Evaluation of a Commercial Enzyme-Linked Immunosorbent Assay for the Diagnosis of Bovine Tuberculosis from Milk Samples from Dairy Cows

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Milk samples from dairy cows provide a ready source of material for measuring antibody responses to Mycobacterium bovis antigens. In this study, we evaluated the IDEXX enzyme-linked immunosorbent assay (ELISA) for the measurement of antibody responses to M. bovis antigens MPB70 and MPB83 in milk samples from New Zealand cattle. Test sensitivities for individual milk and serum samples were assessed in samples collected from 44 M. bovis-infected cows, and test specificities were assessed in milk samples collected from 356 cows from tuberculosis (TB)-free herds. Milk vat samples were collected from 505 herds from regions with relatively high or low prevalences of infection. The ELISA had a sensitivity of 50% and a specificity of 97.5% for milk samples, and the test sensitivities for milk and serum samples were the same. Dilution of the positive test milk samples in milk from noninfected cows at 1/10, 1/20, and 1/50 dilutions reduced the proportions of positive responses to 13/21, 9/21, and 4/21, respectively. Small differences were observed in the ELISA responses of milk samples from individual TB-free cows collected at different times during lactation. No significant differences were detected in the ELISA responses of milk vat samples collected from infected and noninfected herds. This study shows that milk samples can be substituted for serum samples for screening individual cows for M. bovis infection, and pooling of milk samples from 10 to 20 animals can result in a reduction in the sensitivity by approximately 50%. However, screening of milk vat samples is unlikely to be useful in countries with low prevalences of M. bovis in cattle and large herd sizes.

Bovine tuberculosis (TB) caused by Mycobacterium bovis is a major economic problem in many countries, and control of this chronic disease in domestic livestock has often proved difficult. Although the disease has been eradicated from cattle in a number of countries through the implementation of a “test-and-slaughter” control program, this strategy has been less effective in countries which have a wildlife reservoir of M. bovis infection and has been impractical in countries where the strategy is unaffordable or socially unacceptable (1). The test-and-slaughter programs have been based on screening of animals using the tuberculin skin test, which measures delayed hypersensitivity responses to purified protein derivative (PPD) prepared from M. bovis or which, in some countries, compares PPDs prepared from M. bovis and Mycobacterium avium. More recently, the whole-blood gamma interferon (IFN-γ) test has been used in conjunction with the skin test to improve specificities by retesting skin test reactor cattle or to improve specificities by retesting skin test-negative animals from chronically infected herds (2, 3). Although the IFN-γ test has made a valuable contribution in improving the accuracy of diagnosing TB in cattle, additional types of assays are needed, particularly assays which are inexpensive and which can potentially be used on pooled samples from groups of animals or identify animals not responding in cell-based assays.

Serological assays have shown promise for diagnosing M. bovis infections in animals which have not responded in skin tests (4, 5, 6), particularly those which have severe pathology and are more likely to shed M. bovis. A serological assay developed to detect antibodies to M. bovis in milk samples provides advantages in that milk samples are routinely collected for dairy herd improvement testing and can be pooled from groups of animals. In regions of New Zealand which are considered free of bovine TB, the interval between tuberculin skin tests has been extended to 3 years. The use of an inexpensive screening assay such as a pooled milk serological test for bovine TB in the interval between skin tests might provide added assurance that the herds remain free of TB. An economic analysis of the control strategies for bovine TB surveillance indicated that enzyme-linked immunosorbent assay (ELISA) testing of bulk milk samples may be a cost-effective strategy if the testing became feasible (7). Encouraging results for the detection of antibodies to M. bovis in individual and bulk milk samples were recently reported (6, 8), and the detection of antibodies in bulk milk samples has been used in control programs for the diagnosis of brucellosis, enzootic bovine leukosis, and Johne’s disease in cattle (9–11). However, one of the concerns with the use of serological tests for the detection of M. bovis infection in cattle has been the variation in the sensitivities of tests when applied to sera from M. bovis-infected cattle from different countries. Waters et al. (6) reported sensitivities of 74% for the IDEXX serological test for sera from M. bovis-infected cattle from Great Britain (n = 184), 69% from Ireland (n = 130), 46% from the United States (n = 122), and 40% from New Zealand (n = 42). These variations may
have resulted from cattle being at different stages of infection or from differences in the antigenicities or virulence of the *M. bovis* strains. In addition, there might have been differences in how the diagnostic tests were applied; whether blood samples for serology were collected following tuberculin skin testing, possibly boosting antibody responses or blood sample collection for serology, was not related to the application of the skin test. The sensitivities of the serological tests appeared to be lower in countries where control of the disease has been more successful, such as the United States and New Zealand, than in countries with less successful control, such as Ireland and Great Britain. As of June 2012, only 70 cattle and farmed deer herds in New Zealand were classified as being infected with bovine TB (12). The current study was undertaken to determine whether a milk serological test can be a valuable test in a country which has a low incidence of bovine TB in domestic animals and also in which infected animals are generally detected at an early stage of the disease.

**MATERIALS AND METHODS**

**Samples from *M. bovis*-infected cows.** Milk and/or blood samples were collected from 260 cows which had reacted positively in the caudal fold tuberculin skin test as part of the routine testing in the period from September 2010 to May 2012. These animals were from 22 dairy herds located on the West Coast of South Island, New Zealand, a region where TB is endemic, and from 4 herds from other regions which had a history of *M. bovis* infection. The majority of the samples in the 2010–2011 milking season (*n* = 72) were collected in the period of 10 to 30 days after injection of the skin test reagents when blood samples were collected for the whole-blood gamma interferon (IFN-γ) test (Bovigam test; Prionics AG, Schlieren, Switzerland), while samples in the 2011–2012 milking season (*n* = 188) were predominantly collected at the time of reading of the skin test, as this was considered more time efficient. A total of 135 animal necropsies were performed in accordance with the decision to slaughter TB reactor cattle based on the disease history of the herd and results of the whole-blood IFN-γ test using previously described cutoff values (3). Forty-four cows were classified as infected with *M. bovis*, and these animals came from 11 herds, of which 9 were located on the West Coast, 1 in Northland, and 1 in Southland. Thirty-seven of these animals had macroscopic tuberculous-like lesions, and the remaining 7 animals had no detectable tuberculous-like lesions, but *M. bovis* was cultured from their pooled lymph nodes. The definition of *M. bovis* infection was based on the culture of *M. bovis* by Bactec and confirmation by Accuprobe or typical tuberculous-like lesions with histopathological confirmation. Confirmation by histopathology was used only when more than three animals from a herd had tuberculous-like lesions on one occasion, and samples from three animals with lesions had been collected for culture of *M. bovis*. If no lesions were observed in animals from high-risk herds, a pool of lymph nodes (retropharyngeal, bronchial, mediastinal, and tracheobronchial) was submitted for culture of *M. bovis*. Milk and serum samples were collected from 38 of the 44 animals classified as *M. bovis* infected to allow comparisons between antibody responses in milk and serum samples from the same animals collected on the same day. The 216 animals that were tuberculin reactors but were not classified as infected with *M. bovis* were excluded from the study, as it was not certain whether they had been exposed to *M. bovis*.

Milk samples from the animals were centrifuged at 400 × g for 10 min before an aliquot of skim milk below the fat layer was removed and stored at −80°C until assaying. Sera were collected from the blood samples and stored at −20°C until assaying. Samples from tuberculin reactor animals were processed in the AgResearch laboratory. All animal manipulations were approved by an independent animal ethics committee.

**Samples from cows from TB-free regions.** In July 2011, milk samples for serology were randomly collected during routine milk production herd testing from 245 cows from four herds in which the cows calved throughout the year. These herds had been classified as free of TB for >10 years and were located in TB-free regions of New Zealand. Only cows that calved between February and May were selected for the trial. Additional milk samples for serology were collected in the 2011–2012 milking season during routine milk production herd testing from another four herds in which the cows calved in spring and were dried off in late autumn, with all cows calving between July and September. For these herds, milk samples were collected at multiple times during the season to determine whether there were variations in the responses in the milk ELISAs at different stages of lactation. Milk samples were collected from a group of cows in the early (October) and late (April) stages of lactation from three TB-free herds and the early (October), mid (February), and late (April) stages of lactation from a group in another TB-free herd. These herds were located in TB-free regions and had been classified as free of TB for >10 years. Skim milk aliquots from these milk samples and from the bulk milk vat samples were separated in the Livestock Improvement Corporation laboratory.

**Bulk milk vat samples.** Milk samples were collected in April 2012 from the milk vats of 394 dairy herds on the West Coast of South Island (the New Zealand region with the highest prevalence of bovine TB) and 111 herds from Taranaki (the region with no wildlife reservoirs for *M. bovis* infection and a very low prevalence of bovine TB). Forty-eight herds on the West Coast were classified as infected with TB during the period from 1 April 2012 to 1 July 2013, of which 20 herds had been classified as infected with TB during the period of 1 April 2012 to 1 July 2012. The aim was to determine if there was any association between milk vat serology in April 2012 and infected herd status over the subsequent 13 to 14 months (1 April 2012 to 1 July 2013) or close to the time of collection of the milk vat sample (1 April 2012 to 1 July 2012). A herd is classified as infected if one or more cases of TB are diagnosed and the herd is then placed on movement control (13). The infected herd status is lifted when all animals in a herd test negative in two consecutive tuberculin skin tests no less than 6 months apart and, for herds which have been persistently infected, when all animals in a herd test negative in two consecutive tuberculin skin tests followed by all animals testing negative in the whole-blood IFN-γ test. All the Taranaki herds sampled were classified as noninfected for bovine TB in 2012. For the 2011-2012 period, the average dairy cow herd sizes were 393 on the West Coast and 280 in Taranaki (see New Zealand dairy statistics at [http://www.dairynz.co.nz/dairystatistics](http://www.dairynz.co.nz/dairystatistics)).

**IDEXX *M. bovis* antibody ELISA.** The procedure used for determining antibody responses in the ELISA was as described by the manufacturer (IDEXX Laboratories, Westbrook, ME), and all testing was undertaken in the AgResearch laboratory. Briefly, serum samples were diluted 1:50 and milk samples 1:1 in the sample diluent supplied with the kit. Then 100 µl/well of the diluted sample was added to a 96-well microtiter plate precoated with mycobacterial antigens (MPB70 and MPB83) and incubated at room temperature for 60 min. After the plates were washed, 100 µl of a monoclonal anti-bovine IgG-horseradish peroxidase conjugate was added to each well, and the plates were incubated for 30 min, followed by another washing step. 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (100 µl/well) was added to each well, and the plates were incubated for 15 min at room temperature. Color development was stopped by the addition of 100 µl of stop solution, and the plates were read at 450 nm (in an ELISA microtiter reader). Results are expressed as the ratio of the sample optical density (OD) minus the mean kit positive-control OD to the mean kit negative-control OD minus the mean kit negative-control OD (S/P ratio). A positive result was defined by the manufacturer as an S/P ratio of ≥0.3.

To determine if the serological test can identify infected animals from pooled milk samples, milk samples from 21 animals classified as infected with *M. bovis* were individually diluted in skim milk from noninfected cows at dilutions of 1/10, 1/20, and 1/50 before diluting 1:1 in the sample diluent per the kit instructions.

**Statistical analyses.** Spearman’s rank correlation was calculated to determine the relationship between the milk and serum samples of the.
bovis-infected cows using \( \log_{10} \)-transformed S/P ratios. The ELISA responses for milk samples collected at different stages of lactation from the cows from the four TB-free herds were analyzed by a mixed-effects model with the random effect of cow nested within herd and the fixed effect of lactation stage (early and late). Meanwhile, the fourth TB-free herd data were utilized independently to detect the effect of lactation stage (early, mid, or late). The Mann-Whitney U test was utilized for comparing the responses of the milk vat samples from the herds classified as infected with M. bovis with those of the noninfected herds and was performed using Minitab 16. All the other analyses were undertaken using R statistical software. A receiver operating characteristic (ROC) curve was produced by R software, which incorporated data used for estimating sensitivities and specificities, and this provided a graphical plot to illustrate the accuracy of the milk antibody test for the diagnosis of bovine TB.

RESULTS AND DISCUSSION

Forty-four animals were classified as infected with M. bovis, and milk samples were collected from all these animals; serum samples were also collected from 38 of them. Milk samples from 22 of these animals were positive in the IDEXX ELISA (S/P ratio, \( \geq 0.3 \)), which equated to a test sensitivity of 50% (95% confidence limits, 35.8% and 64.2%). The range of responses in the test is shown in Fig. 1. The distinction between the positive and negative responses in the test was reasonably clear, with 17/22 of the positive responses having an S/P ratio of \( >1.0 \) and 18/22 of the negative responses having an S/P ratio of \( <0.1 \). The median S/P ratio for the 22 animals with positive milk ELISA responses was 3.51 (range, 0.33 to 6.47). Of the 7 animals which were M. bovis culture positive but had no detectable tuberculous lesions, 2 were positive in the milk ELISA. It has been reported that serum antibody responses in cattle are boosted following tuberculin skin testing (14, 15, 16). Although this was not tested in the current study, the proportion of positive milk antibody responses from M. bovis-infected cattle in the 2010-2011 milking season was 7/11 for milk samples collected predominantly 2 to 4 weeks after skin testing and 15/33 in the 2011-2012 milking season with collection at the time of the skin test reading.

The test sensitivity for the milk samples was lower than the 88% sensitivity reported by Jeon et al. (8), although the TB status of the animals in that study was not confirmed by slaughter of the animals, and the tuberculin skin test was used as the reference test. However, the test sensitivity in the current study was comparable to the 40% sensitivity reported for serum samples from M. bovis-infected animals from New Zealand tested by the IDEXX test in another study (6). Studies using sera from Irish cattle indicated that IDEXX TB test sensitivities increased markedly with the severity of the disease (6).

Twenty-one of the milk samples which were positive in the serological test were subsequently retested to determine if the samples were positive following pooling at a 1/10, 1/20, or 1/50 dilution in skim milk from noninfected animals. This analysis was undertaken at the end of the study using samples which had been refrozen. All samples, including those diluted in milk from noninfected animals, were diluted 1:1 in sample diluent before testing, as described for the original samples. All samples previously shown to be positive in the test were confirmed as positive (S/P ratio, \( \geq 0.3 \)), while the proportions of the milk samples that were positive when diluted in milk from noninfected animals at 1/10, 1/20 and 1/50 dilutions were 13/21, 9/21, and 4/21, respectively. As the test had an initial sensitivity of only 50%, pooling the milk samples in groups of 10 to 20 animals can lower the detection rate to approximately 25% at the recommended cutoff value. Testing of the pooled milk samples would only be used as a prescreening test, and if a pooled sample was positive, individual samples would need to be retested to identify the ELISA-positive cow. Hence, it may be appropriate to use a lower cutoff value for the pooled (or vat) samples to improve the sensitivity, even at the expense of the specificity; however, this option needs to be explored more fully.

Both milk and serum samples were collected from 38 of the 44 M. bovis-infected animals. There was a strong positive linear correlation between the responses in milk and serum samples from the same animals (Spearman’s rank test, \( \rho = 0.89, P < 0.001 \)) (Fig. 2). For these 38 animals, the test sensitivity was 52% (20/38) for the milk and serum samples, and 17 animals had positive responses in both the milk and serum ELISAs (Table 1). The median for a positive response in these animals was an S/P ratio similar to those for the milk and serum ELISAs (3.99 [range, 0.33 to 6.42] and 3.72 [range 0.39 to 11.59], respectively). High correlations between the milk and serum antibody responses from the same

![FIG 1](http://cvi.asm.org) Distribution of the antibody responses in the IDEXX ELISA for Mycobacterium bovis-infected cows. Results are expressed as the number of animals (count) and the response shown as the sample-negative/positive-negative (S/P) ratio. The dashed line indicates the cutoff S/P ratio of \( \geq 0.3 \).

![FIG 2](http://cvi.asm.org) Correlation between the antibody responses in the serum and milk samples from Mycobacterium bovis-infected cattle measured in the IDEXX ELISA. Results are expressed as \( \log_{10} \)-transformed S/P ratios (Spearman’s rank correlation \( \rho = 0.89, P < 0.001 \)).
animals have also been reported for a TB ELISA (8) and an *M. avium* subsp. *paratuberculosis* ELISA (11).

The test specificity for the milk ELISA was 97.5% (95% confidence limits, 95.2% and 98.7%) for cows from herds classified as TB free; there were 9 false-positive responses from 356 animals. The median S/P ratio for these 9 positive responders was 0.41 (range, 0.30 to 2.20).

In the comparison of antibody responses in the individual animals from the four herds in the early and late lactation stages, there was a slightly lower significant response in the early lactation stage (*P* < 0.05) (Table 2). A comparison of the three time points in individual animals from one herd revealed a slightly but significantly higher response in the early lactation stage than that in the midlactation stage (*P* < 0.05) (Table 2). A negative S/P ratio occurred when the milk sample OD was less than that for the negative control. It was not possible to collect milk samples from infected animals at different time points during lactation, as these animals had to be slaughtered once TB was suspected. However, one animal from a TB-free herd produced a false-positive response in October 2011 (S/P ratio, 0.346) and was again positive in April 2012 (S/P ratio, 0.304). The recommended time for collection of bulk milk samples for the detection of antibodies to *M. avium* subsp. *paratuberculosis* is the early or late lactation stage, when colostral antibodies are increased or when the dilution effect of peak milk production has passed (11). However, the collection of milk samples in the week after calving should be avoided because nonspecific effects may arise, as has been observed in ELISA testing of milk samples collected at this time for antibodies to *Mycobacterium kansasii* (5, 16). All 394 vat samples from the West Coast were also tested by the IDEXX *M. avium* subsp. *paratuberculosis* ELISA in the Livestock Improvement laboratory, and no correlation was demonstrated between TB and *M. avium* subsp. *paratuberculosis* ELISA results (data not shown).

The median milk ELISA result for the milk vat samples collected in April 2012 from the 394 dairy cow herds on the West Coast was an S/P ratio of 0.025 (range, 0.075 to 0.559), and the median for the 111 Taranaki herds was 0.029 (range, 0.042 to 0.007). The 20 West Coast herds classified as infected on 1 April to 1 June 2012 had a median S/P ratio of 0.018 (range, 0.048 to 0.491) compared to the median for herds classified as TB free at those times of 0.026 (range, 0.075 to 0.559). Similarly, for the 48 West Coast herds classified as infected between 1 April 2012 and 1 July 2013, the median S/P ratio was 0.020 (range, 0.048 to 0.057) compared to the median for the TB-free herds during this period of 0.027 (range, 0.075 to 0.559). There were no significant differences between the medians for these two types of herds (*P* > 0.05). There were three West Coast herds which had S/P ratios of ≥0.3 (S/P ratios of 0.559, 0.491, and 0.461). In the herd with the milk vat sample S/P ratio of 0.559, 10 animals from

<table>
<thead>
<tr>
<th>Herd(s)</th>
<th>No. of animals</th>
<th>Early</th>
<th>Mid</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–4</td>
<td>111</td>
<td>−0.028 A (−0.047 to −0.002)</td>
<td>NS</td>
<td>−0.021 B (−0.042 to 0.007)</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>0.01 A (0.005 to 0.017)</td>
<td>0.0004 B (−0.002 to 0.0037)</td>
<td>0.006 AB (0.002 to 0.011)</td>
</tr>
</tbody>
</table>

*Median S/P values (95% confidence intervals) are given. In each row of data, median values followed by the same letters are not significantly different (*P* > 0.05). NS, no samples collected.*

![FIG 3 Receiver operating characteristic curve for antibody responses of milk samples from *Mycobacterium bovis*-infected and noninfected cows. A cutoff S/P ratio of ≥0.3 yielded a sensitivity of 50% and a specificity of 97.5%.

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**TABLE 1** Cross-classification results of ELISAs for antibodies against *Mycobacterium bovis* in serum and milk for 38 confirmed *M. bovis*-infected cattle

<table>
<thead>
<tr>
<th>Milk ELISA result</th>
<th>No. of animals with a serum ELISA result of:</th>
<th>Total no. of results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>17 Positive, 3 Negative</td>
<td>20</td>
</tr>
<tr>
<td>Negative</td>
<td>3 Positive, 15 Negative</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>20 Positive, 18 Negative</td>
<td>38</td>
</tr>
</tbody>
</table>

![TABLE 2 Milk ELISA responses in cows from four TB-free herds for milk samples collected from individual cows at different stages of lactation](http://cvi.asm.org/1815)
a herd of 380 cows had reacted in the tuberculin skin test, but bovine TB was not confirmed in any of these animals at slaughter, indicating a nonspecificity concern, while the herd with a milk vat sample S/P ratio of 0.491 was classified as infected with M. bovis. In 2012, 48 cows from a total of 529 cows in this milking herd were positive in the intradermal tuberculin test. Following retesting with the IFN-γ test, 10 animals were slaughtered, and 2 animals were confirmed as M. bovis infected. For the three herds with positive milk vat antibody responses, two of the herds had a large number of animals which produced false-positive responses for the intradermal test, suggesting that positive milk vat responses may also result from animals producing nonspecific antibody responses to the test antigens. The other issue with the use of milk vat samples for New Zealand herds is that when M. bovis infection is detected in cattle herds in New Zealand, generally only one to two animals are infected. Considering that pooling of milk samples resulted in few animals having positive S/P ratios of ≥0.3 at a 1/50 dilution in milk from noninfected animals, analysis of milk samples is unlikely to be useful unless a TB outbreak involves a large number of animals. Alternatively, a lower cutoff value could be considered for testing milk vat samples. The potential for testing bulk tank milk samples has been shown in another study (6) where the bulk tank milk samples from 14 of 17 Mexican dairies were positive in the IDEXX TB ELISA. The bulk milk tank samples from all 17 dairies were positive for M. bovis DNA using PCR, indicating that there was a very high prevalence of infection or severity of disease in these herds. In contrast, the bulk tank milk samples from 185 TB-free dairies in the United States were negative in the IDEXX ELISA.

One aspect which was not investigated in the current study was the detection of M. bovis-infected animals using the milk ELISA for those that had not reacted in the tuberculin skin test. Others have shown that serum ELISAs can be used to identify M. bovis-infected animals that have been negative in tuberculin skin tests (17).

Findings from the current study indicate that use of the IDEXX TB ELISA on milk samples can be applied for identifying M. bovis-infected cows in a country such as New Zealand, where most infections are likely to be detected early due to close monitoring of herds and where the control program is at an advanced stage. The sensitivity of the IDEXX test for milk samples was similar to that for serum samples collected from the same animals. Furthermore, milk samples may be more convenient for testing when individual milk samples are collected for routine dairy herd improvement testing rather than serum samples, which have to be collected additionally. Milk samples from 10 to 20 animals can be pooled to identify infected animals, although the test sensitivity may decrease by approximately 50%. However, the sensitivity of the test for pooled milk samples could be increased by using a lower cutoff value. The testing of milk vat samples is unlikely to be useful when a small number of infected animals are found in a herd and the herds are large.

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