New Latex Bead Agglutination Assay for Differential Diagnosis of Cattle Infected with *Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis*

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Extensive studies have shown that the current assays used to identify cattle infected with *Mycobacterium bovis* or *Mycobacterium avium* subsp. *paratuberculosis* are not sufficiently sensitive and specific to detect all infected animals, especially animals recently infected with the pathogens. In the present report we show that these limitations might be overcome with a latex bead agglutination assay (LBAA). With the specific immunodominant epitope (ESAT6-p) of *M. bovis*, we developed an LBAA and enzyme immunoassay (EIA) for that purpose and compared them with the “gold standard” culture method and skin test for their efficacy in detecting bovine tuberculosis. When sera from control healthy cows (n = 10), *M. avium* subsp. *paratuberculosis*-positive cattle (naturally infected, n = 16; experimentally infected, n = 8), and *M. bovis*-positive cattle (naturally infected, n = 49; experimentally infected, n = 20) were applied to an EIA and an LBAA developed with ESAT6-p, the two tests showed similar sensitivity (97.1% by EIA, 95.7% by LBAA), high specificity (94.2% by EIA, 100% by LBAA), and a positive correlation (kappa value, 0.85; correlation rate, 95.2%; correlation coefficient, 0.64). Receiver operating characteristic analysis of EIA results and comparison with the culture method determined a suitable cutoff value at 0.469, with an area under the curve of 0.991 (95% confidence interval, 0.977 to 1.0). As LBAA didn’t show any positive reactions with sera from uninfected control cows or *M. avium* subsp. *paratuberculosis*-infected cattle, which were confirmed to be free of *M. bovis* by culture or PCR, LBAA using the ESAT6-p can be a rapid and useful *M. bovis* diagnostic assay. The data suggest that rapid, sensitive, and specific assays can be developed with peptides containing immunodominant epitopes present in proteins uniquely expressed in *M. bovis* or *M. avium* subsp. *paratuberculosis* for differential diagnosis of cattle infected with *M. bovis* or *M. avium* subsp. *paratuberculosis*.

*Mycobacterium bovis*, the causative agent of bovine tuberculosis (bTB) (13), and *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne’s disease (paratuberculosis [pTB]), are of economic importance worldwide and a potential health hazard for animals and humans (9). Efforts to control these diseases have been difficult because of the lack of effective vaccines and the lack of diagnostic assays that can identify infected animals before the appearance of clinical disease. This has been a major problem in the control of pTB because infected animals begin to shed bacteria in feces and contaminate the environment well before signs of clinical disease. Difficulty in controlling the diseases has also been compounded by the presence of reservoirs of *M. bovis* and *M. avium* subsp. *paratuberculosis* in wild animal reservoirs (9, 11, 15, 17, 20). To address these problems, there is a need for a continued effort to develop diagnostic assays with greater sensitivity (Se) and specificity (Sp) that can be used in the field and laboratory, ideally assays that can be formatted for use with multiple species. Since the ESAT6 protein is secreted at an early or active phase of mycobacterial infection but not from *M. bovis* BCG-vaccinated animals (5, 22, 25, 26), synthetic ESAT6 peptides (ESAT6-p) were prepared to establish early specific detection of *M. bovis* serologically. In this report we show that a latex bead agglutination assay (LBAA) with synthetic peptides containing species-specific mycobacterial epitopes, ESAT6-p, may provide an approach to developing rapid diagnostic assays for bTB and pTB.

**MATERIALS AND METHODS**

**Animals.** (i) *M. bovis*-infected cattle. Three different groups of bovine sera were used in this study. A herd surveillance program in Korea has been checking for bTB-positive cows among all the cattle in Korea by using skin tests and then following up with cultures from suspect cows at least two times a year. Within 10 days after a positive skin intradermal test reaction (skin thickness, over 5 mm) in this national herd check program, sera were obtained from 49 cows documented to be naturally infected with *M. bovis*, which was verified by culture of *M. bovis* from intestinal tissue or nasal and tracheal mucus at the time of necropsy.
Although acute miliary TB with developing necrotic focus was observed in tracheobronchial and mediastinal lymph nodes of 10 cows, those cows were not thought to be in an advanced stage of IBT, as only a mild cough was observed as a clinical sign, without weight loss, swelling of lymph nodes in the head, or marked lymphadenomegaly with multiple nodular pale granulomas at necropsy. Additional sera were obtained from 20 calves (four groups of five calves each) experimentally infected with aerosol challenge of 10^5 or 10^6 CFU of either of two different strains of M. bovis, one isolated from white-tailed deer (strain 1315) and the other isolated from cattle (HC2005T). Serial samples of sera were collected at monthly intervals for 4 months pre- and postinfection (1 week preinfection and 27, 78, and 137 days postinfection) (18). Infection was confirmed in 19 calves by tuberculin skin test, isolation of M. bovis, and gross or microscopic tuberculous lesions in the lungs and tracheobronchial and mediastinal lymph nodes at the time of necropsy.

(ii) M. paratuberculosis–infected cattle. Sera were collected from 16 cows naturally infected with M. avium subsp. paratuberculosis, as verified by clinical signs of advanced pTB or by use of the PARACHEK Johnes’ absorbed enzyme-linked immunosorbent assay (EIA; CSL Veterinary, Parkville, Victoria, Australia) and the isolation of M. avium subsp. paratuberculosis from the intestine at necropsy. Additionally, blood samples were obtained from three male Holstein calves inoculated intratracheally with the K10 strain of M. avium subsp. paratuberculosis (28) and five male Holstein calves exposed orally to a field strain (01-13665, Washington State University) of M. avium subsp. paratuberculosis. Those eight calves were not older than 1 year. The experimental M. avium subsp. paratuberculosis infected infection was confirmed by the peripheral M. avium subsp. paratuberculosis colony detection by culture and IS900 PCR with feces and intestinal tissues in three intratracheally M. avium subsp. paratuberculosis–infected calves. The basis for the diagnosis of the immune response to M. avium subsp. paratuberculosis antigens in all eight calves (reference 28 and unpublished data).

All of the M. avium subsp. paratuberculosis–positive cattle were confirmed to be free of M. bovis by M. bovis culture and PCR with intestinal tissue or fecal samples. The sera from naturally and experimentally infected cattle with M. avium subsp. paratuberculosis were included in this study to demonstrate there were no cross-reactive antibodies present in sera from M. avium subsp. paratuberculosis–infected cattle.

(iii) Negative controls. Control sera were obtained from 10 cows from the Washington State University dairy herd, which is free of M. bovis and M. avium subsp. paratuberculosis.

Preparation of ESAT6-p-conjugated latex beads. Latex beads [PS(V/C-COOH); near soap free; 0.85 μm] were obtained from Bangs Labs Inc. The peptide sequence of ESAT6 (KGSGSMTEQQWNFAGIEAAASAIQG) known to contain an epitope recognized by antibodies from infected animals (7, 23) was synthesized as a single peptide with the peptide synthesizer ABI model 431A version 2.00 SFFMoc in the School of Molecular Biosciences at Washington State University (14). An extra lysine, glycine, and serine were added to the N-terminal end of ESAT6-p to enhance hydrophilicity and introduce a moiety for making an antibody to M. avium subsp. paratuberculosis–infected cattle and ascertain serological positive responses in M. avium subsp. paratuberculosis–positive cattle used in this study. Briefly, absorption of serum was done by incubating 25 μl of test and control serum samples and 475 μl of the diluent in the kit at room temperature for 30 min. The EIA plates were incubated with 100 μl of absorbed test or control serum at 37°C for 1 h. After washing with wash buffer, the plates were reacted with 100 μl of 1:1,000 dilution of alkaline phosphatase–conjugated reagent (horseradish peroxidase-labeled antiovine immunoglobulin G (H+L; Zymed) for 1 h. Following washing in PBS, the plates were reacted with 100 μl of a 1:2,500 dilution of biotinylated rabbit-anti bovine immunoglobulin G (H+L; Zymed) for 1 h. After washing in PBS, the plates were reacted with 100 μl of a 1:3,000 dilution of streptavidin–alkaline phosphatase (10 μg/ml; pH 9.5) for 1 h. Following washing in PBS, the plates were reacted with 100 μl of a 1:2,500 dilution of biotinylated rabbit-anti bovine immunoglobulin G (H+L; Zymed) for 1 h. After washing in PBS, the plates were reacted with 100 μl of 3 mM NaOH was added to stop the reaction. Absorbance was read at 405 nm with an M8340 EIA reader (Dynatech). The mean ratio of the OD of wells containing sera from infected animals to the OD of wells containing sera from uninfected control animals.

EIA for antibody to M. avium subsp. paratuberculosis. Serological responses against M. avium subsp. paratuberculosis were evaluated with the commercially available PARACHEK Johnes’ absorbed EIA (CSL Veterinary) with absorbed serum and HerdChek M. paratuberculosis (IDEXX Labs, Westbrook, Maine) to check possible M. avium subsp. paratuberculosis infection in M. bovis–infected cattle and ascertain serological positive responses in M. avium subsp. paratuberculosis–positive cattle used in this study. Brieﬂy, absorption of serum was done by incubating 25 μl of test and control serum samples and 475 μl of the diluent in the kit at room temperature for 30 min. The EIA plates were incubated with 100 μl of absorbed test or control serum at 37°C for 1 h. After washing with wash buffer, the plates were reacted with 100 μl of freshly prepared 1:1,000 dilution of alkaline phosphatase–conjugated reagent (horseradish peroxidase-labeled antiovine immunoglobulin G (H+L; Zymed) for 1 h. After washing in PBS, the plates were reacted with 100 μl of 1:2,500 dilution of biotinylated rabbit-anti bovine immunoglobulin G (H+L; Zymed) for 1 h. After washing in PBS, the plates were reacted with 100 μl of a 1:2,500 dilution of biotinylated rabbit-anti bovine immunoglobulin G (H+L; Zymed) for 1 h. After washing in PBS, the plates were reacted with 100 μl of 3 mM NaOH was added to stop the reaction. Absorbance was read at 405 nm with an M8340 EIA reader (Dynatech). Plasma samples were considered positive if the OD from test samples was above the cutoff value (the mean of the negative controls plus 0.100), the OD from negative control was below 0.150, and the OD from the positive control was between 0.9 and 1.2. Duplicate serum samples were submitted to the diagnostic laboratory for evaluation with HerdChek M. paratuberculosis (IDEXX Labs).

Data analysis. A suitable cutoff value for the EIA was determined with receiver operating characteristics (ROC) analysis (2) based on the results of the gold standard culture for cattle used in this study. The cutoff value for each test used was determined by the performance of ROC analysis of the data using the DeLong method (4). The diagnostic value of the typical EIA was 0.934 with a range of 0.707 to 1.000.

RESULTS

Standardization of the LBAA. Testing of latex beads coated with different concentrations of ESAT6-p showed conjugation of 1 mg of beads with 45 μg of ESAT6-p was best for obtaining specific agglutination, with no agglutination evident with sera from uninfected control cattle (Fig. 1). Examination of plates over a 4-h period revealed agglutination was evident with all test sera by 2 h. There was no change in the patterns of agglutination when the plates were incubated overnight at 4°C.

Sp and Se of EIA and LBAA. After determining the optimal conditions for agglutination, sera from naturally or experimentally M. bovis–infected cattle were tested at four dilutions (1/5, 1/10, 1/20, and 1/40) along with sera from control healthy cows.
and *M. avium* subsp. *paratuberculosis*-infected cattle. Consistent results were obtained using a dilution of 1/20, and the resulting Se and Sp of LBAA are summarized in Table 1. The intensity of agglutination was calculated and analyzed in 10 fields of 1 mm² randomly selected in wells of the LBAA. Positive agglutination reactions were scored as follows: 1, 0 to 25%; 2, 26 to 50%; 3, 51 to 75%; and 4, 76 to 100%.

FIG. 1. Representative pattern of agglutination of latex beads coated with ESAT6 and reacted with sera from an infected cow (a to d; positive agglutination scores of 1 to 4) and an uninfected cow (e; negative agglutination). An Image Analyzer (Olympus) and the Optimas 6.5 program were used to calculate and analyze the intensity of agglutination in 10 fields of 1 mm² randomly selected in wells of the LBAA. Positive agglutination reactions were scored as follows: 1, 0 to 25%; 2, 26 to 50%; 3, 51 to 75%; and 4, 76 to 100%.

FIG. 2. ROC curves for EIA with ESAT6-p. Se and Sp were calculated as follows: Se = (number of serum samples showing positive culture and EIA results at each cutoff value)/(number of *M. bovis* culture positive results). Sp = (number of serum samples showing negative culture and EIA results at each cutoff value)/(number of *M. bovis* culture negative results). Both Se and Sp were based on the results of the *M. bovis* culture method as the standard and compared the two tests (culture versus EIA).

TABLE 1. Overall serological response of sera from naturally or experimentally *M. bovis*-infected cattle in EIA and LBAA utilizing ESAT6-p as coating antigen

<table>
<thead>
<tr>
<th>Assay</th>
<th>Naturally infected cows (n = 49)</th>
<th>Experimentally inoculated calves (n = 20)</th>
<th>Total (n = 69)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIA</td>
<td>49/49 (100)</td>
<td>16/20 (80.0)</td>
<td>65/69 (97.1)</td>
</tr>
<tr>
<td>LBAA</td>
<td>46/49 (93.9)</td>
<td>20/20 (100)</td>
<td>66/69 (95.7)</td>
</tr>
</tbody>
</table>

* Results are expressed as number of sera positive/number of sera tested, with percent sensitivity shown in parentheses. Detailed information on how natural *M. bovis* infection was confirmed, how cattle were inoculated experimentally, and how a positive response in the EIA or LBAA was defined is provided in Materials and Methods.

* There was no false-positive reaction of LBAA with sera from *M. bovis*-negative (naturally or experimentally *M. avium* subsp. *paratuberculosis*-infected or healthy) cattle, but one serum from the group of control healthy cows showed an OD of 0.469, which is the same as the selected cutoff value for the EIA used in this study.

score of 1, 0 to 25%; 2, 26 to 50%; 3, 51 to 75%; 4, 76 to 100% (Fig. 1). Although background agglutination was noted at higher concentrations of serum with some animals, no agglutination was evident with sera from control or *M. avium* subsp. *paratuberculosis*-infected cattle or from calves at the preinfection stage of aerosol *M. bovis* challenge.

To compare Se and Sp and evaluate agreement between LBAA and EIA, the same sera were tested in an EIA format. As shown in Table 1, specific reactivity was obtained when sera were diluted to 1/50. Some sera yielded stronger reactions in EIA as well as in LBAA than other sera, as shown by the OD. After ROC analysis (Fig. 2), the area under the curve and standard error of the area under the curve were 0.991 (95% confidence interval [CI], 0.977 to 1.000) and 0.007, respectively. Among different cutoff values from the ROC analysis, a cutoff value of 0.469 was selected, as the Se of 97.1% and Sp of 94.2% at the chosen cutoff was optimal for our EIA. All *M. bovis*-positive sera from naturally *M. bovis*-infected cows confirmed by the culture of *M. bovis* at necropsy showed an OD which was greater than our selected cutoff value. Out of 20 bovine sera from experimentally *M. bovis*-inoculated calves, 4 sera showed a negative EIA result at the cutoff value.

One calf experimentally infected with an aerosol challenge of $10^7$ CFU of *M. bovis* isolated from cattle (HC2005T) didn’t show any gross or microscopic findings, positive response at tuberculin skin test, or bacteriologic isolation in feces, lymph nodes, or other tissues (18). When sera from that calf were applied, sera from 27 days postinfection showed positive responses to ESAT6-p with the EIA and LBAA developed in this study.
Latex bead technology has been used successfully to develop simple, easy-to-use agglutination assays (12, 16, 27, 29, 30). Recombinant or synthetic peptides derived from species-specific M. bovis or M. avium subsp. paratuberculosis proteins that contain epitopes recognized by antibodies from infected animals could be used singly or multiplexed on a lysine polymer backbone by using currently available technology to detect antibodies specific for M. bovis or M. avium subsp. paratuberculosis (10).

In the present study we have demonstrated that an LBAA offers the potential of developing a relatively rapid assay for detecting animals infected with M. bovis. Serial samples of sera collected pre- and postinfection were applied to our developed EIA and LBAA in order to determine how early the serological response to ESAT6-p appears. Although all the sera from preinfection were negative by EIA and LBAA, 85, 100, and 100% of sera collected at 27, 78, and 137 days, respectively, following aerosol challenge with M. bovis showed a positive serological response to ESAT6-p by EIA or LBAA. Along with this early serological response to ESAT6-p in M. bovis-infected cattle, one of which showed a positive ESAT6-p response before fecal shedding, a tuberculin skin response, or histological changes, the high Se and Sp of EIA and LBAA and the high agreement between EIA and LBAA in order to determine how early the serological response to ESAT6-p appears. Although all the sera from preinfection were negative by EIA and LBAA, 85, 100, and 100% of sera collected at 27, 78, and 137 days, respectively, following aerosol challenge with M. bovis showed a positive serological response to ESAT6-p by EIA or LBAA. Along with this early serological response to ESAT6-p in M. bovis-infected cattle, one of which showed a positive ESAT6-p response before fecal shedding, a tuberculin skin response, or histological changes, the high Se and Sp of EIA and LBAA and the high agreement between EIA and LBAA showed that the LBAA developed in our study can be a reliable and rapid M. bovis diagnostic assay. Importantly, the results suggest that a similar assay can be developed for M. avium subsp. paratuberculosis, following the identification of a specific and immunodominant epitope of M. avium subsp. paratuberculosis. An additional advantage that an LBAA offers is that the same assay can be used with multiple species, since no additional reagents are needed to develop the test. Further studies are now in progress to identify other peptides for use in the LBAA for M. bovis and M. avium subsp. paratuberculosis.

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