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# An estimate of specificity for a Johne's disease absorbed ELISA in northern Australian cattle

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**Objective** To estimate the specificity of an absorbed enzyme-linked immunosorbent assay kit<sup>d</sup> for Johne's disease (JD) when used in mature cattle populations resident in northern Australia.

**Design** Blood samples were collected from beef cattle in northern Queensland, the Northern Territory and northern Western Australia, and from dairy cattle in northern Queensland. The specificity of a serological test for JD was estimated by testing the blood samples with an absorbed ELISA kit. Further samples were collected from cattle with positive ELISA results to determine the presence or absence of infection with *Mycobacterium avium* subsp *paratuberculosis*.

**Procedure** During 1995 and 1996, blood, tissue and gut contents were collected from beef cattle at abattoirs in Queensland and the Northern Territory; and blood and faecal samples were collected from dairy cattle in herds assessed to be most at risk for JD in northern Queensland. The blood samples were tested using an absorbed ELISA kit. Tissues and gut contents from beef cattle that had positive ELISA results were cultured for *M avium* subsp *paratuberculosis*, and tissues were examined histologically. Faecal samples from dairy cattle with positive ELISA results were cultured for *M avium* subsp *paratuberculosis*.

**Results** Estimates of specificity for this absorbed ELISA in mature northern Australian cattle were 98.0% (97.0 to 98.8%, 95% CI) in beef cattle, and 98.3% (96.7 to 99.3%, 95% CI) in dairy cattle.

**Conclusion** Estimates of specificity in this study were lower for beef cattle from the Northern Territory and northern Western Australia and for dairy cattle from northern Queensland than those quoted from studies on cattle in southern Western Australia. This should be considered when serological testing using the JD ELISA is carried out on northern Australian cattle.

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BVL	Berrimah Veterinary Laboratories, Northern Territory Department of Primary Industry and Fisheries
CI	Binomial confidence interval
ELISA	Enzyme-linked immunosorbent assay
JD	Johne's disease
OVL	Oonoonba Veterinary Laboratory, Queensland Department of Primary Industries
QDPI	Queensland Department of Primary Industries
VIAS	Victorian Institute of Animal Science

Johne's disease is a bacterial disease of ruminants including cattle, sheep, goats and deer.<sup>1</sup> The causative agent is *Mycobacterium avium* subsp *paratuberculosis* and the disease is also called paratuberculosis. Animals are usually infected when young and most clinical cases are aged between 3 and 5 years.<sup>1</sup> Affected animals lose weight, develop chronic diarrhoea and die if not culled or destroyed. There is no known cure for the disease.<sup>2</sup>

Two groups of *M avium* subsp *paratuberculosis* isolates have been identified by restriction endonuclease and DNA hybridization analyses - a bovine group and an ovine group.<sup>2</sup> In Australia, isolates from cattle, goats and alpaca are generally bovine JD.<sup>3</sup> This study considers bovine JD.

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There is substantial evidence to indicate that Queensland, the Northern Territory and Western Australia<sup>4</sup> are not endemically infected with bovine JD. Both Queensland and the Northern Territory have been granted Protected Zone status for bovine JD; and Western Australia has been granted Free Zone status. When bovine JD has been detected, it has always been in animals introduced from interstate, and eradication of the infection followed detection. However, in the absence of clinical disease, initial application of the absorbed ELISA indicated that the prevalence of positive serological reactions increased from south to north in Queensland (J Roberts personal communication). A survey in 1992 of north Queensland dairy cattle, conducted by QDPI at OVL, revealed a seroprevalence of about 7% using the absorbed ELISA kit. All animals with positive ELISA tests had negative faecal culture results, and there was no evidence of clinical disease (L Kulpa personal communication).

In Australian cattle, complement fixation tests for JD have been reported to have lower specificity and sensitivity than the absorbed ELISA.<sup>5</sup> The CFT has thus been replaced in recent years in southern states by the absorbed ELISA, for which specificity estimates close to 100% have been reported.<sup>6-8</sup> This study reports the evaluation of specificity for an absorbed ELISA kit, undertaken on cattle originating from northern Queensland, the Northern Territory and northern Western Australia, to assess whether specificity estimates for this population are similar to estimates previously reported. An estimate of the specificity for the absorbed ELISA is important for this population to ensure that Australia's live cattle export trade is not jeopardised by high numbers of false positive test results, and to ensure that the test will be useful for any active surveillance carried out to maintain the status of freedom from infection with *M avium* subsp *paratuberculosis* in these regions.

## Materials and methods

### Beef cattle

Samples from beef cattle in north Queensland were from aged cull cows killed at the Australia Meat Holdings abattoir at Townsville. Sampling was conducted between November 1995 and May 1996 as suitable herds became available at the meatworks. Seventeen collections were made representing 16 properties from 11 of the 12 major beef producing shires in north Queensland. A total of 541 animals were sampled with between 30 and 35 animals from each property.

Samples from beef cattle in the Northern Territory and northern Western Australia were from aged animals from the major cattle export regions of these states. Suitable herds were sampled as they became available at Tenarra Pty Ltd Batchelor Meatworks near Darwin. Samples were collected between November 1995 and October 1996. A total of 508 animals from 17 properties were sampled with between 1 and 50 animals from each property (only two properties had samples taken from less than 20 animals). There were 293 cattle from the Northern Territory and 215 from Western Australia.

### Dairy cattle

The dairy cattle industry in north Queensland is confined to three shires on the Atherton Tablelands and has 202 herds in total. Herds included in the study were those assessed as being most at risk of having JD. This assessment was done in consultation with advisers and private veterinarians and by review of herd health records, past JD ELISA and complement fixation testing, interstate import records and local knowledge. Risk

factors included cases of chronic diarrhoea, wasting, unexplained deaths and introductions from states with endemic JD.

Twenty-five dairy herds were identified as at risk by this initial assessment and, of these, 18 were sampled in the preliminary round of testing that was conducted between August and November 1995. A total of 475 dairy cows from the Atherton Tablelands in north Queensland were included in the study. At least 25 mature cows per herd were sampled, and cows that had a positive or high negative result in the absorbed ELISA were subject to further investigation.

### Specimens

Specimens collected from beef cattle, at slaughter, were blood and tissue samples and gut contents. The tissue samples were:

- proximal colon adjacent to the ileocaecal valve (10 cm x 5 cm sample)
- entire ileocaecal valve
- distal ileum adjacent to the ileocaecal valve (10 cm x 5 cm sample)
- ileum, 20 cm proximal to the ileocaecal valve (10 cm x 5 cm sample)
- ileocaecal lymph nodes
- mesenteric lymph nodes, about 15 cm proximal to the ileocaecal valve.

The gastro-intestinal tract specimens were opened, washed and examined for any gross pathological changes such as hyperaemia or thickening of the mucosa.

Blood samples were collected from dairy cattle and, from animals that had positive or high negative results in the absorbed ELISA, further blood samples and also faecal samples were collected.

### Testing

Specimens collected from the Northern Territory and northern Western Australian beef cattle were sent to BVL for processing and testing, and those from Queensland cattle were sent to OVL.

All cattle sera were tested during 1995 and 1996 with the absorbed ELISA kit. The same 'quality assured' JD ELISA batch (B0301-W5170, expiry date 7 April 1997) was used by the participating laboratories. The manufacturer recommends two discrete categories when interpreting JD ELISA results, these being either positive (greater than the cut-off value) or negative (less than or equal to the cut-off value). The cut-off value is the mean absorbance value of the negative controls plus 0.100. For this study, three result categories were used - a high negative category was included to identify animals for further investigation. The high negative category had absorbance values less than or equal to the cut-off value but greater than the cut-off value minus 0.025. Sera that had positive and high negative results were sent to VIAS for re-testing with the same ELISA batch.

Tissue specimens and gut contents collected from beef cattle whose serum had positive or high negative absorbed ELISA results, were submitted for bacteriological culture and the tissue specimens were processed for histopathology. Tissue from each site was cultured and a histopathological examination was conducted at OVL or BVL, and at VIAS, in accordance with the Australian and New Zealand Standard Diagnostic Procedures.<sup>3</sup>

The dairy cattle that had positive or high negative results in the absorbed ELISA were subject to intensive investigation for

JD. This included collection of animal history, clinical examination, and the collection of three additional samples of blood and faeces. Faecal culture was performed at both OVL and VIAS.

The bacteriological culture technique used for faecal samples from dairy cattle and for tissue samples and gut contents from beef cattle was the 'double incubation' technique.<sup>3</sup>

### Analysis

Values for specificity, binomial confidence intervals and the  $\chi^2$  statistic were calculated using Epi Info version 6.04b. The six studies, as listed in Table 1, were compared using the Tukey-type multiple comparison test.<sup>9</sup>

### Results

Of a total of 1049 beef cattle, 21 had positive test results in the absorbed ELISA. All animals with positive tests were negative for JD at necropsy or subsequent testing. This gave a specificity of 98.0% (97.0 to 98.8%, 95% CI). In Queensland eight positive tests in 541 samples gave a specificity of 98.5% (97.1 to 99.4%, 95% CI), and in the Northern Territory/northern Western Australian beef cattle 13 positive tests in 508 samples gave a specificity of 97.4% (95.7 to 98.6%, 95% CI). There was no significant difference in the proportion of positive tests from these two regions ( $\chi^2$  P-value, 0.21).

Tissue samples and gut contents from all beef cattle with positive (21) and high negative (15) tests were negative on bacteriological culture for *M avium* subsp *paratuberculosis* at OVL, BVL and VIAS. The histopathological examination of tissues from these animals, at VIAS by one of the authors (RC), and by pathologists at OVL or BVL, found no evidence of infection with *M avium* subsp *paratuberculosis*. There was a high degree of parasitism (*Oesophagostomum* spp) with enteritis, in association with eosinophilic infiltration, parasitic granulomas and chronic inflammatory lesions in lymph nodes.

There were eight positive tests in the blood samples from 475 dairy cattle in 18 herds. All of these animals were negative on subsequent culture of faeces, indicating a specificity of 98.3% (96.7 to 99.3%, 95% CI). Of these 18 herds, nine contained animals that were positive (8 cows) or high negative (11 cows) when tested using the absorbed ELISA. Subsequently these 19 cows were subjected to further intensive investigation, which involved three collections of faeces for bacteriological culture, and blood sampling for absorbed ELISA. Retesting was performed in late November 1995, in March 1996 and in June 1996. Two cows did not complete the three rounds of additional testing - one cow was culled due to bracken fern poisoning and concurrent mastitis before March 1996, and one cow died due to misadventure before June 1996. Faecal samples from animals with positive and high negative tests were negative on bacteriological culture for *M avium* subsp *paratuberculosis* at both OVL and VIAS. None of the animals showed clinical signs of JD throughout the test period, and when the remaining 17 were examined in late October 1996, all were healthy.

A  $\chi^2$  statistic calculated for the six studies listed in

Table 1 indicated that the data were significantly different ( $P = 0.003$ ). Subsequent multiple comparison of the pairs of studies listed in Table 1 indicated that the specificity figures calculated for studies 1, 2, 3 and 4 were not different from each other. The specificity figures calculated for studies 1, 3, 4, 5 and 6 were also not different from each other. Therefore it can be stated that the specificity figure for study 2 is different from the specificity figures for study 5 and study 6.

### Discussion

The specificity of the ELISA for detecting antibodies to *M avium* subsp *paratuberculosis* was improved in the mid-1980s by the pre-absorption of test sera with *M phlei*.<sup>10,11</sup> The problem of false positive tests was suggested to be due to cross-reacting antigens in the antigen preparations used to detect antibodies in the assay.<sup>10</sup> *M avium* subsp *paratuberculosis* has antigenic components in common with other species of mycobacteria, and with related organisms such as *Corynebacterium* sp, *Nocardia* sp, and *Actinomyces* sp.<sup>1,10</sup> Reduction of cross-reactive antibodies by absorption with *M phlei* organisms substantially reduced the number of false positive tests. The absorbed ELISA kit used in this study further refined the absorption of cross-reacting antibodies, to make it a rapid test to perform.<sup>7</sup> The kit was released for sale in March 1990,<sup>6</sup> and has been evaluated in other studies (Table 1).

In estimating specificity and sensitivity of a diagnostic test, the samples should be representative of the population in which the test is going to be used.<sup>13</sup> This will avoid the chance that cross-reacting antigens in the environment of the population of interest are not present in the samples from which the estimate of test specificity is made. The specificity will be overestimated for a population if determined from samples taken from another population that does not have cross-reacting antigens, because they can cause false positive tests.

It is likely that previous estimates of specificity for this kit were studied in JD-free populations because the 'gold standard' of bacteriological culture used to assess potential false positive tests in the absorbed ELISA has poor sensitivity.<sup>1</sup> In this study we were also able to estimate specificity without concern about the poor sensitivity of the 'gold standard' because the population is regarded as free from JD. The major difference between this and previous studies is geographical location. This suggests that differences in environmental factors, such as cross-reacting antigens, could cause the higher percentage of false positive tests in beef cattle from the Northern Territory and northern Western Australia, and in north Queensland dairy cattle, compared to the cattle from Western Australia.

**Table 1. Estimates of specificity for absorbed ELISA for Johne's disease in cattle in different geographical areas.**

Study	Specificity (95% CI)	No. of cattle	Population
1	99.0% (96.4 - 99.9%) <sup>a</sup>	196	Dairy cattle certified free of JD; Wisconsin, USA <sup>5,6,12</sup>
2	99.8% (99.3 - 100%) <sup>a</sup>	997	Cattle from a JD free region; Western Australia <sup>5,7</sup>
3	99.0% (97.6 - 99.7%) <sup>a</sup>	485	Cattle that were faecal culture negative; USA <sup>8</sup>
4	98.5% (97.1 - 99.4%)	541	Beef cattle from northern Queensland, current study
5	97.4% (95.7 - 98.6%)	508	Beef cattle from Northern Territory and northern Western Australia, current study
6	98.3% (96.7 - 99.3%)	475	Dairy cattle from northern Queensland, current study

<sup>a</sup>Derived from figures in references.

From this study, it appears that a percentage of false positive test results between 1.2 and 3% (beef) and 0.7 and 3.3% (dairy) can be expected when testing cattle in northern Australia with the absorbed ELISA. The complement fixation test has been used in northern Australia as part of the protocols for the export of breeder cattle. In Queensland, observations on the complement fixation test include a long term (1977 to 1986) estimate of false positive tests in 0.2% of the cattle tested for export or sale (mainly young beef cattle),<sup>14</sup> and false positive tests in 3.6% of dairy cattle in a structured surveillance project (D Pitt unpublished). The absorbed ELISA is quicker to perform than the complement fixation test, and there may be only a marginal advantage in using the absorbed ELISA in northern Australia in terms of reducing the number of false positive tests as both tests have high specificity.

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## BOOK REVIEW

**Anatomy of the Horse: An illustrated text.** 3rd edn. Budras KD, Sack WO and Rock S, Schlutersche, Hannover/ Iowa State University Press, Ames, 2001, 152 pages. Price US\$ 74.95. ISBN 3 87706 620 8.

This is a regional/topographic anatomy of the horse that purports to provide a short description and "copious illustration" of clinically relevant anatomy for veterinary students and practising veterinarians. It is the second of a series that is intended to cover all the major domestic species.

This book appears to have been carelessly translated from the German with minimal editorial input, so the reader has to contend with numerous mistakes of grammar and spelling (often several in a single sentence), which can be somewhat confusing. In general, the illustrations contain far fewer mistakes and omissions than the script and the majority of anatomical terms are correctly spelt, so the book could be of some use as a quick reference as long as the reader is prepared to check on the spelling, and to ignore the mistakes. Further, the list of collaborators and sources for the figures, radiographs, and photographs has been omitted from this edition, which means that there could be problems in correctly attributing material if the book is used as a reference source.

The authors say that they have omitted "clinically irrelevant detail", and have clearly focused on the limb anatomy, some of the anatomy of the head, and the anatomy of structures that may be palpated per rectum. The illustrations are presented in such a way that they could be of use to a surgeon cutting down onto a structure in the leg or in the region of the guttural pouch, but would be of very limited usefulness to anyone using an imaging modality to investigate a specific area, or embarking on thoracic drainage or abdominal surgery. There are also serious limitations to the usefulness as a reference text because of the difficulty of understanding, then following, the referencing system that is used to relate the clinical and functional aspects that are mentioned in Chapter 10 to the anatomical descriptions in the rest of the book. This difficulty could have some educational advantage for veterinary students (if the effort expended can be related to the likelihood that the information is retained), but is likely to prove tedious and frustrating for a practitioner. It is possible, for example, to follow the description for a specific diagnostic nerve block, but it takes some time and concentration to find the referenced information that is distributed through pages 8/9, and pages 108/109 for the forelimb. The cutaneous innervation is shown on page 6 for the forelimb and page 18 for the hind limb, and not related to the other references so the reader would have to already know where and how to test their nerve block's efficacy or where to find the information. Further, the reader is sent back to the forelimb information and not the hindlimb illustrations when seeking information concerning hind limb nerve blocks.

Overall the relatively cheap price and the clinical descriptions make this book of some potential use to veterinary students who could find the referencing difficulties of assistance in helping them to retain information. However it is likely to be too frustrating and too limited in detail to be of any use to practitioners. There are some much easier to use and more complete (but considerably more expensive) practical anatomy books available in English.

### HMS Davies

Dr Helen Davies is a lecturer in anatomy and researcher into equine biomechanics and functional anatomy in the Faculty of Veterinary Science, The University of Melbourne.