

Bayesian Validation of the Indirect Immunofluorescence Assay and Its Superiority to the Enzyme-Linked Immunosorbent Assay and the Complement Fixation Test for Detecting Antibodies against *Coxiella burnetii* in Goat Serum

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Although many studies have reported the indirect immunofluorescence assay (IFA) to be more sensitive in detection of antibodies to *Coxiella burnetii* than the complement fixation test (CFT), the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of the assay have not been previously established for use in ruminants. This study aimed to validate the IFA by describing the optimization, selection of cutoff titers, repeatability, and reliability as well as the DSe and DSp of the assay. Bayesian latent class analysis was used to estimate diagnostic specifications in comparison with the CFT and the enzyme-linked immunosorbent assay (ELISA). The optimal cutoff dilution for screening for IgG and IgM antibodies in goat serum using the IFA was estimated to be 1:160. The IFA had good repeatability (>96.9% for IgG, >78.0% for IgM), and there was almost perfect agreement (Cohen's kappa > 0.80 for IgG) between the readings reported by two technicians for samples tested for IgG antibodies. The IFA had a higher DSe (94.8%; 95% confidence interval [CI], 80.3, 99.6) for the detection of IgG antibodies against *C. burnetii* than the ELISA (70.1%; 95% CI, 52.7, 91.0) and the CFT (29.8%; 95% CI, 17.0, 44.8). All three tests were highly specific for goat IgG antibodies. The IFA also had a higher DSe (88.8%; 95% CI, 58.2, 99.5) for detection of IgM antibodies than the ELISA (71.7%; 95% CI, 46.3, 92.8). These results underscore the better suitability of the IFA than of the CFT and ELISA for detection of IgG and IgM antibodies in goat serum and possibly in serum from other ruminants.

Coxiella burnetii causes Q fever in humans as well as abortions, stillbirths, and infertility in ruminants (1–5). The organism replicates in the placenta of infected ruminants, reaching a level of up to 10⁹ bacteria per gram of placenta tissue (4–6). *C. burnetii* organisms are shed in an extremely high concentration in birth fluids, placental tissues, and membranes of aborted fetuses as well as in milk, urine, and feces of infected ruminant animals around the parturition period (4, 5). The high concentration of *C. burnetii* organisms shed in tissues, fluids, and excreta of infected ruminants is the primary source of human infections (7).

Caprine and ovine infections have been reported to result in severe placentitis and consequently in shedding of higher numbers of *C. burnetii* organisms than infections of cattle (8). Studies have also revealed that goats and sheep shed higher quantities of *C. burnetii* in feces, vaginal mucus, and birth tissues than other livestock (9). Thus, the risk of human transmission is higher when infections occur in herds of small ruminants than when they occur with other livestock. Unsurprisingly, the majority of reported large outbreaks of Q fever have been associated with infected sheep and goat flocks, including a major outbreak of more than 4,000 human Q fever cases in the Netherlands that was linked to sheep and goat farms with over 50 animals (9–14). Infection with *C. burnetii* can be asymptomatic in many animals and may be detected in ruminants only when infection causes abortions and reproductive abnormalities in pregnant animals (1). Delay in diagnosis in livestock slows the implementation of appropriate control strategies, thus increasing the risk of human infection.

Coxiellosis in animals can be diagnosed through microscopic

examination of stained tissues, culture, detection of *C. burnetii* DNA using PCR, and detection of antibodies to *C. burnetii* in blood and milk (15, 16). The microscopic diagnosis of coxiellosis is mainly undertaken on placental tissues using Stamp-Macchiavello coloration or Giemsa stain. The organism can be cultured in cells, embryonated hen eggs, or cell-free media (15, 17). However, microscopy and culture are expensive and require biosafety level 3 facilities. Furthermore, microscopic examination of stained tissues for *C. burnetii* detection is reported to have poor specificity because *C. burnetii* can be confused with other organisms such as *Chlamydia* and *Brucella* (17). The culturing of *C. burnetii* is slow and has been reported to be unsuccessful using samples from

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some individuals despite their being positive by PCR, serology, and microscopy, suggesting that culturing is an unreliable method for *C. burnetii* detection (18).

PCR methods of detecting *C. burnetii* DNA are widely considered to be highly sensitive (19). However, the relatively short period of time during which ruminants shed *C. burnetii* in feces, milk, vaginal mucus, and urine (5, 9) limits the suitability of PCR for the detection of *C. burnetii* infection. For example, goats experimentally infected with *C. burnetii* were reported to shed the bacterium for 14 days in vaginal swabs, 52 days in milk, and 20 days in feces (5). Another study in naturally infected dairy cattle generated reports of scenarios where shedding occurred by one route and not the others, with only 6.4% of the infected animals shedding the organism by all the three shedding routes (vaginal mucus, feces, and milk) (15). Therefore, PCR detection of *C. burnetii* from milk, feces, and vaginal swabs should be attempted only within a short period before and after parturition and should be used alongside other diagnostic methods.

Antibodies to *C. burnetii* in ruminants and humans have been reported to remain in circulation for long periods, thus making serological diagnosis a reliable method of detecting exposure to the organism. Antibody titers in vaccinated dairy cattle were reported to remain four times as high as titers in unvaccinated cattle for at least 20 months (20). Furthermore, antibodies detected following acute Q fever in human patients in the Netherlands were reported to persist for at least a year after the initial diagnosis (20, 21). IgM and IgG antibodies to phase 1 and phase 2 antigens of *C. burnetii* are used to interpret the course of *C. burnetii* infection in animals and humans (22–24). Recent infections can be identified by detection of IgM phase 2 antibodies, which appear early in the course of the disease. Persisting or chronic infections can be identified by detection of IgG antibodies, thus making serology very useful in detection of *C. burnetii* infection and epidemiological investigations (23, 24). Limitations of serology for the diagnosis of coxiellosis include an estimated delay of 2 to 3 weeks between exposure and seroconversion, whereas *C. burnetii* DNA can be detected in peripheral blood cells within days of exposure, with results for seronegative animals being detectable by PCR on blood samples (22, 25). Early detection of *C. burnetii* DNA before seroconversion has not been reported in ruminants, however. In experimental infections with *C. burnetii* in goats, the earliest PCR-positive blood samples were obtained 28 days after exposure (26). Paired samples collected 4 weeks apart should be obtained to ensure that seronegative animals are diagnosed appropriately (27, 28). The occurrence of seronegative animals shedding *C. burnetii* beyond the 4-week period during which seroconversion might be expected to occur could be due to lack of sensitivity of the serological tests rather than to a true absence of antibodies in infected animals (9, 29). Indeed, some of these studies have used a mixed-antigen enzyme-linked immunosorbent assay (ELISA) that has been reported to lack sensitivity for IgG phase 2 antibodies (30). Further studies are therefore necessary to investigate the occurrence of seronegative animals shedding *C. burnetii* in excreta.

The World Organisation for Animal Health (OIE) recommends the complement fixation test (CFT) for serological diagnosis of coxiellosis in animals (31) despite this assay being widely reported to have very low diagnostic sensitivity (DSe) (32) and to have nonspecific reactions on some samples leading to uninterpretable results. The indirect immunofluorescence assay (IFA) is the human reference test (27, 33) and has been reported to have a

diagnostic sensitivity of between 98% and 100% and a diagnostic specificity (DSp) of 95%. The ELISA is reported to have a similarly high specificity but a lower sensitivity than the IFA in diagnosing human Q fever using serum samples (33, 34).

A number of studies have reported that IFA and ELISA are more sensitive than CFT for diagnosis of coxiellosis in ruminants (32, 35–37). As yet, estimates of DSe and DSp have not been published for the IFA for use in ruminants. The OIE guidelines for the validation of diagnostic tests (38) require a clear description of the optimization process and setting of cutoff values as well as establishing the analytical and diagnostic performance of the assay for any given diagnostic purpose. Bayesian latent class analysis has been reported to provide reliable estimates of DSe and DSp in situations where the reference test (the “gold standard”) is imperfect, as is the case with diagnosis of coxiellosis, where the reference test (CFT) is known to have poor DSe (32, 39). In this study, we aimed to validate the indirect immunofluorescence assay for detection of antibodies to *C. burnetii* in goat serum in infected herds, as well as for declaring freedom of disease in herds of unknown infection status. The specific objectives included using Bayesian latent class analysis to estimate the DSe and DSp of the IFA in detecting antibodies against *C. burnetii* in goat serum.

MATERIALS AND METHODS

Development of an IFA for detection of antibodies against *C. burnetii* in goat serum. Microscope slides were coated with phase 1 (Henzerling strain from the Australian Q vax vaccine) and phase 2 (Nine Mile) *C. burnetii* antigens grown in Vero cells as previously described (40). Fluorescein-labeled anti-goat IgG and anti-sheep IgM (KPL, USA) were used to detect IgG and IgM antibody-antigen complexes, respectively, as described previously (41). All samples and conjugates were diluted in 2% casein–phosphate-buffered saline (PBS) to limit nonspecific binding. All samples and controls were tested in duplicate on every slide.

Briefly, serum samples made to a starting dilution of 1:40 and 2-fold serial dilutions of 1:40 were incubated with antigen in duplicate for a period of 40 min at 37°C before unbound serum was removed by washing with 10% PBS. Secondary conjugated antibodies were applied for a period of 40 min at 37°C, and then unbound antibodies were removed by washing with 10% PBS. The slides were observed using UV light microscopy at ×400 magnification. Seropositive samples were identified by the presence of fluorescence, while negative samples produced no fluorescence.

We obtained 12 goat serum samples from New Zealand (NZ [a country declared *C. burnetii*-free by the OIE]) for negative controls and used 2 goat serum samples from a known *C. burnetii*-positive farm that we had pretested and found to have positive CFT titers (16 and 32) for positive controls. All the negative-control sera also tested negative using CFT (Serion Virion) and ELISA (IDEXX Q fever antibody ELISA kit).

To establish an initial dilution cutoff value for goat serum, we tested 2-fold serial dilutions of 1:5 to 1:160 of all the negative-control sera with conjugate at a 1:50 dilution. The 1:50 conjugate dilution was established using checkerboard dilutions of the conjugates within the recommended manufacturer’s range of 1:10 to 1:100. The lowest dilutions of conjugate and negative-control sera that did not produce fluorescence were chosen as the initial cutoff dilutions for a true positive sample.

Serum samples were then collected from 84 randomly selected goats in a 250-goat herd in Victoria, Australia. (All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Melbourne [University of Melbourne Animal Ethics Committee approval number 1413118].) The herd had previously tested positive for *C. burnetii* antibodies in serum using CFT testing and had also tested positive for *C. burnetii* DNA in air samples, vaginal swabs, and placenta samples using

PCR (42). There were also 22 laboratory-confirmed human cases of acute Q fever associated with the farm (42). Two-fold serial dilutions of the serum samples, from 1:40 to 1:40,960, were prepared and tested in duplicate using the IFA to determine the endpoint titer above which no antibodies to *C. burnetii* were detected in each of the samples. A starting dilution of 1:40 was previously published as optimal for detecting *C. burnetii* antibodies in goat serum using IFA (32).

Analytical performance of the developed IFA for detection of antibodies against *C. burnetii* in goat serum. To assess the reliability of interoperator readings, each of the wells of the 84 serum samples from the randomly selected goats and of the 14 control serum samples was read by two technicians (43) and the level of agreement beyond that expected due to chance effects alone was estimated using Cohen's kappa test statistic (K) (44). In total, 1,280 wells were read in duplicate (364 for the phase 1 IgG test, 364 for phase 2 IgG, 276 for phase 1 IgM, and 276 for phase 2 IgM). To assess the robustness and repeatability of the test, 32 of the 84 serum samples from the randomly selected goats were retested after 3 months of storage at 4°C and K values were estimated for the paired samples. Kappa values were interpreted according to the Landis and Koch descriptors, with $K \leq 0$ considered to represent poor agreement, $0 < K \leq 0.20$ slight agreement, $0.20 < K \leq 0.40$ fair agreement, $0.40 < K \leq 0.60$ moderate agreement, $0.60 < K \leq 0.80$ substantial agreement, and $0.80 < K < 1.00$ almost perfect agreement (45).

Comparison of the diagnostic performances of the IFA, CFT, and ELISA methods for detecting antibodies against *C. burnetii* in goat serum. The 12 negative-control sera and the 84 field samples from the infected herd were tested using the CFT and a commercially available ELISA (IDEXX CHEKIT). The CFT was used as it is configured to detect IgG and IgM antibodies to phase 2 *C. burnetii*. This testing was performed at the Victorian State Government veterinary diagnostic laboratory (AgriBio, Department of Economic Development, Jobs, Transport and Resources [DEDJTR]).

A IDEXX CHEKIT ELISA kit was used to detect IgG antibodies to phase 1 and phase 2 *C. burnetii* in all serum samples according to the manufacturer's instructions. The IDEXX ELISA kit was modified to detect IgM antibodies by replacing the anti-ruminant IgG conjugate with mouse anti-sheep IgM monoclonal antibody (clone 25.69; isotype IgG1 from AbD Serotec) and peroxidase-conjugated sheep anti-mouse IgG, as previously described (36). The plates were blocked with 10 mg/ml bovine serum albumin (BSA)–PBS. All sera and conjugated antibodies were diluted in 5 mg/ml BSA–PBS–0.05% Tween 20. The anti-sheep IgM was used at the optimum dilution of 1:600 (see Table S1 in the supplemental material). The anti-mouse IgG was used at the optimum dilution of 1:3,000 (see Table S1), while control and test serum samples were used at a dilution of 1:400. To determine the cutoff value for the IgM ELISA, the negative-control NZ sera were tested at the optimum serum and conjugate dilutions and their absorbance was measured at 450 nm. The mean optical density (OD) values and standard deviations (SD) of the results from the known negative samples were calculated. Corrected OD (COD) values were then calculated using the following formula:

$$\text{COD} = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})}{(\text{OD}_{\text{positive}} - \text{OD}_{\text{blank}})} \quad (1)$$

where $\text{OD}_{\text{sample}}$ represents the mean OD of two ELISA plate wells containing the same sample, OD_{blank} represents the mean OD of the two ELISA plate wells containing only the diluent (5 mg/ml BSA–PBS), and $\text{OD}_{\text{positive}}$ represents the mean OD of the two ELISA plate wells containing diluted positive controls.

Cutoff values for the ELISA were calculated using COD mean and SD values obtained from the negative-control sera. The 84 samples from the *C. burnetii*-positive farm samples and the 12 negative-control samples were all tested by the modified ELISA (modELISA) to detect total IgM antibodies (IgM antibodies to both phase 1 and phase 2 *C. burnetii*).

Statistical analysis. Bayesian latent class models were constructed to estimate the cutoff titer that maximized diagnostic sensitivity and speci-

ficity, as assessed using Youden's index ($Y = \text{DSe} + \text{DSp} - 1$), following the OIE-recommended approach (38, 46, 47). Separate models were constructed to compare pairs of tests, assuming both tests in each pair were conditionally dependent (i.e., based on similar biological phenomena) and that neither was a gold standard. Comparisons were made only between the different combinations of antigen and immunoglobulin classes that the CFT and ELISA are designed to detect: IgG and IgM to phase 2 only for the CFT, IgG only for phases 1 and 2 for the ELISA, and IgM only for phases 1 and 2 for the modELISA. This approach makes no assumptions about the infection status of tested animals. Indeed, the model is constructed to estimate four latent probabilities (that samples testing doubly positive [+ +], positive and negative [+ -], negative and positive [- +], or doubly negative [- -] on the two tests are truly positive) to enable inference of the diagnostic specifications of both tests without perfect knowledge. A two-population model was implemented with the assumption of different true animal-level prevalences for the 12 known *C. burnetii*-negative NZ samples and the 84 samples from the infected herd.

Prior information about the diagnostic specificity and sensitivity of each assay was modeled using independent and informative unimodal beta distributions based on published diagnostic sensitivities of 93.1%, 93.1%, 85.7%, and 20.6% for the IFA, IDEXX ELISA, modELISA, and CFT, respectively, and diagnostic specificities of 91.2%, 91.2%, 97.6%, and 97.3% for the IFA, IDEXX ELISA, modELISA, and CFT, respectively (see Table S2 in the supplemental material for detailed prior specifications) (32–35, 48).

Diagnostic specificity and sensitivity of the IFA were specified as diffuse prior distributions, following the method of Branscum et al. (46), to represent a lack of knowledge of the test's specifications. Dependence parameters were specified as “uninformed” independent uniform distributions, and Bayesian inferences were based on the joint posterior distribution, numerically approximated using the program WinBUGS (49), implemented with R2WinBUGS package (50) in the R statistical package (51), running 110,000 model iterations, discarding the first 10,000 iterations as burn-in, and thinning by 10 to minimize autocorrelation. Parameters for beta prior distributions were estimated using the epiR library (52). K , prevalence and bias adjusted kappa (PABAK), and the proportions of positive and negative agreement for each comparison were directly calculated as model outputs among the samples in the group known to be negative and the samples in the group from the infected herd. Final inferences were presented as the 50%, 2.5%, and 97.5% quantiles of the marginal posterior distributions for each of the parameters, corresponding to a posterior median point estimate and 95% confidence interval (95% CI), respectively. Analyses were repeated, applying different cutoff titers for dichotomizing the IFA results as test-positive results, which enabled estimation of the two-way receiver operating characteristic (ROC) curve and globally optimal cutoff value (as assessed using Youden's index). A sensitivity analysis was performed to test for the influence of the priors on the final results, inputting vague (“flat”) priors with wider confidence intervals and comparing all model outputs.

RESULTS

Analytical performance of the IFA for detection of antibodies against *C. burnetii* in goat serum. No fluorescence was observed using any of the negative-control samples with the IFA conjugates (IgG and IgM) and antigens (phase 1 and phase 2) at a 1:160 serum dilution (Table 1). The overall observed level of agreement between the two experienced technicians' readings was 94.4% (95% CI, 93.0, 95.5), and overall agreement beyond chance between the readings of the two technicians was $K = 0.88$ (95% CI, 0.83, 0.94).

Test-specific observed levels of agreement between readings of two technicians are reported in Table 2. The repeatability of the IFA was 100% (95% CI, 89.3, 100) for IgG phase 1, 96.9% (95% CI, 84.3, 99.4) for IgG phase 2, and 78.1% (95% CI, 61.2, 89.0) for both IgM phase 2 and IgM phase 1 (Fig. 1). The K values corre-

TABLE 1 Assay optimization to establish the serum dilution without nonspecific binding to anti-goat IgG and anti-sheep IgM conjugates when 12 known negative goat sera were tested for antibodies against phase 1 and 2 *C. burnetii* antigen using the indirect immunofluorescence assay^a

Sample ID or parameter	IFA result at indicated sample dilution or no. of samples											
	Phase 2 antigens						Phase 1 antigens					
	5	10	20	40	80	160	5	10	20	40	80	160
Anti-goat IgG conjugate												
370	±	–	–	–	–	–	–	–	–	–	–	–
371	±	±	–	–	–	–	–	–	–	–	–	–
372	±	±	±	–	–	–	–	–	–	–	–	–
373	±	±	±	–	–	–	–	–	–	–	–	–
374	±	±	–	–	–	–	–	–	–	–	–	–
375	–	–	–	–	–	–	–	–	–	–	–	–
924	±	±	±	–	–	–	–	–	–	–	–	–
925	–	–	–	–	–	–	–	–	–	–	–	–
927	±	±	±	±	±	–	–	–	–	–	–	–
928	–	–	–	–	–	–	–	–	–	–	–	–
930	±	±	–	–	–	–	–	–	–	–	–	–
936	±	±	±	±	±	–	–	–	–	–	–	–
No. of samples with ± result	9	8	5	2	2	0	0	0	0	0	0	0
Anti-sheep IgM conjugate												
370	–	–	–	–	–	–	±	±	±	±	–	–
371	±	±	±	±	–	–	±	±	±	±	–	–
372	±	±	±	–	–	–	±	–	–	–	–	–
373	±	±	±	–	–	–	–	–	–	–	–	–
374	±	±	–	–	–	–	±	±	±	±	–	–
375	±	±	±	–	–	–	±	±	±	±	±	–
924	±	±	±	–	–	–	–	–	–	–	–	–
925	±	±	±	±	–	–	–	–	–	–	–	–
927	±	±	±	±	–	–	±	±	–	–	–	–
928	±	±	±	±	–	–	±	±	±	±	–	–
930	±	±	±	–	–	–	–	–	–	–	–	–
936	±	±	±	–	–	–	±	±	±	±	±	–
No. of samples with ± result	11	11	10	4	0	0	8	7	6	6	3	0

^a –, negative; ±, nonspecific binding; ID, identification; IFA, indirect immunofluorescence assay.

sponding to the levels of agreement between the two tests were 0.93 (95% CI, 0.58, 1.00) and 1.00 (95% CI, 0.65, 1.00) for IgG phase 2 and IgG phase 1 and were 0.57 (95% CI, 0.26, 0.89) and 0.58 (95% CI, 0.27, 0.89) for IgM phase 2 and IgM phase 1, respectively.

Comparison of the diagnostic performances of the IFA and IDEXX ELISA methods for detecting IgG antibodies against *C. burnetii* in goat serum. Comparisons of the CFT and the ELISA to the IFA are described in Table 3. Only one sample was positive on ELISA but negative on both IFA and CFT, and two other samples were inconclusive on ELISA but positive on IFA and CFT (Table 3). All of the negative-control samples from New Zealand (declared *C. burnetii*-free by the OIE) tested negative on all three tests.

Compared to the IDEXX ELISA, the IFA had the greater specificity and sensitivity (Youden's index) for detecting both IgG

phase 2 and 1 antibodies in goat sera at a cutoff of 1:160 total antibody titer (Fig. 2). The observed agreement between the IFA and the ELISA at the 1:160 cutoff titer was 81.1% (95% CI, 72.7, 88.0); the proportion of agreement beyond chance was 0.60 (95% CI, 0.43, 0.74). The sensitivity of the IFA (94.8%; 95% CI, 80.3, 99.6) was greater than that of the ELISA (70.1%; 95% CI, 52.7, 91.0) in detecting IgG phase 1 and phase 2 *C. burnetii* antibodies in goat serum. The IFA and IDEXX ELISA had comparable diagnostic specificity results (Table 4). Bayesian estimates of the specificity and sensitivity for the IFA and IDEXX ELISA, and prior probability distributions, are presented in Table 4.

Comparison of the diagnostic performances of the IFA and modelELISA for detecting IgM antibodies against *C. burnetii*. The mean COD + 2 SD of the negative-control sera tested with the modelELISA was 0.448, while the mean COD + 3 SD of the nega-

TABLE 2 Level of agreement between readings of results of the indirect immunofluorescence assay against phase 1 and 2 *C. burnetii* antigens in goat serum reported by two technicians^a

Test	% positive agreement (n)	% negative agreement (n)	% observed agreement (95% CI)	K (95% CI)
IgG phase 2	86.2 (145)	98.2 (219)	93.4 (90.4, 95.5)	0.86 (0.76, 0.96)
IgG phase 1	95.2 (165)	99.0 (199)	97.3 (95.0, 98.5)	0.94 (0.84, 1.00)
IgM phase 2	75.9 (83)	96.9 (193)	90.6 (86.6, 93.5)	0.77 (0.65, 0.88)
IgM phase 1	98.3 (61)	94.8 (215)	95.7 (92.6, 97.5)	0.88 (0.76, 1.00)

^a CI, confidence interval; K, Cohen's kappa.

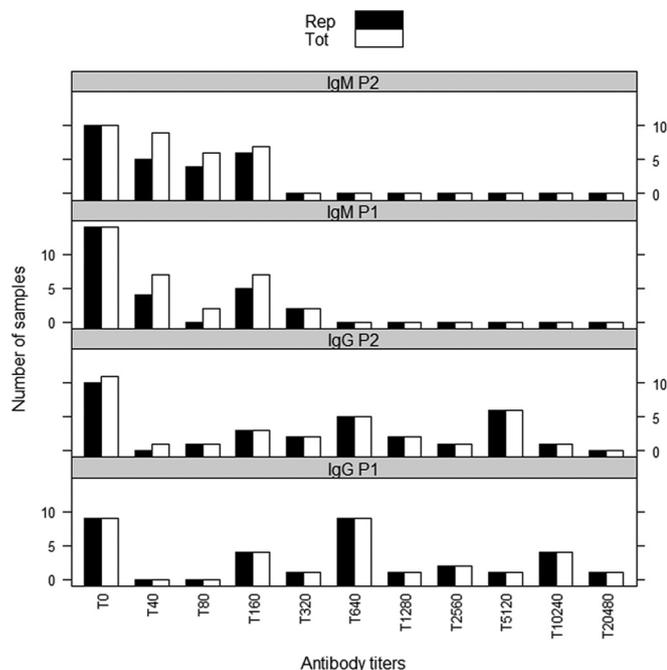


FIG 1 The number of samples at different antibody titers that produced the same results when tested with the indirect immunofluorescence assay, 3 months later. The black bars (Rep) show the number of samples with same results, while the white bars (Tot) represent the total number of samples retested. T, antibody titer.

tive-control sera was 0.530. Therefore, with the modelISA samples that had a COD of <0.448 were interpreted as representing negative results, while test samples that had a COD of ≥ 0.530 were interpreted as positive. Test samples that had a COD of at least 0.448 but less than 0.530 were interpreted as representing inconclusive results. The Bland-Altman test of agreement performed on all the negative-control sera and the three positive-control sera showed no statistically significant difference between duplicate modelISA results on the same sample [$P(t) = 0.813$].

Compared to the modelISA results, the highest specificity and sensitivity (Youden's index) of the IFA in detecting both IgM phase 2 and 1 antibodies in goat sera were obtained at a cutoff titer of 1:160 (Fig. 3). The IFA had a higher diagnostic sensitivity (88.8%; 95% CI, 58.2, 99.5) for IgM (phase 1 and phase 2) antibodies than the modelISA (71.7%; 95% CI, 46.3, 92.8) at the 1:160 cutoff titer. Bayesian estimates of the specificity and sensitivity for the IFA and modelISA, and prior probability distribu-

TABLE 3 Comparison of the indirect immunofluorescence assay, CFT (Serion Virion), and ELISA (IDEXX) in detecting IgG antibodies to *C. burnetii* in 96 goat serum samples at the optimum cutoff of 1:160 for the IFA as estimated with Bayesian latent class analysis^a

Sample category	No. of samples			
	ELISA +	ELISA -	CFT +	CFT -
IFA +	22	14	12	24
IFA -	1	59	0	60
ELISA +			10	13
ELISA -			2	71

^a +, positive; -, negative; IFA, indirect immunofluorescence assay. All samples from New Zealand (declared *C. burnetii*-free by the OIE) tested negative in all three tests.

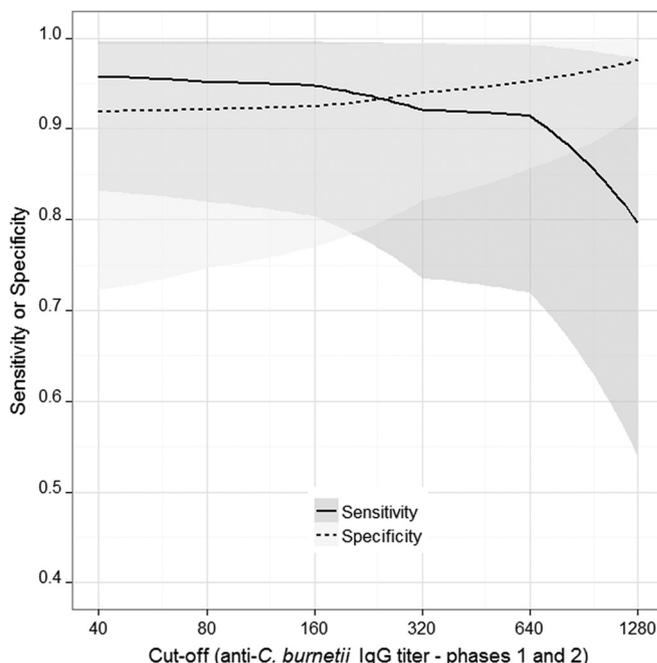


FIG 2 Bayesian estimates of the diagnostic sensitivity and specificity of the *C. burnetii* IFA at different cutoff titers in goat sera compared to the sensitivity and specificity of the IDEXX ELISA. Shading represents 95% confidence intervals; the solid line (representing the sensitivity of the IFA) crosses the dashed line (representing the specificity of the IFA) at the dilution where the highest Youden index (best combined sensitivity and specificity of the IFA) is obtained.

tions, are also presented in Table 4. The comparison between the IFA and modelISA showed less agreement beyond chance (K) than the comparison between the IFA and IDEXX ELISA (for IgG) (Table 4). Given the relatively low animal-level prevalence of IgM antibodies, PABAK was a more appropriate measure and estimated higher agreement values than K .

Comparison of the performances of the IFA, CFT, and ELISA in detecting antibodies to *C. burnetii*. The ELISA (IDEXX and modelISA) had significantly higher diagnostic sensitivities than the IFA for IgG (78.1%; 95% CI, 59.7, 94.9) and IgM (79.6%; 95% CI, 56.6, 94.8) antibodies against phase 2 *C. burnetii* compared to IgG (71.5%; 95% CI, 53.9, 92.5) and IgM (71.7%; 95% CI, 46.0, 92.8) against phase 1 *C. burnetii* (see Table S3 in the supplemental material). The IFA was more sensitive (84.5%; 95% CI, 54.4, 98.7) than the CFT (29.8%; 95% CI, 17.0, 44.8) in detecting IgG and IgM antibodies to phase 2 at the 1:160 cutoff (Table 4). Bayesian estimates of the specificities and sensitivities as well as prior distributions, prevalence estimates, and levels of agreement between the IFA and CFT are presented in Table 4.

Estimates of sensitivity and specificity of the IFA versus the ELISA and the IFA versus the CFT as well as the IFA versus modelISA on the basis of vague prior probability distributions are presented as additional material (see Table S4 in the supplemental material). The use of flat priors resulted in diagnostic sensitivity results for the IFA (65.4%; 95% CI, 21.8, 95.6) and modelISA (64.7%; 95% CI, 33.2, 90.1) for IgM antibodies that were significantly lower than the sensitivity estimates obtained using published priors (Table 4). The diagnostic specifications (sensitivity and specificity) of all other tests were highly comparable irrespective of whether flat or informative priors were utilized.

TABLE 4 Bayesian estimates of diagnostic sensitivity and specificity of the IFA in comparison to the ELISA (IDEXX) in detection of IgG and IgM antibodies to *C. burnetii* at a 1:160 dilution of goat serum^a

Antibody type(s)	Test	% sensitivity (95% PI)	% specificity (95% PI)	% prevalence 1 (95% PI)	% prevalence 2 (95% PI)	% agreement ^b (95% PI)	<i>K</i> ^b (95% PI)	PABAK ^b (95% PI)
IgG phases 1 + 2	IFA	94.8 (80.3, 99.6)	92.5 (77.1, 99.3)	40.4 (26.0, 53.6)	0.00 (0.00, 1.20)	81.1 (72.7, 88.0)	0.60 (0.43, 0.74)	0.62 (0.45, 0.76)
	ELISA	70.1 (52.7, 91.0)	96.2 (88.9, 99.2)					
IgM phases 1 + 2	IFA	88.8 (58.2, 99.5)	92.4 (83.0, 99.2)	16.0 (8.6, 25.4)	0.00 (0.00, 1.20)	73.5 (64.5, 81.1)	0.27 (0.09, 0.45)	0.47 (0.29, 0.62)
	modELISA	71.7 (46.3, 92.8)	80.7 (71.2, 89.8)					
Phase 2 only (IgM/IgG)	IFA	84.5 (54.4, 98.7)	94.4 (79.8, 99.5)	41.4 (26.0, 63.7)	0.00 (0.00, 1.30)	71.4 (62.2, 79.1)	0.30 (0.16, 0.46)	0.43 (0.24, 0.58)
	CFT	29.8 (17.0, 44.8)	96.8 (89.2, 99.5)					

^a IFA, immunofluorescence assay; PI, predictive interval; PABAK, prevalence- and bias-adjusted *K*; modELISA, modified IDEXX ELISA. Prevalence 1 data represent estimated animal-level true prevalences in samples from a known infected herd. Prevalence 2 data represent estimated animal-level true prevalences in samples from New Zealand. Agreement data represent proportions of agreement. *K* data represent Cohen's kappa values.

^b Data represent estimates and include results from negative controls from New Zealand (declared *C. burnetii*-free by the OIE).

DISCUSSION

The CFT is still widely used for the serological detection of *C. burnetii* in animals despite its poor sensitivity (31, 32), possibly because it is the only test that has been fully validated and standardized for use in detecting antibodies to *C. burnetii* in animals across different laboratories worldwide. We have adapted and validated the highly sensitive and specific human IFA for use in serological diagnosis of coxiellosis in goats. We have also generated diagnostic sensitivity and specificity estimates of the test for detecting IgG and IgM antibodies to phase 1 and phase 2 *C. burnetii* antigens.

Unlike the CFT, the IFA can be used to distinguish IgG antibodies from IgM antibodies to phase 1 and phase 2 *C. burnetii*, an

attribute that has been used to differentiate recent infections from past exposure in humans (23, 24). Our validated IFA could potentially also be used in veterinary diagnostics to differentiate recent infections from chronic infections in infected goat herds to assist with outbreak investigations or to establish freedom from *C. burnetii* infection. We have also produced diagnostic sensitivity and specificity estimates of the IFA based on its performance in samples from infected and disease-free populations.

The finding of the higher sensitivity of the IFA than of the ELISA for detection of antibodies against *C. burnetii* in goat serum obtained in this study is similar to what has been previously published for human Q fever serology (33, 34). There have also been reports of low sensitivity to IgG phase 2 antibodies in ELISA kits coated with mixed (both phase 1 and phase 2) antigens (53). A study done to compare the performances of three ELISA kits (mixed antigen, phase 1, and phase 2) in detecting antibodies against *C. burnetii* in cattle sera resulted in reports of poor performance of the mixed-antigen ELISA compared to that of the phase 1 and phase 2 ELISA (53). This “mixed-antigen effect” could explain the lower sensitivity of the ELISA than of the IFA in detecting IgG antibodies observed in our study (Table 4). We also observed that the ELISA was more sensitive to IgG antibodies against phase 2 antigen than against phase 1, which could possibly be a result of the type of antigen used in that kit.

None of the commercially available tests were able to separately quantify IgM antibodies in goat sera; the IDEXX ELISA measures only IgG antibodies, and the CFT titers represent a combination of IgG and IgM antibodies. Our validated IFA, which provides specificity and sensitivity estimates for detecting IgM antibodies to phase 1 and phase 2 *C. burnetii* antigens, is a novel test for veterinary diagnostics. The sensitivity of the modELISA for detection of IgM antibodies is comparable to the sensitivity of the IDEXX ELISA for detection of IgG antibodies, which is possibly because the modELISA and the IDEXX ELISA contain the same antigen, “mixed phase 2 and phase 1 *C. burnetii*.” The estimates of lower sensitivity of the ELISA and IFA for detection of IgM antibodies obtained using “flat” priors could possibly be due to low prevalence of IgM antibodies in the samples used for this study, a scenario that has been reported previously in human diagnostics (33).

The low sensitivity of the CFT obtained in our study is similar to what has been reported in other studies; it can be explained by the fact that, in ruminants, only IgG1 antibodies fix complement and the presence of IgG2 and IgM antibody types inhibits IgG1

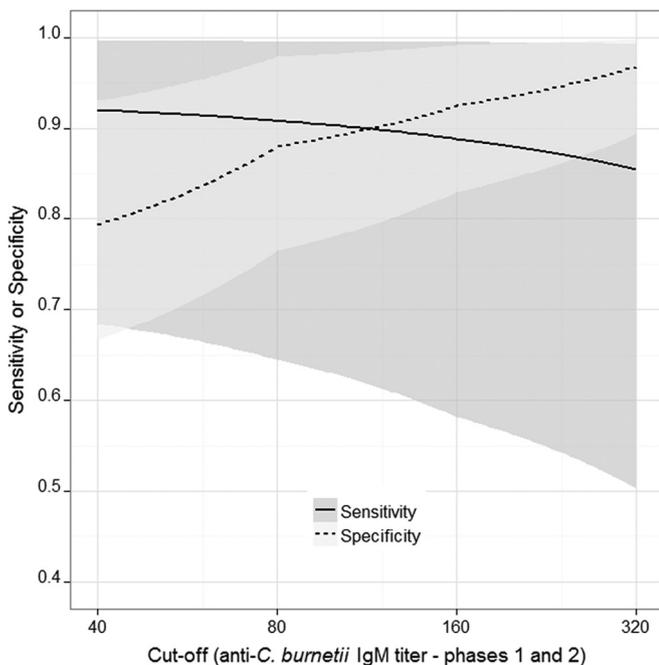


FIG 3 Diagnostic sensitivity and specificity of the *C. burnetii* IFA at different cutoff titers estimated using Bayesian latent class analysis for goat sera compared to the sensitivity and specificity of the modified IgM ELISA. Shading represents 95% confidence intervals; the solid line (representing the sensitivity of the IFA) crosses the dashed line (representing the specificity of the IFA) at the dilution where the highest Youden index (best combined sensitivity and specificity of the IFA) is obtained.

from fixing complement (32, 54, 55). The CFT is thus likely to result in many false-negative sample results, which make the test unsuitable for estimating prevalences and identifying exposed or infected ruminants. However, due to its high specificity and the fact that it is not species specific, the CFT is still useful for identifying infected herds and declaring herds to be free of disease. However, a larger sample size would be required because of the low sensitivity of the CFT. This low sensitivity explains why the agreement between the CFT and the highly sensitive IFA was only fair ($K = 0.30$), with only 13% of the samples positive for both tests at a 1:160 serum dilution (Table 3).

Accumulated evidence shows that New Zealand is free of Q fever (27, 56, 57). Therefore, any fluorescence observed in New Zealand samples was considered representative of false fluorescence. The negative-control samples showed no fluorescence at a 1:160 serum dilution, which was used as a cutoff titer for absolute positivity. Our Bayesian latent class model also produced the highest Youden's index at 1:160 for both IgG and IgM, which reinforces the idea of the reliability of a cutoff titer value of 160. All samples with end titer values below 160 were treated as representative of negative results. Furthermore, the high kappa coefficient (Table 2) and the high observed proportion of agreement (95.5%) between the readings of the two technicians (45) confirmed that there was limited human bias in the readings. This is important, as evaluation of IFA results is a subjective process and may require the operator to differentiate background fluorescence from true fluorescence. Much of the disagreement occurred at dilutions close to the endpoint titer where weakly positive titers were classified as negative by either of the technicians.

All samples had almost perfect repeatability over time (Fig. 1) for detection of antibodies against IgG phase 1 and phase 2 *C. burnetii*. Most of the poor repeatability results were for IgM titers, which were generally low, between 0 and 160, in the tested population. These low titers were difficult to distinguish from those seen with the negative controls, which also had background fluorescence at these titers. This further supports the use of 1:160 as a reliable cutoff titer for determining a true positive result and suggests that the IFA is unreliable for detecting *C. burnetii* antibody titers below 1:160 in goat sera.

By providing sensitivity and specificity estimates of three serological tests used in Q fever diagnostics, our results are a yardstick for the serological diagnosis of coxiellosis in goats. The IFA is highly sensitive and specific and should be used as a reference diagnostic test for coxiellosis in goats and other livestock. Furthermore, the ability of the IFA to differentiate IgG and IgM antibodies could be a useful tool in identifying recently infected animals and associated risk factors for infection as well as for designing *C. burnetii* disease control programs in goat herds. On the other hand, the ELISA is easier to perform and may be the quickest way to test high numbers of samples, while the high specificity of the CFT makes it suitable as a confirmatory test for samples that tested positive in other tests. However, because of its low diagnostic sensitivity, the CFT is likely to give unreliable estimates of prevalences of *C. burnetii* antibodies in goats and other livestock.

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We declare that we have no conflicts of interest.

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