Immunochromatographic Test for Simultaneous Serodiagnosis of *Babesia caballi* and *B. equi* Infections in Horses

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Received 6 December 2005/Returned for modification 30 January 2006/Accepted 6 March 2006

An immunochromatographic test for the simultaneous detection of *Babesia caballi*- and *B. equi*-specific antibodies (BceICT) was developed using a recombinant *B. caballi* 48-kDa rhoptry protein (rBc48) and a recombinant truncated *B. equi* merozoite antigen 2 (rEMA-2t). An evaluation of the ability of the BceICT to detect antibodies in sera from uninfected horses and experimentally infected horses showed high sensitivities and specificities of 93.5% (38/41 sera) and 92.9% (22/24 sera), respectively, for the anti-*B. caballi* antibody and 94.1% (22/19 sera) and 88.2% (21/24 sera), respectively, for the anti-*B. equi* antibody. Results from the detection of antibodies in field-collected sera indicated that the BceICT results correlated well with those of enzyme-linked immunosorbent assays (ELISA), showing 96.1% correspondence (67/73 sera) for *B. caballi* and 95.9% correspondence (70/73 sera) for *B. equi*, and that the BceICT results also corresponded with the ICT for *B. caballi* and for *B. equi*, both of which were 98.2% (55/56 sera). The comparable results of the ICT and ELISA and the simplicity and rapidity of the performance of the ICT suggest that the BceICT would be a feasible test for the simultaneous serodiagnosis of both agents of equine Babesiosis in the field.

Materials and Methods

rEMA-2t. rEMA-2 was expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase, as described previously (5). The fusion protein was purified using glutathione Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). The leader protein, glutathione S-transferase, was cleaved by thrombin protease.

rBc48. Bc48 was prepared as described previously, with some modification (7, 8). Briefly, the Bc48 gene inserted into pBluescript SK (+) vectors was subcloned into pGEX-4T (Amersham) of the bacterial expression vector after digestion with EcoRI and XhoI. The *E. coli* (BL21 strain) colony transformed with pGEX-4T/Bc48 was cultured on a small scale overnight in Luria-Bertani (LB) medium (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, and 0.5% 5 N NaOH) with 50 µg/ml of ampicillin sodium at 37°C. The overnight culture was then diluted to 1:100 in an LB medium for a large-scale culture at 25°C. When the optical density at 600 nm (OD600) reached 0.50, *E. coli* was induced to express the rBc48 protein by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside and incubation for another 4 h at 25°C. The purification procedure for rBc48 was the same as that for rEMA-2.

Conjugates. After dialysis in a 5 M phosphate buffer at the proper pH (6.5 for rEMA-2t and 8.0 for rBc48), rEMA-2t and rBc48 were diluted to their optimal concentrations, 200 µg/ml and 125 µg/ml, respectively, and mixed gently with gold colloid particles (British BioCell International, SDX, United Kingdom) at the optimal pH. The ratio of volumes was 1:10. The mixtures were incubated at room temperature for 10 min without disturbance. Then, 0.05% polyethylene glycol 20,000 (PEG) and 1% bovine serum albumin (BSA) were added to stabilize and block the conjugate particles. After centrifugation at 18,000 × g for 20 min, 90% of the supernatants were discarded, and the pellets were resuspended in the remaining supernatants by sonication and then washed with phosphate-buffered saline containing 0.5% BSA and 0.05% PEG. Following the second centrifugation, the pellets were resuspended in phosphate-buffered saline with 0.5% BSA and 0.05% PEG until the OD600 reached 5. After the two conjugates were mixed and diluted in 10 mM Tris-HCl (pH 8.2) with 5% sucrose, the mixture was sprayed onto glass fiber (Schleicher & Schuell, NH) and dried in a vacuum overnight.

Rabbit anti-rEMA-2t IgG. A rabbit was immunized with 1 ml of rEMA-2t (2 mg/ml) mixed with 1 ml of complete Freund’s adjuvant (Difco, Detroit, MI) by multiple intradermal injections into its dorsum. Two booster injections were given in a 2-week interval, with the same dose of antigen mixed with incomplete Freund’s adjuvant (Difco). The rabbit was bled 10 days after the last booster. The immunoglobulin G fraction was purified from blood serum with an Econo-
RESULTS

Detection of specific antibodies against *B. caballi* and *B. equi* in sera from experimentally infected horses. The results of experiments for the detection of specific antibodies are summarized in Tables 1 and 2. The sensitivities and specificities of the BceICT were 83.3% (10/12 sera) and 92.9% (52/56 sera), respectively, for the detection of the antibody against *B. caballi* and 94.1% (16/17 sera) and 88.2% (45/51 sera), respectively, for the detection of the antibody against *B. equi* infection. The specificity of the BceICT for detecting antibodies to *B. caballi* (83.3%) and *B. equi* (94.1%) were equal to those of *B. caballi* ELISA (BcELISA) and *B. equi* ELISA (BeELISA). On the other hand, the specificity of the BceICT for detecting antibodies to *B. caballi* (92.9%) and *B. equi* (88.2%) were slightly lower than those of BcELISA (100%) and BeELISA (100%).

**Table 1.** Comparison of BceICT with BcELISA in the detection of specific antibodies against *B. caballi* in equine sera

| BeELISA result | Uninfected sera (n = 39) | B. equi-infected sera (n = 17) | B. caballi-infected sera (n = 12) | Field sera (n = 73) |
|----------------|--------------------------|-------------------------------|----------------------------------|-------------------|
| +              | 1 38 2 14 0              | 0 0 0 0 0                   | 10 0 10 10 2                   | 19 5 19 19 19     |
| -              | 0 38 3 0 0              | 0 0 0 0 0                   | 2 0 2 2 2                     | 1 1 1 1 1         |
| Total          | 1 38 3 14 0             | 10 0 10 10 2                | 21 20 20 20 20                |

**Table 2.** Comparison of BceICT with BeELISA in the detection of specific antibodies against *B. equi* in equine sera

| BeELISA result | Uninfected sera (n = 39) | B. equi-infected sera (n = 17) | B. caballi-infected sera (n = 12) | Field sera (n = 75) |
|----------------|--------------------------|-------------------------------|----------------------------------|-------------------|
| +              | 0 0 0                   | 0 0 0                        | 39 1 0                         | 39 1 39 39 39     |
| -              | 2 37 0                  | 1 1 1                        | 8 2 8 2 2                     | 2 2 2 2 2         |
| Total          | 2 37 0 1 1             | 8 2 8 2 2                     | 41 32 41 32 41                |

a The sensitivity of both BeELISA and BceICT for detecting antibody to *B. equi* was 94.1% (16/17), and the specificities of BeELISA and BceICT were 100% (51/51) and 88.2% (45/51), respectively.
phase, which are conjugated with gold particles, and antigens and antibodies in the immoblie phase. The captured antigen and antibody complex then develops a colored line. As soon as the test strip is available, the performance is as simple as loading the sample onto the strip, and the result can be determined in a few minutes with the naked eye, according to the colored lines. No equipment or testing skills are required. Therefore, this test is more practical to use in the field than any colored lines. No equipment or testing skills are required.

Detection results of the specific antibodies in the known \( \text{B. } \text{caballi} \)-infected and uninfected sera indicate

| Anti-\( \text{B. } \text{caballi} \) antibody | Anti-\( \text{B. equi} \) antibody |
|--------------------------------|----------------|
| No. of positive sera (%)     | No. of negative sera (%) |
|                                |                                |
| BeICT +                       | 18 (32.1)                     |
|                               | 1 (1.8)                       |
| BeICT −                       | 0                             |
|                               | 37 (66.1)                     |

Total \((n = 56)\) 18 (32.1) 38 (67.9) 27 (48.2) 29 (51.8)

\( * \) The percentage of results that corresponded with those of BeICT was 98.2% for both BeICT and BeICT.

**ACKNOWLEDGMENTS**

This study was supported by grants from the 21st Century COE Program (A1) and Ministry of Education, Culture, Sports, Science, and Technology, Japan, and by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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