Sensitivity and Specificity of Two Enzyme-linked Immunosorbent Assays and a Quantitative Real-time Polymerase Chain Reaction for Bovine Paratuberculosis Testing of a Large Dairy Herd

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ABSTRACT

Serum and fecal samples from a large California herd were used to estimate the diagnostic sensitivities and specificities of two ELISA kits and a fecal quantitative real time PCR (qPCR) for *paratuberculosis*. Both ELISA kits were marketed by the same company but one (*Mycobacterium paratuberculosis*; MP) was advertised as having increased sensitivity over an older kit that is no longer available (HerdChek; HC). Because of the large amount of data accumulated using the older kit, there was concern that the transition to a new test kit would provide significant complications in following herd level seroprevalence rates for Johne's disease. Furthermore, both kits diagnostic accuracy in large herds was not known. A 3 test (2 dependent, 1 independent) no gold standard analysis of the ELISA and qPCR results from a large dairy herd in California was conducted. Results of this study showed an improvement in the new ELISA kit compared to the previous one in terms of sensitivity (34% compared to 31%, respectively) and specificity (96% compared to 94%). The fecal qPCR assay had a sensitivity of 68% and a specificity of 96%. Prevalence of *paratuberculosis* in the study herd was 10.5%. Estimates reported here may differ from previous studies due to the differences in source herds, reference tests used and results variability due to laboratory and over time.

INTRODUCTION

Enzyme linked immunosorbent assays (ELISA) are commonly used to detect and estimate the concentration of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in cattle serum.[9,10] In 2010, the Herd Chek (HC) ELISA kit[a] marketed in the US for identifying MAP seropositive cattle was replaced by the *Mycobacterium paratuberculosis* (MP) antibody test kit[b] (Institute Pourquier, Montpellier, France) previously used in Europe.[9] Both kits' colorimetric antigen-antibody reaction is measured in optical density and interpreted relative to a plate's positive and negative controls by calculation of the sample-to-positive (S/P) ratio.[1] However, the MP kit has a higher cut-off for positive samples (≥ 0.7 as positive) compared to the HC kit (≥ 0.25 as positive). In addition, a suspect category was introduced in the interpretation of the MP kit results, perhaps in response to the previously documented variability in repeat HC ELISA testing of serum samples.[1] The higher cut-off for positive results and the additional suspect category in the MP kit compared to the HC kit raises questions about potential changes in test results interpretation under field conditions. Furthermore, a decade's worth of experience has been accumulated by diagnosticians, researchers and practitioners using the HC kit for identification of MAP seropositive cattle as part of USDA's Demonstration herd program which can be a valuable guide for the interpretation of MP kit results. In addition, use of quantitative real-time PCR (qPCR) testing to identify MAP-containing fecal or environmental samples has been supported by its high correlation with the widely accepted ante-mortem reference diagnostic test, culture on Herold's egg yolk medium (HEYM).[2,5] The diagnostic sensitivity and specificity of the MP kit in comparison to the older kit and qPCR on large dairy herds has not been evaluated. Specific reasons for the lack of sensitivity and specificity estimates for such MAP antibody and antigen detection tests from large dairy herds may be due to the difficulty identifying MAP-free large dairy herds to estimate specificity, and confirmation of MAP-infection to estimate sensitivity. Hence, a comparison of diagnostic accuracy between old and new ELISA kits is warranted, provided well documented samples from cows with known MAP serological and fecal shedding status are available. Such sensitivity and specificity estimates would allow the use of historical test results to track Johne's prevalence rates in herds that utilized HC kit and may use MP the replacement kit.

Another important aspect of the accuracy of a diagnostic test is that sensitivity and specificity estimates may vary by the population tested.[6,8,12] Hence, studies based on large herds may offer more accurate estimates of a diagnostic test's accuracies in such populations. The difficulty and expense in confirming disease-free or disease status of cows in large dairy herds can be overcome by the use of no gold standard methods developed by Hue and Walter ,[14] Georgiadis et al[13] or Branscum et al[7]. Furthermore, a Bayesian approach allows for inclusion of dependent tests such as the 2 ELISA kits in question by relaxing the conditional independence assumption and requiring prior information about the tests. Hence, the investigation reported here utilized a Bayesian approach for estimation of diagnostic sensitivity and specificity of MP, HC and qPCR assays in a known MAP infected dairy herd. A repository of serum samples from a whole herd test in a large California dairy was tested using both ELISA kits. As part of the whole herd test, fecal samples were collected simultaneously for MAP antigen detection using fecal qPCR. The objectives of this study were to estimate and compare the sensitivities and

a. HerdChek, IDEXX Laboratories Inc., Westbrook, ME
b. Mycobacterium paratuberculosis Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME
c. VetAlert, Tetracore Inc., Rockville, MD
d. Microsoft Excel® 2007, Microsoft Corp., Redmond, WA.

Table 1. *Prior estimates for model parameters to estimate the diagnostic sensitivity and specificity of two ELISAs and a quantitative real time PCR for Mycobacterium avium subsp. paratuberculosis testing*

Input Parameter		Mode (%)	Source	Percentile, limit	Beta distribution
MP ELISA	Sensitivity	27.95	Collins et al*	95th, 70	(1.9650,3.4877)
	Specificity	99.00	Collins et al*	5th , 85	(20.6368, 1.1984)
HC ELISA	Sensitivity	28.92	Collins et al*	95th, 50	(5.6019,12.3105)
	Specificity	95.26	Collins et al*	5th, 85	(35.0828,2.6959)
qPCR	Sensitivity	72.00	Alinovi et al†	95th, 90	(5.0861, 2.5890)
	Specificity	96.00	Alinovi et al†	5th, 90	(71.0559, 3.9190)
Herd prevalence		5.28	Herd test‡	95th, 30	(1.5551, 10.9573)

** Collins MT, Wells SJ, Petrini KR, et al.: 2005, Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. Clin Diagn Lab Immunol 12:685-692.*
†Alinovi CA, Ward MP, Lin TL, et al.: 2009, Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of Mycobacterium avium ssp. paratuberculosis. Vet Microbiol 136:177-179.
‡ Based on a whole herd survey using quantitative real-time PCR of fecal samples and pooled fecal samples (n=10) conducted in 2007

specificities of the MP, HC kits, and fecal qPCR when testing cows in a large dairy herd. A secondary objective of the study was to estimate the correlation and agreement between the MP and HC kits.

MATERIALS AND METHODS

Study herd

A California herd of 3577 Jersey cows was utilized for this study. The study herd had a 3.5% apparent seroprevalence of MAP based on routine testing of adult cows at the start of the dry period using the HC kit as part of the Voluntary Bovine Johnes Disease Control Program. Herd seroprevalence was confirmed by a whole herd serum ELISA test in 2007.

Sample selection

A repository of samples from a 2007 cross-sectional study to identify MAP shedders was used for the current study. The repository consisted of serum and fecal samples collected simultaneously from 3577 cows on a California dairy.[4] For the current study, 988 individual cow fecal and serum samples were identified using a stratified sampling design described elsewhere.[4]

Diagnostic testing

Serum ELISA testing for MAP antibodies using the HC and MP kits in 2010 was performed by a single laboratory[e] using the manufacturers recommended procedures[a,b]. Fecal samples were tested for MAP DNA at a testing laboratory[f] as described previously.[3] Results of the qPCR were truncated at 50 Ct the maximum number of cycles run. All test results (HC and MP ELISA and qPCR) were housed and matched in a relational database.[g]

Interpretation of diagnostic test results

QPCR results were interpreted using the manufacturer's cut-off of < 42 cycles-to-threshold (Ct) as positive for MAP DNA. For the MP kit, the manufacturer's recommended cut-off of ≥ 0.7 S/P ratio for a positive sample was observed otherwise suspect (0.6< S/P < 0.7) and negative (≤ 0.6 S/P) test results were interpreted as negative or not positive (< 0.7 S/P ratio). For the HC kit results, the manufacturer's recommended cut-off of ≥ 0.25 S/P ratio for a positive test

e. California Animal Health and Food Safety Laboratory, Davis, CA
f. Johne's Research Laboratory, New Bolton Center, University of Pennsylvania, PA
g. Microsoft Access, 2007, Microsoft Corp., Redmond, WA.
h. Winbugs version 1.4.3

Table 2. *Test results of 988 cows on a California dairy tested for Mycobacterium avium subsp. paratuberculosis (MAP) antibodies using two ELISA and MAP DNA in fecal samples using quantitative real time PCR*

MP ELISA	HC ELISA	qPCR	Number of cows
Negative	Negative	Negative	776
Negative	Negative	Positive	70
Negative	Positive	Negative	5
Negative	Positive	Positive	3
Positive	Negative	Negative	12
Positive	Negative	Positive	14
Positive	Positive	Negative	24
Positive	Positive	Positive	84

result was observed.

Statistical analysis

Bayesian estimates of sensitivity and specificity were derived using a software package.[h,15] using a 3 test (2 dependent, 1 independent) 1 population no gold standard analysis.[7] Briefly, beta distributed priors for the sensitivity and specificity of each of the assays and prevalence of MAP in the study herd were prescribed as detailed in Table 1. The distribution of model estimates (posterior) were obtained by employing a 2 chain model each with a unique set of initial values and traced to the end of the MC simulation to assess convergence at 100,000 iterations with the first 10,000 discarded. Subsequently, estimates reported were those from an additional 100,000 iterations to verify that estimates were stationary. Winbugs diagnostics assessed included the Brooks-Gelman-Rubin statistic, quantile and autocorrelation plots.

After categorizing both kits test results as positive or negative, the agreement between both kits was estimated using the survey-weight adjusted Kappa coefficient. Variance for Kappa was estimated using Taylor linearization[11] and used to test the hypothesis that kit agreement was not due to random chance.

RESULTS

Table 2 summarizes results of qPCR and the two ELISA kits for all 988 cows. A total of 171 of the 988 fecal samples tested positive by qPCR. A total of 87 and 98 serum

Table 3. *Sensitivity and specificity of two ELISA kits for identification of Mycobacterium avium subsp. paratuberculosis seropositive cows and a quantitative real time PCR assay for identification of MAP in fecal samples.*

Diagnostic Assay	Median Sensitivity* %	95% probability limits		Median Specificity† %	95% probability limits	
		lower	upper		lower	upper
MP ELISA‡	34.2	8.4	70.0	95.8	84.9	99.6
HC ELISA§	30.6	15.0	50.1	93.6	85.1	98.2
qPCR¦	67.7	37.5	90.0	95.2	90.0	98.2

** Proportion† Proportion ‡ b. Mycobacterium paratuberculosis Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME*

§ HerdChek, IDEXX Laboratories Inc., Westbrook, ME

¦ VetAlert, Tetracore Inc., Rockville, MD

Table 4. *Survey weighted agreement proportions between a ELISA kit and its newly marketed replacement for antibodies against Mycobacteirum avium subspecies paratuberculosis in 988 serum samples representative of a 3577 Jersey dairy herd in California.*

		Herdcheck* ELISA		
		Negative (%)	Positive (%)	
Mycobacterium paratuberculosis	Negative (%)	90.3	0.2	90.5
ELISA† (replacement)	Positive (%)	1.1	8.4	9.5
		91.4	8.6	

** HerdChek, IDEXX Laboratories Inc., Westbrook, ME*
† Mycobacterium paratuberculosis Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME

samples of cows with fecal samples that tested positive by qPCR also tested positive for MAP antibodies using the HC and MP kits, respectively. Bayesian estimates of the sensitivities and specificities of the three diagnostic tests are presented in Table 3. The study herd prevalence was estimated at 10.5% with 95% credible interval of 1.7% to 30.0%. The survey-adjusted Kappa coefficient for both kits agreement beyond chance was 92.1% (P value <0.01) which showed that the kits had excellent agreement beyond chance. The survey weighted agreement proportions for both ELISA results of the 988 serum samples representative of the 3577 cows are presented in Table 4.

DISCUSSION

Serum ELISA testing for exposure to MAP in adult cattle has suffered from relatively low sensitivity but good specificity. Results of this study showed that the new MAP ELISA kit (MP) had a slight improvement in diagnostic sensitivity and specificity compared to the previous kit (HC). The estimated sensitivity of the new kit was 34% compared to 31% for the previous kit. Similarly, the new kit's specificity was higher compared to that of the previous kit (96% compared to 94%, respectively). However, estimates for the new kit's sensitivity and specificity were less than that claimed by the manufacturer. (http://www.idexx.com/pubwebresources/pdf/en_us/livestock-poultry/map-ab-competitive-information-sheet.pdf). Such a difference may be a reflection of differences in study design between the original validation performed by the manufacturer and the study reported here. Two study design factors that could potentially influence the sensitivity calculations are the population of animals sampled and the method used to determine the 'true' infection status of the individual animals. In the study reported here animals from a single herd were sampled. The conditions of the original validation work are uncertain but it is likely that samples from animals of known MAP infection status were used for the positive group and animals originating from herds with extensive negative test results were used for the negative group. Such a divergent population could yield different test performance results than would be calculated from a large herd with a MAP prevalence such as was used in this study.

Collins et al provided a detailed comparison of several ELISA assays including those reported in this study.[9] However, this study differs in its target population being a large herd, a common characteristic of California dairies. In addition, the stratified random sample used for the study reported here represented all the animals in the herd and hence included negative and positive animals with different shedding stages. Furthermore, laboratory related variability in assay results was minimized by arranging for sample testing consecutively at the same laboratory and by the same technical staff.

To estimate diagnostic test sensitivity, suspect results can be classified as negative to avoid a potentially false increase in the sensitivity estimate. However, such an approach may also result in an increase in false negative animals. Similarly, when estimating specificity, suspect results can be classified as positive in an effort to avoid a potentially false increase in specificity. This approach has the potential to cause a false positive result. In the study reported here sensitivity and specificity were both estimated after classifying suspect results as negative. However, specificity estimates for both kits did not change significantly when suspects were classified as positive (data not shown). Previous studies have shown that the older kit had variability in test results for individual samples when tested multiple times.[1] The new test kit includes a suspect category which reflects the uncertainty inherent in any test of a biological phenomena.

Johne's disease control programs have been ongoing in many states for more than 10 years and one concern with the transition from a popular ELISA test kit to another with which there has been less experience was how test results from the two kits would compare. The two kits use a different S/P ratio for the positive cut-off value and therefore the numerical values cannot be considered equivalent. Results of this study confirm the manufacturer's claim of improved sensitivity and specificity. Furthermore, the results of this study show that the two kits have good agreement (kappa = 92.1%) with comparable categorical results (positive, not-positive) and there should not be significant classification problems from the use of results from the two kits in following herd level seroprevalence over time.

CONFLICTS OF INTEREST

The authors declare that they had no conflicts of interest with respect to their authorship or the publication of this manuscript.

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