low pH on antigen binding to the coated wells would account for this. A *t*-test (paired 2 samples for means) showed that there was no significant difference, after buffering, between the ELISA results on Day 1 and Day 3 [t statistic $= 0.99$, $P\ (t \leq t)$ one-tail $= 0.17$].

The ELISA results of increasingly buffered subsamples of 3 hay extracts are depicted in Fig. 5*b*. The ELISA results were relatively stable between pH 7.0 and 7.5 for 2 of the extracts, and were progressively reduced with pH reduction from 7 down to 4, with a steeper slope below pH 5.8 in the other extract.

Cut-off values between negative and low positive and positive

The Abs_{450} results for the 100 negative samples ranged from 0.07 to 0.15 with a mean of 0.1 ± 0.021 (3 s.d.), and the EU ranged from 0 to 3.06 with a mean of 0.67 ± 2.6 (3 s.d.). The range of results for the 20 samples containing 1 gall/kg was from 9 to 100 EU, with a mean of 55 ± 35 EU (1 s.d.). The cut-off between negative and 1 gall/kg (weak positive) was conservatively chosen to be 10 EU. The results for the 15 samples containing 10 galls/kg ranged from 126 to 2250 EU. The mean was 1114 ± 606 (1 s.d.) and the median was 1032 EU. The cut-off between weak positive and positive (used only as an indication of the severity of contamination of the sample) was conservatively designated as 100 EU.

Limit of detection of the ARGT export ELISA and variation in gall antigen content

At a dilution of 1 in 10^5, all replicates of 1 gall/mL were clearly distinguishable from the negative controls (PBST and overnight extract of uninfected hay). The range of Abs_{450} values at 1 in 10^5 was 0.38–2.91 (mean 1.40 ± 0.83 [1 s.d.]), after subtracting the negative control values (0.08–0.38). This represents more than a 7-fold variation in antigen content

Table 2. Treatment mean Abs_{450} and 5% least significant differences* for T1 to T3
Extraction and incubation times decreased from T1 to T3

Sample Abs_{450}	Unit	T1	T2	T3	5% l.s.d.*
Average Abs_{450} <2	$Log_e(Abs_{450})$	−0.0123	−0.2111	−0.638	0.062
	Retransformed Abs_{450} ($e^{logeAbs450}$)	0.988	0.810	0.528	
Average Abs_{450} >2	Abs_{450}	1.212	1.018	0.666	0.067

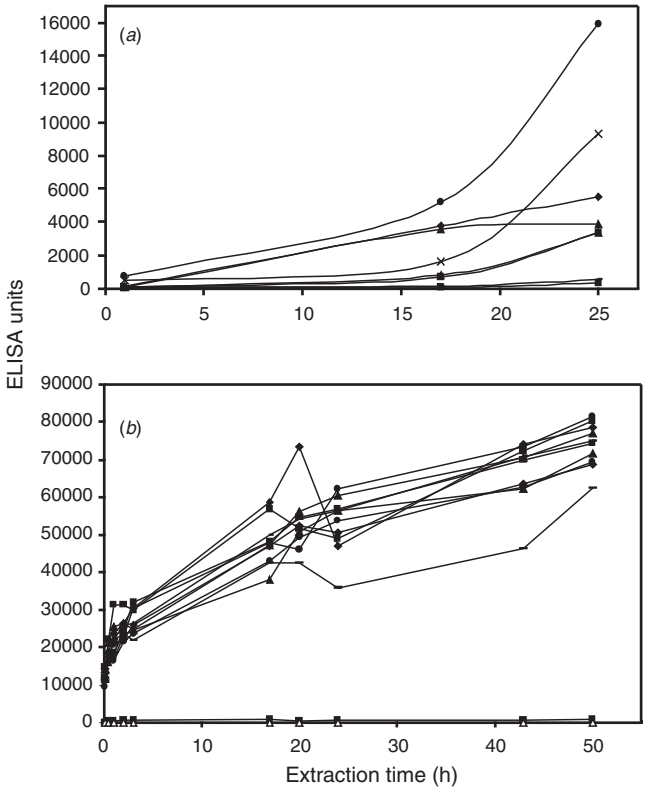

Fig. 4. Samples from infected paddocks were soaked in 5 times their weight of water in tough plastic bags. Samples were collected at intervals during soaking. Before collecting each sample, the hay and water were mixed by kneading. The graphs show ARGT export ELISA results of (*a*) 8 hay samples soaked for 25 h and (*b*) 12 hay samples soaked for 50 h. Results are expressed in ELISA units.

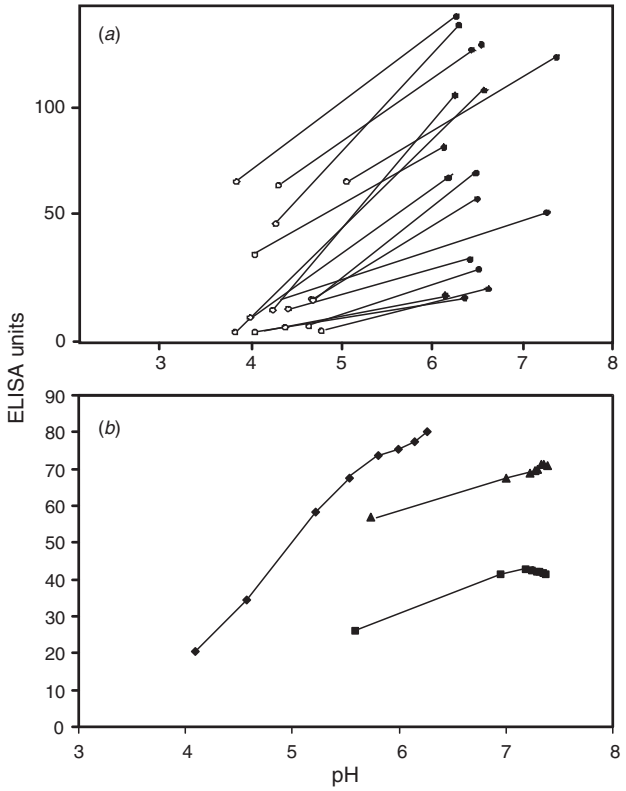

Fig. 5. (*a*) Each line represents one sample. The coordinates of the ends of the line represent pH and ELISA units before (○) and after (●) raising the pH with 1/10 volume of sample buffer. (*b*) ELISA results of 3 hay extracts, each divided into subsamples that were buffered with increasing volumes of sample buffer. Volumes of the subsamples were equalised with water to equalise the antigen concentration.

Table 3. Experiment to test the effect of fermentation and low pH on bacterial antigen

	Day 1 (straight after 17-h soak)				Day 2 (extracts at 37°C for 1 day)				Day 3 (extracts at 37°C for 2 days)			
	Before pH change		Sample pH raised		Before pH change		Sample pH raised		Before pH change		Sample pH raised	
#	pH	EU[A]	pH	EU[B]	pH	EU[A]	pH	EU[B]	pH	EU[A]	pH	EU[B]
1	4.2	32	6.3	64	4.1	31	6.2	67	4.2	34	6.2	73
2	4.4	40	6.4	70	4.3	46	6.4	73	3.9	24	5.3*	58*
3	3.9	5	6.2	19	3.9	6	6.1	20	3.9	6	6.1	19
4	4.0	22	6.3	44	3.9	24	6.3	44	4.0	24	6.3	44
5	4.3	53	6.4	106	4.3	56	6.3	113	3.7	26	4.6*	58*
6	4.0	30	6.5	64	4.0	34	6.4	64	4.0	35	6.5	67
7	4.1	32	6.1	49	4.1	15	6.1	36	4.1	17	6.1	37
8	4.2	840 500	7.3	912 500	4.2	882 500	7.3	899 500	4.1	941 500	7.2	888 500
9	5.6	1348	7.4	1339	5.0	1405	7.2	1398	5.3	1451	7.2	1440
10	5.1	567	7.4	590	4.2	651	7.1	680	4.0	714	6.9	715

*The pH was not raised above 6.0 by SB and results are accordingly low.
[A]ELISA units (EU), 100 µL sample + 10 µL of 0.2 M PBS (inadequate to correct the pH) to equalise volume with that in wells with SB, see below.
[B]ELISA units (EU), 100 µL sample + 10 µL of sample buffer (SB) to correct pH.

between galls, as the response curve of Abs_{450} to antigen concentration is non-linear above ~2.7. The limit of detection in this experiment was 1 in $\geq 10^5$, or 1 gall in ≥100 L.

The range of results for the 10 samples representing 1 average gall/kg hay was from 68 to 272 EU, with a mean of 176 ± 71 (1 s.d.) and a median of 199 EU. The limit of

detection for bacterial galls in hay samples, when soaked in 5 times their weight in water, was therefore less than 1 average gall/kg. An average gall was easily detected, being well above the 10 EU cut-off.

Variation in weight of galls

The weights of 100 bacterial galls ranged from 0.3 to 2.4 mg. The median was 0.9 mg and the average weight was 0.96 ± 0.443 (1 s.d.) mg.

Discussion

The test described in this paper is an antigen capture sandwich ELISA based on polyclonal trapping and monoclonal detection of an *R. toxicus*-specific polysaccharide antigen. The novel aspect of the test is its ability to detect this highly water-soluble *R. toxicus* antigen by using a specific Mab.

In specificity testing, isolates of *Rathayibacter tritici* and *Rathayibacter rathayi* were selected as examples of one serologically distinct group and the isolates of *Arthrobacter ilicus*, *Rathayibacter michiganense* subsp. *insidiosum*, and *Rhodococcus fascians* as examples of 3 other serologically distinct groups of plant pathogenic bacteria identified by linkage cluster analysis after indirect ELISA (Riley 1987). 'Corynebacterium agropyri' was tested as another plant pathogen. The Mab was specific for *R. toxicus*. The polyclonal antibody reacted with all the above species, but was more reactive with *R. toxicus* than the other species at 1 in 1600 and 1 in 6400. The dilution used in the ELISA, 1 in 2000, was chosen to optimise the reactivity with *R. toxicus* while minimising the reactivity with the other species.

The experiments to find bacteria that could potentially interfere in the ELISA did not discover any species that may cause false positives, but showed potential for false negative results if extracts are stored under conditions that promote rapid bacterial growth. *B. subtilis* is a common bacterial species. When present at levels equivalent to or higher than McFarlands opacity = 1 (3×10^8/mL) in hay extracts, it could cause false negative results if the amount of *R. toxicus* contamination is very low. When the *R. toxicus* contamination is moderate to high, the ELISA Abs_{450} may be reduced, but *R. toxicus* antigen will still be detected. The concentration of *B. subtilis* in hay extracts is unlikely to be as high as that reached in the experiments described here, if extraction is performed in cool conditions and the extracts are stored frozen if there is any delay before testing.

Prior to the development of the ELISA described here, the MRT and the pre-flowering test (also an ELISA) were the tests in use. The MRT was used as a benchmark when developing the ELISA test in this study. The MRT is relatively quick for a small number of samples, but due to the time required to thresh, clean, and count the seeds, it is not as time-efficient as the ELISA when large numbers of samples are to be processed. In addition the dust from handling and threshing toxic ryegrass is potentially hazardous. The MRT is based on a bacterial gall count, will often miss shattered galls. and cannot detect gummosis (bacterial slime). Unlike the MRT, the ELISA cannot detect nematode galls that are not infected with bacteria. The ELISA can detect the *R. toxicus* antigen whether in galls, shattered galls, or gummosis. It was evident that this occurred when comparisons were made between the 2 tests, as there were several samples with a gall count of 0 and ELISA Abs_{450} above 0.5. This was one reason for the low correlation between the 2 tests. A second reason is that, even if the sample is mixed well before division into 2 portions, if there is only 1 gall per kg sample, one portion will have one gall and the other will have none. Variation in gall antigen content would also reduce the correlation between the 2 tests, especially for the samples with 1–3 galls/30 mL. In samples with higher gall counts, the variation in gall antigen content would be averaged out. The fourth reason is that the linear range of the response curve of Abs_{450} to antigen concentration was between about 0.2 and 2.0. The ELISA absorbance readings above 2.0 do not accurately represent the amount of antigen present. The samples were tested without dilution and some had very high gall counts. In the work on samples spiked with 3 or 12 galls, it was later found that even samples with 3 galls per 30 mL sample required dilution to 1 in 100 or 1 in 1000 to obtain an Abs_{450} in the linear range of the ELISA response curve for test configuration T1. If the samples had been tested at various 10-fold dilutions and EU calculated as described for the ARGT export ELISA, accounting for the dilution, the correlation between EU and gall count would be better than that between Abs_{450} and gall count. The MRT, the pre-flowering test, and the ELISA described in this paper all have the drawback that they do not measure the toxin. The risk of ARGT is more directly related to the toxin content of the samples.

During ELISA development, 3 test configurations were compared, with varying times for soaking the samples and for incubation of the extracts in the test plates. Repeatability was acceptable, even for T1, for which all ELISA results were based on diluted solutions and probably due to dilution were considerably more variable than results for T2 and T3. Sensitivity increased with increased soaking time from 10 min to overnight and increased incubation time from 10 to 60 min. The results indicated, however, that high levels of *R. toxicus* contamination can be detected even with a short assay protocol.

The incubation times and extraction times chosen for the ARGT export ELISA were to optimise sensitivity and convenience. Continuing the extraction for more than 17 h allowed the extraction of more antigen, but this increases the time for growth of other bacteria present in the hay, leading to a reduction in pH of the extract and potential interference in the test as described above. All that is required by the Australian Quarantine and Inspection Service is certainty of detecting antigen when it is present in the sample. The 17-h soaking time for extracting the antigen

from hay may be longer than the minimum time required to be certain of distinguishing negative from positive samples. It is possible that a reduced soaking time could be validated by further experimentation.

A proportion of the oaten hay extracts had a low pH due to fermentation both during extraction of the antigen and during storage of extracts before submission to the laboratory. The low pH interfered with the binding of *R. toxicus* antigen to the coating antibody in the test wells. For this reason it was decided to buffer all samples in the test wells to ensure detection of the antigen when present. When positive hay extracts were incubated at 37°C for 2 days the pH of the extracts went down. When the extracts were then buffered to raise the pH above 6.0, the ELISA results were not significantly different from those before incubation, indicating that the antigen had not deteriorated. The pH experiments, as well as the experiments with bacteria isolated from hay extracts (bearing in mind that some of the isolates grew at 4°C and room temperature), emphasised the desirability of performing the extractions in cool conditions and storing the hay extracts frozen until use.

The cut-off value between negative and low positive (10 EU) was set at the lower end of the range for 20 samples containing 1 gall/kg hay. A later experiment to determine the limit of detection, using 10 samples of 10 galls/kg hay tested at a dilution of 1 in 10, produced a wider range of results and a higher mean. The galls for the above 2 experiments were collected in different areas at different times, which may partly account for the different mean and median results and ranges. The cut-off is well below the result for one 'average gall', whether that is taken to be the mean or the median result.

The limit of detection when one gall was crushed in 1 mL of water ($\leq$1 gall in 100 L or $\leq$0.05 gall in 5 L) was smaller than the limit of detection when galls in 1 kg hay were soaked in 5 L of water (a fraction of an average gall/kg). This probably reflects a difference in antigen extraction efficiency between the 2 methods, and possibly the difference in the source and age of the galls. Less antigen would be released from galls in wet hay in a plastic bag, mixed by kneading, than when crushed in water. Both methods showed considerable variation among galls. This is partly due to the 8-fold variation demonstrated in the weights of 100 galls. In practical terms, the limit of detection of the ARGT export ELISA is well below 1 average gall/kg.

The ELISA described here is simple and cost-effective and can handle a large number of samples. Like the MRT and the pre-flowering test, the accuracy of the ARGT ELISA is only as good as the sample collection procedure. All 3 tests have an inherent field sampling error. The distribution of bacterial galls in a paddock is frequently not uniform. Sampling procedures to minimise the risk of failing to detect *R. toxicus* when present in hay and straw for export have been described (Elson 2002*b*).

This laboratory tests approximately 20 000 hay samples for export annually. Since regular testing with the ELISA described in this paper was instigated in 1996, there have been no cases of ARGT in Japan reported to the Australian Quarantine and Inspection Service.

The implications of the development of the ARGT export ELISA for agriculture are firstly that cost-effective testing of large numbers of samples for the presence of *R. toxicus* is possible, with a 24-h turn-around time. Secondly, with the ability to detect amounts of bacterial antigen equivalent to fractions of an average gall (whether low concentrations are due to gummosis, or to breaking up of galls during sample collection and processing, or to pooling of hay samples from several bales), the sensitivity of detection has increased compared with the MRT. Thirdly, the test has been useful in demonstrating *R. toxicus* in hay fed to Western Australian animals showing signs of ARGT, thus providing supporting information for farmers, field veterinarians, and veterinary pathologists in diagnosis of the clinical disease.

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References

Bird AF, Stynes BA (1977) The morphology of a *Corynebacterium* sp., parasitic on annual rye grass. *Phytopathology* **67**, 828–830.

Brink RH, Dubach P, Lynch DL (1960) Measurement of carbohydrates in soil hydrolysates with anthrone. *Soil Science* **89**, 157–166.

Campbell AM (1991) 'Monoclonal antibodies and immunosensor technology.' (Ed. PC van der Vliet) (Elsevier: Amsterdam)

Crowther JR (2001) 'The ELISA guidebook.' (Humana Press: Totowa, NJ)

Edgar JA, Frahn JL, Cockrum PA, Anderton N, Jago MV, Culvenor CCJ, Jones AJ, Murray K, Shaw KJ (1982) Corynetoxins causative agents of annual ryegrass toxicity; their identification as tunicamycin group antibiotics. *Journal of the Chemical Society. Chemical Communications* **1982**, 222–224. doi: 10.1039/c39820000222

Elson R (2002*a*) Code of practice for minimisation of the risk of corynetoxins contamination of hay and straw for export. Department of Agriculture, Fisheries and Forestry – Australia and Australian Quarantine and Inspection Service.

Elson R (2002*b*) Sampling procedures for minimisation of the risk of corynetoxins contamination of hay and straw. Department of Agriculture, Fisheries and Forestry – Australia and Australian Quarantine and Inspection Service.

Galfrè G, Howe SC, Milstein C, Butcher GW, Howard JC (1977) Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature* **266**, 550–552. doi: 10.1038/266550a0

Galfrè G, Milstein C (1981) Preparation of monoclonal antibodies: strategies and procedures. In 'Methods in enzymology'. Vol. 73. (Academic Press, Inc.: New York)

Gwynn R, Hadlow AJ (1971) Toxicity syndrome in sheep grazing Wimmera ryegrass in Western Australia. *Australian Veterinary Journal* **47**, 408.

Harlow E, Lane D (1988) 'Antibodies. A laboratory manual.' (Cold Spring Harbour Laboratory: New York)

Jacobs SWL (2001) The genus *Lachnagrostis* (Gramineae) in Australia. *Telopea* **9**, 439–448.

Lane D (1991) 16S/23S rRNA sequencing. In 'Nucleic acid techniques in bacterial systematics'. (Eds E Stackebrandt, M Goodfellow) pp. 115–147. (John Wiley and Sons: Chichester, UK)

Lumsden J (1969) Hypothesis testing and sampling distributions. In 'Elementary statistical method'. pp. 90–91. (University of Western Australia Press: WA)

McIntosh GH, Rac R, Thomas MR (1967) Toxicity of parasitised Wimmera ryegrass, *Lolium rigidum*, for sheep and cattle. *Australian Veterinary Journal* **43**, 349–353.

McKay AC (1985) Control of annual ryegrass toxicity. PhD thesis, University of Adelaide, Australia.

McKay AC, Ophel KM (1993) Toxigenic *Clavibacter*/*Anguina* associations infecting grass seedheads. *Annual Review of Phytopathology* **31**, 151–167. doi: 10.1146/annurev.py.31.090193.001055

McKay AC, Ophel KM, Reardon TB, Gooden JM (1993) Livestock deaths associated with *Clavibacter toxicus*/*Anguina* sp., infection in seedheads of *Agrostis avenacea* and *Polypogon monspeliensis. Plant Disease* **77**, 635–641.

McKay A, Riley IT (1993) Sampling ryegrass to assess the risk of annual ryegrass toxicity. *Australian Veterinary Journal* **70**, 241–243.

Riley IT (1987) Serological relationships between strains of coryneform bacteria responsible for annual ryegrass toxicity and other plant-pathogenic bacteria. *International Journal of Systematic Bacteriology* **35**, 153–159.

Riley IT (1992) Paddock sampling for management of annual ryegrass toxicity. *Journal of Agriculture Western Australia* **33**, 51–56.

Riley IT, McKay AC (1991) Inoculation of *Lolium rigidum* with *Clavibacter* sp., the toxigenic bacteria associated with annual ryegrass toxicity. *Journal of Applied Bacteriology* **71**, 302–306.

Riley IT, Ophel KM (1992) *Clavibacter toxicus* sp. nov., the bacterium responsible for annual ryegrass toxicity in Australia. *International Journal of Systematic Bacteriology* **42**, 64–68.

Roberts WD, Bucat J (1992) The Surveillance of Annual Ryegrass Toxicity in Western Australia October 1991–May 1992. Animal Industries Division, Epidemiology Branch, Department of Agriculture, Western Australia.

Sasaki J, Chijimatsu M, Susuki K (1998) Taxonomic significance of 2,4-diaminobutyric acid isomers in the cell wall peptidoglycan of actinomycetes and reclassification of *Clavibacter toxicus* as *Rathayibacter toxicus* comb. nov. *International Journal of Systematic Bacteriology* **48**, 403–410.

Schneider DJ (1981) First report of annual ryegrass toxicity in the Republic of South Africa. *The Onderstepoort Journal of Veterinary Research* **48**, 251–255.

Stynes BA, Bird AF (1983) Development of annual ryegrass toxicity. *Australian Journal of Agricultural Research* **34**, 653–660. doi: 10.1071/AR9830653

Tjissen P (1985) 'Practice and theory of enzyme immunoassays'. Vol. 15 of series: Laboratory techniques in biochemistry and molecular biology. (Eds RH Burdon, PH van Knippenberg) (Elsevier: Amsterdam)

Vogel P, Petterson DS, Berry PH, Frahn JL, Anderton N, Cockrum PA, Edgar JA, Jago MV, Lanigan GW, Payne AL, Culvenor CCJ (1981) Isolation of a group of glycolipid toxins from seedheads of annual ryegrass (*Lolium rigidum* Gaud.) infected by *Corynebacterium rathayi. Australian Journal of Experimental Biology and Medical Science* **59**, 455–467.

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