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Age-Stratified Bayesian Analysis To Estimate Sensitivity and Specificity of Four Diagnostic Tests for Detection of *Cryptosporidium* Oocysts in Neonatal Calves[▽]

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There is no gold standard diagnostic test for the detection of bovine cryptosporidiosis. Infection is usually highest in 2-week-old calves, and these calves also excrete high numbers of oocysts. These factors may give rise to variations in the sensitivity and specificity of the various diagnostic tests used to detect infection in calves of various ages. An age-stratified Bayesian analysis was carried out to determine the optimum diagnostic test to identify asymptomatic and clinical *Cryptosporidium* sp. infection in neonatal calves. Fecal samples collected from 82 calves at 1 week, 2 weeks, 3 weeks, and 4 weeks of age were subjected to the following tests: microscopic examination of smears stained with either phenol-auramine O or fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* monoclonal antibody, nested-PCR, and quantitative real-time PCR. The results confirmed a high prevalence of *Cryptosporidium* sp. infection, as well as a high level of oocyst excretion, in 2-week-old calves. The sensitivities of all the tests varied with the age of the calves. Quantitative real-time PCR proved to be the most sensitive and specific test for detecting infection irrespective of the age of the calf. The microscopic techniques were the least sensitive and exhibited only moderate efficiency with 2-week-old calves excreting large numbers of oocysts, the majority of which were diarrheic. It was concluded that, when interpreting the results of routine tests for bovine cryptosporidiosis, cognizance should be taken of the sensitivity of the tests in relation to the age of the calves and stage of infection.

Morbidity due to neonatal enteritis prevents the economical production of cattle (4, 25, 32). The zoonotic protozoan *Cryptosporidium parvum* is one of the main causes of diarrhea in young calves (5, 25). Infected animals can exhibit clinical signs ranging from asymptomatic infection to profuse diarrhea and dehydration, and in some cases death (7). The control and prevention of this disease have economic benefits for both animal and public health. In general, the effectiveness of a disease control program depends on the sensitivity and specificity of the tests used to identify asymptomatic and clinically infected animals. Knowledge of the most appropriate diagnostic test to use at the various stages of an infection is important for regulators designing national surveillance and control programs and for practicing veterinarians dealing with outbreaks of clinical disease and instituting on-farm control protocols. At present, there is no gold standard test for the detection of *Cryptosporidium* spp., including *C. parvum* oocysts in feces (10, 17). However, this lack of a gold standard test need not inhibit the comparison of the relative merits of any group of diagnostic tests.

A Bayesian model, which estimates the sensitivity and specificity of various tests in the absence of a gold standard, can help in identifying the most suitable test for routine surveillance and diagnosis (1). This method has been validated on data for a number of important zoonotic and veterinary pathogens, including *Cryptosporidium* spp. (10, 12). In the case of bovine cryptosporidiosis, two PCR assays, *Cryptosporidium* PCR (C-PCR) and *Cryptosporidium* oocyst wall protein PCR (COWP-PCR), were found to be more sensitive than microscopic and enzyme-linked immunosorbent assays for the detection of infection in calves up to 10 weeks of age (10). Since that study, quantitative real-time PCR (qPCR), which is generally considered to have high sensitivity, has become the method of choice for the detection and quantification of various pathogens in well-equipped veterinary clinical laboratories. Despite this, microscopy methods, such as immunofluorescence and phenol-auramine O staining (PAO) of fecal smears, continue to be used routinely in less sophisticated veterinary diagnostic centers. It was therefore considered important to reevaluate the sensitivity and specificity of the routine diagnostic tests with the newly developed qPCR.

The pathogenesis of a given pathogen influences the sensitivity and specificity of diagnostic tests. In the case of bovine cryptosporidiosis, age is an important determinant of susceptibility, as the prevalence of *C. parvum* infection is usually higher in 2-week-old calves than in other age groups (10, 26, 28, 33). Thus, it was considered that a better estimation of the

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relative merits of the various tests might be obtained if the test results were stratified according to the age of the animals.

The objective of the present study was to estimate and compare the sensitivities and the specificities of four diagnostic tests for detection of *Cryptosporidium* sp. oocysts in fecal samples from neonatal calves grouped according to their age using a Bayesian model. The four tests were two molecular methods, qPCR and nested-PCR (nPCR), and two microscopic methods, immunofluorescence assay (IFA) and PAO. None of them was *C. parvum* specific. An additional goal was to identify the most appropriate test for detecting calves with asymptomatic infection so that they can be isolated, before they develop clinical signs, from healthy animals in order to control the spread of the disease in a herd.

MATERIALS AND METHODS

Study design and parameters recorded. A cohort study to monitor *Cryptosporidium* sp. infection in neonatal calves was carried out on a dairy herd with a history of cryptosporidiosis. A high prevalence of neonatal cryptosporidiosis caused by *C. parvum* had been confirmed through routine and molecular testing during the previous year (V. De Waele, unpublished data). The dairy herd consisted of 400 cows, with calving throughout the year. Cows were moved to a calving pen approximately 1 week prior to calving. Newborn calves were fed 2 liters of their dam's colostrum and separated from their mothers within 12 h of birth. The calves were kept in individual calf pens made of aluminum with a slatted wooden base. The pens were washed with disinfectant (Hyperox; DuPont, United Kingdom), and the floor was covered with straw before the introduction of the calves. Fresh straw was added daily. Every 2 weeks, the old bedding was removed and the floor was washed with disinfectant (Hyperox; DuPont, United Kingdom) and covered with fresh straw. Each calf received 2.5 liters of whole milk twice daily. Water and a ration containing soy, wheat, and citrus pulp, mixed on the farm, were supplied *ad libitum*.

A total of 82 Holstein Friesian calves born on the farm during the period from 2 March to 28 April 2003 were enrolled in the study. Information, such as the date of birth and sex, was recorded for each calf. Fecal samples (2 g) were collected from each of the 82 calves on four occasions, i.e., in the first, second, third, and fourth weeks of life. The consistency of the feces was recorded at the time of collection using the following scoring system: 0 for a solid or pasty sample, 1 for a liquid sample, and 2 for a watery sample. The mean fecal score was calculated by taking the mean of the scores for each sampling week. Diarrheic feces (feces scored as liquid or watery) were also tested for the presence of other common neonatal enteropathogens, including *Escherichia coli* K99, *Salmonella* spp., rotavirus, and coronavirus, using routine bacteriological culture and a commercial immunofluorescence kit (Bio-X Diagnostics Sprl, Belgium). In addition, serum was collected from each calf during the first week of life. This was tested for transfer of maternally derived immunoglobulins using the zinc sulfate turbidity test (ZST) (19). Fecal samples were also collected within 7 days postpartum from the cows whose calves were included in the experiment.

Oocyst concentration. Oocysts were concentrated from the calf samples (2 g) by filtering 10 ml of a 1:5 feces/distilled water (dH_2O) suspension through a 45-μm filter, followed by centrifugation at 1,050 × *g* for 5 min. The top 9 ml of the supernatant was discarded, leaving a final volume of 1 ml.

PAO method. A smear was made by adding 100 μl of the concentrated fecal suspension to a glass microscope slide. This was allowed to dry at room temperature. The smear was fixed in methanol for 3 min and exposed to formalin vapor in a humidity chamber at 37°C for 30 min. This smear was then stained with the phenol-auramine O solution for 10 min (20). Finally, the slide was washed briefly in dH_2O, counterstained with potassium permanganate for 30 s, washed in dH_2O. and allowed to dry at room temperature. The smear was examined at ×400 magnification using a fluorescence microscope (Olympus, Japan) containing a filter cube with an emission of 530 nm and an excitation wavelength of 490 nm. A smear was considered positive if at least one *Cryptosporidium* sp. oocyst was identified on the stained slide. For each positive slide, a semiquantitative estimation of the number of oocysts was obtained by examining 10 arbitrarily chosen fields at ×400 magnification and using the following scoring system: 0 for no oocysts observed on the smear, 1 for less than one oocyst per field, 2 for one to five oocysts per field, 3 for six to 50 oocysts per field, and 4 for over 50 oocysts per field. The mean oocyst score was calculated by taking the mean of the scores for each of the four sampling weeks.

IFA. A second 100-μl aliquot of the concentrated fecal suspension was added to a well (14-mm diameter) on a microscope slide and stained with 50 μl of fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* monoclonal antibody (Cellabs Pty Ltd., Australia) according to the procedure described by McEvoy et al. (18). The slide was examined at ×400 magnification using a fluorescence microscope (Olympus, Japan) containing a filter cube with an emission of 530 nm and an excitation wavelength of 490 nm. A slide was considered positive if at least one *Cryptosporidium* sp. oocyst was identified on the stained well. For each positive slide, the approximate number of oocysts per gram of feces (OPG) was calculated using the mean number of oocysts present in 10 arbitrarily chosen fields at ×400 magnification and corrected for the total surface of the smear and the dilution factor of the original sample. No correction was made for the fluidity of the feces.

Extraction of DNA. DNA was extracted from 500 μl of fecal suspension with a commercial kit (FastDNA Spin Kit for Soil; Qbiogene Inc.) according to the manufacturer's protocol (15).

nPCR and sequencing. *Cryptosporidium* sp. DNA was detected using a nested PCR that amplified a segment of the small-subunit 18S rRNA gene of approximately 830 bp (36). Secondary PCR products from 10 randomly selected positive fecal samples were sequenced to confirm the presence of *Cryptosporidium* sp. oocysts. Purification and sequencing of the amplicons were carried out by a commercial company (MWG Biotech AG, Germany). The amplified PCR products were sequenced in both directions using forward and reverse primers from the secondary PCR. The sequences were assembled and aligned with reference sequences from GenBank using the SeqMan II module within the Lasergene software (DNAStar Inc., Madison, WI).

qPCR. The real-time PCR assay used in this study was based on the procedure described by Jothikumar et al. using primers and probes targeting the 18S rRNA gene of *Cryptosporidium* spp. (16). The original protocol was modified as follows: each 20-μl reaction mixture contained 10 μl of 2× Jumpstart ReadyMix (Sigma, United Kingdom), 4 mM $MgCl_2$, 0.25 μM primers JVAF and JVAR, 0.1 μM TaqMan probe JVAP18S, 0.4 mg/ml bovine serum albumin (BSA), 1 μM carboxy-X-rhodamine (ROX), and 5 μl DNA. The 96-well clear plates (ABgene; ThermoScientific) were manually sealed with adhesive tape (Sigma, United Kingdom). Real-time PCR amplifications were performed using a thermocycler (Mx3000p; Stratagene) with the following conditions: denaturation at 94°C for 2 min, followed by 45 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 1 min, and extension at 72°C for 30 s. There was no DNA in the negative-control wells. In addition, six serial dilutions of a certified *C. parvum* DNA (ATCC PRA-67D) were used to generate a calibration curve in each plate. The concentration of DNA in the serial dilutions was measured with a spectrophotometer (Nanodrop ND1000; ThermoScientific) and covered 6 $\log_{10}$ concentrations of DNA. All samples and controls were tested in duplicate. The results from the different runs were analyzed using the "multiple experiment analysis" option of the MxPro qPCR software (Stratagene). The duplicate quantification values (*Cq* values) of all qPCR-positive samples were entered into a hierarchical Bayesian model to estimate the adjusted DNA concentration in each aliquot (V. De Waele, M. Berzano, N. Speybroeck, D. Berkvens, G. M. Mulcahy, and T. M. Murphy, submitted for publication). The DNA concentration of each sample was then obtained by calculating the mean DNA concentration of the duplicate aliquots. The number of oocyst equivalents per gram of feces was calculated on the premise that one oocyst contains 40 fg of genomic DNA (14).

Thirty-two samples arbitrarily selected from those collected during the first and second weeks of life were tested with a second qPCR using primers and probe targeting the COWP gene (14). The original protocol was modified as follows. Each 20-μl reaction mixture contained 10 μl 2× Sigma Jumpstart ReadyMix (Sigma, United Kingdom), 4 mM $MgCl_2$, 0.4 μM primers P702_F and P702_R, 0.2 μM TaqMan probe P702_P, 0.4 mg/ml BSA, 1 μM ROX, and 5 μl DNA. The 96-well clear plates (ABgene; ThermoScientific) were manually sealed with adhesive film (Sigma, United Kingdom). Real-time PCR amplifications were performed using the real-time Mx3005 thermocycler (Stratagene) with the following conditions: initial denaturation at 95°C for 2 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 20 s. There was no DNA in the negative-control wells. In addition, six serial dilutions of certified *C. parvum* DNA (ATCC PRA-67D) were used to generate a calibration curve as described for the previous qPCR. All samples were tested in duplicate, and the number of oocyst equivalents per gram of feces was calculated as previously described for the 18S rRNA assay (14).

Oocyst detection in feces collected from cows. Fecal samples (5 g) from the cows were initially concentrated by sucrose density flotation (3). The fecal suspension obtained was then tested for the presence of *Cryptosporidium* sp.

DNA using the extraction and nPCR procedures described for the neonatal calves.

Estimation of the sensitivities and specificities of the four diagnostic tests in 1-, 2-, 3-, and 4-week-old calves. The Bayesian model, described by Berkvens et al. (1), was modified to take into account potential age stratification related to test sensitivity and specificity (see Appendix). The model-building strategy consisted of incorporating probabilistic prior information, derived from previously published studies and expert opinion, to reduce the number of parameters to be estimated (10, 12, 16). Different levels of correlation between tests were considered likely to be present for the different groups of calves. Two-week-old calves were more likely to excrete high levels of oocysts, which would imply a higher correlation between the results of the diagnostic tests than in the other age groups. Based on the prior ranges available, different Bayesian models were run in WinBUGS 1.4 (31). After a burn-in of 5,000 iterations, the model was run for an additional 10,000 iterations. The convergence of the various models was determined by means of the Gelman-Rubin convergence diagnostic test (9). Their efficiencies were assessed by verifying that the Monte Carlo standard error of each parameter of interest was less than 5% of the sample standard deviation. A model selection was carried out using statistical tools, which validated the subjectivity in the prior information incorporated in the model, such as the deviance information criterion (DIC), effective number of parameters estimated (p_D), and Bayesian P value (1, 30). The values of the DIC and p_D parameters evaluated in the posterior mean of the multinomial probability were assessed for agreement with those evaluated in the posterior mean of the parameters of the model (12). The estimated Bayesian P values had to have a value below 0.55 and to tend toward zero when severe constraints on the priors were applied in order to indicate a good model fit (13).

In addition, the posttest probability of infection after a given test result, i.e., the probability of an animal testing positive or negative actually being infected with *Cryptosporidium* spp., was calculated for the four age groups of calves based on the estimated prevalence, sensitivity, and specificity of the diagnostic tests (34).

Estimation of the quantity of oocysts excreted. The quantity of oocysts excreted by calves was estimated using the microscopic methods and qPCR. The precision of the qPCR between the quantification values (*Cq* values) of the standard samples was assessed within and between runs using the concordance correlation coefficient on STATA/MP 10.0 software (Stata Corporation, College Station, TX). The precision of the qPCR was further evaluated using a hierarchical Bayesian model (29). A Bayesian model for estimating a master calibration curve was modified to allow for within and between run variability (De Waele et al., submitted). Additional estimations were included in the model to determine if the intercepts and slopes of the master calibration curve were significantly different from those of the calibration curves of the runs. If no significant difference was found between the master calibration curve and the calibration curve of the runs, the master calibration curve was further used to determine the concentration of DNA (ng/μl) in the samples. Diffused prior distributions were used to estimate the model parameters. The model was run on WinBUGS 1.4. (31). The burn-in phase was 5,000 iterations, and the model was run for a further 10,000 iterations to obtain estimates of the slopes and intercepts of the master calibration curve and the calibration curves for each run. The convergence and efficiency of the models were assessed as described above for the previous Bayesian model. The precision of the DNA concentrations in duplicate aliquots was assessed using the coefficient of variation.

In order to further assess the quantification of oocysts by the qPCR targeting the 18S gene, 32 calf samples were tested with an additional qPCR that targeted the COWP gene, and the results obtained from both qPCR assays were compared for agreement. The amounts of *Cryptosporidium* sp. DNA (ng/μl) estimated by both qPCRs were analyzed with STATA/MP 10.0 software (Stata Corporation, College Station, TX) using the Pearson correlation coefficient and the concordance correlation coefficient.

Analysis of risk factors associated with cryptosporidiosis. Any significant effect ($P < 0.05$) that the variables, such as diarrhea, sex, ZST test result, and *Cryptosporidium* status of the dam, may have on the number of calves excreting oocysts was tested with the generalized estimating equation (GEE) models using STATA/MP 10.0 software (Stata Corporation, College Station, TX) (6). The GEE models were selected for the analysis to take into account repeated observations for each animal. The binomial family, logistic link, and exchangeable correlation matrix were assumed. A logistic regression was also used to test for any significant effect ($P < 0.05$) of the same variables on the number of calves shedding oocysts during the first week of life using STATA/MP 10.0 software (Stata Corporation, College Station, TX).

TABLE 1. Combinations of results obtained using four different diagnostic tests for the detection of *Cryptosporidium* spp. in fecal samples collected from 66 calves at 1, 2, 3, and 4 weeks of age

Diagnostic test result[a]				No. of samples exhibiting combination in wk:			
qPCR	nPCR	IFA	PAO	1	2	3	4
+	+	+	+	0	38	9	2
+	+	+	−	4	3	9	5
+	+	−	+	1	9	9	7
+	+	−	−	11	6	21	10
+	−	+	+	0	5	1	1
+	−	+	−	0	1	1	2
+	−	−	+	0	0	0	1
+	−	−	−	14	1	6	12
−	+	+	+	0	0	1	0
−	+	+	−	1	0	1	1
−	+	−	+	0	2	0	0
−	+	−	−	7	1	0	5
−	−	+	+	0	0	0	2
−	−	+	−	0	0	0	2
−	−	−	+	0	0	4	1
−	−	−	−	28	0	4	15

[a] +, positive; −, negative (results in test).

RESULTS

Estimation of the sensitivities and specificities of the four diagnostic tests in 1-, 2-, 3-, and 4-week-old calves. A fecal sample was collected weekly from each of the 82 calves recruited for the study for the first 4 weeks of life. The feces were examined for the presence of *Cryptosporidium* sp. oocysts using four routine diagnostic tests, resulting in 16 combinations of positive and negative results for each sampling point (Table 1). The results from 264 samples taken from 66 calves were subjected to statistical analysis. The results from 16 other calves were not included in this analysis because, on some sampling occasions, an insufficient quantity of feces was collected to allow completion of all the diagnostic tests. All the calves excreted *Cryptosporidium* sp. oocysts on one or more sampling occasions, and a diarrheic sample was collected from 38% (25/66) of the calves on at least one occasion.

Several Bayesian models with different constraints were constructed to estimate the sensitivity and specificity of the four diagnostic tests. One of the converged models was selected based on validation criteria of the three statistical indices (DIC, p_D, and Baysian P value) which assessed the subjectivity of the prior information and the fitness of the model (Tables 2 and 3). The priors for the 2-week-old age group were included in the specificity of the qPCR, nPCR, and IFA, which were greater than or equal to 80%, between 70 and 90%, and greater than or equal to 70%, respectively. In the age groups other than the 2-week-old group, additional prior information on the specificity and sensitivity of the tests was included in the mode to enable it to reach convergence. In the 1-week-old group, the prior for the specificity of the qPCR was increased to at least 90% and the same constraint on the specificity of the IFA was added on the PAO (≥70%). Additional prior information on the sensitivities of the qPCR (≥80%), nPCR (40 to 80%), IFA (≤50%), and PAO (≤10%) was included to further improve the model. The other age groups (3 and 4 weeks old)

TABLE 2. Parameters (*th*) estimated in the age-stratified Bayesian model with the priors selected for calves aged 1, 2, 3, and 4 weeks

Conditional probabilities[a]		Model priors for wk[b]:			
Parameter (*th*)	Parameter definition	1	2	3	4
*th*1 = Prev	$Pr(D^+)$	0–1	0–1	0–1	0–1
*th*2 = Se_1	$Pr(T_1^+ \mid D^+)$	0.8–1	0–1	0–1	0.8–1
*th*3 = Sp_1	$Pr(T_1^- \mid D^-)$	0.9–1	0.8–1	0.8–1	0.8–1
*th*4	$Pr(T_2^+ \mid D^+ \cap T_1^+)$	0.4–0.8	0–1	0–1	0.4–0.8
*th*5	$Pr(T_2^+ \mid D^+ \cap T_1^-)$	0.4–0.7	0–1	0.4–0.7	0.4–0.7
*th*6	$Pr(T_2^- \mid D^- \cap T_1^-)$	0.7–0.9	0.7–0.9	0.7–0.9	0.7–0.9
*th*7	$Pr(T_2^- \mid D^- \cap T_1^+)$	0.3–0.6	0.3–0.6	0.3–0.6	0.3–0.6
*th*8	$Pr(T_3^+ \mid D^+ \cap T_1^+ \cap T_2^+)$	0–0.5	0–1	0–1	0–1
...[c]					
*th*12	$Pr(T_3^- \mid D^- \cap T_1^- \cap T_2^-)$	0.7–1	0.7–1	0.7–1	0.7–1
...					
*th*16	$Pr(T_4^+ \mid D^+ \cap T_1^+ \cap T_2^+ \cap T_3^+)$	0–0.1	0–1	0–1	0–0.4
...					
th24	$Pr(T_4^- \mid D^- \cap T_1^- \cap T_2^- \cap T_3^-)$	0.7–1	0–1	0–1	0–1
...					
*th*28	$Pr(T_4^- \mid D^- \cap T_1^- \cap T_2^+ \cap T_3^+)$	0–1	0–1	0–1	0–1
...					
*th*31	$Pr(T_4^- \mid D^- \cap T_1^+ \cap T_2^+ \cap T_3^+)$	0–1	0–1	0–1	0–1

[a] T_1, quantitative real-time PCR; T_2, nested PCR; T_3, immunofluorescence assay; T_4, phenol-auramine O staining method. Prev, prevalence; Se_1, sensitivity of the quantitative real-time PCR; Sp_1, specificity of the quantitative real-time PCR. D^+ indicates presence and D^- indicates absence of *Cryptosporidium* infection. T^+ indicates positive and T^- indicates negative results in a test. $Pr(T_1^+ \mid D^+)$, probability of a positive result with the quantitative real-time PCR (T_1^+) when the animal is infected with *Cryptosporidium* (D^+). $Pr(T_2^+ \mid D^+ \cap T_1^+)$, probability of a positive result with the nested PCR (T_2^+) when the animal is infected with *Cryptosporidium* (D^+) and tested positive with the quantitative real-time PCR (T_1^+).

[b] The range (*a*–*b*) denotes that *a* is the lower limit and *b* is the upper limit of the parameter interval.

[c] . . ., the missing formula follows the same pattern as the previously listed formula.

required similar but less severe constraints on the test sensitivities than the 1-week-old age group.

The estimated prevalence of infection varied depending on age; the lowest (48%) was in 1-week-old calves, and the highest value (98%) was in 2-week-old calves, with over 30% of them exhibiting clinical signs of enteritis (Table 4). Similarly, the sensitivities and, to a lesser extent, the specificities of the different tests varied with the age of the calves (Table 4). The sensitivities of examining PAO and IFA stained smears and the nPCR were lowest (5%, 18%, and 54%, respectively) in the 1-week-old calves and highest (79%, 71%, and 87%, respectively) in the 2-week-old calves. The sensitivities of those tests decreased thereafter for the 3- and 4-week-old age groups. Overall, qPCR had the highest sensitivity and specificity, which varied between 89 and 95% for all age groups.

The probability of a positive test result indicating an animal with an actual infection varied with the test employed and the age of the calves (Table 5). A 1-week-old calf positive by the qPCR test had a 95% probability of being infected with *Cryptosporidium* spp., while with positive PAO stained smears, it was only 40% (Table 5). The accuracy of the microscopic staining techniques in detecting actual infection increased after the first week of age, and positive 2- and 3-week-old calves had at least 81% probability of being infected. Similarly, a 1-week-old calf negative by qPCR had a 9% probability of actually being infected, while if negative by PAO, the animal still had a 49% chance of being infected.

TABLE 3. Bayesian *P* values, effective numbers of estimated parameters, and deviance information criteria used to validate the age-stratified Bayesian model estimating the sensitivities and specificities of four diagnostic tests of *Cryptosporidium* sp. infection in 1-, 2-, 3-, and 4-week-old calves

Wk	Bayesp[a]	Parent nodes		Multinomial	
		p_D	DIC	p_D	DIC
1	0.539	3.484	36.348	3.591	36.455
2	0.514	6.552	42.633	6.824	42.904
3	0.451	7.229	50.577	7.782	51.130
4	0.331	7.113	56.336	7.764	56.988

[a] Bayesp, Bayesian *P* value.

Estimation of the quantity of oocysts excreted. During the study, it was shown that at least 53.5 fg of certified *C. parvum* DNA (ATCC PRA-67D) could be detected in all the 18S rRNA qPCR runs. Ten arbitrarily selected amplicons from the nPCR runs that were also positive by qPCR were sequenced and were shown to have 100% identity with *C. parvum* (GenBank accession number AF093490) (35).

The efficiency of the qPCR assay, calculated from the data from 10 runs, varied between 81.6 and 101.6%. The concordance correlation coefficients within and between runs were at least 0.997. The *Cq* values of the standard samples in 10 runs were included in a Bayesian model in order to estimate the master calibration curve. The model converged, and the intercept and slope of the master calibration curve were not statistically different from those of the calibration curves from each individual run. Therefore, the master calibration curve with an intercept of 20.640 (credible interval [CI], 20.480 to 20.810) and a slope of −3.421 (CI, −3.525 to −3.320) was used to estimate the DNA concentrations of all aliquots. The coefficients of variation (0.007) between duplicate DNA concentrations were satisfactory. The DNA concentration of each sample was transformed into an oocyst equivalent per gram of feces, and the mean number of oocysts per gram of feces for the different age groups was calculated and compared to the

TABLE 4. Estimated prevalence of *Cryptosporidium* sp. infection and sensitivity and specificity, with 95% credible intervals, of four diagnostic tests used to detect *Cryptosporidium* sp. infection in 1-, 2-, 3-, and 4-week-old calves

Parameter[a]	Diagnostic test	Wk 1		Wk 2		Wk 3		Wk 4	
		Mean	CI	Mean	CI	Mean	CI	Mean	CI
Prev (%)		48	34–63	98	92–100	86	73–97	63	45–79
Se (%)	qPCR	90	81–99	95	88–99	94	83–100	89	80–99
	nPCR	54	42–69	87	79–94	83	73–92	59	45–73
	IFA	18	8–30	71	60–81	37	25–49	27	15–42
	PAO	5	2–8	79	69–87	37	26–49	22	14–31
Sp (%)	qPCR	95	90–100	90	80–99	89	80–99	89	80–99
	nPCR	79	70–88	76	66–87	77	67–87	77	67–87
	IFA	94	87–99	76	59–93	76	59–91	78	64–91
	PAO	93	85–98	50	17–83	50	24–77	77	62–89

[a] Prev, prevalence; Se, sensitivity; Sp, specificity.

number obtained with the IFA and PAO microscopic techniques (Table 6). Both the microscopic methods and the qPCR assay demonstrated an increase in the mean of oocyst excretion by 2-week-old calves of up to 3×10^6 and 3×10^9 oocysts per gram, respectively.

The efficiency of the qPCR targeting the COWP gene was 99.6%. The two qPCR assays had 81% (26/32) agreement for the detection of *Cryptosporidium* sp. DNA. However, six samples were positive by the 18S rRNA assay and not by the COWP assay despite the oocyst concentration (calculated from the results of the 18S rRNA test) ranging from 5×10^3 to 6×10^6 oocysts per gram of feces. When comparing the quantitative measurements of the two qPCRs for the 18S rRNA and COWP genes, the Pearson's coefficient was estimated at 0.995, while the concordance correlation coefficient was estimated at 0.683. In those samples ($n = 17$) that were positive in both assays, the estimation of the amount of oocysts excreted was always higher with the qPCR assay for the COWP gene than with the assay for 18S rRNA gene.

Analysis of risk factors associated with cryptosporidiosis. Over half (56%) of the calves had inadequate absorption of immunoglobulins, as their ZST values were below 15 units. In addition to the presence of *Cryptosporidium* spp., other common neonatal enteropathogens were also present on the farm, and 17% of the experimental calves had mixed infections of *Cryptosporidium* spp. with either *E. coli*, rotavirus, or coronavirus. Only 41 cows whose calves were used in this study were tested to determine if they were excreting *Cryptosporidium* sp. oocysts; the reason was that it was not always possible to match a calf with its mother once the cow had been reintroduced into the milking herd. Four of the dams were positive, but the concentration of DNA in the nPCR amplicon was insufficient to allow species identification by either restriction fragment length polymorphism (RFLP) or sequencing. The GEE models confirmed that calves, especially 2-week-old animals, with diarrhea excreted more oocysts and were more at risk of being infected with *Cryptosporidium* spp. than nondiarrheic calves. Calves born during the month of April were more at risk of being infected in their first week than calves born earlier, in March (odds ratio = 3.06; $P = 0.03$). Other risk factors, such as sex, ZST test result, and *Cryptosporidium* status of the dam, had no significant effect on the presence of infection in the young calves. However, it is likely that the low ZST values increased the susceptibility of the majority of calves to the other neonatal enteropathogens.

DISCUSSION

Diagnostic tests with a high level of sensitivity and specificity are necessary for accurate diagnosis of clinical and subclinical infection. Identification of asymptomatic individuals in the early stages of infection prior to the development

TABLE 5. Estimated posttest probabilities of cryptosporidiosis, with 95% credible intervals, of four diagnostic tests used to detect *Cryptosporidium* sp. infection in 1-, 2-, 3-, and 4-week-old calves

Time	Diagnostic test	Posttest probability of infection (%)							
		Wk 1		Wk 2		Wk 3		Wk 4	
		Mean	CI	Mean	CI	Mean	CI	Mean	CI
After a positive test	qPCR	95	86–100	100	99–100	98	94–100	92	82–100
	nPCR	70	51–86	99	97–100	95	90–99	80	62–93
	IFA	74	45–94	99	97–100	90	76–99	67	38–89
	PAO	40	14–71	98	94–100	81	62–97	62	37–84
After a negative test	qPCR	9	0–23	72	15–99	28	1–82	18	1–41
	nPCR	35	21–51	88	60–100	57	29–89	47	29–68
	IFA	45	31–60	94	79–100	83	68–96	61	42–79
	PAO	49	35–64	95	79–100	88	75–98	63	45–80

TABLE 6. Mean fecal score, mean oocyst score estimated from phenol-auramine O (PAO)-stained smears, mean number of oocysts per gram of feces estimated from immunofluorescence assay (IFA) and quantitative real-time PCR (qPCR), and mean concentration of *Cryptosporidium* DNA in fecal samples collected from 1-, 2-, 3- and 4-week-old calves

Age (wk)	Mean fecal score	Mean oocyst score using PAO	Mean no. (range) of oocysts per g of feces		Mean amt (range) of *Cryptosporidium* sp. DNA (ng/μl) using qPCR
			IFA	qPCR	
1	0.08	0.02	3,788 (0–50,000)	124,312 (0–1,925,900)	0.00622 (0–0.09630)
2	0.41	2.18	365,909 (0–2,550,000)	303,161,391 (0–2,187,000,000)	15.15807 (0–109.35000)
3	0.09	0.77	61,363 (0–850,000)	19,433,188 (0–529,500,000)	0.97166 (0–26.47500)
4	0.02	0.38	11,364 (0–150,000)	12,115 (0–201,810)	0.00061 (0–0.01009)

of overt clinical signs is important because they can be a source of disease for the remaining, healthy population. Accurate tests are also required in nonclinical population-based medicine, disease surveillance, prevalence estimations, and epidemiological studies. Information gleaned from such studies can be applied in the development of risk management and disease modeling.

The sensitivities and specificities of diagnostic tests for parasites vary depending on the pathogenesis of the different life cycle stages, the susceptibility of the host, and the prevalence of the etiological agent in the population being studied (2, 23, 24). In the case of bovine cryptosporidiosis, newborn calves are most susceptible to *C. parvum* infection. The four tests in this study identified *Cryptosporidium* oocysts only to the genus level. However, sequencing 10 randomly selected amplicons from the nPCR indicated that *C. parvum*, a species that is known to be highly infectious, was present among the calves. This study confirms previous published reports that the prevalence and severity of infection depend on a calf's age: although some of the calves became infected in the first week, the peak of infection occurred in the second week (10, 26, 28).

There is no gold standard diagnostic test for cryptosporidiosis (10, 17). A previous report on a Bayesian statistical analysis of the test properties of six diagnostic tests, including two enzyme-linked immunosorbent assays (ELISAs), carbolfuchsin smear staining, immunofluoresence microscopy, and two PCR assays, identified PCR as the most sensitive assay for determining *Cryptosporidium* sp. infection in calves up to 10 weeks of age (10). The results of the present study also indicated that the two molecular assays were more sensitive than the microscopy methods for detecting infection in calves during the first 4 weeks of their lives. The difference in the mean numbers of oocysts detected by IFA and qPCR further highlighted the known poor recovery obtained when using microscopic methods (22). Immunofluorescence and phenol-auramine O staining of fecal smears gave satisfactory results only with 2-week-old calves exhibiting signs of clinical disease and excreting large numbers of oocysts. Immunofluorescence microscopy is the diagnostic method of choice for cryptosporidiosis in most veterinary diagnostic laboratories, and as this study has shown, it is an efficient investigative tool only during disease outbreaks. In addition, its specificity is low and it does not allow species discrimination, unlike the more sensitive nested and real-time PCR assays (8, 37). qPCR proved to be the most sensitive of all the tests during the neonatal period and appears to be the ideal method for identifying animals in the early stages of infection before clinical signs become evident in the second week of a calf's life. The assay has the added advantage that the level of oocyst excretion can be easily quantified and an assessment of the potential of affected animals to act as a source of infection for other calves and to contaminate the environment, including surface waters, can be readily made.

The performance (sensitivity and specificity) of diagnostic tests in different epidemiological and pathogenic scenarios has practical implications for the design of surveillance and/or control programs for *C. parvum*. It is important that the test selected for these studies be appropriate to the level of infection in the target population. The ideal test is one that is 100% sensitive and specific. However, in many situations, the choice of diagnostic test often depends on the resources available. Veterinarians must be aware of the limitations of the various diagnostic procedures when interpreting results. As sensitivity and specificity may be difficult to interpret, further information about the test, such as the posttest probability of infection, may help in the interpretation of the results vis-à-vis an animal's disease status (34). In this study, qPCR detected infection in all of the calves, including recently infected asymptomatic animals, and it may be the ideal test for on-farm control programs and epidemiological investigations, as demonstrated in previous studies (14, 21, 27). The disadvantages to using this procedure are that expensive equipment and reagents and trained personnel are required to carry it out. The microscopy methods lack sensitivity and are only suitable for identifying animals excreting large numbers of *Cryptosporidium* sp. oocysts and usually showing clinical signs of enteritis, which as this study has shown on a commercial farm with an on-going disease problem, occurs in 2-week-old calves.

Despite good farming practices, rearing calves singly in clean pens with individual feeders and ensuring they receive some colostrum at birth, all the calves excreted *Cryptosporidium* sp. oocysts at some stage of the study. The analysis of the factors that may have increased the susceptibility of calves to infection was inconclusive and indicated only that animals born toward the latter end of the study were more likely to become infected in their first week of life and to progress to exhibiting clinical signs of disease by the second week. This was probably due to the buildup of environmental contamination over the course of the experiment, and calves that were born later in the study experienced greater infection pressure.

It is likely that stratifying the data by factors other than age, such as breed and sex, may have changed the performance of the diagnostic tests. It was shown previously that stratification according to the presence of clinical signs, such as diarrhea, changed the sensitivity of an IFA detecting *Giardia duodenalis* in dog feces (11).

In conclusion, this study has shown that the sensitivity and

specificity of the routine tests used to detect *Cryptosporidium* sp. oocysts varied with the age of neonatal calves and also with the stage of infection. It is suggested that cognizance should be taken of these parameters and that the most appropriate test be selected for diagnosing asymptomatic and clinically ill calves. Quantitative real-time PCR proved to be the best assay for the detection of both clinical and subclinical infection. It had the added benefit that the level of oocyst excretion could be quantified, and thus, it has the potential to be used outside clinical veterinary medicine as a tool for monitoring environmental and surface water contamination from slurries and dung.

APPENDIX

Age-stratified Bayesian model to estimate the prevalence, sensitivity, and specificity of four diagnostic tests detecting *Cryptosporidium* sp. oocysts in neonatal calves.

```
# Model four tests four strates.
model
{
result1[1:16] ~ dmulti(pr1[1:16], n1)
result2[1:16] ~ dmulti(pr2[1:16], n2)
result3[1:16] ~ dmulti(pr3[1:16], n3)
result4[1:16] ~ dmulti(pr4[1:16], n4)

# Tests results probabilities.
pr1[1]<-th1[1]*th1[2]*th1[4]*th1[8]*th1[16]+(1-th1[1])*(1-th1[3])*
   (1-th1[7])*(1-th1[15])*(1-th1[31])
pr1[2]<-th1[1]*th1[2]*th1[4]*th1[8]*(1-th1[16])+(1-th1[1])*
   (1-th1[3])*(1-th1[7])*(1-th1[15])*th1[31]
pr1[3]<-th1[1]*th1[2]*th1[4]*(1-th1[8])*th1[17]+(1-th1[1])*
   (1-th1[3])*(1-th1[7])*th1[15]*(1-th1[30])
pr1[4]<-th1[1]*th1[2]*th1[4]*(1-th1[8])*(1-th1[17])+(1-th1[1])*
   (1-th1[3])*(1-th1[7])*th1[15]*th1[30]
pr1[5]<-th1[1]*th1[2]*(1-th1[4])*th1[9]*th1[18]+(1-th1[1])*
   (1-th1[3])*th1[7]*(1-th1[14])*(1-th1[29])
pr1[6]<-th1[1]*th1[2]*(1-th1[4])*th1[9]*(1-th1[18])+(1-th1[1])*
   (1-th1[3])*th1[7]*(1-th1[14])*th1[29]
pr1[7]<-th1[1]*th1[2]*(1-th1[4])*(1-th1[9])*th1[19]+(1-th1[1])*
   (1-th1[3])*th1[7]*th1[14]*(1-th1[28])
pr1[8]<-th1[1]*th1[2]*(1-th1[4])*(1-th1[9])*(1-th1[19])+
   (1-th1[1])*(1-th1[3])*th1[7]*th1[14]*th1[28]
pr1[9]<-th1[1]*(1-th1[2])*th1[5]*th1[10]*th1[20]+(1-th1[1])*
   th1[3]*(1-th1[6])*(1-th1[13])*(1-th1[27])
pr1[10]<-th1[1]*(1-th1[2])*th1[5]*th1[10]*(1-th1[20])+(1-th1[1])*
   th1[3]*(1-th1[6])*(1-th1[13])*th1[27]
pr1[11]<-th1[1]*(1-th1[2])*th1[5]*(1-th1[10])*th1[21]+(1-th1[1])*
   th1[3]*(1-th1[6])*th1[13]*(1-th1[26])
pr1[12]<-th1[1]*(1-th1[2])*th1[5]*(1-th1[10])*(1-th1[21])+
   (1-th1[1])*th1[3]*(1-th1[6])*th1[13]*th1[26]
pr1[13]<-th1[1]*(1-th1[2])*(1-th1[5])*th1[11]*th1[22]+(1-th1[1])*
   th1[3]*th1[6]*(1-th1[12])*(1-th1[25])
pr1[14]<-th1[1]*(1-th1[2])*(1-th1[5])*th1[11]*(1-th1[22])+
   (1-th1[1])*th1[3]*th1[6]*(1-th1[12])*th1[25]
pr1[15]<-th1[1]*(1-th1[2])*(1-th1[5])*(1-th1[11])*th1[23]+
   (1-th1[1])*th1[3]*th1[6]*th1[12]*(1-th1[24])
pr1[16]<-th1[1]*(1-th1[2])*(1-th1[5])*(1-th1[11])*(1-th1[23])+
   (1-th1[1])*th1[3]*th1[6]*th1[12]*th1[24]
pr2[1]<-th2[1]*th2[2]*th2[4]*th2[8]*th2[16]+(1-th2[1])*(1-th2[3])*
   (1-th2[7])*(1-th2[15])*(1-th2[31])
…
pr3[1]<-th3[1]*th3[2]*th3[4]*th3[8]*th3[16]+(1-th3[1])*(1-th3[3])*
   (1-th3[7])*(1-th3[15])*(1-th3[31])
…
pr4[1]<-th4[1]*th4[2]*th4[4]*th4[8]*th4[16]+(1-th4[1])*(1-th4[3])*
   (1-th4[7])*(1-th4[15])*(1-th4[31])
…

# Conditional probabilities.

# Priors.
th1[1] ~ dbeta(1,1)
th1[2] ~ dbeta(1,1)I(0.8,1)
th1[3] ~ dbeta(1,1)I(0.9,1)
th1[4] ~ dbeta(1,1)I(0.4,0.8)
th1[5] ~ dbeta(1,1)I(0.4,0.7)
th1[6] ~ dbeta(1,1)I(0.7,0.9)
th1[7] ~ dbeta(1,1)I(0.3,0.6)
th1[8] ~ dbeta(1,1)I(0,0.5)
th1[9] ~ dbeta(1,1)I(0,0.5)
th1[10] ~ dbeta(1,1)I(0,0.5)
th1[11] ~ dbeta(1,1)I(0,0.5)
th1[12] ~ dbeta(1,1)I(0.7,1)
th1[13] ~ dbeta(1,1)I(0.7,1)
th1[14] ~ dbeta(1,1)I(0.7,1)
th1[15] ~ dbeta(1,1)I(0.7,1)
th1[16] ~ dbeta(1,1)I(0,0.1)
th1[17] ~ dbeta(1,1)I(0,0.1)
th1[18] ~ dbeta(1,1)I(0,0.1)
th1[19] ~ dbeta(1,1)I(0,0.1)
th1[20] ~ dbeta(1,1)I(0,0.1)
th1[21] ~ dbeta(1,1)I(0,0.1)
th1[22] ~ dbeta(1,1)I(0,0.1)
th1[23] ~ dbeta(1,1)I(0,0.1)
th1[24] ~ dbeta(1,1)I(0.7,1)
th1[25] ~ dbeta(1,1)I(0.7,1)
th1[26] ~ dbeta(1,1)I(0.7,1)
th1[27] ~ dbeta(1,1)I(0.7,1)
th1[28] ~ dbeta(1,1)
th1[29] ~ dbeta(1,1)
th1[30] ~ dbeta(1,1)
th1[31] ~ dbeta(1,1)
th2[1] ~ dbeta(1,1)
th2[2] ~ dbeta(1,1)
th2[3] ~ dbeta(1,1)I(0.8,1)
th2[4] ~ dbeta(1,1)
th2[5] ~ dbeta(1,1)
th2[6] ~ dbeta(1,1)I(0.7,0.9)
th2[7] ~ dbeta(1,1)I(0.3,0.6)
th2[8] ~ dbeta(1,1)
th2[9] ~ dbeta(1,1)
th2[10] ~ dbeta(1,1)
th2[11] ~ dbeta(1,1)
th2[12] ~ dbeta(1,1)I(0.7,1)
th2[13] ~ dbeta(1,1)
th2[14] ~ dbeta(1,1)
th2[15] ~ dbeta(1,1)
th2[16] ~ dbeta(1,1)
th2[17] ~ dbeta(1,1)
th2[18] ~ dbeta(1,1)
th2[19] ~ dbeta(1,1)
th2[20] ~ dbeta(1,1)
th2[21] ~ dbeta(1,1)
th2[22] ~ dbeta(1,1)
th2[23] ~ dbeta(1,1)
th2[24] ~ dbeta(1,1)
th2[25] ~ dbeta(1,1)
th2[26] ~ dbeta(1,1)
th2[27] ~ dbeta(1,1)
th2[28] ~ dbeta(1,1)
th2[29] ~ dbeta(1,1)
th2[30] ~ dbeta(1,1)
th2[31] ~ dbeta(1,1)
th3[1] ~ dbeta(1,1)
th3[2] ~ dbeta(1,1)
th3[3] ~ dbeta(1,1)I(0.8,1)
th3[4] ~ dbeta(1,1)
th3[5] ~ dbeta(1,1)I(0.4,0.7)
th3[6] ~ dbeta(1,1)I(0.7,0.9)
th3[7] ~ dbeta(1,1)I(0.3,0.6)
th3[8] ~ dbeta(1,1)
```

```
th3[9] ~ dbeta(1,1)
th3[10] ~ dbeta(1,1)
th3[11] ~ dbeta(1,1)
th3[12] ~ dbeta(1,1)I(0.7,1)
th3[13] ~ dbeta(1,1)
th3[14] ~ dbeta(1,1)
th3[15] ~ dbeta(1,1)
th3[16] ~ dbeta(1,1)
th3[17] ~ dbeta(1,1)
th3[18] ~ dbeta(1,1)
th3[19] ~ dbeta(1,1)
th3[20] ~ dbeta(1,1)
th3[21] ~ dbeta(1,1)
th3[22] ~ dbeta(1,1)
th3[23] ~ dbeta(1,1)
th3[24] ~ dbeta(1,1)
th3[25] ~ dbeta(1,1)
th3[26] ~ dbeta(1,1)
th3[27] ~ dbeta(1,1)
th3[28] ~ dbeta(1,1)
th3[29] ~ dbeta(1,1)
th3[30] ~ dbeta(1,1)
th3[31] ~ dbeta(1,1)
th4[1] ~ dbeta(1,1)
th4[2] ~ dbeta(1,1)I(0.8,1)
th4[3] ~ dbeta(1,1)I(0.8,1)
th4[4] ~ dbeta(1,1)I(0.4,0.8)
th4[5] ~ dbeta(1,1)I(0.4,0.7)
th4[6] ~ dbeta(1,1)I(0.7,0.9)
th4[7] ~ dbeta(1,1)I(0.3,0.6)
th4[8] ~ dbeta(1,1)
th4[9] ~ dbeta(1,1)
th4[10] ~ dbeta(1,1)
th4[11] ~ dbeta(1,1)
th4[12] ~ dbeta(1,1)I(0.7,1)
th4[13] ~ dbeta(1,1)
th4[14] ~ dbeta(1,1)
th4[15] ~ dbeta(1,1)
th4[16] ~ dbeta(1,1)I(0,0.4)
th4[17] ~ dbeta(1,1)I(0,0.4)
th4[18] ~ dbeta(1,1)I(0,0.4)
th4[19] ~ dbeta(1,1)I(0,0.4)
th4[20] ~ dbeta(1,1)I(0,0.4)
th4[21] ~ dbeta(1,1)I(0,0.4)
th4[22] ~ dbeta(1,1)I(0,0.4)
th4[23] ~ dbeta(1,1)I(0,0.4)
th4[24] ~ dbeta(1,1)
th4[25] ~ dbeta(1,1)
th4[26] ~ dbeta(1,1)
th4[27] ~ dbeta(1,1)
th4[28] ~ dbeta(1,1)
th4[29] ~ dbeta(1,1)
th4[30] ~ dbeta(1,1)
th4[31] ~ dbeta(1,1)
```

 # Parameters

 # Compute Prevalence (prev).

```
prev1<-th1[1]
prev2<-th2[1]
prev3<-th3[1]
prev4<-th4[1]
```

 # Compute sensitivity (se) and specificity (sp).

```
se1[1]<-th1[2]
sp1[1]<-th1[3]
se1[2]<-th1[2]*th1[4]+(1-th1[2])*th1[5]
sp1[2]<-th1[3]*th1[6]+(1-th1[3])*th1[7]
se1[3]<-th1[2]*(th1[4]*th1[8]+(1-th1[4])*th1[9])+(1-th1[2])*
   (th1[5]*th1[10]+(1-th1[5])*th1[11])
sp1[3]<-th1[3]*(th1[6]*th1[12]+(1-th1[6])*th1[13])+(1-th1[3])*
   (th1[7]*th1[14]+(1-th1[7])*th1[15])
se1[4]<-th1[2]*(th1[4]*(th1[8]*th1[16]+(1-th1[8])*th1[17])+
   (1-th1[4])*(th1[9]*th1[18]+(1-th1[9])*th1[19]))+(1-th1[2])*
   (th1[5]*(th1[10]*th1[20]+(1-th1[10])*th1[21])+(1-th1[5])*
   (th1[11]*th1[22]+(1-th1[11])*th1[23]))
sp1[4]<-th1[3]*(th1[6]*(th1[12]*th1[24]+(1-th1[12])*th1[25])+
   (1-th1[6])*(th1[13]*th1[26]+(1-th1[13])*th1[27]))+(1-th1[3])*
   (th1[7]*(th1[14]*th1[28]+(1-th1[14])*th1[29])+(1-th1[7])*
   (th1[15]*th1[30]+(1-th1[15])*th1[31]))
se2[1]<-th2[2]
…
se3[1]<-th3[2]
…
se4[1]<-th4[2]
…
```

 # Bayesp1.

```
# compute G01
for (i in 1:16)
{
d1 <-result1*log(max(result1,1)/(pr1*n1))
}
G01<-2*sum(d1[])
# generate multinomial from current estimates
result1b[1:16] ~ dmulti(pr1[1:16],n1)
# compute Gt1
for (i in 1:16)
{
d1b <-result1b*log(max(result1b,1)/(pr1*n1))
}
Gt1<-2*sum(d1b[])
# Compute Bayesp1
bayesp1<-step(G01 - Gt1)
```

 # Bayesp2

 # Bayesp3

 # Bayesp4.

```
}
```

 # Data

#qPCR 18S, PCR, IFA and PAO.

list(result1 = c(0, 4, 1, 11, 0, 0, 0, 14, 0, 1, 0, 7, 0, 0, 0, 28), n1 = 66, result2 = c(38, 3, 9, 6, 5, 1, 0, 1, 0, 0, 2, 1, 0, 0, 0, 0), n2 = 66, result3 = c(9, 9, 9, 21, 1, 1, 0, 6, 1, 1, 0, 0, 0, 0, 4, 4), n3 = 66, result4 = c(2, 5, 7, 10, 1, 2, 1, 12, 0, 1, 0, 5, 2, 2, 1, 15), n4 = 66).

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REFERENCES

1. **Berkvens, D., N. Speybroeck, A. Adel, and E. Lesaffre.** 2006. Estimating disease prevalence in a Bayesian framework using probabilistic constraints. Epidemiology **17:**145–153.
2. **Brenner, H., and O. Gefeller.** 1997. Variation of sensitivity, specificity, likelihood ratios and predictive values with disease prevalence. Stat. Med. **16:** 981–991.
3. **Bukhari, Z., and H. V. Smith.** 1995. Effect of three concentration techniques on viability of *Cryptosporidium parvum* oocysts recovered from bovine feces. J. Clin. Microbiol. **33:**2592–2595.
4. **de la Fuente, R., A. Garcia, J. A. Ruiz-Santa-Quiteria, M. Luzon, D. Cid, S. Garcia, J. A. Orden, and M. Gomez-Bautista.** 1998. Proportional morbidity rates of enteropathogens among diarrheic dairy calves in central Spain. Prev. Vet. Med. **36:**145–152.
5. **de la Fuente, R., M. Luzon, J. A. Ruiz-Santa-Quiteria, A. Garcia, D. Cid, J. A. Orden, S. Garcia, R. Sanz, and M. Gomez-Bautista.** 1999. *Cryptosporidium* and concurrent infections with other major enteropathogens in 1 to 30-day-old diarrheic dairy calves in central Spain. Vet. Parasitol. **80:**179–185.
6. **Dohoo, I., W. Martin, and H. Stryhn.** 2003. Generalised estimating equations, p. 527–531. *In* M. S. McPike (ed.), Veterinary epidemiologic research. AVC, Inc., Charlottestown, Canada.
7. **Fayer, R., L. Gasbarre, P. Pasquali, A. Canals, S. Almeria, and D. Zarlenga.**

1998. *Cryptosporidium parvum* infection in bovine neonates: dynamic clinical, parasitic and immunologic patterns. Int. J. Parasitol. **28:**49–56.
8. **Fayer, R., M. Santin, and J. M. Trout.** 2007. Prevalence of *Cryptosporidium* species and genotypes in mature dairy cattle on farms in eastern United States compared with younger cattle from the same locations. Vet. Parasitol. **145:**260–266.
9. **Gelman, A., and D. B. Rubin.** 1992. Inference from iterative simulation using multiple sequences. Stat. Sci. **7:**457–511.
10. **Geurden, T., D. Berkvens, P. Geldhof, J. Vercruysse, and E. Claerebout.** 2006. A Bayesian approach for the evaluation of six diagnostic assays and the estimation of *Cryptosporidium* prevalence in dairy calves. Vet. Res. **37:**671–682.
11. **Geurden, T., D. Berkvens, S. Casaert, J. Vercruysse, and E. Claerebout.** 2008. A Bayesian evaluation of three diagnostic assays for the detection of *Giardia duodenalis* in symptomatic and asymptomatic dogs. Vet. Parasitol. **157:**14–20.
12. **Geurden, T., E. Claerebout, J. Vercruysse, and D. Berkvens.** 2008. A Bayesian evaluation of four immunological assays for the diagnosis of clinical cryptosporidiosis in calves. Vet. J. **176:**400–402.
13. **Goris, N., N. Praet, D. Sammin, H. Yadin, D. Paton, E. Brocchi, D. Berkvens, and K. De Clercq.** 2007. Foot-and-mouth disease non-structural protein serology in cattle: use of a Bayesian framework to estimate diagnostic sensitivity and specificity of six ELISA tests and true prevalence in the field. Vaccine **25:**7177–7196.
14. **Guy, R. A., P. Payment, U. J. Krull, and P. A. Horgen.** 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl. Environ. Microbiol. **69:**5178–5185.
15. **Jiang, J., K. Alderisio, A. Singh, and L. Xiao.** 2005. Development of procedures for direct extraction of *Cryptosporidium* DNA from water concentrates and for relief of PCR inhibitors. Appl. Environ. Microbiol. **71:**1135–1141.
16. **Jothikumar, N., A. J. Da Silva, I. Moura, Y. Qvarnstrom, and V. R. Hill.** 2008. Detection and differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan assays. J. Med. Microbiol. **57:**1099–1105.
17. **Kehl, K. S., H. Cicirello, and P. L. Havens.** 1995. Comparison of four different methods for detection of *Cryptosporidium* species. J. Clin. Microbiol. **33:**416–418.
18. **McEvoy, J. M., G. Duffy, E. M. Moriarty, C. J. Lowery, J. J. Sheridan, I. S. Blair, and D. A. McDowell.** 2005. The prevalence and characterisation of *Cryptosporidium* spp. in beef abattoir water supplies. Water Res. **39:**3697–3703.
19. **McEwan, A. D., E. W. Fisher, and I. E. Selman.** 1970. A turbidity test for the estimation of immune globulin levels in neonatal calf serum. Clin. Chim. Acta **27:**155–163.
20. **Nichols, G., and B. T. Thom.** 1984. Letter. Lancet **i:**735.
21. **Parr, J. B., J. E. Sevilleja, S. Amidou, C. Alcantara, S. E. Stroup, A. Kohli, R. Fayer, A. A. M. Lima, E. R. Houpt, and R. L. Guerrant.** 2007. Detection and quantification of *Cryptosporidium* in HCT-8 cells and human fecal specimens using real-time polymerase chain reaction. Am. J. Trop. Med. Hyg. **76:**938–942.
22. **Pereira, M., E. R. Atwill, and T. Jones.** 1999. Comparison of sensitivity of immunofluorescent microscopy to that of a combination of immunofluorescent microscopy and immunomagnetic separation for detection of *Cryptosporidium parvum* oocysts in adult bovine feces. Appl. Environ. Microbiol. **65:**3236–3239.
23. **Rosenbaum Nielsen, L., and A. Kjær Ersbøll.** 2004. Age-stratified validation of an indirect *Salmonella* Dublin serum enzyme-linked immunosorbent assay for individual diagnosis in cattle. J. Vet. Diagn. Invest. **16:**212–218.
24. **Sánchez, R. G., D. R. Labarthe, R. N. Forthofer, and A. Fernandez-Cruz.** 1992. National standards of blood pressure for children and adolescents in Spain: international comparisons. Int. J. Epidemiol. **21:**478–487.
25. **Santin, M., and J. M. Trout.** 2008. Livestock, p. 451–483. *In* R. Fayer and L. Xiao (ed.), *Cryptosporidium* and cryptosporidiosis. CRC Press, Inc., Boca Raton, FL.
26. **Santín, M., J. M. Trout, and R. Fayer.** 2008. A longitudinal study of cryptosporidiosis in dairy cattle from birth to two years of age. Vet. Parasitol. **155:**15–23.
27. **Shahiduzzaman, M., V. Dyachenko, J. Keidel, R. Schmaschke, and A. Daugschies.** 2010. Combination of cell culture and quantitative PCR (cc-qPCR) to assess disinfectants efficacy on *Cryptosporidium* oocysts under standardized conditions. Vet. Parasitol. **167:**43–49.
28. **Silverlås, C., U. Emanuelson, K. de Verdier, and C. Bjorkman.** 2009. Prevalence and associated management factors of *Cryptosporidium* shedding in 50 Swedish dairy herds. Prev. Vet. Med. **90:**242–253.
29. **Sivaganesan, M., S. Seifring, M. Varma, R. A. Haugland, and O. C. Shanks.** 2008. A Bayesian method for calculating real-time quantitative PCR calibration curves using absolute plasmid DNA standards. BMC Biotechnol. **9**.
30. **Spiegelhalter, D. J., N. G. Best, B. P. Carlin, and A. der Linde.** 2002. Bayesian measures of model complexity and fit (with discussion). J. R. Stat. Soc. B **64:**583–640.
31. **Spiegelhalter, D. J., A. Thomas, N. G. Best, and D. Lunn.** 2003. Winbugs version 1.4 user manual. MRC Biostatistics Unit, Cambridge, United Kingdom.
32. **Thompson, H. P., J. S. G. Dooley, J. Kenny, M. McCoy, C. J. Lowery, J. E. Moore, and L. Xiao.** 2007. Genotypes and subtypes of *Cryptosporidium* spp. in neonatal calves in Northern Ireland. Parasitol. Res. **100:**619–624.
33. **Trotz-Williams, L. A., S. W. Martin, K. E. Leslie, T. Duffield, D. V. Nydam, and A. S. Peregrine.** 2007. Calf-level risk factors for neonatal diarrhea and shedding of *Cryptosporidium parvum* in Ontario dairy calves. Prev. Vet. Med. **82:**12–28.
34. **van Stralen, K. J., V. S. Stel, J. B. Reitsma, F. W. Dekker, C. Zoccali, and K. J. Jager.** 2009. Diagnostic methods I: sensitivity, specificity, and other measures of accuracy. Kidney Int. **75:**1257–1263.
35. **Xiao, L., L. Escalante, C. Yang, I. Sulaiman, A. A. Escalante, R. J. Montali, R. Fayer, and A. A. Lal.** 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl. Environ. Microbiol. **65:**1578–1583.
36. **Xiao, L., C. Bern, J. Limor, I. Sulaiman, J. Roberts, W. Checkley, L. Cabrera, R. H. Gilman, and A. A. Lal.** 2001. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J. Infect. Dis. **183:**492–497.
37. **Xiao, L., R. Fayer, U. Ryan, and S. J. Upton.** 2004. *Cryptosporidium* taxonomy: recent advances and implications for public health clinics. Microbiol. Rev. **17:**72–97.