

Evaluation of an IgG Enzyme-Linked Immunosorbent Assay as a Serological Assay for Detection of *Mycoplasma bovis* Infection in Feedlot Cattle

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Mycoplasma bovis is a pathogen of emerging significance in cattle throughout the world that is causing a range of diseases, including mastitis, arthritis, and pneumonia. The limited availability and efficacy of current diagnostic and prophylactic tools for its control and its increasing antimicrobial resistance are contributing to its increasing importance in beef and dairy cattle. We have developed an indirect IgG enzyme-linked immunosorbent assay (ELISA) based on a recombinant fragment of the MilA protein and have shown its potential as an effective diagnostic tool. To more comprehensively estimate the diagnostic sensitivity and specificity of this IgG ELISA for detection of infection with *M. bovis* in cattle and to define a suitable cutoff for use in the field, we further assessed its performance in experimentally infected calves in a closed beef herd and by applying Bayesian latent class modeling to laboratory testing results from 7,448 cattle entering Australian feedlots. The most effective cutoff points were estimated to be 68.6 antibody units (AU) for experimentally infected calves and to be 58.7 AU for a closed adult herd. Under field conditions, in feedlot cattle the globally optimal cutoff was estimated to be 105 AU. At this cutoff, the diagnostic sensitivity was 94.3% (95% probability interval [PI], 89.9% to 99.6%) with a diagnostic specificity of 94.4% (95% PI, 90.3% to 99.6%). Applying this 105 AU cutoff, 13.1% of cattle were seropositive for infection with *M. bovis* on entry into feedlots, and 73.5% were seropositive when followed up approximately 6 weeks later suggesting a high risk of infection shortly after entry into feedlots.

Mycoplasma bovis is regarded to be an emerging cause of bovine respiratory disease (BRD) in veal calves, and infection can also cause polyarthritis and/or otitis media. In adult cattle, *M. bovis* is mainly associated with mastitis, keratoconjunctivitis, and reproductive tract disorders. Outbreaks of pneumonia induced by *M. bovis* have been reported throughout the world (1–3) and cause economic loss due to reduced weight gain and increased medication costs (4). The importance of *M. bovis* has been increasing because of increasing resistance to antimicrobial agents (5) and the lack of an effective vaccine (6). Transmission can occur through fomites, contact, or aerosol, and subclinically infected calves are believed to be carriers for life, so comingling of animals from different farms and age groups, combined with transport stress, results in outbreaks shortly after arrival in feedlots (7). Therefore, identification of infected source herds before introduction of animals to a naive group may help to prevent outbreaks (6).

The gold standard for diagnosis of *M. bovis* infection is culture of the organism, but this is time-consuming and laborious. A *M. bovis*-specific PCR assay has been developed (8), but such assays are unlikely to be useful for herd screening, as excretion is only detectable for a limited time after infection. Serological diagnosis can be sensitive and inexpensive if an appropriate target protein can be identified, but there are few commercially available *M. bovis* enzyme-linked immunosorbent assay (ELISA) kits and their performances have not been fully validated. We have developed an ELISA based on a recombinant fragment of MilA and assessed its sensitivity and specificity in experimentally infected calves (9).

In the studies reported here, we aimed to evaluate the performance of this *M. bovis* MilA IgG ELISA as a diagnostic assay in

young experimentally infected calves, beef cattle, and feedlot cattle and to assess its potential as a serological assay for detection of prior infection with *M. bovis* under field conditions.

MATERIALS AND METHODS

Cattle serum samples. (i) **Experimental study.** Serum samples were collected from ninety 1-month-old Friesian cross calves from a single farm in Victoria, Australia, in two similar experiments conducted in 2010 and 2011 that we have described previously (9). Briefly, experiment 1 contained 24 calves that were allocated into three groups. Group 1 contained 4 calves that were exposed to an aerosol of culture medium on day 0, group 2 contained 12 calves that were exposed to an aerosol of *M. bovis* strain 3683 on day 18, and group 3 contained 8 calves that were exposed to a temperature-sensitive experimental vaccine strain (vaccine A) of *M. bovis* on days 0 and 3 and were challenged together with calves from group 2 with *M. bovis* strain 3683 18 days after the initial vaccination. Each calf

Received 13 September 2015 Returned for modification 23 October 2015

Accepted 13 February 2016

Accepted manuscript posted online 24 February 2016

Citation Wawegama NK, Markham PF, Kanci A, Schibrowski M, Oswin S, Barnes TS, Firestone SM, Mahony TJ, Browning GF. 2016. Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for detection of *Mycoplasma bovis* infection in feedlot cattle. *J Clin Microbiol* 54:1269–1275.
doi:10.1128/JCM.02492-15.

Editor: B. W. Fenwick

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.02492-15>.

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received an estimated dose of $10^{5.2}$ color-changing units of *M. bovis* strain 3683, and the aerosol was performed as described previously (10). Serum samples were collected from all of the calves on days 0, 7, 14, 18, 28, 35, and 42, and all of the calves were euthanized and necropsied on day 42 (9). In experiment 2, sixty-six 1-month-old Friesian cross calves were allocated into three groups. Group 1 contained five calves that were exposed to an aerosol of mycoplasma culture medium, group 2 contained 30 calves that were exposed to an aerosol of *M. bovis* strain 3683, and group 3 contained 31 calves that were vaccinated and challenged as described in experiment 1. Aerosol infection and sampling was performed as described for Experiment 1.

All of the calves were tested for bovine viral diarrhoea virus (BVDV) by PCR (Gribbles Veterinary, Clayton, Victoria, Australia) to ensure that none were persistently infected with this virus, and they were also screened for *M. bovis* by quantitative PCR (qPCR) (11) prior to the commencement of the experiments and shown to be free of detectable infection with either of these pathogens. In each experiment, the three groups were separately housed and observed for clinical signs throughout the experiments. At necropsy, group 2 calves in the two experiments had various degrees of lobular pulmonary consolidation and those in group 3 had mild lobular lesions, but there were no lesions in the uninfected calves. Culture and qPCR of swabs from the trachea, bronchi, and lesions of the calves in groups 2 and 3 confirmed the presence of *M. bovis*, while culture and qPCR of the trachea and bronchi of the uninfected calves confirmed the absence of *M. bovis* from these animals (10).

The two experiments were conducted with the approval of the University of Melbourne Animal Ethics Committee, application numbers 0911327 and 1111970.

(ii) Extensively managed closed beef cattle herd. Serum samples were collected from 52 adult cattle in a beef herd in central Victoria, Australia. The herd had been tested annually for 3 years using serology and viral culture for bovine herpesvirus 1 and bovine viral diarrhoea virus, and the animals were found to be free of these pathogens and had had no history of respiratory disease over the last 3 years. No heifers had been introduced into the herd from other farms, so the herd was considered likely to be free of infection with *M. bovis*. Serum samples were collected with the approval of the CSL/Zoetis Animal Ethics Committee under application number 960-0.

(iii) Feedlot cattle. A prospective longitudinal study was conducted to evaluate possible risk factors for BRD in feedlot cattle (12). The study population consisted of 35,160 cattle that were inducted (the process of tagging, weighing, treating, and entering animal identifiers into the feedlot system) into 14 feedlots across Australia between March 2009 and December 2011. These cattle were managed in 170 cohorts (animals assembled together in a feedlot pen following induction). All animals were blood sampled at induction and approximately 42 days later (follow-up). The serum samples were aspirated and frozen at -20°C until analyzed. A nested case-control study was conducted within the cohort study in which 3,725 cattle were diagnosed with BRD between 7 and 35 days after their cohort had been assembled (i.e., no other animals were added) and had induction and follow-up blood samples collected with a sampling interval of 60 days or less. The controls were 3,725 cattle that had not been diagnosed with BRD or any other diseases by day 35 after their cohort had been assembled, were still with their cohort at that time, and had induction and follow-up blood samples collected with a sampling interval of 60 days or less. Cases were randomly selected from all cattle meeting the case criteria (4,442 animals, i.e., 15.8% of 28,082 animals eligible as either cases or controls), and controls were randomly selected from all cattle meeting the control criteria (23,640 animals, i.e., 84.2% of 28,082 animals eligible as either cases or controls) (13). Following completion of the case-control study, there was sufficient serum from one or both of the induction sample or follow-up sample from all except for 2 control animals, so samples from 7,448/7,450 animals were used in the current study. These cattle were managed in 161 cohorts across 14 feedlots. Thirteen days prior to induction, they were in 867 different groups (group 13s) at locations with

687 unique property identification codes (PICs), although some farm enterprises may be assigned multiple PICs.

Approval for research conducted in Queensland, South Australia and Western Australia was covered by the University of Queensland Animal Ethics approval certificates SVS/383/07/MLA, SVS/495/08/MLA, and SVS/125/10/MLA (NF). Research in New South Wales (NSW) was approved by the University of New England Animal Ethics Committee under AEC09/027.

IgG ELISA for antibodies against *M. bovis*. The *M. bovis* MilA IgG ELISA described previously (9) was used to test serum samples collected from all three sources. The concentration of antibody against *M. bovis* in each sample was calculated in empirical antibody units (AU) by comparison with a set of standards included on each ELISA plate using DeltaSoft 3 (Biometallic).

Assessment of commercial ELISA kits for diagnosis of *M. bovis* infection. Two commercially available ELISA kits, BIO K302 and BIO K260 from BioX Diagnostics (Belgium), were used to test a subset ($n = 70$) of serum samples from experiment 2 to compare the results obtained with these tests with those obtained with the *M. bovis* MilA IgG ELISA. Serum samples from group 1 (uninfected) and 2 on day 18 (the day of challenge) and day 42 (24 days after challenge) were tested, and the results were calculated according to the manufacturer's guidelines.

Data analysis. (i) Descriptive statistics. The data from the two experimental studies were combined, and descriptive statistics were calculated using GraphPad Prism 5 for the three treatment groups at each time point. Differences in antibody concentration between the groups on each day of sampling were compared using Mann-Whitney tests.

The cutoff value for the IgG ELISA was first empirically estimated using the experimental data by determining the 95th percentile point for the serum antibody concentrations of all of the uninfected calves in the two experiments by including one serum sample selected at random from each of the calves in group 1, one serum sample selected at random from between day 0 and day 18 from group 2, and the serum samples from day 0 from group 3 (90 samples in total). Based on this cutoff, the diagnostic sensitivity was estimated using day 42 results from groups 2 and 3 ($n = 81$), and the diagnostic specificity was estimated using a randomly selected sample point for each calf (group 1 calves at any time point, group 3 calves before vaccination, and group 2 calves before challenge [$n = 90$]). A cutoff value was similarly estimated (the 95th percentile point) using the serum samples from the extensively managed beef cattle herd, which was considered to be negative for *M. bovis*.

The results obtained with the commercial *M. bovis* ELISAs (BIO K302 and BIO K260) and the *M. bovis* MilA IgG ELISA with serum samples from calves in groups 1 and 2 on day 18 and day 42 from experiment 2 ($n = 70$) were compared, and, for each test, diagnostic test performance was estimated, with all animals at day 42 exposed to an aerosol of *M. bovis* strain 3683 considered to be truly positive ($n = 30$). All animals at day 18 and all group 1 animals at day 18 and day 42 were considered to be truly negative ($n = 40$).

(ii) Bayesian latent class modeling. The diagnostic specificity and sensitivity of the *M. bovis* MilA IgG ELISA were estimated, as well as the seroprevalence at induction to the feedlots and at follow-up, following a Bayesian latent class modeling approach for one test in eight populations in the absence of a gold standard (14, 15). The populations were defined on the basis of sampling point for *M. bovis* serology (induction versus follow-up) and the 14 feedlots (aggregated into the following 4 regional groups: NSW Riverina, 6 feedlots; mid-NSW, 1 feedlot; Darling Downs southern Queensland, 5 feedlots; South Australia and Western Australia, 2 feedlots). This approach makes no assumptions about the status of the animals, that is, the "true" statuses of these animals are incorporated into the model as latent variables. Prevalence was assumed to be different in each population, and diagnostic specificity and sensitivity were assumed to be constant over time. We applied noninformative beta prior distributions, with both shape parameters set to one, for the diagnostic specificity

TABLE 1 *M. bovis*-specific antibody concentrations in experimentally infected calves

Group no. and description	No. of calves	Median <i>M. bovis</i> -specific antibody concn in antibody units (25% and 75% quartiles) by no. of days after challenge (for groups 2 and 3) ^a			
		0	10	17	24
1. Uninfected	9	30 (13.7, 35) ^A	28 (13.6, 45) ^A	23.5(17.8, 34.7) ^A	18.5 (17, 29.5) ^A
2. <i>M. bovis</i> strain 3683	42	20.6 (13, 28.6) ^A	16 (10, 23) ^A	18.8 (13, 29) ^A	218.6 (108, 380.7) ^B
3. Vaccine A	39	18 (10, 31) ^A	24 (15, 53) ^A	78 (37, 295.5) ^B	284 (138.7, 880) ^B

^a Values marked with the same superscript letters are not significantly different ($P < 0.05$).

and sensitivity of the IgG ELISA and the seroprevalence at induction into the feedlot and at follow-up (for each feedlot group).

Bayesian inferences were based on the joint posterior distribution, numerically approximated using the open source JAGS software (version 9.3.1; <http://mcmc-jags.sourceforge.net/>), which was implemented in parallel on the Victorian Life Sciences Computational Initiative cluster with the R2jags package (version 0.5-7; <http://cran.ms.unimelb.edu.au/web/packages/R2jags/index.html>) in the R statistical package (version 3.2.0; The R Project for Statistical Computing, <https://www.r-project.org/>). A total of 210,000 model iterations were run, discarding the first 10,000 iterations as burn-in and thinning by 20 to minimize autocorrelation. Final inferences were presented as the 50%, 2.5%, and 97.5% quantiles of the marginal posterior distributions for each inferred parameter, corresponding to a posterior median point estimate and 95% probability interval (95% PI), respectively.

Iterative analyses were performed by applying different cutoff values for the *M. bovis* IgG ELISA to dichotomize the feedlot cattle serology results as test positive or test negative. This enabled estimation of the two-way receiver operator characteristic (ROC) curve and the globally optimal cutoff, which was considered in this case as the point on the ROC curve with the highest Youden index (i.e., the highest combined diagnostic sensitivity and specificity) (16).

Finally, true and apparent seroprevalence on entry and at follow-up were estimated by BRD status, applying the optimal cutoff (17), and then reweighting based on the sampling fractions of cases and controls for the fully enumerated target population. Confidence intervals (CI) were adjusted using variance inflation factors (VIFs) to account for clustering by immediate source (group 13s) on entry to feedlots and by cohort at follow-up (as described earlier). The VIFs were adjusted to test for heterogeneity of source populations, and, where appropriate, the most conservative VIFs were applied. One-way analysis of variance (ANOVA) was used to estimate between- and within-group variance and, thereby, intra-class correlation coefficients (ICC or ρ) (18). The heterogeneity of the prevalence of antibody against *M. bovis* across each of the immediate sources was assessed by inspecting histograms, and then mixture analyses were implemented with the CAMAN library in R (R package version 0.72; <http://cran.ms.unimelb.edu.au/web/packages/CAMAN/index.html>) to estimate the appropriate amount of variance inflation.

As many of the cattle studied were sampled twice (on entry and follow-

TABLE 2 Number of experimentally infected calves serologically positive for *M. bovis* with *M. bovis*-MilA IgG ELISA on each day of sampling

Group no. and description	No. of calves	No. calves positive on day (days after challenge for groups 2 and 3) ^a :						
		0	7	14	18	28 (10)	35 (17)	42 (24)
1. Uninfected	9	1	0	0	0	0	0	0
2. <i>M. bovis</i> strain 3683	42	3	0	0	0	0	4	35
3. Vaccine A	39	4	1	0	2	7	22	38

^a Days after exposure to an aerosol of *M. bovis* strain 3683 are shaded. Note that calves in group 3 were vaccinated on day 0.

up), a sensitivity analysis was performed to test for the influence of violation of the assumption of independence by repeating the latent class modeling and comparing estimates based on unpaired (independent) random samples of half of the available data at each time point.

RESULTS

Experimental study. Results obtained with calves in groups 1 and 2 in the two experiments have been described previously (9). The results obtained after inclusion of data from samples collected from group 3 are summarized in Table 1. The median concentration of antibody (in antibody units) in the serum samples from the calves in group 3 was significantly higher than that in the serum samples from the animals in groups 1 and 2 on day 35 (17 days after challenge), but by day 42 (24 days after challenge) the calves in group 2 had developed serum antibody concentrations similar to those of the calves in group 3. The 95th percentile point for serum antibody concentrations in uninfected calves from all three groups was 68.6 AU, so this was used as the cutoff for all subsequent analyses on serum samples from experimentally infected calves. At day 42 (24 days after challenge), 35 of 42 animals in group 2 and 38 of 39 in group 3 were seropositive for *M. bovis* infection in the *M. bovis* MilA IgG ELISA (Table 2).

Based on the known infection status of the experimental animals, the estimated diagnostic sensitivity of the *M. bovis* MilA IgG ELISA at day 42 (24 days after challenge) was 90% (95% CI, 82% to 95%) and the diagnostic specificity of the assay was 96% (95% CI, 89% to 98%).

A comparison of the results obtained with the two commercial ELISAs and the *M. bovis* MilA IgG ELISA is presented in Tables 3 and 4. The diagnostic sensitivity of the *M. bovis* MilA IgG ELISA was 87%, while that of the BIO K302 ELISA was 37% and that of the BIO K260 ELISA was 13% (Table 5). All tests had comparable and high diagnostic specificities. There was only slight agreement between the BIO K302 ELISA and the *M. bovis* MilA IgG ELISA (Cohen's kappa coefficient, 0.28 [95% CI, 0.04 to 0.52]).

TABLE 3 Results summary of *M. bovis* MilA IgG ELISA and commercially available ELISAs BIO K302 and BIO K260 (BioX Diagnostics, Belgium) in detecting *M. bovis* seropositive animals from experiment 2 on day 18 and day 42 for groups 1 and 2 only

Test result ($n = 70$)	No. <i>M. bovis</i> positive	No. <i>M. bovis</i> negative
IgG ELISA positive	26	4
IgG ELISA negative	4	36
BIO K260 positive	4	0
BIO K260 negative	26	40
BIO K302 positive	11	2
BIO K302 negative	19	38

TABLE 4 Comparison of the *M. bovis* MilA IgG ELISA results with the two commercially available ELISAs BIO K302 and BIO K260 (BioX Diagnostics, Belgium) in detecting *M. bovis* seropositive animals from experiment 2 on day 18 and day 42 for groups 1 and 2 only

IgG ELISA result (<i>n</i> = 70)	No. BIO K302 positive	No. BIO K302 negative	No. BIO K260 positive	No. BIO K260 negative
	Positive	10	20	4
Negative	3	37	0	40

Extensively managed beef cattle herd. The 95th percentile point for the serum samples from adult cattle in the extensively managed beef herd was 58.7 AU. Using this cutoff, all but two of the animals were seronegative for infection with *M. bovis*. The specificity of the ELISA in this beef herd, based on the assumption that it was free of *M. bovis*, was therefore 96% (95% CI, 87% to 100%).

Feedlot cattle. Of the 7,448 cattle that entered the feedlots, testing was completed on 7206 cattle at induction and 7289 at follow-up, with paired samples tested for 7062 cattle. The distributions of the antibody concentrations at the two time points are shown in Fig 1 by BRD status. Using these data and Bayesian latent class analysis, the optimal cutoff was estimated to be 105 AU (Fig 2). At this cutoff, the diagnostic sensitivity of the *M. bovis* IgG ELISA was 94.3% (95% probability interval, 89.9% to 99.6%) and the diagnostic specificity was 94.4% (95% PI, 90.3% to 99.6%). Applying the 105 AU cutoff, the apparent *M. bovis* seroprevalence on entry to the feedlot was 9.3% in animals that developed BRD between 7 and 35 days after their cohort was assembled compared to 13.8% among controls (13.1% overall in the target population of 28,082 animals that entered the feedlot and were deemed eligible for the case-control study), and at follow-up the apparent seroprevalence had risen to 85.8% in BRD cases and 71.1% in controls (Table 6). The Bayesian model predicted true seroprevalence at induction into the feedlot to have been 4.2% in BRD cases and 9.3% in controls (8.4% overall in the target population); these values increased to 90.5% in cases and 73.9% in controls at follow-up (76.5% overall). The marginal posterior probability distributions are presented as supplemental figures (see Fig. S1 in the supplemental material), including the noninformative priors to show the extent of prior learning.

There was considerable heterogeneity in *M. bovis* seroprevalence by immediate source (group 13s) at induction in the BRD cases and noncases (see Table 6; $VIF_{\text{hetero}} \gg VIF$). VIF_{hetero} is the VIF adjusted to account for heterogeneity by immediate source. Among BRD cases at follow-up, clustering effects by cohort alone were comparable to those seen after adjusting for heterogeneity by source group ($VIF_{\text{hetero}} \approx VIF$). In contrast, among noncases at follow-up, there was stronger clustering by cohort than after adjustment for heterogeneity by source group ($VIF_{\text{hetero}} \ll VIF$).

When considered by feedlot group, considerable heterogeneity was observed in seroprevalence on entry and at follow-up (see Table S1 in the supplemental material). The 12 feedlots in NSW and Queensland had comparable low estimates of true seroprevalence on entry (range of 4.8% to 6.1%), whereas the feedlots in South Australia and Western Australia had a relatively high seroprevalence on entry (34.5%). The true seroprevalence estimates at follow-up ranged from 62.1% for the single feedlot in mid-NSW

TABLE 5 Performances of the commercial ELISAs and the *M. bovis* MilA IgG ELISA

Parameter	% (95% CI) for:		
	BIO K302	BIO K260	IgG ELISA
Relative sensitivity	37 (22, 54)	13 (5, 30)	87 (70, 95)
Relative specificity	95 (83, 99)	100 (91, 100)	90 (77, 96)

to 95.7% for the group of five feedlots in the Darling Downs (Queensland).

Sensitivity analysis based on two randomly selected unpaired samples of the available data at each time point (*n* = 3,724 for each) yielded an optimal cutoff of 102 AU. At this cutoff, based on the abridged data set, there was little change in either the estimated diagnostic specifications (sensitivity, 95.2% [95% PI, 91.5% to 99.7%]; specificity, 94.6% [95% PI, 90.4% to 99.6%]) or the seroprevalence estimates for each of the feedlot groupings (see Table S2 in the supplemental material).

DISCUSSION

There have been several seroepidemiological studies of *M. bovis* infection conducted in feedlot or dairy cattle in North America and Europe (19–21), but this is the first study to our knowledge looking at the seroepidemiology of *M. bovis* in Australian feedlot cattle. There have been preliminary studies of *M. bovis* mastitis in Australian dairy cattle but no analysis of the prevalence of infection in beef cattle even though it is known to cause outbreaks of pneumonia in feedlot cattle (22, 23). The main reason for the lack of epidemiological studies is probably the lack of well-validated serodiagnostic assays. We have shown previously that our *M. bovis* MilA IgG ELISA is a suitable serodiagnostic assay for detecting seroconversion after infection with *M. bovis* in experimentally infected calves (9), but this study aimed to determine reliable cutoff points that can be used under field conditions and estimate test performance (diagnostic sensitivity and specificity) at these cutoffs. While there are two commercially available ELISAs for serological detection of infection with *M. bovis*, we have shown here, using a subset of serum samples from calves with known infection status, that our *M. bovis* MilA IgG ELISA has considerably greater sensitivity and comparable specificity to these two assays. For this reason, we did not use these commercial ELISAs for analyzing serum samples from field studies.

The cutoff estimated using serum samples from experimentally infected calves (68.6 AU) was similar to that estimated using serum samples from a closed beef herd (58.7 AU). A higher cutoff (105 AU) was estimated by modeling data from a large number of animals sampled under field conditions, and we consider this the most appropriate cutoff to apply globally across field conditions in relatively similar populations to those studied. Other cutoffs may be more appropriate in different contexts. Higher diagnostic sensitivity may be favored over specificity, and therefore, a lower cutoff may be more appropriate when screening, when attempting to rule-out a diagnosis, or when testing for extremely rare diseases (such as toward the end of an eradication program); however, when ruling in a diagnosis (confirmatory testing), a higher cutoff may be appropriate to ensure higher diagnostic specificity at the expense of sensitivity.

As our analysis estimated that the true seroprevalence was only 8.4% at induction, some of the source herds may have been either

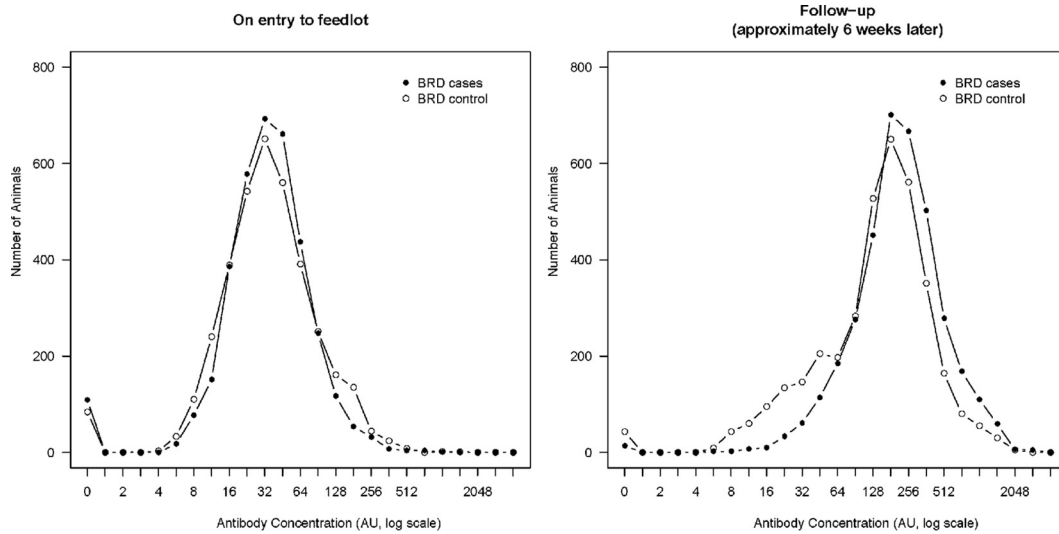


FIG 1 Distributions of anti-*M. bovis* IgG ELISA concentrations (in antibody units) for 7,206 cattle at induction to feedlots (A) and 7,289 cattle at follow-up approximately 6 weeks later (B) by bovine respiratory disease (BRD) case status.

free of *M. bovis* or have had very low animal-level prevalence of infection. The increase in estimated true seroprevalence to 76.5% within the approximately 6-week period to follow-up indicates a high rate of infection of naive animals, presumably as a result of transmission from animals already in the feedlot or from those animals infected prior to arrival. A previous European study reported that 55% of calves that were introduced into feedlot herds seroconverted in a *M. bovis* ELISA within 7 weeks and that 50% of

episodes of respiratory disease were attributable to *M. bovis* infection (24). Our results show that there is a similar increase in seropositivity in Australian feedlots over a similar period of time. That there would be a high rate of infection is not surprising, as there is no specific control for *M. bovis* infection in feedlots in Australia.

Heterogeneity of the seroprevalence among the immediate

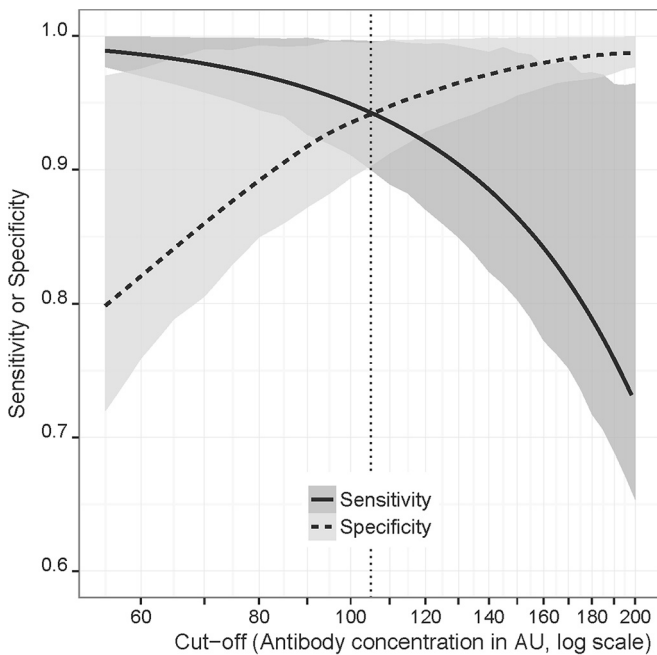


FIG 2 Diagnostic sensitivity and specificity of the *M. bovis* MilA IgG ELISA at different antibody concentration cutoffs (in antibody units) estimated using Bayesian latent class analysis for 7,206 cattle at induction to feedlots and 7,289 at follow-up. Shading represents 95% probability intervals, and the dotted reference line at 105 AU indicates the cutoff with the highest Youden index (best combined sensitivity and specificity).

TABLE 6 Seroprevalence estimates for feedlot cattle using the *M. bovis* IgG ELISA by bovine respiratory disease status^a

Cattle group	True seroprevalence (%) ^b	Apparent seroprevalence (%) ^b	ρ	VIF	VIF _{hetero}
On entry to feedlot (day 0)					
BRD cases (n = 3,577)	4.2 (1.3, 7.2)	9.3 (6.7, 12.0)	0.144	1.7	7.6
BRD control (n = 3,629)	9.3 (6.0, 12.5)	13.8 (10.9, 16.7)	0.252	2.1	6.5
At follow-up (~42 days later)					
BRD cases (n = 3,651)	90.5 (86.8, 94.1)	85.8 (82.6, 89.0)	0.162	5.7	8.0
BRD control (n = 3,638)	73.9 (68.4, 79.4)	71.1 (66.3, 76.0)	0.453	10.8	6.4
Overall (reweighted) estimates (n = 28,082; 15.8% BRD cases)					
On entry to feedlot	8.4 (5.2, 11.6)	13.1 (10.3, 15.9)			
At follow-up	76.5 (71.3, 81.7)	73.5 (69.9, 77.0)			

^a Estimated based on a cutoff of 105 antibody units (sensitivity, 94.3%; specificity, 94.4%). ρ , intraclass correlation coefficient; VIF, variance inflation factor estimated based on clustering by immediate source (group 13s) on entry to feedlots and by pen at follow-up; VIF_{hetero}, VIF adjusted to account for heterogeneity by immediate source.

^b Seroprevalence estimates in parentheses represent 95% confidence intervals adjusted by the most conservative VIF estimates.

source groups observed here on entry (and possibly residual effects among BRD cases at follow-up) suggests that the immediate source groups themselves fall into subpopulations. Some immediate source groups on entry may therefore have been derived from different populations with respect to *M. bovis* prevalence. This is reasonable to expect, noting that the study population has been sampled from pens within feedlots that themselves have been formed from multiple groups of animals (group 13s) that originated from herds in widespread locations around Australia. The increase in unadjusted VIF estimates at follow-up indicates the importance of clustering by cohort within the feedlot from the time of induction. Mixing of source groups (within cohorts postinduction) presumably led to a more homogeneous cluster seroprevalence distribution among noncases at follow-up. In cluster-based study designs, the variance inflation due to heterogeneity needs to be distinguished from the inflation due to clustering in general before proceeding to calculate confidence limits around prevalence estimates (18).

The heterogeneity that we detected in seroprevalence by feedlot group at the two time points indicates that there may be different epidemiological factors at play in the different regions. Of particular interest is the observation that the feedlots with the highest seroprevalence on entry were not those with the highest seroprevalence at follow-up. Further research is therefore warranted into risk factors associated with *M. bovis* infection, especially those that are modifiable.

As there are no vaccines of proven efficacy currently available to control *M. bovis*, alternative control measures are clearly needed. The ELISA described here may be used to identify infected source herds and thus reduce the risk of introduction of infected cattle into feedlots, as our studies show that this *M. bovis* MilA IgG ELISA is able to detect seroconversion after infection with *M. bovis* in calves as well as prior infection in adult cattle under field conditions.

It is impossible to estimate sensitivity and specificity with a single test without additional information when true disease status is unknown (25). Bayesian latent class models for data based on two or more tests, or two or more sampled populations, can include sufficient information to estimate all parameters, that is, be identifiable. Although the populations in this study were not truly independent, in that many of the same animals were sampled twice, in sensitivity analyses the estimates appeared highly robust to violation of this assumption.

The high sensitivity and specificity of this assay under field conditions suggests that it is likely to be useful for screening source herds to aid in the development of enhanced biosecurity measures for *M. bovis* in beef herds and also as a tool for epidemiological investigations to better estimate the impact of *M. bovis* on animal welfare and productivity and to identify risk factors for infection and disease, areas that have been identified as priorities for better understanding and for controlling this pathogen (6).

ACKNOWLEDGMENTS

We thank Liza Rosenbaum Nielsen (University of Copenhagen) and Associate Peter Mansell (Faculty of Veterinary and Agricultural Sciences, The University of Melbourne) for comments on the manuscript.

This study was supported in part by an Australian Research Council Linkage Project Grant and by Zoetis Australia Pty Ltd. and in part by grant B.FLT.0224 from Meat and Livestock Australia with matching funds provided by the Australian Government. The analyses were also supported by

Victorian Life Sciences Computation Initiative (VLSCI) grant VR0277 on its Peak Computing Facility at the University of Melbourne, an initiative of the Victorian Government, Australia.

FUNDING INFORMATION

This work, including the efforts of Anna Kanci, Sally Oswin, Timothy John Mahony, Glenn F. Browning, Nadeeka Kumari Wawegama, and Philip Francis Markham, was funded by Zoetis Australia Pty. Ltd. This work, including the efforts of Nadeeka Kumari Wawegama, Philip Francis Markham, Anna Kanci, and Glenn F. Browning, was funded by Australian Research Council (LP0989620). This work, including the efforts of Meghan Schibrowski, Tamsin S. Barnes, and Timothy John Mahony, was funded by Meat and Livestock Australia (B.FLT.0224).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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