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

Xiaohong Huang,1,2 Xuenan Xuan,1 Rodolfo A. Verdida,1 Shoufa Zhang,3 Naoaki Yokoyama,1 Longshan Xu,2 and Ikuo Igarashi1*

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan; Fujian Provincial Center for Diseases Control and Prevention, Fuzhou, Fujian Province 350001, China; and Department of Veterinary Medicine, Yanbian University, Longjing, Jilin Province 133400, China

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Equine piroplasmosis, caused by Babesia caballi and Babesia equi, is an important protozoan disease worldwide from both veterinary and economic viewpoints (2). Various serodiagnostic tests have been developed for the disease, such as the complement fixation test (1, 11, 12), the indirect immunofluorescent antibody test (1, 11, 12), the enzyme-linked immunosorbent assay (ELISA) (1, 3, 5, 7, 8, 10, 13, 14), the competitive-inhibition ELISA (ELISA) (9), and the immunochromatographic test (ICT) (6). In our previous studies, ELISAs for the serodiagnoses of Babesia caballi and Babesia equi infections demonstrated many advantages, such as higher sensitivity and specificity, lower cost of materials, and greater objectivity in the determination of results (5, 8), over the complement fixation test, indirect immunofluorescent antibody test, and competitive-inhibition ELISA. Compared with ELISA, however, the ICT is relatively simple, can be performed quickly, and has the listed advantages of ELISA (6).

Babesia caballi and Babesia equi have overlapping geographical distributions (4). In such areas, an individual horse may be infected by both species. Therefore, a test capable of detecting the antibodies induced by both types of parasites would be desirable. Here, we report an ICT for the simultaneous detection of Babesia caballi- and B. equi-specific antibodies (BceICT) was developed using a recombinant Babesia caballi 48-kDa rhoptry protein (rBc48) and a recombinant truncated B. equi merozoite antigen 2 (rEMA-2t) as antigens for the simultaneous serodiagnosis of both agents of equine babesiosis in the field.

**MATERIALS AND METHODS**

**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 (Amerham) from 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 0.1% 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 Bc48 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-2t.

**Conjugates**. After dialysis in a 5 mM phosphate buffer at the proper pH 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 OD520 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 (IgG) fraction was purified from blood serum with an Econo-
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 sensitivity 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%).

**DISCUSSION**

The ICT is a nitrocellulose membrane-based immunocassay that relies on the migration of a liquid across the surface of the membrane by the capillary mechanism and the capture of the antibodies in the sample using the antigens in the mobile adhesive card (Schleicher & Schuell) with other components, such as an absorptive pad, a conjugate pad, and a sample pad, cut into 6-mm-wide strips using a BioDot cutter (BioDot, Inc., CA), as shown in Fig. 1 (lane 1). Detection was performed by pipetting 100 μl of serum onto the sample pad. In the preliminary test, color in the control line took a maximum of 7 min to develop; color in the test lanes took a maximum of 15 min to develop, and the results did not change when the sample pad was read later than 15 min. Therefore, results were determined 15 min after the application of serum samples and recorded as (i) positive for both equine babesiosis species (Fig. 1, lane 2); (ii) positive for *B. caballi* and negative for *B. equi* (Fig. 1, lane 3); (iii) negative for *B. caballi* and positive for *B. equi* (Fig. 1, lane 4); and (iv) negative for both *B. caballi* and *B. equi* (Fig. 1, lane 5).

**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 sensitivity 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**

<table>
<thead>
<tr>
<th>BcELISA result</th>
<th>Uninfected sera (n = 39)</th>
<th>B. equi-infected sera (n = 17)</th>
<th>B. caballi-infected sera (n = 12)</th>
<th>Field sera (n = 73)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>17</td>
<td>12</td>
<td>73</td>
</tr>
</tbody>
</table>

* The sensitivity of both BcELISA and BceICT for detecting antibody to *B. equi* was 94.1% (16/17), and the specificities of BcELISA and BceICT were 100% (51/51) and 88.2% (45/51), respectively.
TABLE 3. Comparison of BceICT with BcICT and BeICT in the detection of specific antibodies against B. caballi and B. equi infections in field sera

<table>
<thead>
<tr>
<th>BcICT or BeICT detection result</th>
<th>Anti-B. caballi antibody</th>
<th>Anti-B. equi antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of positive sera (%)</td>
<td>No. of negative sera (%)</td>
</tr>
<tr>
<td>BcICT +</td>
<td>18 (32.1)</td>
<td>1 (1.8)</td>
</tr>
<tr>
<td>BcICT −</td>
<td>0</td>
<td>37 (66.1)</td>
</tr>
<tr>
<td>BeICT +</td>
<td>26 (46.4)</td>
<td>0</td>
</tr>
<tr>
<td>BeICT −</td>
<td>0</td>
<td>29 (51.8)</td>
</tr>
</tbody>
</table>

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

* The percentage of results that corresponded with those of BceICT was 92.8% for both BcICT and BeICT.

phase, which are conjugated with gold particles, and antigens and antibodies in the immobile 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 other test. In our previous studies, the BcICT and BeICT were developed for the detection of antibodies to B. caballi (unpublished data) and B. equi (6). Both of the tests showed results that were comparable with those of ELISAs. To combine the two ICTs into one test, we developed a BceICT for the simultaneous detection of antibodies against infection by two species of Babesia. Using this test, some materials used for the preparation of test strips, sera, manpower, and time required could be reduced by one-half.

Detection results of the specific antibodies in the known B. caballi- and B. equi-infected and uninfected horses indicate that the sensitivity and specificity of the BceICT was 83.3% and 92.9%, respectively, for anti-B. caballi antibody and 94.1% and 88.2%, respectively, for anti-B. equi antibody. No significant differences were observed in sensitivity between BcICT and BeELISA and between BcICT and BeELISA. However, the specificity of the BceICT was less than those of BeELISA and BeELISA. The nonspecific reaction in the BcICT for the detection of B. equi infection was observed mainly in sera from B. caballi-infected horses, in reverse, and that for the detection of B. caballi infection was observed mainly in sera from B. equi-infected horses. Therefore, these nonspecific reactions may be due to an antigen or antibody cross-reaction rather than the effect of some physical or chemical factors. The reaction may occur when the two conjugates are mixed. Other possibilities are related to the storage of the sera, for example, the length of the storage period, the quantity of preservative added, or the conditions for the preparation of the test strips. If further discrimination between the two species is necessary, ELISAs could be carried out to examine the BcICT-positive sera.

The high correspondence of BcICT results with ICT or ELISA results were also found for B. caballi and B. equi infections, respectively, in sera collected from horses in the field. The correspondence of the BcICT with BeELISA, BeELISA, BcICT, and BeICT were 91.8%, 95.9%, 98.2%, and 98.2%, respectively (Tables 1, 2, and 3). These results for B. equi infection were very comparable with those in previous studies (6).

In conclusion, the present study indicates that the BcICT employing antigen bound to nitrocellulose membranes has a high specificity and sensitivity for detecting antibodies to both B. caballi and B. equi. The results of the BcICT are easily obtained and comparable with those from ELISA. Therefore, the BcICT is a feasible field test for the simultaneous serodiagnosis of both types of equine babesiosis, even though some improvements of the BcICT and an evaluation on a larger scale are necessary.

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