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Validation of an indirect immunofluorescence assay (IFA) for the detection of IgG antibodies against *Coxiella burnetii* in bovine serum

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ABSTRACT

There is limited knowledge of the true prevalence and distribution of coxiellosis in dairy and beef cattle populations in Australia. For this to occur, apparent prevalence estimates need to be reliably adjusted, accounting for diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of the test used. However, there are few tests available with known diagnostic specifications suitable to inform screening and surveillance activities in the Australian context. We initially modified and optimised a human indirect immunofluorescence assay (IFA) test for the detection of IgG antibodies against phase I and/or phase II *Coxiella burnetii* in bovine sera and determined an optimal screening dilution cut-off to be 1:160. Direct comparison of the modified IFA with the commercial IDEXX enzyme-linked immunosorbent assay (ELISA) kit (Q Fever Ab Test IDEXX Laboratories, United States of America) was performed by testing 458 serum samples from four distinct cattle populations across the east coast of Australia and New Zealand. Cross classified test results were then analysed using Bayesian latent class modelling, to validate the tests in the absence of a gold standard reference test. Results from this analysis indicate that the IFA, at a 1:160 serum dilution, has an estimated DSe of 73.6% (95% Credible Interval (CrI) 61.1, 85.9) and DSp of 98.2% (95% CrI 95.1, 99.7). The commercial IDEXX ELISA kit was found to have a higher DSe of 87.9% (95% CrI 73.9, 96.4) and similar DSp of 97.7% (95% CrI 93.2, 99.7). Evaluation of the diagnostic performance of the IFA and ELISA methods, specifically for use in cattle will enable more accurate interpretation of prevalence estimates of *C. burnetii* exposure to be reported for cattle in Australia and other countries.

1. Introduction

Coxiella burnetii is a zoonotic bacterium able to infect multiple species, including but not limited to livestock and humans. It is the causative agent of Q fever in humans and coxiellosis in animals (Woldehiwet, 2004). Domesticated ruminants, including cattle, sheep and goats are considered to be important sources of human Q fever (Maurin and Raoult, 1999). Infection with *C. burnetii* may cause reproductive disease in ruminants and has been associated with bovine abortion, premature delivery and birth of weak neonates in dairy cattle in Europe (Agerholm, 2013; Hansen et al., 2011). Subclinical infection in cattle may persist for weeks to months, with bacterial shedding possible through multiple secretions including placental fluids, vaginal mucus, milk and faeces (Guatteo et al., 2011). Estimations of the true prevalence and distribution of coxiellosis in dairy and beef cattle populations in Australia are limited due to minimal surveillance and minimal standardisation of diagnostic test methods.

The *C. burnetii* bacterium exhibits two phase variations, phase I and

Abbreviations: IFA, indirect immunofluorescence assay; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; DSe, diagnostic sensitivity; DSp, diagnostic specificity; CrI, credible interval; CI, confidence interval

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phase II, with both being used as antigens for serological assays. Antibodies against *C. burnetii* can be detected in bovine serum and milk and serology is considered a reliable diagnostic method for herd-level investigations (OIE, 2018; Roest et al., 2013). Identification of IgG antibodies against *C. burnetii* provides evidence of past exposure and/or recent infection (Natale et al., 2012). In human studies, detection of elevated levels of phase I and phase II antibodies are used to differentiate between the acute and chronic or persistent forms of Q fever disease (Eldin et al., 2016; Tozer et al., 2011). The detection of phase-specific antibody patterns could have potential to identify ruminants that are chronically shedding bacteria (Lucchese et al., 2015). There is also preliminary evidence that a phase I IgG seroconversion may indicate protective immunity in goats following previous exposure to *C. burnetii* (Canevari et al., 2018; Muleme et al., 2017). However, a clear association between *C. burnetii* phase-specific antibody response and clinical disease in cattle has not been well established.

The validation of affordable and reliable serological tests may encourage surveillance and epidemiological studies of bovine coxiellosis in Australia. The most commonly used serological methods are the complement fixation test (CFT), indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) (Brom Van den et al., 2015; Natale et al., 2012; Porter et al., 2011). The only commercially available serological test for use in cattle in Australia is a multi-species ruminant ELISA kit (Q Fever Ab Test IDEXX Laboratories, United States of America). This kit can be prohibitively expensive for large-scale cattle screening and there are no current validation data available from the manufacturer for the test's performance in bovines. While the CFT has been standardised and validated for use in veterinary laboratories, it is consistently reported to have very low sensitivity (ranging from 29.8%–36.7%), is laborious to perform and prone to non-specific reactions resulting in inconclusive test results (Emery et al., 2012; Horigan et al., 2011; Kittelberger et al., 2009; Muleme et al., 2016; OIE, 2018). Currently, there is no gold standard for sero-diagnosis of coxiellosis in livestock.

The IFA is the reference test for human Q fever diagnostics in Australia and was found to have greater sensitivity than the CFT and provide a more cost-effective option to monitor antibody dynamics of patients (Worswick and Marmion, 1985). While there are no commercially available IFA kits for use in ruminants, Muleme et al. (2016) recently validated an IFA for detecting IgG and IgM antibodies against *C. burnetii* in goat serum. They estimated the diagnostic sensitivity (DSe) of the IFA, at a 1:160 serum dilution cut-off, to be 94.8% (Credible Interval (CrI) 80.3–99.6); this was higher than the DSe of the ELISA (70.1%; CrI 52.7–91.0) and CFT (29.8%; CrI 17.0–44.8). The diagnostic specificity (DSp) for detecting IgG was found to be similar across all three serological tests (92.5%, 96.2%, 96.8%, respectively). Therefore, in goats, the IFA appeared to have superior sensitivity compared to both the IDEXX ELISA kit and CFT test methods (Muleme et al., 2016).

The objectives of this study were to modify, optimise and validate an IFA for the detection of phase-specific IgG antibodies against *C. burnetii* in cattle serum for the purpose of estimating prevalence of exposure in epidemiological investigations. Additionally, a commercially available ELISA was evaluated for application in seroprevalence studies in Australian cattle.

2. Materials and methods

Initially, we modified and optimised a human IFA test to detect IgG antibodies against *C. burnetii* in bovine serum. Then, a direct comparison of the modified IFA with the commercial ELISA kit (Q Fever Ab Test IDEXX Laboratories, United States of America) was accomplished by testing serum samples from distinct cattle populations across Australia and New Zealand. Finally, we used Bayesian latent class analysis to estimate the diagnostic test accuracy (DSe and DSp) of the serological tests in the absence of a gold standard (OIE, 2016a).

2.1. Serum samples

2.1.1. Sample collection, storage and animal ethics

Serum samples tested during this validation study were originally collected as a part of either university research projects (The University of Queensland Animal Ethics approval SVS/115/11/MLA (NF) and The University of Sydney Animal Ethics approval 593), or by government department staff for regulatory testing that did not require animal ethics approval (Victorian Government Department of Economic Development, Jobs, Transport and Resources and the New Zealand Ministry of Primary Industries). The use of all samples for the current study was approved by The University of Queensland animal ethics ANRFA/SVS/100/16. Samples were stored frozen at -20 °C or − 80 °C depending on the intended length of storage.

2.1.2. Control sera for IFA optimisation

Positive bovine control sera (freeze-dried) for the IFA were sourced from the Australian National Quality Assurance Program (ANQAP; AgriBio, DEDJTR, Bundoora, Victoria). The negative control sera were obtained from New Zealand, which is considered by the World Organisation for Animal Health (OIE) to be free of *C. burnetii* (OIE, 2018). The Animal Health Laboratory, Wallaceville, New Zealand Ministry of Primary Industries had tested these serum samples using the ELISA (Q Fever Ab Test, IDEXX) to confirm negative status, which we subsequently repeated.

Ten bovine sera were sourced from the Australian Rickettsial Reference Laboratory (ARRL), Geelong, Victoria, to be used as a proficiency panel to compare inter-laboratory IFA test results. These sera were from individual cattle tested by the ARRL using both IFA and ELISA methods.

2.1.3. Samples for the diagnostic test comparison of IFA and ELISA

A total of 458 bovine sera were used to compare the IFA and the ELISA test methods. Serum samples were sourced from four distinct populations. The first population was represented by serum samples (n = 156) collected from a suspected *C. burnetii*-infected dairy-cattle herd in New South Wales, Australia. At the time of sample collection, this herd recorded *C. burnetii* positive individual cow composite milk samples using PCR (*C. burnetii* DNA target *IS1111a*) with 34 out of 155 (21.9%) samples confirmed positive for *C. burnetii* on sequencing (K. Bosward, unpublished data).

The second population was represented by samples (n = 159) previously collected for the purpose of infectious disease testing within a longitudinal epidemiological study investigating causes of poor reproductive performance in beef-cattle (McGowan et al., 2014). Systematic sampling methods were used to collect blood samples from cattle within the three properties enrolled in central Queensland, Australia. Central Queensland is a rural region of Queensland with endemic human Q fever. Although these herds did not have *C. burnetii* PCR testing performed, beef-cattle from this region have previously been reported to have a seroprevalence of approximately 16% (Cooper, 2011).

The third population were samples from a dairy-cattle herd (n = 96) with no history of suspected infection in Victoria, Australia. Although there have been limited published studies, cattle from Victoria have reported a low prevalence (0.5%) from previous and recent serological surveys (Hore and Kovesdy, 1972; Tan, 2018). The fourth population were the negative-reference sera (n = 47) from New Zealand.

2.2. Serological test methods

2.2.1. Development of the IFA slides

Menzel-Glaser 40-well microscope slides (Tasman Scientific, Belgrave Heights, Victoria, Australia) were cleaned with 100% methanol. Slides were then air dried and labelled accordingly. Phase I and phase II *C. burnetii,* Nine Mile strain, antigen were reconstituted as per

manufacturer's instructions with 1 mL of distilled water (Virion/Serion, 97076 Würzburg, Germany). Briefly, reconstituted antigen was diluted with 0.5% chicken yolk sac into three different working stock solutions (Tozer et al., 2011), phase I, phase II and a combined-phase (both phase I and phase II) at the same concentrations in solution. Antigen solutions were spotted onto the wells of separate slides and allowed to air dry. Slides were fixed by immersion in 100% methanol for 5–10 min, then air dried. Slides were stored at −20 °C for long term storage. Anti-bovine IgG polyclonal (whole molecule) fluorescein isothiocyanate (FITC) antibody, raised in rabbit (Sigma- Aldrich, St Louis, MO 63103 USA, product number: F7887) was used to detect IgG antibody-antigen complexes.

Each batch of slides were quality controlled using the IFA protocol outlined below including positive and negative control sera. The batch was considered to have passed quality control if the positive control test sera fluoresced to +/- one titre of its known titre and the negative control showed minimal or no background fluorescence.

2.2.2. Brief overview of IFA method

The IFA method for detecting human phase specific antibodies against *C. burnetii* (Tozer et al., 2011) was modified and optimised for testing bovine serum. Briefly, IFA slides, test sera and reagents were brought to room temperature. Test sera were diluted in 2% casein-phosphate-buffered saline to minimise non-specific binding (Muleme et al., 2016). Diluted test sera were individually placed on the slides in duplicate and incubated in a humidity chamber for a 30 min at 37 °C. If *C. burnetii* antibodies were present in the test serum, they would adhere to the antigen coated to the slide during this initial incubation. The slides were washed in 10% phosphate-buffered saline (PBS) for 5 min, three times and allowed to air dry. Anti-bovine IgG-FITC conjugate diluted in 0.05% Evans blue dye, was added and again incubated in a humidity chamber for 30 min at 37 °C. If IgG antibody-antigen complexes were present, the FITC conjugate would couple with the immunoglobulin complex during the second incubation period. The slides were washed three times removing any excess unbound reagents in 10% PBS for 5 min each time. The slides were air dried and coverslips mounted. Examination of the slides was performed with an immunofluorescent microscope (Nikon Eclipse E600) under a 40x lens (total 400x magnification), with oil immersion under a 100x lens (total 1000x magnification) used for closer inspection. If the test serum contained IgG antibodies against *C. burnetii*, the immune complex would produce an apple green fluorescence of the individual bacteria adhered to the slide, signifying a positive result.

Checkerboard dilutions were used to determine optimum cut-off points for both serum and conjugate dilution. A two-fold serial dilution of 1:40 to 1:2560 was used with negative and positive control sera in duplicate with a range of IgG FITC-conjugate dilutions from 1:300 to 1:600. All negative control sera (n = 47) were then individually titrated from 1:40 to 1:2560; the lowest dilution that produced no background fluorescence was considered as the lowest possible cut-off (the sera screening dilution).

2.2.3. Screening of sera for the diagnostic test comparison

Individual sera from all four populations (n = 458) were screened in duplicate at 1:80 dilution with 2% casein buffer loaded onto slides coated with combined phase I and II antigens. Samples fluorescing at this initial screening dilution on combined-phase slides, were then re-screened and titrated at doubling dilutions from 1:80 to 1:1280 on individual slides prepared with separate phase I and phase II antigens respectively.

2.2.4. Brief overview of ELISA method

The IDEXX ELISA Q Fever Ab Tests were performed as per the manufacturer's guidelines (IDEXX, 2017). All reagents and control sera for the ELISA were provided as a part of the commercial kit. Briefly, micro-well plates coated with combined phase I and phase II *C. burnetii* antigen were provided in the commercial kit. Test sera and control sera were diluted at 1:400 and loaded into the wells in duplicate. After incubation and washing steps, a peroxidase labelled anti-ruminant IgG conjugate was applied to the wells. After further incubation and washing steps, tetramethylbenzidine (TMB) substrate was applied to the wells allowing the development of colour. The amount of *C. burnetii* IgG antibodies present in the serum sample was directly proportional to the final colour outcome. Test results were achieved by reading the ELISA plate through a microplate spectrometer (SpectraMax 340PC384 Microplate Reader) at 450 nm. The optical density (OD) of the test and control samples were compared using the formula provided to determine the sample to positive (S/P) ratio expressed as a percentage:

$$\frac{S}{P}\ \%= 100 \times \frac{OD\ sample - OD\ negative\ control}{OD\ positive\ control - OD\ negative\ control}$$

Cut-off for S/P% values were taken from the kit insert as follows: negative result S/P% < 30%; suspect results 30% ≥ S/P% < 40%; positive result S/P% ≥ 40%.

2.2.5. Analytical performance of the IFA

The proficiency panel of 10 bovine sera were screened using the IFA method at doubling dilutions beginning at 1:40 to 1:5120. End-point titres were determined for IgG against both phase I and phase II *C. burnetii* antigen individually. Screening test results from two independent readers were recorded and compared to those from the reference laboratory to determine the reliability of interpretation of IFA method and hence to investigate subjectivity between operators.

2.2.6. Comparison of phase variation on IFA slides on a subset of samples

An additional comparison was performed with a subset of samples (the New South Wales dairy herd; n = 146). Test sera were screened, in duplicate, on slides coated with combined-phase antigen (a mixed preparation of phase I and II antigen) and also in duplicate, on slides individually coated with phase I and phase II antigen separately. Sera results obtained for the IFA slide preparation of combined-phase antigen were compared with sera results from IFA slide preparations of phase I and phase II separately. A positive to either phase I or phase II or both phases was considered positive for the separate slide preparations. These cross-classified test results were then analysed.

2.3. Statistical analysis

Cohen's kappa test statistic (κ) and prevalence and bias adjusted kappa (PABAK) test statistic were calculated using STATA/SE 15.0® (Stata Statistical Software, Stata Corporation, College Station, TX, USA) and WinPepi (Abramson, 2011) to determine the agreement beyond chance of paired test results (Byrt et al., 1993). Interpretation of kappa statistics were as follows: > 0.8 excellent agreement; > 0.6 - 0.8 substantial agreement; > 0.4 - 0.6 moderate agreement; > 0.2 - 0.4 slight agreement; 0 - 0.2 poor agreement (Thrusfield, 2007).

Bayesian latent class modelling was used to evaluate the performances of the IFA and the ELISA for the detection of IgG antibodies against *C. burnetii* in bovine serum in the absence of a gold standard reference test (Branscum et al., 2005). This analysis assumes the exposure status of each sample as unknown and hence "latent"; the model estimates the probability that each of the four possible test combinations (T1+ T2+ ; T1+ T2- ; T1- T2+ and T1- T2-) represents a true positive sample, thus allowing inference on the diagnostic accuracy of both tests to be made (Branscum et al., 2005). Correlation between the two tests was assumed given both were serological assays designed to detect antibody responses targeting similar antigens of *C. burnetii*. The conditional dependence model for two tests over two populations, as described by Branscum et al. (2005) was adapted to account for four populations. DSe and DSp of the two tests were assumed constant across the four populations; prevalence of *C. burnetii* exposure was assumed distinct in the four populations.

Table 1
Table of prior distributions used in the Bayesian latent class model.

	Test specifications	Detailed prior specifications			
	Mode	5th Percentile		Parameters for prior distribution	Reference
ELISA DSe	0.813	0.54		Beta (8.97, 2.833)	(Horigan et al., 2011)
ELISA DSp	0.874	0.55		Beta (7.252, 1.901)	(Horigan et al., 2011)
IFA DSe	0.948	0.8		Beta (4.793, 1.208)	(Muleme et al., 2016)
IFA DSp	0.925	0.7		Beta (5.053, 1.329)	(Muleme et al., 2016)
	Population prevalence				
	Mode	95th Percentile	99th Percentile	Parameters for prior distribution	Reference
NSW	0.32	0.6		Beta (3.773, 6.892)	(Kittelberger et al., 2009)
QLD	0.2	0.5		Beta (2.637, 7.548)	(Cooper et al., 2011)
VIC	0.01	0.2		Beta (1.136, 14.521)	(Hore and Kovesdy, 1972)
NZ[a]	0.00		0.001	Beta (1,500)	(OIE, 2018)
	0.00		0.01	Beta (1, 458.211)	

Key: IFA, indirect immunofluorescence assay; ELISA, Enzyme-linked immunosorbent assay; NSW, New South Wales; QLD, Queensland; VIC, Victoria; NZ, New Zealand; DSe, diagnostic sensitivity; DSp, diagnostic specificity; [a] A mixture distribution with two priors was used to allow for the possibility of zero infection prevalence. The first prior modelled the probability of the population being infected (akin to a zero-inflation term), and the second prior modelled the prevalence if the population was infected.

Priors for DSe and DSp of the IFA and ELISA were incorporated into the model using independent and informative unimodal beta distributions based on published literature. Unimodal beta distributions were estimated using the "epi.betabuster" function implemented within the "epiR" library (Stevenson, 2017) in R (R Core Team, 2017) as shown in Table 1. The IFA was estimated to have a DSe with mode of 94.8% and DSp with a distribution centred on 92.5% using data published from goats in Australia (Muleme et al., 2016). The ELISA was estimated to have DSe with mode of 81.3% and DSp with mode of 87.4%, as published by Horigan et al. (Horigan et al., 2011). Prior information about the assumed prevalence estimates of *C. burnetii* exposure in the four cattle populations were also taken from published literature available at the time or knowledge of infection status of the herd (Table 1). Diffuse priors centred on the point estimates from published sources were included in the model. These priors were made sufficiently diffuse so that the data could overwhelmingly inform the posterior distributions. The New Zealand population was assumed free of infection; hence, a mixture distribution derived from a Bernoulli distribution and Beta prior distribution was used to account for the high probability that the population was not infected at all (Branscum et al., 2005).

Bayesian inferences were based on the joint posterior distribution, approximated using the computer software JAGS (version 4.3.0; Plummer, 2003), implemented with R2jags package (Yu-Sung and Masanao, 2015) in R. The model was run with two independently initiated chains, each of 120000 iterations, discarding the first 20000, then thinning by 10 to reduce autocorrelation (based on assessment of convergence using the *Gelman-Rubin* test statistic and autocorrelation; Gelman and Rubin, 1992; Plummer et al., 2006). Diagnostic sensitivity and specificity of the two tests, true prevalence of the four populations, and Youden's index were selected for monitoring. The Markov Chain Monte Carlo (MCMC) accuracy was assessed by visual inspection of the autocorrelation function and estimation of the effective sample size (Kruschke, 2015).

Final estimates were reported as the median and 95% Credible Interval (CrI) of the joint posterior distribution. Modelling was repeated assuming different dilution cut-offs (1:80, 1:160, 1:320, 1:640, 1:1280) for the IFA. This allowed estimation of a two-way receiver operator characteristic (ROC) curve, which interpreted with Youden's index allowed identification of the IFA cut-off with the highest combined DSe and DSp (i.e. the globally optimal cut-off). To test the influence of the priors on the final model outputs, less-informed priors were used to perform sensitivity analysis (Appendix A, Table S1.1) (Kostoulas et al., 2017).

3. Results

3.1. Optimisation of the IFA

The optimal dilution of anti-bovine IgG FITC conjugate was found to be 1:600 with 0.05% Evans blue counterstain. At serum dilutions $\geq$ 1:160 with 2% casein buffer, no fluorescence was observed from the 47 known-negative samples. Twenty six of the 47 (57%) negative samples did show fluorescence at the 1:40 serum dilution. At the 1:80 dilution, 12 (26%) of these samples showed fluorescence against phase I, and 17 (36%) against phase II. A serum dilution of 1:160 was therefore considered optimal, this eliminated false positive results due to background fluorescence.

3.2. Analytical test performance of the IFA

Titration results of the proficiency panel sera for phase I and phase II IgG against *C. burnetii* are presented in Table 2. Sample titrations for all 10 sera were in agreement with the reference laboratory within +/- one titre. When considered dichotomised using the 1:160 dilution cut-off to categorise samples as positive or negative, results were in complete agreement.

In total, 477 individual IFA wells were read by two independent

Table 2
Indirect immunofluorescence assay (IFA) end point titre results for IgG antibodies against *C. burnetii* for a proficiency panel of 10 bovine sera across two laboratories.

Proficiency panel	IFA Titre		IFA Titre	
	Phase I IgG		Phase II IgG	
Sample number	Internal laboratory	Reference laboratory	Internal laboratory	Reference laboratory
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	*40	*0	*40	*0
5	*640	*320	160	160
7	*640	*320	*2560	*1280
10	640	640	2560	2560
8	1280	1280	1280	1280
6	1280	1280	2560	2560
9	2560	2560	> 5120	> 5120

Key: * Indicates were a difference of $\pm$ one titre was identified between laboratories.

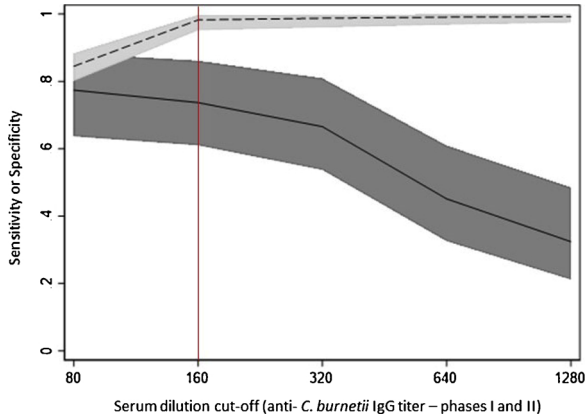

Fig. 1. Estimates from the Bayesian latent class model for the diagnostic sensitivity and diagnostic specificity of the IFA at different cut-off titres of 1:80, 1:160, 1:320, 1:640, 1:1280. Solid black line represents diagnostic sensitivity, dashed line represents diagnostic specificity and shaded areas represent 95% credible intervals. Red reference line at 160 indicates the cut-off with the highest Youden's index (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

readers within the one laboratory to assess the subjectivity of operator readings. Overall observed agreement between the two independent readers was 97.9% (κ = 0.91; 95% CI: 0.83, 1.00), representing almost perfect agreement beyond chance.

3.3. Comparison of phase variation combination on IFA slides for screening sera

Samples from 146 cattle were screened in duplicate on slides coated with combined phase I and II antigen, and in duplicate on slides coated separately with individual phase I and phase II antigens. Screening sera results obtained from both groups of slides were compared. Analysis showed excellent overall agreement with 95.2%; agreement beyond chance represented by κ = 0.85 (95% CI: 0.74, 0.96) and PABAK = 0.90 (95% CI: 0.88, 0.98). It was therefore concluded that the combined antigen coated slides were highly comparable with separate phase antigen prepared slides to detect anti-bovine *C. burnetii* IgG.

3.4. Comparison of the IFA and ELISA

Youden's index was highest for the IFA at the 1:160 dilution (Fig. 1), confirming this to be the optimal global cut-off dilution for a positive serum sample. A comparison of test results attained with the developed IFA and the commercial ELISA across four cattle populations, are presented in Table 3.

At a cut-off of 1:160, the Bayesian estimates of the DSe of the IFA (73.6%; 95% CrI 61.1, 85.9) was lower than the ELISA (87.9%; 95% CrI 73.9, 96.4) in detecting IgG phase I and/or phase II antibodies to *C. burnetii* in bovine serum. The DSp of the IFA and ELISA were both very high, 98.2% (95% CrI 95.1, 99.7) and 97.7% (95% CrI 93.2, 99.7), respectively. Plots contrasting the prior and posterior distributions for DSe and DSp of the ELISA and IFA tests are provided in Fig. S1 (Appendix A). Table 4 shows a summary of the median and 95% credible interval posterior estimates of test performance variables and the estimated true prevalence within the cattle populations. Posterior estimates of the correlation coefficient (ρ) of test outcomes for positive samples was ρ+ = 0.287 (95% CrI 0.008, 0.701) and for negative samples was ρ- = -0.013 (95% CrI -0.199, 0.353). Model diagnostics were all satisfactory. Sensitivity analysis demonstrated that the prior estimate of the DSe of the IFA had the most influence on the model. When the model was re-run using less informed priors (Appendix A, Table S2), the posterior estimate for the DSe of the IFA was reduced from 73.6% to 66.5% (Appendix A, Table S3).

4. Discussion

In this study, an IFA, which is the reference diagnostic test for human Q fever serology, was modified and evaluated for the detection of IgG antibodies against *C. burnetii* in cattle sera alongside an existing commercially available ELISA. Cut-off values for the IFA were determined during the test optimisation process and the DSe and DSp at this optimal cut-off value was then estimated. This study reports the diagnostic test performance of the IFA and a commercial (IDEXX) ELISA, for the detection of *C. burnetii* IgG antibodies in cattle sera. Knowledge of diagnostic test performance enables correct interpretation of serological test results by allowing them to be adjusted appropriately to account for diagnostic test imperfections.

While the OIE (World Organisation for Animal Health) Q fever guidelines acknowledge the ELISA and IFA as both suitable tests for herd level prevalence studies, many laboratories continue to use the CFT despite consistent reports in the literature of low DSe in both cattle and goats (ranging from 29.8% to 36.7%) (Horigan et al., 2011; Kittelberger et al., 2009; Muleme et al., 2016; OIE, 2018). There is no lack of analytical specifications of test performance and standardised serological methods for *C. burnetii* serology in ruminants. Although, prior to this study, there were few diagnostic specifications that could be validly utilised to reliably adjust apparent prevalence estimates or inform screening and surveillance activities in cattle in the Australian context.

The IFA sera dilution cut-off point (1:160) determined using positive and negative controls was in agreement with a recent study that validated an IFA for use in goats (Muleme et al., 2016). Diagnostic characterisation of the assay at multiple cut-off points indicated the highest Youden's index at the same cut-off, indicating this to have the highest combined DSe and DSp. These results suggest that the IFA, modified to detect bovine IgG antibodies against *C. burnetii*, may not be reliable for serum dilutions below 1:160. At this screening cut-off, it

Table 3
Cross classified test results from the IFA (1:160 cut-off) and ELISA (IDEXX) across four cattle populations.

	Cattle populations							
	Population 1 (n = 156)		Population 2 (n = 159)		Population 3 (n = 96)		Population 4 (n = 47)	
	ELISA +	ELISA -	ELISA +	ELISA -	ELISA +	ELISA -	ELISA +	ELISA -
IFA +	31	2	16	7	7	0	0	0
IFA -	16	107	6	130	2	87	0	47

Key: IFA, indirect immunofluorescence assay; ELISA, Enzyme-linked immunosorbent assay; +, test positive; -, test negative; Population 1, sera samples from New South Wales dairy cattle; Population 2, sera samples from Queensland beef cattle; Population 3, sera samples from Victorian dairy cattle; Population 4, negative control sera from New Zealand.

Table 4
Bayesian estimates of the diagnostic sensitivity and specificity for the IFA and ELISA and estimated true prevalence for the three cattle populations for the detection of IgG antibodies against *C. burnetii* in bovine serum.

Test	Sensitivity (95% CrI)	Specificity (95% CrI)	True prevalence (%)		
			Population 1 (95% CrI)	Population 2 (95% CrI)	Population 3 (95% CrI)
IFA*	0.736 (0.611, 0.859)	0.982 (0.951, 0.997)	30.3 (22.0, 39.3)	15.8 (9.4, 23.4)	7.5 (2.2, 14.0)
ELISA (IDEXX)	0.879 (0.739, 0.964)	0.977 (0.932, 0.997)			

Key: IFA, indirect immunofluorescence assay; ELISA, enzyme-linked immunosorbent assay; CrI, Credible Interval; Population 1, represent sera samples from a New South Wales dairy herd; Population 2, represent sera samples from > 1 beef cattle herds in Queensland and insufficient data was available for multi-level estimation of herd- and animal-level true prevalence; Population 3, represent sera samples from a Victorian dairy herd; * IFA results taken using the 1:160 dilution cut-off; point estimates are the posterior medians from the model output.

was determined that the estimated DSe of the IFA was lower than the ELISA in detecting IgG against phase I and/or phase II *C. burnetii* in bovine serum; however, DSp was comparable between the tests.

At the time of this study, no published literature was available reporting the DSe and DSp of an IFA for the detection of anti- *C. burnetii* immunoglobulins specifically in cattle. There are publications that report crude seroprevalence results in cattle using IFA methods, however the DSe and DSp of the tests were not evaluated or reported (Lyoo et al., 2017; Vaidya et al., 2010). In fact, in one of the studies the IFA is assumed to have 100% DSe and 100% DSp and is used as a reference test to compare other diagnostic methods (ELISA and PCR) (Vaidya et al., 2010). Such an approach introduces bias and leads in inaccurate interpretations of test parameters and prevalence estimates.

Several publications have performed test comparisons using different ELISA kits; results for test DSe and DSp were similar to findings from this study. In Denmark, Bayesian latent class analysis was used to analyse the diagnostic performance of the IDEXX ELISA kit for use in bovine milk and blood samples; the ELISA, based on serum samples, was reported to have a DSe of 84.0% and DSp 99.0% (Paul et al., 2013). Horigan et al. (2011) also investigated the diagnostic accuracy of three ELISA tests for use in cattle serum, comparing multiple populations with the ELISAs and a CFT. They report the DSe and DSp of an unspecified commercial ELISA to be 81.3% and 87.4%, respectively (Horigan et al., 2011). In another study, the DSes of two ELISAs for cattle sero-diagnosis were estimated to be significantly higher than CFT; ELISA-1 97%, ELISA-2 97% and CFT 34%; although the specificity of the CFT was superior to both ELISAs (Lucchese et al., 2016).

Rousset et al. (2007) published similar results in goats; with an ELISA and an IFA having overall good agreement for detecting *C. burnetii* antibodies in goat sera, however the ELISA was able to detect more positives than the IFA. Unfortunately, that study only looked at crude comparisons of the two tests and did not attempt to estimate the test performance of either assay against a gold standard or by using alternative methods such as latent class analysis.

Bayesian latent class methods were used in this study to validate the modified IFA and the commercial ELISA, by estimating the DSe and DSp of the tests. In the absence of a gold standard reference test, results from multiple tests can be used to evaluate test performances (Toft et al., 2005; Tu et al., 1999). This method of test validation has been applied increasingly in the last decade in veterinary diagnostics and is recommended by the OIE when insufficient reference samples are available that are representative of the population where the test is intended to be used (Horigan et al., 2011; OIE, 2018; Paul et al., 2014). Latent class analysis was similarly used to estimate the DSes and DSps of two ELISA tests and a CFT for the sero-diagnosis of coxiellosis in cattle, confirming other reports that ELISAs have much higher DSes than the CFT (Lucchese et al., 2016).

Prior estimates of IFA and ELISA test sensitivity and specificity were based on the best available published literature. After performing sensitivity analyses with less informed priors, it became apparent that the posterior DSe estimate for the IFA was most influenced by the high prior IFA DSe estimate. It seems plausible that the IFA test specifications for use in goats may not be applicable for cattle. This may be important when considering serological tests for use in ruminants; species-specific test validation is not always reported. As recommended in the standard reporting guidelines for Bayesian latent class models (STARD-BLCM), the final model estimates were derived from the models using informed priors that were defined prior to the analysis (Kostoulas et al., 2017). The results from the sensitivity analysis are included in Appendix A Table S3.

The model outputs of true prevalence estimates presented is this study represent crude test results adjusted for the estimated DSes and DSps of the tests. However, insufficient data were available for multi-level estimation of herd- and animal-level true prevalence in populations that had more than one herd. It should be noted that due to the non-representative structure of sampling from some of the study populations, the external validity of the true prevalence estimates is limited. Therefore, the true prevalence estimates presented here are not intended to be used for inference of regional or state-wide prevalence.

An additional limitation of the current study is that the IFA only detects IgG and needs to be modified and validated to detect IgM antibodies, as previously undertaken in validation for goats (Muleme et al., 2016), therefore current or very recent infections may be underreported. A further study, to validate the IFA for detection of anti- *C. burnetii* IgM in cattle sera, is warranted.

While these results found the IFA to be less sensitive than the ELISA, it may have other benefits that make this serological method appealing for research or surveillance. Firstly, when testing large numbers of cattle sera, a validated in-house IFA such as described here, has lower consumable costs than the commercial IDEXX ELISA kit. Crude budget calculations at the time of the study were performed and it was estimated that the consumable costs for testing one serum sample (in duplicate) with this ELISA was A$8.30 (Australian dollars) compared to A$0.70 per sample with the in-house IFA test. However, the IFA method is more labour intensive and does not currently have an automated reading method. It has been demonstrated in this study, that screening sera on IFA slides coated with a combined preparation of phase I and phase II antigens can accurately detect bovine IgG antibodies, further reducing costs. If positive at screening, samples can then be tested on separate, individual antigen coated slides to determine phase-specific antibody titres.

For this Bayesian latent class analysis, the DSe and DSp of the IFA was estimated using combined test results from separate phase I and phase II IFA slides. At present, the IDEXX ELISA cannot distinguish between different phase-specific antibody responses as it is pre-coated with a combined antigen preparation of phase I and phase II. The separated phase I and phase II IFA may therefore be a useful tool for investigating phase-specific serological patterns in relation to disease

status in cattle with the potential to identify chronically shedding animals (Lucchese et al., 2015). Phase specific serological patterns have long been used to aid in the diagnosis and interpretation of different stages of infection in humans (Eldin et al., 2016).

As per the OIE (World Organisation for Animal Health) guidelines, this IFA is suitable for the purpose of estimating prevalence of *C. burnetii* exposure in cattle, for further analysis to identify risk factors and possibly as a confirmatory test (OIE, 2016b). This IFA may not be appropriate for testing cattle herds for freedom of disease, as the sensitivity may be sufficiently low to result in unacceptable risk of false negatives (Thrusfield, 2007). Overall, having knowledge of the estimated test performance of each serological method enables accurate interpretation of test results, which reduces bias and misleading conclusions to be drawn from either under or over estimation of infection.

Conflict of interests

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2019.104698.

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