Short communication

Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* ssp. *paratuberculosis*

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A R T I C L E   I N F O

A B S T R A C T

A goal of Johne's disease control programs is to accurately detect *Mycobacterium avium* ssp. *paratuberculosis* (MAP) infected cattle as quickly as possible to reduce disease transmission. A newly introduced real-time PCR provides results rapidly, but its accuracy in the field has not been evaluated. Fecal and serum samples collected from dairy cows in northern Indiana were used to estimate the sensitivity and specificity of a newly licensed real-time PCR test for direct fecal detection of *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Results of the real-time PCR were evaluated in parallel with solid and liquid media culture systems and a serum ELISA for detection of MAP antibodies to determine the accuracy of the real-time PCR and the tests' potential usefulness in the field. A total of 143 samples were tested by all four methods. Using prior published estimates for sensitivity and specificity of each of the tests and Bayesian methodology, the sensitivity and specificity of the real-time PCR test was estimated to be 0.60 and 0.97, respectively. The accuracy of real-time PCR (0.90) was comparable to both solid (0.91) and liquid (0.93) culture. Because real-time PCR accuracy is comparable to standard culture methods, it is a useful new test. In addition, test results are obtained as rapidly as an ELISA, but are more accurate than the ELISA (0.82). This makes real-time PCR an attractive test and should shorten the quarantine period required for new purchases of unknown MAP-status animals into herds participating in an MAP control program.

1. Introduction

To date, accurate ante-mortem diagnostic tests to detect Johne's disease (JD) and *Mycobacterium avium* ssp. *paratuberculosis* (MAP) infection remain time-consuming. Numerous studies have been conducted to evaluate the accuracy (sensitivity and specificity) of the tests available to detect MAP (Collins et al., 2006). In general, MAP detection tests are designed for either diagnostic (identify an infected animal) or surveillance (identify an infected herd) purposes. However, these tests are not always applied in the manner for which they were designed (Tavornpanich et al., 2006). For example, the ELISA is intended as a herd-level surveillance tool, and yet it is often used for diagnostic purposes. One assumes the reason for this is primarily the short turn-around time and cost. Results for MAP–ELISA are available in one day, versus 8–12 weeks for MAP culture; median ELISA cost was $5, and median culture cost was $19 in 2006 (Collins et al., 2006).

Recently, the USDA licensed a real-time PCR test kit (Tetracore's VetAlert™ Johne's). This kit is approved to detect MAP in cattle through direct detection in fecal samples (www.tetracore.com). Results are available in one

day and the cost to the producer is expected to be $20–25 a sample. The test kit is designed to aid diagnosis of Johne's disease; it can also be used in surveillance when testing pooled fecal samples.

Although a new MAP detection method has been added to the list of approved Johne's test methods, the test has yet to be formally evaluated in the field. Our purpose was to evaluate the accuracy of real-time PCR to detect MAP in dairy cattle, compared to more conventional solid media, commercial liquid media, as well as a serum ELISA.

2. Materials and methods

2.1. Animal selection

Cows were selected using a case-control design. Dairy cows previously identified as fecal culture positive (cases) were targeted for sampling during routine JD testing. Control samples were the next cow sampled after the case. If the next cow was a case, the next two animals were then selected as controls. Laboratory personnel were blinded to the case status. Cow information, such as age, days in lactation, calving date and breed were collected and tabulated (Microsoft Excel, 2003).

Cattle were selected from two northern Indiana dairy herds participating in the national Voluntary Johne's Disease Herd Status Program. All cows, except for one Jersey, were Holstein breed. Herd characteristics were one open enrollment herd milking 900 cows, and one closed herd milking 300 cows.

Fecal samples were collected from the rectum of enrolled cows and processed the next business day at the Purdue University Animal Disease Diagnostic Laboratory after overnight storage at 4 °C. Whole blood samples were collected from the tail vein, stored at 4 °C overnight, and processed the next business day following manufacturer's guidelines (Biocor ELISA, Omaha, NE). All samples were collected under Purdue University Animal Care and Use Committee guidelines.

2.2. Laboratory methods

Laboratory methods used have been previously described (Stabel et al., 2004; Tavornpanich et al., 2004). These authors provide an excellent description of methodology for both solid and liquid culture using Herrold's egg-yolk media (HEYM) and Trek ESP. Each fecal sample processed on HEYM culture was plated onto four media tubes and maintained for 16 weeks. Real-time PCR methodology followed instructions included with the Tetracore VetAlert™ kit, using a Corbett Research Rotor-Gene 3000 thermocycler (BioRad iCycler iQ cycling® program settings applied).

2.3. Statistical methods

Estimates of test sensitivity and specificity were made using a Bayesian statistical (no gold standard) approach via the TAGS program (www.epi.ucdavis.edu/diagnostictests/tags.html). Assumptions made were based on published sensitivity and specificity for the evaluated tests. HEYM was assumed to have a sensitivity of 0.60 and a specificity of 0.95, liquid culture was assumed to be slightly more sensitive and specific than solid media (sensitivity 0.65 and specificity 0.99), and serum ELISA was assumed to be 0.30 sensitive and 0.95 specific (Collins et al., 2006; Tavornpanich et al., 2004). Sensitivity and specificity data for the real-time PCR was provided by the manufacturer (www.tetracore.com/real-time-pcr-detection) −0.71 and 0.95, respectively. Conditional independence was assumed between tests (Pouillot et al., 2002). The prevalence of infection was assumed to be 0.3. Accuracy was calculated from resultant values of TAGS calculations: (sensitivity × prevalence) + specificity × (1 − prevalence). Evaluation of differences in test outcome by median age and by lactation was performed using a non-parametric Wilcoxon Rank Sum Test and the Mann–Whitney U statistic. A type I error of 0.05 was used for statistical significance.

3. Results

Fecal and blood samples were obtained from 143 northern Indiana dairy cows. Cows were tested from two dairy herds; combined, the herds amounted to more than 1200 milking cows. One herd was entirely Holstein, the other was a mixture of Holsteins, Jerseys, and first generation Holstein/Jersey crosses; however, only Holsteins and one Jersey were tested in the second herd. Lactations sampled ranged from 1st to 8th (median – 1st lactation). Cow age ranged from 3 to 11 years (median – 4 years). The raw data for the four tests evaluated are shown in Table 1.

One hundred and two samples were negative on all tests (71.3%). Eight samples (5.6%) were positive on HEYM and real-time PCR tests. Seven samples were positive on all tests (4.9%). Six samples (4.2%) were positive on real-time PCR alone. Five samples each (3.5%) were positive on HEYM and ESP; another five were positive by real-time PCR and ESP tests (Table 1).

The median age of cows test positive by HEYM was significantly ($P < 0.02$) greater than the median age of cows test negative, five versus four years, respectively. No other significant ($P > 0.05$) differences in age or lactation

Table 1

Test results for each of four laboratory tests (solid and liquid culture using Herrold's egg-yolk media – HEYM, real-time PCR – RT-PCR, Trek ESP and the Biocor ELISA) used to evaluate 143 dairy cows for MAP infection (0 – MAP test negative, 1 – MAP test positive).

Diagnostic test				Number of samples
HEYM	RT-PCR	ESP	ELISA	
0	0	0	0	102
1	0	0	0	4
0	1	0	0	6
1	1	0	0	8
0	0	1	0	2
1	0	1	0	5
0	1	1	0	5
1	1	1	0	2
1	0	1	1	1
0	1	1	1	1
1	1	1	1	7

status were found between positive and negative cows for any of the other test methods evaluated.

Prevalence of fecal MAP infection, estimated by TAGS, was 0.24. TAGS-estimated sensitivities for solid culture, liquid culture and real-time PCR were 0.72, 0.65 and 0.72, respectively. TAGS-estimated specificities were 0.98, 0.99 and 0.96, respectively. Estimated serum ELISA sensitivity was 0.26, and specificity was 1.00. When data from the two herds were analyzed separately, no significant differences ($p > 0.05$) in the TAGS estimated sensitivities and specificities of solid culture, liquid culture and real-time PCR were found.

Accuracy of each of the tests, based on the above calculated sensitivities, specificities, and prevalence were 0.91 (solid culture), 0.93 (liquid culture), 0.90 (real-time PCR) and 0.82 (serum ELISA).

4. Discussion

Test sensitivity for culture methods and real-time PCR, as well as test accuracy, are comparable. This clearly demonstrates that in field applications, real-time PCR is as useful as solid or liquid culture methods while providing the producer with test results within hours, not weeks. Serum ELISA, although not as accurate as the other tests evaluated, continues to be a useful test because of its rapid turn-around. Now, with real-time PCR, more accurate results can be available as fast as for ELISA.

The goal of Johne's testing is to accurately identify MAP infected cattle as quickly as possible to reduce transmission to offspring and to the infected animal's cohort. Although currently available MAP detection tests are not perfect, rapidity of results remains essential to decision making. Waiting 8–16 weeks for results of fecal culture is now unnecessary with the introduction of the real-time PCR test to the laboratory, especially given that the sensitivity and specificity of real-time PCR is essentially the same as fecal culture. Judicious use of the real-time PCR will give the producer results in 24 h, as is the case with the MAP–ELISA test, but with more accuracy than the ELISA. Large holding areas for newly purchased cattle placed into quarantine uses valuable resources. If MAP infected cattle can be identified in a matter of days, rather than waiting for results of fecal culture, the quarantine period can be substantially reduced. Due to the increased cost of the real-time PCR test, compared to ELISA, pooling of fecal samples may provide increased cost effectiveness.

Results of the current study agree with published reports of sensitivity and specificity for solid and liquid culture (Collins et al., 2006). Liquid culture methods have already halved the time to results for culture methods. Because real-time PCR accuracy is comparable to culture methods, time to results can again be shortened greatly, helping producers make rapid animal management decisions.

Use of the Bayes' theorem to evaluate data in this study reflects our current understanding of available MAP detection tests: no ante-mortem test has high enough sensitivity to be considered a "gold" standard for all herds and infection levels. The advantage of using Bayesian methodology to evaluate these tests is that results from a gold standard test are not needed. However, because of the use of information from all tests performed to estimate sensitivity and specificity, no evaluated test would be expected to be perfect. Thus, in some instances, one test might detect MAP whereas another test might not, leading to apparent false-negative and false-positive test results. Because the only truly specific test is histopathology, and in the field this information is usually unavailable, minor discrepancies between test results are expected and might be explained as a consequence of the statistical methodology used, rather than a real biological phenomenon.

In recent reviews, Johne's disease tests have frequently been evaluated using Bayes' theorem methods. Whilst this approach assigns equal opportunity for test performance, it assumes conditional independence between the tests being evaluated. This assumption may not be valid when evaluating liquid and solid culture techniques, since both methods use the same initial preparation steps. However, our results agree with those of previous researchers using traditional statistical analysis methods; therefore, violation of this assumption is unlikely to have substantially biased results in the present study.

5. Conclusion

The real-time PCR test for detection of presence of MAP in bovine feces is comparable to culture methods, and gives fast results for better on-farm decision making.

Acknowledgements

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