

Development of an Immunochromatographic Test with Recombinant EMA-2 for the Rapid Detection of Antibodies against *Babesia equi* in Horses

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An immunochromatographic test (BeICT) for the rapid detection of antibodies against *Babesia equi* was developed. It clearly differentiated *B. equi*-infected horses from *B. caballi*-infected and uninfected horses. The agreement with enzyme-linked immunosorbent assay results was 96.7% in the detection of field sera. The results suggest that BeICT is rapid, simple, reliable, and suitable for use to detect *B. equi* infection in the field.

Equine piroplasmiasis, caused by *Babesia equi*, is a tick-borne hemoprotozoan disease of horses characterized clinically by fever, anemia, and icterus (4). With the development of international trade, the disease is becoming more and more economically important. Because the parasites are generally not detectable by direct microscopic examination during the latent phase, development and improvement of an immunodiagnostic method are very important to prevent the introduction of infected animals into piroplasma-free countries, such as Australia, the United States, and Japan (3).

Previously, we developed an enzyme-linked immunosorbent assay (ELISA) and Western blot analysis by using a recombinant truncated *B. equi* merozoite antigen-2 (rEMA-2t) (5). The two tests were proven to be highly sensitive, specific, and economical with regard to antigen, which are improvements over the existing tests, such as the complement fixation test, the indirect fluorescent-antibody test, and ELISA with parasite lysate or competitive ELISA with recombinant antigen (1, 2, 6, 7, 8). However, they are still not only time-consuming and labor-intensive but also require professional personnel and special laboratory materials and equipment and are thus unsuitable for use in the field.

The immunochromatographic test (ICT) is a nitrocellulose membrane (NC)-based immunoassay. The procedure may be as simple as just loading the sample as soon as the test strip is available. From the colored lines, the result can be evaluated by the naked eye within a few minutes. In the present study, an ICT (BeICT) for the detection of antibody against *B. equi* infection in horses was developed with rEMA-2t and evaluated. To our knowledge, this is the first report on the application of an ICT for immunodiagnosis of *B. equi* infection.

Expression and purification of rEMA-2t were done as described previously (5). rEMA-2t (200 µg/ml) was conjugated

with a gold colloid (British BioCell International, Cardiff, United Kingdom) at pH 6.5 by gentle mixing (1:10, vol/vol) and incubation at room temperature for 10 min. Polyethylene glycol 20,000 (PEG) at 0.05% and bovine serum albumin (BSA) at 1% were then added to stabilize and block the conjugate particles. After centrifugation at 18,000 × *g* for 20 min, the supernatant was discarded and the pellet was resuspended by sonication and washed with phosphate-buffered saline containing 0.5% BSA and 0.05% PEG. After the second centrifugation, the pellet was resuspended in phosphate-buffered saline with 0.5% BSA and 0.05% PEG. The concentration of the conjugate was adjusted until the absorbance at 520 nm reached 5. The conjugate was diluted in 10 mM Tris-HCl (pH 8.2) with 5% sucrose, sprayed onto glass fiber (Schleicher & Schuell, Inc., Keene, N.H.), and dried in a vacuum overnight. A rabbit was immunized with rEMA-2t mixed with Freund's complete or incomplete adjuvant (Difco Laboratories, Detroit, Mich.) by multiple intradermal injections into the back. The immunoglobulin G (IgG) fraction was purified from its serum with an Econo-Pac protein A kit (Bio-Rad Laboratories, Richmond, Calif.). rEMA-2t (0.5 mg/ml) and rabbit anti-rEMA-2t IgG (1.5 mg/ml) were, respectively, jetted linearly onto a test area and a control area of NC with a plastic backing (Schleicher & Schuell) by using a BioDot's Biojet 3050 quanti-dispenser (BioDot Inc.). The membrane was then dried at 50°C for 30 min and blocked in 0.5% casein in 50 mM boric acid buffer (pH 8.5) for 30 min. After a wash with 50 mM Tris-HCl (pH 7.4) containing 0.5% sucrose and 0.05% sodium cholate, the membrane was dried in air overnight. Sequentially, the NC, absorbent pad, conjugate pad, and sample pad were assembled on an adhesive card (Schleicher & Schuell) and cut into 6-mm-wide strips with a BioDot cutter as demonstrated in Fig. 1, lane 1. Detection was performed by pipetting 100 µl of serum onto the sample pad. The results could be judged within 15 min and recorded as shown in lanes 2 and 3 of Fig. 1. Theoretically, this BeICT is able to detect all classes of immunoglobulin, such as IgG, IgM, and IgA, at the same time.

Sera from 11 *B. equi*-infected horses at 30 to 244 days postinfection (dpi), 8 *B. caballi*-infected horses at 28 to 395 dpi, and

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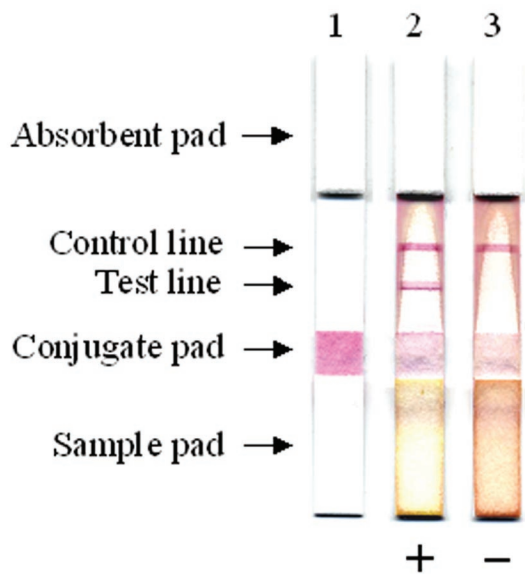


FIG. 1. Examples of BeICT strips before (lane 1) and after (lanes 2 and 3) testing. Symbols: +, positive result; -, negative result.

20 uninfected horses, provided by the Equine Research Institute, the Japan Racing Association, and the Onderstepoort Veterinary Institute, were examined by BeICT. The antibody responses were detected in all *B. equi*-infected horse sera but in none of those from *B. caballi*-infected and uninfected horses, which indicates that the BeICT has high sensitivity and specificity.

Sequential sera from two horses infected experimentally with *B. equi*, provided by the Japan Racing Association, were subjected to the BeICT and an ELISA, which is a highly sensitive and specific test, as described previously (5). The antibodies were detectable by BeICT by 4 dpi (Fig. 2B), earlier than the IgG antibody responses by ELISA (Fig. 2A), which might be because the BeICT is able to detect all classes of immunoglobulin so that it is more sensitive. Moreover, in the BeICT, the reactions of sera at 6 to 12 dpi were stronger than those later than 12 dpi, which was different from those in the ELISA (Fig. 2). This might be because the total level of specific immunoglobulin (including various classes, such as IgG, IgM, and IgA) produced during this period was higher.

The BeICT was evaluated for the detection of antibodies against *B. equi* infection in sera from 61 horses in Jilin Province, China. The results (Table 1) were comparable to those obtained by ELISA. The concordance of the two tests was 96.7% (59 of 61). One ELISA-negative serum was positive by the BeICT, which might be because the horse was at a very early stage of *B. equi* infection, when some classes of immunoglobulin, such as IgM, were detectable but IgG antibody was not. One serum that was weakly positive by ELISA (optical density at 415 nm = 0.1) was negative by BeICT. This suggests that BeICT is reliable.

The stability of the ICT strips was tested. The results showed that they were still effective after storage at 4 or 37°C for at least 8 months or at 45°C for at least 1 month.

Our results suggest that the BeICT meets the "gold standard," namely, (i) it is sensitive enough both for early detection

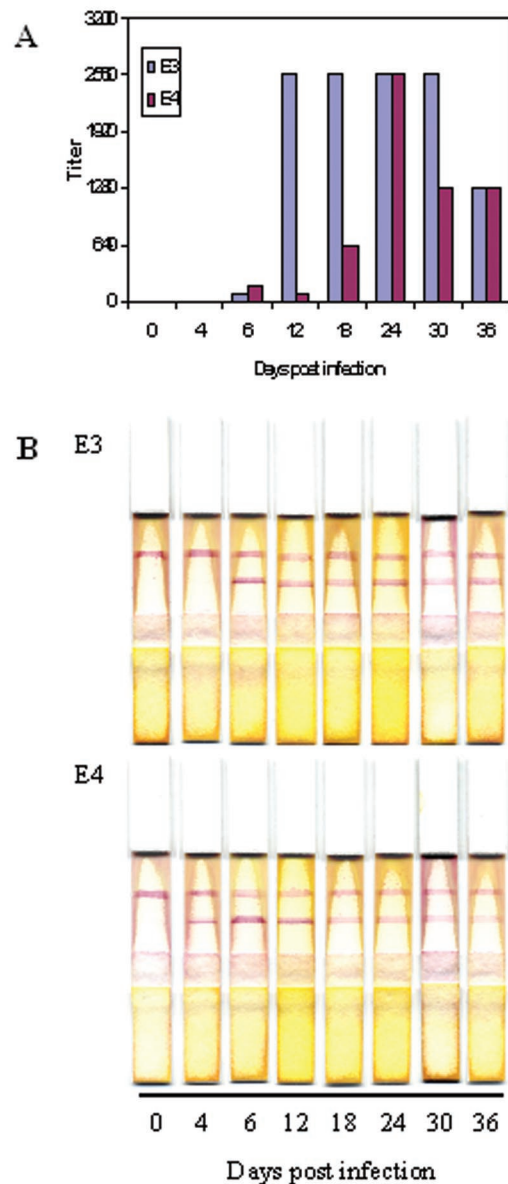


FIG. 2. Specific antibody responses in sequential sera from two horses (E3 and E4) experimentally infected with *B. equi* detected by ELISA and BeICT, respectively. A, IgG antibody titers examined by ELISA; B, antibody responses examined by BeICT.

TABLE 1. Comparison of BeICT and ELISA for detection of antibodies to *B. equi* in horses in Jilin, China

BeICT result	No. (%) of samples with following ELISA result:		Total no. (%)
	+	-	
+	27 (44.3)	1 (1.6)	28 (45.9)
-	1 (1.6)	32 (52.5)	33 (54.1)
Total no. (%) of samples	28 (45.9)	33 (54.1)	61 (100)

of acute infections and for detection of latent infections, (ii) it is specific enough to differentiate between the parasite species *B. equi* and *B. caballi*, and (iii) it is economical with regard to materials and time (2). The latter advantage especially makes BeICT superior to ELISA and other existing tests, because no special expertise or equipment will be required as soon as the strips are commercially available and the test takes less than 15 min to be completed. Furthermore, the ICT strip is quite stable during long storage under ordinary conditions. It would be a suitable diagnostic tool for the detection of *B. equi* infection under field conditions. Further improvements would include the use of whole blood samples in an ICT to simplify the test even more. A larger-scale evaluation with various horse sera is also necessary.

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